Further Studies on the Protection of Histidine Side Chains in Peptide Synthesis: The Use of the π-Benzyloxymethyl Group [1]

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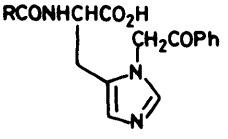
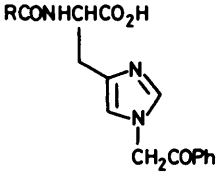
Further studies on the use in peptide synthesis of $N(\pi)$-phenacyl protection for histidine side chains have shown that whilst this prevents the side chain-induced racemisation which can occur if there is a lone pair of electrons available at the π-nitrogen, there are concomitant drawbacks. As an alternative approach to the racemisation problem, the effect of halogenation of the heterocyclic ring carbons (to diminish the availability of the π-nitrogen lone pair) has been investigated. This gives derivatives which are convenient in both classical and solid-phase applications, the halogen modifying groups being removed at the last stage by catalytic hydrogenolysis over a rhodium catalyst. Racemisation is suppressed as expected, but it is not eliminated completely: direct blockade of the π-nitrogen appears to be indispensable for its complete prohibition. Protection of the π-nitrogen with a benzyloxymethyl group has now been found to be much more satisfactory than the use of the phenacyl group for this purpose. A π-benzyloxymethyl substituent not only prohibits side chain-induced racemisation but also gives derivatives with convenient physical properties which can be incorporated into well established classical and solid-phase strategies without the need for any novel or additional operations or changes in protocol. The protecting group is stable to basic conditions, to trifluoroacetic acid, and to aqueous solutions of carboxylic acids, but is cleaved cleanly and rapidly by hydrogen bromide in trifluoroacetic acid or by catalytic hydrogenolysis. $N(\alpha)$-t-Butoxycarbonyl-$N(\pi)$-benzyloxymethyl-L-histidine has been prepared in good yield by a simple procedure from an easily accessible intermediate and isolated as a crystalline solid; its use has been demonstrated by a number of exercises including a solid-phase synthesis of 5-isoleucine-angiotensin II and a classical synthesis of trihistidine.

We have shown,[2] by comparison of the isomers (1) and (2), that the side chain-induced racemisation which can occur on activation of alkoxycarbonylhistidine derivatives for coupling can be eliminated by locating the blocking group specifically at $N(\pi)$. Protecting groups for the heterocyclic ring had previously been used without regard to (or, usually, definite knowledge of) location, although in most cases derivatisation would have occurred mainly or exclusively at $N(\tau)$. We were able to demonstrate the use of (1) and (3) in some simple exercises in peptide synthesis. Further experience has



(1) R = $PhCH_2O$

(3) R = Bu^tO



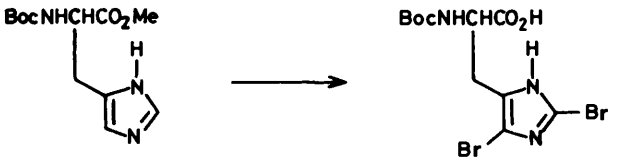
(2) R = $PhCH_2O$

now shown that the solution of the histidine racemisation problem in this way is achieved only at the expense of creating other problems. In particular, the final cleavage of the phenacyl group with zinc–acetic acid leads consistently to difficulties in removing the last traces of zinc ions from the free peptide. Photolysis of the phenacyl group is very clean in trivial cases,[2] but hardly seemed worth pursuing for peptides of any complexity. Electrolytic cleavage without the simultaneous competing reduction to the corresponding alcohol which we observed previously [2] can be performed by conducting the electrolysis at 1.00 V instead of 1.30 V,[3] but we have dismissed this as being too finely balanced to be a reliable general method. Furthermore, persistent side reactions, one of which is exemplified by the model reaction shown in Scheme 1, are observed in the presence of reactive acylating agents. Finally, the solubility of the t-butoxycarbonyl derivative (3) in non-polar organic solvents is low and the use of dimethylformamide for coupling

Scheme 1 Conditions: Boc_2O–DMF then MeOH

reactions, which is rather unsatisfactory with Merrifield resins, is obligatory. Some π-4-chloro- and π-4-methoxyphenacyl-histidine derivatives have been prepared,[4] but these do not appear to offer significant advantages over the unsubstituted compounds.

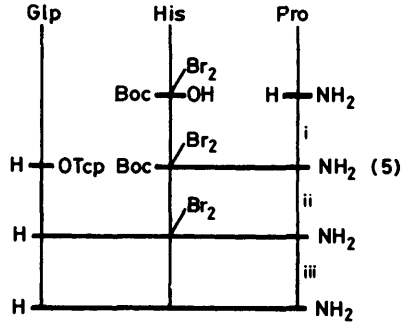
We were prompted to investigate an alternative approach to the prevention of side chain-induced racemisation by the work of Brundish and Wade.[5] These workers had prepared a number of specifically tritiated peptides with high tritium levels by catalytic tritiolysis of halogenated peptides which had been obtained by synthesis with amino-acids halogenated in their aromatic or heteroaromatic side chains. We reasoned that halogenation of the imidazole ring in histidine derivatives should diminish the availability of the lone pair of electrons on the π-nitrogen for participation in side chain-induced racemisation. t-Butoxycarbonyl-L-dibromohistidine (4) was prepared as shown in Scheme 2 and



(4)

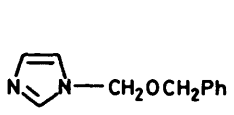
Scheme 2 Conditions: Br_2–Et_3N–$CHCl_3$ then aq. NaOH–MeOH

used in some simple exercises such as the synthesis of thyroliberin outlined in Scheme 3. The dibromo-protection proved very convenient in these examples, but when the dipeptide (5) was prepared under conditions designed to exaggerate the danger of racemisation—

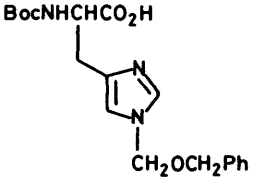


SCHEME 3 Conditions: i, DCCI–1-hydroxybenzotriazole (HOBt); ii, CF_3CO_2H then active ester coupling; iii, H_2–Rh(C)–Pd(C)–IR 45–80% aq. AcOH

activation of (4) with dicyclohexylcarbodi-imide in dimethylformamide at 0 °C for two hours before addition of prolineamide—the histidine in the dipeptide (5) obtained proved to be 15% D. Under the same conditions t-butoxycarbonyl-L-di-iodohistidine led to dipeptide containing 20% D-histidine. Whilst these results are an improvement on $N(\alpha)$-t-butoxycarbonyl-$N(\tau)$-benzyl-L-histidine, which suffers complete racemisation when activated in this way, it is clear that direct blockade of the π-nitrogen is indispensable for the complete eradication of the danger of side chain-induced racemisation. We have therefore renewed our search for substituents which can be located there, but which at the same time have the other *desiderata* for a satisfactory histidine side-chain protecting group: convenience in use, stability during peptide synthesis, and susceptibility to clean removal at the end of the synthesis. The properties of *N*-benzyloxymethylimidazole (6), which are summarised



(6)



(7)

in Table 1, seemed the most promising of a series of *N*-substituted imidazoles which we screened (*cf.* *N*-benzyloxymethyl blockage of pyrroles [6]). $N(\alpha)$-t-Butoxycarbonyl-$N(\pi)$-benzyloxymethyl-L-histidine (8) was prepared as shown in Scheme 4; it can also be prepared *via* $N(\alpha)$-t-butoxycarbonyl-$N(\tau)$-trityl-L-histidine methyl ester, by analogy with our procedure [2] for the preparation of (3).* The τ-isomer (7) was also prepared for comparison; it was found to be optically labile, suffering

* Compound (8), 'Boc-His(πBom)-OH,' is also now commercially available, from Cambridge Research Biochemicals Ltd., Button End Industrial Estate, Harston, Cambridgeshire, CB2 5NX.

