Synthesis of [4,5-DehydroLeu²]- and [3,4-DehydroPro⁶]-Locust Adipokinetic Hormone

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The syntheses of [4,5-dehydroLeu²]- and [3,4-dehydroPro⁶]-locust adipokinetic hormone (LAKH) by the coupling of *N*-terminal hexapeptides prepared by the solid-phase method with a common C-terminal tetrapeptide synthesised in solution is described. At a dose level of 20 pmol, the former is at least as biologically active as LAKH, but the latter has only *ca.* 20% of the activity. Difficulties encountered in the incorporation of a 4,5-dehydroleucine residue into peptides are discussed.

The blocked decapeptide adipokinetic hormone (LAKH; 1) † isolated from the corpora cardiaca of the locust species *Locusta migratoria* and *Schistocerca gregaria*,[1] is concerned with the regulation of lipid utilisation during flight. Synthetic studies [2,3] have confirmed its structure, and the biological activity of a number of analogues and truncated sequences have been examined as part of an exploration of structure–activity relationships.[2] As the first insect neurohormone to be characterised, the mode of action of LAKH is of particular interest to insect physiologists. We now report the synthesis of two further analogues, [4,5-dehydroLeu²]- and [3,4-dehydroPro⁶]-LAKH, which were prepared for a dual purpose. As well as being interesting analogues in their own right, these decapeptides were also designed as precursors for the preparation of tritium-labelled LAKH.

The effect of replacing proline residues by 3,4-dehydroproline has been earlier explored in the peptide hormones oxytocin,[4] arginine vasopressin,[5] thyroliberin,[6] and also in bradykinin [7] and bradykinin potentiating peptide.[8] The biological activities observed for the analogues range from the 40- to 100-fold increase seen with bradykinin potentiating peptide to the 4-fold decrease found with [3,4-dehydroPro⁷]-bradykinin. In the main, however, activities were enhanced or unchanged. It is uncertain whether enhancement of agonist activity in these circumstances is due to conformational changes, increased binding affinities, or resistance to enzymic degradation. In view of the possible involvement of the proline residue of LAKH in a β-bend,[3] the preparation of [3,4-dehydroPro⁶]-LAKH was felt to be of interest in this context.

The high activities of bradykinin potentiating peptide analogues containing dehydroproline has tentatively been ascribed to preferential π–π interaction of the deformed electron cloud of the double bond with binding sites on the enzyme.[8] If this is the case, the question arises as to the effect of alkene linkages in other amino-acid side chains. As a first step in examining this question, the synthesis of [4,5-dehydroLeu²]-LAKH was undertaken. The double bond in 4,5-dehydroleucine is sufficiently remote from the peptide backbone to be unlikely to affect attack by enzymes or the overall conformation of the molecule. Although 4,5-dehydroleucine itself is well known, no peptides containing this residue have hitherto been reported.

3,4-Dehydroproline was prepared from pyrrole-2-carboxylic acid; [9] the alternative method based on 1,2-diethoxycarbonylpyrrolidin-4-one [10] proved less satisfactory in our hands (this part of the work preceded the publication of the synthesis of L-3,4-dehydroproline from L-4-hydroxyproline [11]). The amino-acid was resolved as its *N*-t-butyloxycarbonyl derivative with *R*(+)-α-methyl-*p*-nitrobenzylamine.[6] Both the m.p. and optical rotation of the *N*-t-butyloxycarbonyl-L-3,4-dehydroproline prepared were somewhat lower than the literature value, but the compound gave good analytical figures and the method of Manning and Moore [12] showed an optical purity of greater than 99.5%. Synthesis of 4,5-dehydroleucine was effected by alkylation of ethyl acetamidomalonate. Subsequent partial hydrolysis of the product gave *N*-acetyl-4,5-dehydro-DL-leucine, which was resolved with hog kidney acylase I [13] to give free 4,5-dehydro-L-leucine.

Glp·Leu·Asn·Phe·Thr·Pro·Asn·Trp·Gly·Thr·NH_2
1 2 3 4 5 6 7 8 9 10

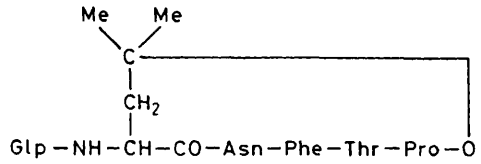
(1)

This amino-acid was also found to have an optical purity of at least 99.5%. Preparation of *N*-t-butyloxycarbonyl-L-4,5-dehydroleucine by the method of Schnabel [14] at pH 9.75 was found to give almost completely racemic product. However, the magnesium oxide buffering method [15] afforded the desired material. *N*-t-Butyloxycarbonyl-L-4,5-dehydroleucine proved to be optically stable at pH 10.2 at 30 °C for 16 h, so racemisation appears to take place concurrently with acylation. Since the same reagent, t-butyl azidoformate, is used in both procedures, the unexpected racemisation is difficult to explain. *N*-Fluoren-9-ylmethyloxycarbonyl-L-4,5-dehydroleucine was prepared using fluoren-9-ylmethyl chloroformate [16] in dioxan–10% aqueous sodium carbonate without difficulty.

The strategy adopted for the synthesis of the decapeptide analogues involved the final coupling of two *N*-terminal hexapeptides, each containing a dehydro-residue, with a common *C*-terminal tetrapeptide. The latter had already been prepared for use in the first synthesis of LAKH by stepwise addition to threonine amide,[2] but the reliability of the final coupling was improved. Deprotection of *N*-t-butyloxycarbonyl-tryptophanyl-glycyl-threoninamide was carried out with hydrogen chloride in ethyl acetate in preference to trifluoroacetic acid, and *N*-t-butyloxycarbonylasparagine 2,4,5-trichlorophenyl ester was used for coupling in preference to the free acid in the presence of *N*,*N*′-dicyclohexylcarbodi-imide. Purification of the protected tetrapeptide by h.p.l.c. proved essential; recrystallisation alone did not eliminate a persistent impurity.

The *N*-terminal hexapeptide containing 3,4-dehydroproline was prepared by solid-phase synthesis using *N*-t-butyloxycarbonylamino-acids and a conventional chloromethylated copoly(styrene–2% divinylbenzene) resin. To avoid losses

† In this paper, all amino-acid residues are of the L-configuration unless otherwise stated. Abbreviations for amino-acids and their use in the formulation of derivatives follow the revised recommendations of the I.U.P.A.C.–I.U.B. Commission on Biochemical nomenclature entitled 'Symbols for Amino-Acid Derivatives and Peptides. Recommendations (1971).'



(2)

through dioxopiperazine