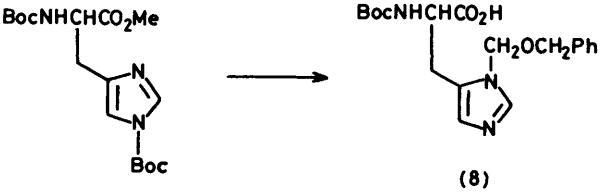
TABLE 1

Experiments on the stability of 1-benzyloxymethylimidazole

Conditions [a]	Result [b]
40% CF_3CO_2H–CH_2Cl_2, 1 week	Very slight reaction
CF_3CO_2H, 1 week	Slight reaction: some imidazole formed
48% HBr–AcOH, overnight	Incomplete cleavage to give imidazole
Sat. HBr–CF_3CO_2H, overnight [c]	Complete cleavage to give imidazole
6M-HCl, 110 °C, 18 h	Complete cleavage to give imidazole
1 equiv. 1M NaOH, MeOH, 1 week	No reaction
1 equiv. $PhCH_2NH_2$, MeOH, 1 week	No reaction
Sat. NH_3–MeOH, 1 week	No reaction
Excess of N_2H_4, MeOH, 24 h	Slight reaction
1 equiv. DCCI,[d] DMF, 1 week	No reaction
H_2–Pd(C), MeOH, overnight [c]	Complete cleavage to give imidazole
H_2–Pd(C), 80% AcOH, overnight [c]	Complete cleavage to give imidazole

[a] Solutions of (6) were approximately 0.2M. [b] As judged by t.l.c. and/or 300 MHz ^1H n.m.r. spectroscopy. [c] Subsequent experience with $N(\pi)$-benzyloxymethylhistidine derivatives has shown that with them shorter periods suffice for complete cleavage of the protecting group. [d] DCCI = dicyclohexylcarbodi-imide; DMF = *NN*-dimethylformamide.

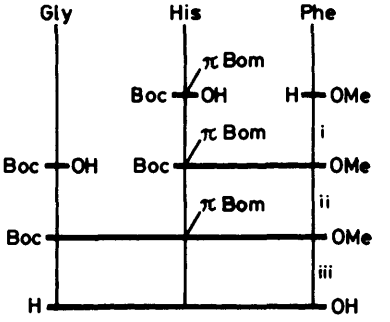
essentially complete racemisation under conditions which had no detectable effect on the π-isomer (8). The application of (8) is illustrated by the syntheses sum-



(8)

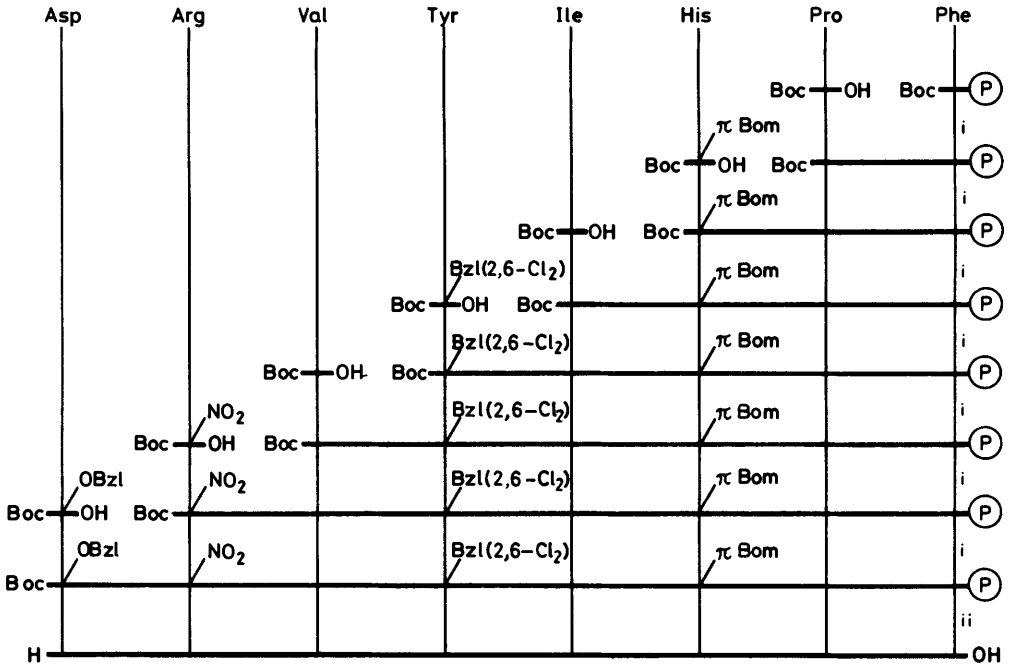
SCHEME 4 Conditions: $PhCH_2OCH_2Cl$–CH_2Cl_2 then aq. NaOH–MeOH on the $N(\alpha)$-t-butoxycarbonyl-$N(\pi)$-benzyloxymethyl-L-histidine methyl ester hydrochloride which is formed

marised in Schemes 5—8, all of which proceeded smoothly without any significant problems.



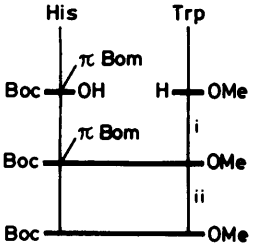
SCHEME 5 Conditions: i, DCCI–HOBt; ii, CF_3CO_2H then DCCI–HOBt coupling; iii, aq. NaOH–MeOH, then H_2–Pd(C)–80% aq. AcOH, then CF_3CO_2H, then IR 45. π-Bom = π-benzyloxymethyl

The tripeptide synthesis shown in Scheme 5 serves to illustrate the ground rules for the use of (8), but otherwise calls for no comment. The angiotensin II synthesis in

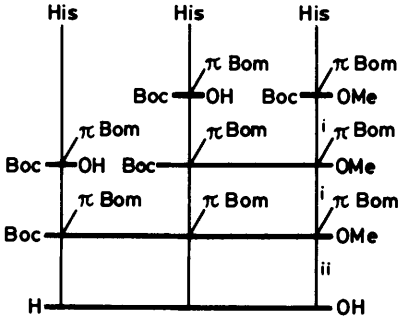


SCHEME 6 Conditions: i, Conventional solid-phase deprotection and coupling; ii, HBr–CF_3CO_2H–anisole, then IR 45 (acetate form), then H_2–Pd(C)–80% aq. AcOH. The encircled P represents the Merrifield resin

Scheme 6 demonstrates its application in solid-phase work; excellent incorporations were obtained at every coupling (Table 2) and the free octapeptide ultimately



SCHEME 7 Conditions: i, DCCI–HOBt; ii, H_2–Pd(C)–80% aq. AcOH



SCHEME 8 Conditions: i, CF_3CO_2H then DCCI–HOBt coupling; ii, aq. NaOH–MeOH, then H_2–Pd(C)–80% aq. AcOH, then CF_3CO_2H, then IR 45 (acetate form) then further hydrogenation

obtained after straightforward purification procedures was indistinguishable from authentic material. Cleavage of the π-benzyloxymethyl group, whether by hydrogenolysis or acidolysis, presumably releases formaldehyde. Some problems might have been anticipated on this score, but in fact have yet to be observed, even with the deprotection of histidine–tryptophan peptides (*e.g.* Scheme 7). Finally, even the trihistidine synthesis outlined in Scheme 8 was free of difficulty; so far as we are aware the synthesis of histidine oligomers such as this has not been attempted previously.

TABLE 2

Amino-acid analyses [a] *en route* to angiotensin II by the solid-phase synthesis in Scheme 6

Cycle no.	Phe	Pro	His	Ile	Tyr	Val	Arg	Asp
2	1.05	1.00	0.94					
3	0.97	1.00	0.99	0.97				
4	1.02	0.98	1.06	0.98	0.95			
5	1.03	1.01	1.02	1.01	0.98	1.04		
6	0.96	0.99	1.11	0.98	1.05	1.09	1.03	
7	0.95	0.91	1.09	0.95	1.03	1.00	0.97	0.98

[a] After hydrolysis of the peptide–resin conjugate with conc. hydrochloric acid–propionic acid (1 : 1) containing 0.1% phenol for 17 h at 110 °C.

The histidine side-chain protecting group in (8) has exactly the same balance of properties as the standard benzyl-based protecting groups which are widely used for the protection of other functional side chains. Furthermore (8) is soluble with ease in solvents such as dichloromethane. It is therefore possible to use it in the well tried strategy of classical and solid-phase peptide synthesis which uses $N(\alpha)$-t-butoxycarbonyl 'temporary' protection for stepwise chain extension from the *C*-terminus in conjunction with benzyl-based side chain protection for side chain functionality. No novel operations or changes in protocol are necessary. Another useful feature of π-benzyloxymethyl protection is that histidine is released quantitatively under the standard

conditions for hydrolysing peptides prior to amino-acid analysis. It appears to us that the protecting group has a practically ideal combination of features. It is chemically simple and thus unlikely to be prone to side-reactions. It is stable to the conditions of the routine operations of both the standard Merrifield solid-phase strategy and one of the most important classical strategies. The key t-butoxycarbonyl derivative (8), which is an easily prepared compound, is freely soluble in a wide range of organic solvents and can be activated and coupled without racemisation or other side reactions. Analytical control of intermediates presents no special problem, and final removal of the protecting group together with other benzyl-based side-chain protection is fast and clean.

The 4-bromo-(benzyloxymethyl) analogue of (8) has also been prepared and its use in some simple exercises has been investigated; the bromine substituent makes no significant difference to the characteristics of the side-chain protecting group and confers no additional advantages.

EXPERIMENTAL

M.p.s were determined with a Kofler hot-stage apparatus. N.m.r. spectra were recorded with a Bruker WH 300 spectrometer operating at 300 MHz. Mass spectra were recorded on a Varian CH7, a V.G. Micromass 16F, or a V.G. Micromass ZAB 1F instrument. Optical rotations were measured on a Perkin-Elmer 241 automatic polarimeter in a 1 dm cell. All the compounds for which elemental analyses and/or mass spectral molecular weights are reported were obtained in a chromatographically homogeneous state and had n.m.r. spectra consistent with their formulation as single isomers of the structures stated. Samples for amino-acid analysis were prepared, except where otherwise stated, by hydrolysis with 6M-hydrochloric acid at 110 °C for 24 h (with 0.1% phenol added when tyrosine residues were present) and analysed on a Jeol JLC 5AH instrument. Thin-layer chromatography was performed on Merck Kieselgel $60F_{254}$ plates using the following systems for elution: A ($CHCl_3$–MeOH, 9 : 1); B ($CHCl_3$–MeOH, 4 : 1); C ($CHCl_3$–MeOH, 19 : 1); D (CH_2Cl_2–MeOH–AcOH–H_2O, 15 : 10 : 2 : 3); E ($CHCl_3$–MeOH–AcOH, 17 : 2 : 1); F (Bu^nOH–AcOH–H_2O, 4 : 1 : 1); G ($CHCl_3$–MeOH–AcOH, 10 : 2 : 1); H (Bu^nOH–AcOH–pyridine–water, 30 : 6 : 24 : 20). Development of chromatograms was by quenching of u.v. fluorescence, chlorine–starch–potassium iodide, Pauly's reagent, or Sakaguchi's reagent as appropriate. All reactions and operations were performed at room temperature unless otherwise stated. All hydrogenations were performed at atmospheric pressure. All the dimethylformamide used was freshly redistilled.

Reaction of 1-*Phenacylimidazole with Di-t-butyl Dicarbonate*.—A solution of 1-phenacylimidazole[7] (1.86 g, 10 mmol) in dimethylformamide (10 ml) was treated with a solution of di-t-butyl dicarbonate (4.36 g, 20 mmol) in dimethylformamide (5 ml). After 2 h the solvent was removed and the residue was dissolved in methanol (20 ml). After 2 h the solvent was removed and the major component of the mixture (t.l.c.-A, R_F 0.4) was isolated by chromatography on a silica gel column with chloroform–methanol (19 : 1) as eluant. Crystallisation from ether gave *t-butyl* (*imidazol*-1-*yl*)*acetate* (0.97 g, 53%), m.p. 116 °C (Found: C, 59.5; H, 7.8; N, 15.4; M^+ 182. $C_9H_{14}N_2O_2$ requires C, 59.3; H, 7.7; N, 15.4%; *M* 182). The compound gave no visible reaction with Pauly's reagent.

1-*Benzyloxymethylimidazole* (6).—A mixture of benzyl chloromethyl ether (1.4 ml, 10 mmol) and imidazole (1.4 g, 20 mmol) in methyl cyanide (40 ml) was heated under reflux for 3 h. Evaporation gave an oily residue which was distributed between dichloromethane (50 ml) and water (25 ml). The organic layer was washed twice with water (25 ml) and dried. Evaporation gave 1-benzyloxymethylimidazole (6) as a chromatographically homogeneous (t.l.c.-A) pale yellow syrup (1.05 g, 87%). The corresponding *picrate* (m.p. 94 °C) was obtained in the usual way (Found: C, 49.2; H, 3.6; N, 16.6. $C_{17}H_{15}N_5O_7 \cdot 0.75H_2O$ requires C, 49.2; H, 3.6; N, 16.9%).

N(α)-*t-Butoxycarbonyl*-2,5-*dibromo*-L-*histidine Methyl Ester*.—Triethylamine (12 ml, 86 mmol) was added to a stirred solution of *N*(α)-t-butoxycarbonyl-L-histidine methyl ester[2] (8.0 g, 28 mmol) in chloroform (100 ml) and the mixture was cooled to 0 °C. Bromine (4.0 ml, 76 mmol), dissolved in chloroform (50 ml), was added during 2 h, and the mixture was allowed to attain room temperature overnight. T.l.c.-B indicated that the starting material had been entirely consumed. The solution was washed with 1M-sodium thiosulphate (3 × 50 ml) and water (50 ml), and dried. Evaporation and flash chromatography[8] (Merck Kieselgel 60, $CHCl_3$–MeOH, 19 : 1) of the residue gave, after reprecipitation from ethyl acetate–ether N(α)-*t-butoxycarbonyl*-2,5-*dibromo*-L-*histidine methyl ester* (3.08 g, 25%) as a pale yellow powder of m.p. 65—69 °C, $[\alpha]_D^{20}$ −6.7° (*c* 1.0, MeOH) (Found: C, 34.6; H, 4.3; N, 9.4; M^+ 425, 427, 429. $C_{12}H_{17}Br_2N_3O_4 \cdot 0.25EtOAc$ requires C, 34.7; H, 4.2; N, 9.35%; *M* 425, 427, 429).

N(α)-*t-Butoxycarbonyl*-2,5-*dibromo*-L-*histidine* (4).—Sodium hydroxide (1M; 15 ml) was added to a solution of *N*(α)-t-butoxycarbonyl-2,5-dibromo-L-histidine methyl ester (3.08 g, 7.2 mmol) in methanol (4 ml) and the mixture was stirred for 1 h. T.l.c.-B indicated complete consumption of starting material. Water (50 ml) was added and the mixture was extracted with chloroform (2 × 20 ml). The pH was adjusted to 5 by addition of 1M-hydrochloric acid and the mixture was extracted with chloroform (5 × 10 ml). The combined extracts were dried and evaporated to give, after repeated trituration with ether and evaporation, N(α)-*t-butoxycarbonyl*-2,5-*dibromo*-L-*histidine* (2.18 g, 73%) as a pale yellow powder of m.p. 92—94 °C, $[\alpha]_D^{20}$ −1.1° (*c* 1.0, MeOH) (Found: C, 33.0; H, 3.9; N, 9.45; M^+ 411, 413, 415. $C_{11}H_{15}Br_2N_3O_4 \cdot 0.25Et_2O$ requires C, 33.4; H, 4.05; N, 9.7%; *M* 411, 413, 415).

N(α)-*t-Butoxycarbonyl*-2,5-*dibromo*-L-*histidyl*-L-*proline-amide* (5).—1-Hydroxybenzotriazole (183 mg, 1.2 mmol) and dicyclohexylcarbodi-imide (247 mg, 1.2 mmol) were added to a solution of *N*(α)-t-butoxycarbonyl-2,5-dibromo-L-histidine (413 mg, 1.0 mmol) and L-prolineamide hydrochloride (151 mg, 1.0 mmol) in dimethylformamide (2 ml) at 0 °C. The ' pH ' was adjusted to 9 by addition of triethylamine (150 μl, 1.1 mmol). After 2 h at 0 °C the mixture was allowed to attain room temperature overnight. The precipitated dicyclohexylurea was removed by filtration and the solvent by evaporation, giving a yellow oil which was dissolved in ethyl acetate (5 ml) and set aside at 0 °C for 1 h. Further dicyclohexylurea was removed and the solution was washed with saturated sodium hydrogen carbonate (2 × 10 ml) and then extracted with 5% citric acid (4 × 5

ml). The combined citric acid extracts were covered with an equal volume of ethyl acetate and the pH was adjusted to 8.5 by addition of sodium hydrogen carbonate. The organic phase was separated and the aqueous phase was extracted with ethyl acetate (3 × 20 ml). The combined organic extracts were dried and evaporated to give, after repeated trituration with ether and evaporation, *N*(α)-t-butoxycarbonyl-2,5-dibromo-L-histidyl-L-prolineamide (258 mg, 70%) as a white hygroscopic powder of m.p. 98—105 °C, $[\alpha]_D^{20}$ +4.9° (*c* 1.0, MeOH) (Found: M^+ 507, 509, 511. Calc. for $C_{16}H_{23}Br_2N_5O_4$: *M* 507, 509, 511). Enzymic assay [9] after hydrogenolysis as in the example below and hydrolysis indicated that less than 4% of the dibromohistidine in the peptide was D.

L-Pyroglutamyl-2,5-dibromo-L-histidyl-L-prolineamide.—The preceding protected dipeptide (96 mg, 0.16 mmol) was dissolved in trifluoroacetic acid (5 ml). After 0.5 h at room temperature the trifluoroacetic acid was evaporated off and the residue was, after trituration with ether, dissolved in dimethylformamide (2 ml) and cooled to 0 °C. L-Pyroglutamic acid 2,4,5-trichlorophenyl ester [10] (52.4 mg, 0.16 mmol) was added and the ' pH ' was adjusted to 8 by addition of triethylamine (90 μl, 0.65 mmol). After 16 h the solvent was evaporated off and the residue was triturated with ether. The required dibromotripeptide was isolated by chromatography on a column (2.5 × 100 cm) of Sephadex G 15 swollen and eluted with 25% aqueous acetic acid and reprecipitated from methanol–ether to give L-pyroglutamyl-2,5-dibromo-L-histidyl-L-prolineamide (161 mg, 79%) as a white hygroscopic powder of m.p. 179—181 °C, $[\alpha]_D^{20}$ −29.4° (*c* 0.5, 1M-AcOH) (Found: M^+ 518, 520, 522. Calc. for $C_{16}H_{20}Br_2N_6O_4$: *M* 518, 520, 522).

Thyroliberin.—5% Rhodium on carbon (20 mg), 5% palladium on carbon (20 mg), and a few beads of Amberlite IR 45 (acetate form) anion exchange resin were added to a solution of the preceding dibromotripeptide (27 mg, 0.05 mmol) in 80% aqueous acetic acid (1 ml). Hydrogen gas was passed through the stirred mixture for 6 h, after which t.l.c.-D indicated complete conversion into thyroliberin. The mixture was filtered and evaporated, and thyroliberin (14 mg, 76%) was isolated as a white powder by chromatography on a column (2.5 × 100 cm) of Sephadex G10 swollen and eluted with 25% aqueous acetic acid: amino-acid analysis Glu 1.04, His 1.00, Pro 1.07 (Found: C, 40.5; H, 6.8; N, 12.3. Calc. for $C_{16}H_{22}N_6O_4 \cdot 4AcOH \cdot 6H_2O$: C, 40.6; H, 7.0; N, 11.8%). Measurement of the specific rotation using concentration values corrected for the presence of water and excess of acetic acid gave a value $[\alpha]_D^{20}$ −69° (*c* 0.11, 1M-AcOH) for thyroliberin sesquiacetate, *cf.* lit.[11] $[\alpha]_D^{20}$ −68° (*c ca.* 1, 1M-AcOH) for thyroliberin sesquiacetate of proven chemical and optical purity. The product was indistinguishable from authentic material by t.l.c. in several systems and by n.m.r. spectroscopy at 300 MHz.

Coupling of N(α)-*t-Butoxycarbonyl*-N(τ)-*benzyl-L-histidine with L-Prolineamide under Conditions designed to exaggerate Racemisation.*—*N*(α)-t-Butoxycarbonyl-*N*(τ)-benzyl-L-histidine [12] (172 mg, 0.50 mmol) was added to a solution of dicyclohexylcarbodi-imide (113 mg, 0.55 mmol) in dimethylformamide (4 ml) which was stirred at 0 °C. After 120 min a precooled solution of L-prolineamide hydrochloride (75 mg, 0.50 mmol) and triethylamine (70 μl, 0.50 mmol) in dimethylformamide (2 ml) was added. After a further 2 h at 0 °C the mixture was allowed to attain room temperature overnight. The dicyclohexylurea was removed by filtration and the solvent was evaporated off. A solution of the residue in chloroform (25 ml) was washed with saturated sodium hydrogen carbonate (2 × 30 ml) and brine (20 ml), dried, and evaporated to give the crude protected dipeptide as a white powder (154 mg, 86%) which was homogeneous by t.l.c.-E. This crude protected dipeptide was dissolved in 80% aqueous acetic acid (5 ml); 5% palladium on carbon (150 mg) was added and the mixture was hydrogenated for 72 h which was sufficient for complete consumption, as judged by t.l.c.-E, of the fully protected dipeptide. Filtration and evaporation gave crude *N*(α)-t-butoxycarbonyl-histidyl-L-prolineamide as a white solid (132 mg) with a D-histidine content, determined by enzymic assay [9] after hydrolysis, of 45%.

Coupling of N(α)-*t-Butoxycarbonyl-2,5-di-iodo-L-histidine with L-Prolineamide under Conditions designed to exaggerate Racemisation.*—*N*(α)-t-Butoxycarbonyl-2,5-di-iodo-L-histidine [13] (50 mg, 0.10 mmol) was coupled with L-prolineamide under the conditions of the preceding experiment, and the resulting mixture was worked up similarly except that the chloroform solution was in this case also washed with 10% citric acid (2 × 5 ml) before drying and evaporation. This gave the di-iododipeptide (35 mg, 58%) as a white powder which was homogeneous by t.l.c.-E. A portion of this material (3 mg) was added to a suspension of 5% rhodium on calcium carbonate (3 mg) and 5% palladium on carbon (4 mg) in dimethylformamide (1 ml) through which hydrogen was passed for 2 h. Filtration and evaporation gave crude *N*(α)-t-butoxycarbonylhistidyl-L-prolineamide as a pale yellow powder with a D-histidine content, determined by enzymic assay [9] after hydrolysis, of 20%.

Coupling of N(α)-*t-Butoxycarbonyl-2,5-dibromo-L-histidine with L-Prolineamide under Conditions designed to exaggerate Racemisation.*—*N*(α)-t-Butoxycarbonyl-2,5-dibromo-L-histidine (41.3 mg, 0.10 mmol) was coupled with L-prolineamide and the reaction mixture was processed as in the preceding experiment giving chromatographically homogeneous (t.l.c.-E) dibromodipeptide (29 mg, 57%). A portion (3 mg) was dissolved in 80% aqueous acetic acid (3 ml) in which 5% rhodium on carbon (5 mg), 5% palladium on carbon (5 mg), and a few beads of Amberlite IR 45 (acetate form) anion exchange resin were suspended. Hydrogen was passed for 2 h. Filtration and evaporation gave crude *N*(α)-t-butoxycarbonylhistidyl-L-prolineamide with a D-histidine content, determined by enzymic assay [9] after hydrolysis, of 15%.

N(α)-*t-Butoxycarbonyl*-N(π)-*benzyloxymethyl-L-histidine* (8).—*Route* 1; N(α),*N*(τ)-*Bis-t-butoxycarbonyl-L-histidine methyl ester.* Triethylamine (70 ml, 0.5 mol), followed by di-t-butyl dicarbonate (120 g, 0.55 mol), was added to a suspension of L-histidine methyl ester dihydrochloride (60.5 g, 0.25 mol) in methanol (200 ml) and the mixture was set aside overnight. The solvent was removed, chloroform (250 ml) was added, and the mixture was extracted with 10% citric acid (2 × 50 ml). Drying and evaporation gave an oil which solidified when triturated with light petroleum (b.p. 40—60 °C) to give *N*(α),*N*(τ)-bis-t-butoxycarbonyl-L-histidine methyl ester (83 g, 90%) of m.p. 96 °C, $[\alpha]_D^{20}$ +25.6° (*c* 1.0, CCl_4) (Found: C, 55.1; H, 7.2; N, 11.3. Calc. for $C_{17}H_{27}N_3O_6$: C, 55.3; H, 7.3; N, 11.4%), lit.,[12] m.p. *ca.* 90 °C. This intermediate may be stored for short periods at 0 °C but for best results with subsequent reactions it should be used as soon as possible.

N(α)-*t-Butoxycarbonyl*-N(π)-*benzyloxymethyl-L-histidine methyl ester hydrochloride.* A solution of the preceding ester (32 g, 0.082 mol) and freshly redistilled benzyl chloromethyl ether (18 ml, 0.13 mol) in dichloromethane (200 ml) was set

aside overnight. Evaporation, dissolution in methanol (30 ml), and addition of ether (400 ml) gave a slightly turbid solution from which the *protected ester hydrochloride* (24 g, 69%) crystallised when set aside overnight: m.p. 152 °C, $[\alpha]_D^{20}$ −19.1° (*c* 1.0, MeOH) (Found: C, 56.5; H, 6.7; N, 9.6. $C_{19}H_{25}N_3O_5$·HCl requires C, 56.4; H, 6.6; N, 9.9%).

N(α)-*t-Butoxycarbonyl*-N(π)-*benzyloxymethyl*-L-*histidine* (8). *Method* 1. Sodium hydroxide (1M; 120 ml) was added to a solution of the preceding ester hydrochloride (22 g, 0.052 mol) in methanol (50 ml). After 15 min water (1 l) was added and the pH was adjusted to 4.5 by the dropwise addition of 1M-hydrochloric acid. The solution was extracted with chloroform (3 × 100 ml) and the combined organic extracts were dried. Removal of the solvent gave an oil which was dissolved in ethyl acetate (50 ml). Evaporation gave the *acid* (8) (17 g, 87%) of m.p. 155 °C, $[\alpha]_D^{20}$ +6.9° (*c* 0.5 MeOH) (Found: C, 60.3; H, 6.6; N, 11.1; *M* + H$^+$ 376. $C_{19}H_{25}N_3O_5$ requires C, 60.8; H, 6.7; N, 11.2%; *M* 375).

N(α)-*t-Butoxycarbonyl*-N(π)-*benzyloxymethyl*-L-*histidine* (8).—*Route* 2; N(α)-*t-Butoxycarbonyl*-N(π)-*benzyloxymethyl*-N(τ)-*triphenylmethyl*-L-*histidine methyl ester imidazolium chloride*. Benzyl chloromethyl ether (1.21 g, 7.7 mmol) was added to a solution of *N*(α)-t-butoxycarbonyl-*N*(τ)-triphenylmethyl-L-histidine methyl ester [14] (3.58 g, 7 mmol) in ether (20 ml). The *imidazolium salt* (1.9 g, 41%) separated overnight as a white crystalline material which was collected and washed well with ether, m.p. 138—141 °C, $[\alpha]_D^{20}$ −9.2° (*c* 0.6, MeOH) (Found: C, 70.0; H, 6.3; N, 6.3; *m/e* 632. $C_{39}H_{42}ClN_3O_5$ requires C, 70.1; H, 6.3; N, 6.3%; $C_{39}H_{42}N_3O_5$ requires *m/e* 632).

N(α)-*t-Butoxycarbonyl*-N(π)-*benzyloxymethyl*-L-*histidine methyl ester*. Silver acetate (0.38 g, 2.28 mmol) was added to a solution of the preceding imidazolium salt (1.5 g, 2.25 mmol) in 80% aqueous acetic acid (5 ml) and the mixture was stirred overnight. Filtration and evaporation gave a syrup to which saturated sodium hydrogen carbonate (20 ml) was added. Extraction with ether (5 × 20 ml), drying, and evaporation gave crude product which was purified by flash chromatography (Merck Kieselgel 60, $CHCl_3$–MeOH, 19 : 1). Removal of solvents and trituration with light petroleum (b.p. 40—60 °C) gave the *protected ester* (0.49 g, 56%) of m.p. 103 °C, $[\alpha]_D^{20}$ −10.8° (*c* 0.55, MeOH) (Found: C, 61.7; H, 6.9; N, 10.8; *M*$^+$, *M* + H$^+$ 389, 390. $C_{20}H_{27}N_3O_5$ requires C, 61.5; H, 6.9; N, 10.65%; *M* 389).

N(α)-*t-Butoxycarbonyl*-N(π)-*benzyloxymethyl*-L-*histidine* (8). *Method* 2. Sodium hydroxide (1M; 3.7 ml) was added to a solution of the preceding methyl ester (1.25 g, 3.2 mmol) in methanol (10 ml). After 15 min the mixture was processed as in Method 1 to give analytically pure acid (8) (0.95 g, 79%) which was identical in every respect with that obtained by that Method.

N(α)-*t-Butoxycarbonyl*-N(τ)-*benzyloxymethyl*-L-*histidine Methyl Ester*.—A solution of *N*(α)-t-butoxycarbonyl-L-histidine methyl ester (1.35 g, 5 mmol), benzyl chloromethyl ether (0.7 ml, 6 mmol), and triethylamine (0.7 ml, 5 mmol) in dichloromethane (10 ml) was set aside overnight. Evaporation afforded an oil which was dissolved in dichloromethane (30 ml), to give a solution which was extracted with 10% citric acid (2 × 20 ml) dried, and concentrated to an oil. Chromatography on a silica gel column with chloroform–methanol (19 : 1) as eluant under slight pressure gave the ester (0.36 g, 19%) as an oil (Found: *M*$^+$, *M* + H$^+$ 389, 390. Calc. for $C_{20}H_{27}N_3O_5$; *M* 389).

N(α)-*t-Butoxycarbonyl*-N(τ)-*benzyloxymethyl*-L-*histidine* (7).—Sodium hydroxide (1M; 1.0 ml) was added to a solution of the preceding methyl ester (0.325 g, 0.84 mmol) in methanol (2 ml). After 0.5 h water (140 ml) was added and the pH was adjusted to 4.5 by the dropwise addition of 1M-hydrochloric acid. The solution was extracted with dichloromethane (5 × 15 ml) and the combined organic extracts were dried and evaporated to give an oil which was redissolved in ethyl acetate (10 ml). Evaporation afforded the *acid* (7) as a crisp meringue (0.23 g, 73%): m.p. *ca.* 50 °C, $[\alpha]_D^{20}$ +93.9° (*c* 1.0, $CHCl_3$) (Found: C, 60.6; H, 6.8; N, 10.5; *M*$^+$, *M* + H$^+$ 375, 376. $C_{19}H_{25}N_3O_5$·0.3EtOAc requires C, 60.4; H, 6.8; N, 10.5%; *M* 375).

Polarimetric Comparison of the Optical Lability of (7) *and* (8).—The acid (7) or (8) (37.5 mg) was dissolved in dimethylformamide (2 ml), di-isopropylcarbodi-imide (18.6 μl, 1 equiv.) was added, the solution was cooled to 4 °C, and the optical rotation of the mixture was observed using a cell maintained at 4 °C. The rotation of the mixture made up with (7) decreased by *ca.* 50% in 30 min but the rotation of the mixture made up with (8) remained practically unchanged after 2 h. No net chemical reaction could be detected in either case (*cf.* ref. 15). The same results were obtained with the 4-bromo-analogues of (7) and (8).

N(α)-*t-Butoxycarbonyl*-N(π)-*benzyloxymethyl*-L-*histidyl*-L-*phenylalanine Methyl Ester*.—A mixture of *N*(α)-t-butoxycarbonyl-*N*(π)-benzyloxymethyl-L-histidine (0.375 g, 1.0 mmol), 1-hydroxybenzotriazole (0.168 g, 1.1 mmol), and dicyclohexylcarbodi-imide (0.248 g, 1.2 mmol) in dimethylformamide (5 ml) was set aside at 0 °C for 1 h and then at room temperature for 1 h. To this was added a solution of L-phenylalanine methyl ester hydrochloride (0.237 g, 1.1 mmol) and triethylamine (0.167 ml, 1.2 mmol) in dimethylformamide (3 ml). The mixture was set aside overnight, filtered, and evaporated to give a gum which was dissolved in ethyl acetate (20 ml). The ethyl acetate solution was extracted with 1M-sodium hydrogen carbonate (2 × 15 ml), water (15 ml), and 10% citric acid (5 × 15 ml). The combined citric acid extracts were neutralised (solid sodium hydrogen carbonate) and extracted with ethyl acetate (5 × 15 ml). The ethyl acetate extracts were combined, dried, and evaporated to give an oil which was dissolved in ether and evaporated to give the *protected dipeptide* as a crisp meringue (0.41 g, 81%), m.p. 45—47 °C, $[\alpha]_D^{20}$ −10.4° (*c* 0.5, MeOH) (Found: C, 64.6; H, 6.9; N, 10.4; *M*$^+$, *M* + H$^+$ 536, 537. $C_{29}H_{36}N_4O_6$ requires C, 64.9; H, 6.7; N, 10.45%; *M* 536).

t-Butoxycarbonylglycyl-N(π)-*benzyloxymethyl*-L-*histidyl*-L-*phenylalanine Methyl Ester*.—Solution A: dicyclohexylcarbodi-imide (0.144 g, 0.7 mmol) was added to a solution of t-butoxycarbonylglycine (0.123 g, 0.7 mmol) and 1-hydroxybenzotriazole (0.107 g, 0.7 mmol) in dimethylformamide (5 ml) and the mixture was set aside at 0 °C for 1 h and then at room temperature for 1 h. Solution B: a solution of the preceding protected dipeptide (0.375 g, 0.7 mmol) in trifluoroacetic acid (5 ml) was set aside at room temperature for 0.5 h, the trifluoroacetic acid was evaporated off, the residue after ether trituration was dissolved in dimethylformamide (3 ml), and the ' pH ' was adjusted to 9 by addition of triethylamine.

Solution B was added to solution A and the mixture was set aside overnight. Filtration and evaporation gave an oil which was dissolved in ethyl acetate (25 ml). The ethyl acetate solution was extracted with 1M-sodium hydrogen carbonate (2 × 15 ml), water (15 ml), and 10% citric acid

(5 × 15 ml). The combined citric acid extracts were neutralised with solid sodium hydrogen carbonate and extracted with ethyl acetate (5 × 15 ml). The ethyl acetate extracts were combined, dried ($MgSO_4$), and evaporated to give an oil which was dissolved in ether and evaporated giving the *protected tripeptide* as a crisp meringue (0.30 g, 85%) of indefinite m.p., $[\alpha]_D^{20}$ −13.95° (*c* 0.5, MeOH). Amino-acid analysis: Gly 0.98; His 1.00; Phe 0.96 (Found: C, 62.5; H, 6.7; N, 11.6. $M + H^+$ 594. $C_{31}H_{39}N_5O_7$ requires C, 62.7; H, 6.6; N, 11.8%; *M* 593).

Glycyl-L-histidyl-L-phenylalanine.—Sodium hydroxide (1M; 0.186 ml) was added to a solution of the protected tripeptide (0.10 g, 0.17 mmol) in methanol (1 ml). After 1 h water (25 ml) was added, the pH was adjusted to 4 with 1M-hydrochloric acid, and the solution was extracted with dichloromethane (5 × 15 ml). The combined dichloromethane extracts were dried and evaporated to give a solid residue which was chromatographically homogeneous by t.l.c.-G. A solution of the residue in 80% acetic acid (10 ml) was hydrogenated in the presence of 10% palladium on carbon (20 mg). Filtration and evaporation after 4 h gave a solid which was dissolved in trifluoroacetic acid (5 ml). The solution was set aside for 0.5 h at room temperature and then evaporated. A solution of the residue in 25% aqueous acetic acid (3 ml) was passed through a column of Amberlite IR 45 ion-exchange resin (acetate form) and then applied to a column of Sephadex G10, which was swollen and eluted with 25% aqueous acetic acid. Fractions giving a positive Pauly reaction were pooled and evaporated; the residue was repeatedly dissolved in water and evaporated giving the *tripeptide* (45 mg, 68%) of indefinite m.p., $[\alpha]_D^{20}$ −2.1° (*c* 0.4, 1M-AcOH). Amino-acid analysis: Gly 0.96; His 1.00; Phe 0.96 (Found: C, 52.0; H, 6.0; N, 16.8; $M + H^+$ 360. $C_{17}H_{21}N_5O_4 \cdot 0.5AcOH \cdot 1.5H_2O$ requires C, 51.9; H, 6.25; N, 16.8%; *M* 359).

Solid-phase Synthesis of 5-Isoleucine-angiotensin II.—*N*(α)-t-Butoxycarbonyl-L-phenylalanine was coupled to a Merrifield 1% crosslinked polystyrene resin in dimethylformamide at 60 °C [16] to give a resin which, after washing with dimethylformamide, was shown by nitrogen analysis to contain 0.8 mmol/g of protected amino-acid. The resin (0.42 g) was placed in a glass vessel and agitated by passing nitrogen gas upwards through a glass sinter. Peptide synthesis was carried out using a manual procedure, each cycle of the synthesis comprising treatment of the resin with (all solvent volumes 5 ml): (a) dichloromethane 3 × 2 min; (b) propan-2-ol 3 × 2 min; (c) dichloromethane (10% v/v) 3 × 2 min; (d) trifluoroacetic acid in dichloromethane (40% v/v) 1 × 1 min; (e) trifluoroacetic acid in dichloromethane (40% v/v) 1 × 30 min; (f) dichloromethane 3 × 2 min; (g) propan-2-ol 3 × 2 min; (h) dichloromethane 3 × 2 min; repeat (d)—(h); (i) triethylamine in dichloromethane 3 × 2 min; (j) dichloromethane 5 × 2 min; (k) coupling with 4 equiv. of t-butoxycarbonylamino-acid and 4.4 equiv. of dicyclohexylcarbodi-imide in dichloromethane (5 ml), except in the case of *N*(α)-t-butoxycarbonyl,*N*(ω)-nitroarginine when dimethylformamide (5 ml) was used. Coupling was performed once for 4 h, then once for 16 h [except in the case of *N*(α)-t-butoxycarbonyl-*N*(π)-benzyloxymethyl-L-histidine which was coupled once only for 4 h]. Amino-acid analyses were performed on hydrolysates after each cycle except the first with the results shown in Table 2. Half of the final protected peptide-resin conjugate was washed with trifluoroacetic acid (10 ml) and then suspended in trifluoroacetic acid (5 ml) containing methoxybenzene (1 ml). A stream of hydrogen bromide gas was passed for 1 h. Filtration and evaporation gave crude partially protected peptide hydrobromide which was triturated with ether, dissolved in 25% aqueous acetic acid, and passed several times through a column of Amberlite IR 45 (acetate form) ion exchange resin, with 25% aqueous acetic acid as eluant. Evaporation gave a hygroscopic solid which was dissolved in 80% aqueous acetic acid (10 ml). The solution was hydrogenated for 24 h in the presence of 10% palladium on carbon (50 mg) after which t.l.c.-F revealed only one major component. The solvent was removed and the oily residue (180 mg) was dissolved in 25% aqueous acetic acid and fractionated on a Sephadex G25 gel column swollen and eluted with 25% aqueous acetic acid. The Pauly-active fractions were combined and the gel filtration was repeated twice. T.l.c.-F still showed trace impurities so the peptide was dissolved in 0.0185M-trimethylammonium acetate buffered to ' pH ' 4.2 and applied to a Whatman CM52 carboxymethyl cellulose cation exchange column (0.9 × 30 cm) and eluted with a linear ' pH ' and concentration gradient of 0.0185M ' pH ' 4.2 to 0.185M ' pH ' 5.2 trimethylammonium acetate. The major Pauly active component was collected and the aqueous buffer was evaporated. The residue was repeatedly evaporated from water and finally dried *in vacuo* to give 5-isoleucine-angiotensin II as a hygroscopic white solid (80 mg, 34%) which was indistinguishable from an authentic sample by t.l.c. in several systems or by 300 MHz ^1H n.m.r. spectroscopy. The specific rotation was $[\alpha]_D^{20}$ −65.5° (*c* 0.5, 1M HCl), calculated using concentration values for the monoacetate determined by amino-acid analysis after hydrolysis of the solution in the presence of an internal standard: lit.,[17] $[\alpha]_D^{20}$ −67° (*c* 0.4, 1M HCl) for the monoacetate. Amino-acid analysis: Asp, 1.06; Arg, 1.01; Val, 1.01; Tyr, 1.00; Ile, 0.99; His, 1.00; Pro, 0.99; Phe, 1.06 (Found: C, 51.3; H, 6.5; N, 14.1. Calc. for $C_{50}H_{71}N_{13}O_{12} \cdot 3.5AcOH \cdot 3.75H_2O$: C, 51.7; H, 7.0; N, 13.75%).

L-Tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine.—The preceding synthesis was carried out on the same scale as far as the incorporation of the fifth residue and the crude pentapeptide hydrobromide was obtained by treatment with hydrogen bromide in trifluoroacetic acid in the manner described above. A white solid which was essentially homogeneous by t.l.c.-D was obtained after passage of a solution in 25% aqueous acetic acid through a column of Amberlite IR 45 (acetate form) ion exchange resin, evaporation, and trituration with ether. Chromatography on a column of Sephadex G 25 swollen and eluted with 25% aqueous acetic acid, combination of the Pauly-active fractions, evaporation, reprecipitation from methanol–ether, and drying at 0.1 mmHg overnight gave the *pentapeptide* as a white solid (88 mg, 30%) of indefinite m.p., $[\alpha]_D^{20}$ −2.8° (*c* 0.25, 1M-AcOH). Amino-acid analysis: Tyr, 0.95; Ile, 0.98; His, 1.06; Pro, 0.98; Phe, 1.02 (Found: C, 49.8; H, 6.2; N, 10.3; $M + H^+$ 676. $C_{35}H_{45}N_7O_7 \cdot 2AcOH \cdot 8H_2O$ requires C, 49.8; H, 6.5; N, 10.4%; *M* 675).

N(α)-t-*Butoxycarbonyl*-N(π)-*benzyloxymethyl-L-histidyl-L-tryptophan Methyl Ester*.—1-Hydroxybenzotriazole (243 mg, 1.60 mmol) and dicyclohexylcarbodi-imide (328 mg, 1.60 mmol) were added to a solution of *N*(α)-t-butoxycarbonyl-*N*(π)-benzyloxymethyl-L-histidine (500 mg, 1.33 mmol) and L-tryptophan methyl ester (339 mg, 1.40 mmol) in dimethylformamide (2 ml)–chloroform (8 ml) stirred at 0 °C which had just previously been adjusted to ' pH ' 9 by the addition of triethylamine. After 1 h at 0 °C the mixture was allowed

to attain room temperature overnight. Filtration and evaporation gave a yellow oil which was dissolved in chloroform (10 ml) and cooled to 0 °C for 1 h after which a second crop of dicyclohexylurea was filtered off. The filtrate was diluted with chloroform (10 ml) and washed with saturated sodium hydrogen carbonate (2 × 10 ml) before extraction with 10% citric acid (4 × 20 ml). The citric acid extracts were combined and adjusted to pH 8.5 by the addition of solid sodium hydrogen carbonate before extraction with chloroform (4 × 50 ml). The combined organic extracts were dried and evaporated to give, after trituration with ether, a white solid. Crystallisation from methanol–ether gave N(α)-*t-butoxycarbonyl*-N(π)-*benzyloxymethyl*-L-*histidyl*-L-*tryptophan methyl ester* (476 mg, 61%) of m.p. 90—92 °C, $[\alpha]_D^{20}$ +3.1° (*c* 1.0, MeOH) (Found: C, 64.0; H, 6.3; N, 12.0; M^+ 575. $C_{31}H_{37}O_6N_5 \cdot 0.5H_2O$ requires C, 63.7; H, 6.5; N, 12.0%; *M* 575).

N(α)-*t-Butoxycarbonyl*-L-*histidyl*-L-*tryptophan Methyl Ester*.—The preceding protected dipeptide (100 mg, 0.17 mmol) was dissolved in 80% aqueous acetic acid (5 ml). Palladium on carbon (5%, 30 mg) was added and the suspension was hydrogenated for 2 h after which t.l.c.-E indicated that all the starting material had been consumed. Filtration and evaporation gave, after trituration with ether, N(α)-*t-butoxycarbonyl*-L-*histidyl*-L-*tryptophan methyl ester* as a white solid (58 mg, 73%) of m.p. 164—165 °C, $[\alpha]_D^{20}$ −3.7° (*c* 1.0 in MeOH) (Found: C, 56.1; H, 6.95; N, 11.9; M^+, $M + H^+$ 455, 456. $C_{23}H_{29}O_5N_5$.2AcOH requires C, 56.3; H, 6.6; N, 12.2%; *M* 455).

N(α)-*t-Butoxycarbonyl*-N(π)-*benzyloxymethyl*-L-*histidyl*-N(π)-*benzyloxymethyl*-L-*histidine Methyl Ester*.—A solution of *N*(α)-t-butoxycarbonyl-*N*(π)-benzyloxymethyl-L-histidine methyl ester hydrochloride (1.0 g, 2.35 mmol) in trifluoroacetic acid (10 ml) was set aside for 0.5 h. The solvent was then evaporated off and the residue was treated with ether (50 ml). After 1 h at 4 °C the ether was decanted and fresh ether (50 ml) was added. The mixture was set aside at 4 °C overnight, after which the ether was removed by decantation. The oily residue was dissolved in dimethylformamide (3 ml) and the ' pH ' was adjusted to 9 by the addition of triethylamine. This solution was immediately added to an ice-cooled solution of dicyclohexylcarbodi-imide (0.59 g, 2.85 mmol), 1-hydroxybenzotriazole (0.4 g, 2.6 mmol), and *N*(α)-t-butoxycarbonyl-*N*(π)-benzyloxymethyl-L-histidine (0.98 g, 2.6 mmol) in dimethylformamide (5 ml). The solution was set aside overnight, filtered, and evaporated to give a solid which was suspended in dichloromethane (30 ml), extracted with 10% sodium carbonate (15 ml), dried, and evaporated to give an oil which was dissolved in ethyl acetate (5 ml) and set aside at 4 °C overnight. Further dicyclohexylurea was then filtered off and the solution was evaporated to give an oil. Flash chromatography on a column of silica gel with chloroform–methanol (9 : 1) as eluant gave the *fully protected dihistidine* (1.05 g, 67%) as a powder of m.p. 54—59 °C, $[\alpha]_D^{20}$ −12.1° (*c* 1.2, MeOH) (Found: C, 61.3; H, 6.6; N, 12.6; M^+, $M + H^+$ 646, 647. $C_{34}H_{42}N_6O_7 \cdot H_2O$ requires C, 61.4; H, 6.6; N, 12.65%; *M* 646).

L-*Histidyl*-L-*histidine*.—1M-Sodium hydroxide (0.56 ml, 0.56 mmol) was added to a solution of the preceding protected dipeptide (0.3 g, 0.45 mmol) in methanol (1 ml). Water (50 ml) was added after 15 min and the pH was adjusted to 4.5 by the dropwise addition of 1M-hydrochloric acid. The solution was extracted with ethyl acetate (6 × 15 ml) and the combined ethyl acetate extracts were dried and evaporated. A solution of the residue in 80% aqueous acetic acid (20 ml) was hydrogenated overnight in the presence of 10% palladium on carbon (50 mg). Filtration and evaporation gave an oily residue which was dissolved in trifluoroacetic acid (5 ml) and set aside for 0.5 h. The solvent was evaporated off and the residue was treated with ether (3 × 50 ml) which was removed by decantation. The residue was dissolved in 25% aqueous acetic acid (2 ml) and passed through a column of Amberlite IR 45 anion exchange resin (acetate form). Chromatography on a column of Sephadex G 25 swollen and eluted with 25% aqueous acetic acid and collection of the Pauly-active fractions gave, after drying at 0.1 mmHg overnight, *dihistidine* (66 mg, 38%) as a hygroscopic powder of indefinite m.p., $[\alpha]_D^{20}$ −8.7° (*c* 0.5, 1M-AcOH) (Found: C, 42.5; H, 6.2; N, 22.0; $M + H^+$ 293. $C_{12}H_{16}N_6O_2 \cdot 3H_2O \cdot 0.75$AcOH requires C, 42.1; H, 6.5; N, 21.8%; *M* 292).

N(α)-*t-Butoxycarbonyl*-N(π)-*benzyloxymethyl*-L-*histidyl*-N(π)-*benzyloxymethyl*-L-*histidyl*-N(π)-*benzyloxymethyl*-L-*histidine Methyl Ester*.—Solution A: a solution of the preceding protected dipeptide (0.774 g, 1.2 mmol) in trifluoroacetic acid (10 ml) was set aside for 0.5 h; the solvent was evaporated off and the residue was treated with ether (50 ml); after 1 h at 4 °C the ether was decanted and replaced with fresh ether (50 ml); the mixture was set aside at 4 °C overnight and the ether was removed by decantation; the residue was dissolved in dimethylformamide (5 ml).

Solution B: a solution of *N*(α)-t-butoxycarbonyl-*N*(π)-benzyloxymethyl-L-histidine (0.45 g, 1.2 mmol), dicyclohexylcarbodi-imide (0.272 g, 1.32 mmol), and 1-hydroxybenzotriazole (0.184 g, 1.2 mmol) in dimethylformamide (5 ml) was stirred at 0 °C for 0.5 h and then at room temperature for 0.5 h.

The ' pH ' of solution A was adjusted to 9 by the addition of triethylamine and it was immediately added to solution B. The resultant solution was set aside overnight. Filtration and evaporation gave an oil which was dissolved in ethyl acetate (10 ml) and set aside at 4 °C overnight. Further dicyclohexylurea was filtered off and the solution was evaporated. A solution of the residue in dichloromethane (40 ml) was washed with 10% sodium carbonate, dried, and evaporated giving the *fully protected trihistidine* (0.70 g, 63%) as a crisp meringue: m.p. 58—64 °C, $[\alpha]_D^{20}$ −27.6° (*c* 0.5, MeOH) (Found: C, 62.75; H, 6.4; N, 13.65; M^+ 903. $C_{48}H_{57}N_9O_9 \cdot H_2O$ requires C, 62.5; H, 6.4; N, 13.7%; *M* 903).

L-*Histidyl*-L-*histidyl*-L-*histidine*.—Sodium hydroxide (1M; 0.6 ml) was added to a solution of the preceding fully protected tripeptide (0.5 g, 0.54 mmol) in methanol (2 ml). The solution was stirred for 15 min and then water (50 ml) was added and the ' pH ' was adjusted to 4.5 by the dropwise addition of 1M-hydrochloric acid. The solution was extracted with ethyl acetate (6 × 15 ml) and the combined ethyl acetate extracts were dried and evaporated. A solution of the residue in 80% aqueous acetic acid (20 ml) was hydrogenated overnight in the presence of 10% palladium on carbon (50 mg). The residue, after filtration and evaporation, was dissolved in trifluoroacetic acid (5 ml). After 0.5 h the trifluoroacetic acid was evaporated off and the resulting oil was treated with ether (3 × 50 ml) which was removed by decantation. The residue was dissolved in 25% acetic acid (2 ml) and passed through a column of Amberlite IR 45 anion exchange resin (acetate form). T.l.c.-H at this stage showed the presence of 2 major components (R_F 0.05 and 0.1) in approximately equal amounts.

The hydrogenation procedure was repeated for 2 h which sufficed to consume the remainder of the component of R_F 0.1. Fractionation on a column of Sephadex G 25 swollen and eluted with 25% aqueous acetic acid and combination of the principal Pauly-active fractions, evaporation and overnight drying at 0.1 mmHg gave *trihistidine* (146 mg, 42%) as a powder of m.p. 107—115 °C, $[\alpha]_D^{20}$ +11.9° (*c* 1.0, 1M-AcOH) (Found: C, 46.6; H, 5.5; N, 19.4; M^+ 429. $C_{18}H_{23}N_9O_4 \cdot 3.5AcOH$ requires C, 46.95; H, 5.8; N, 19.7%; *M* 429).

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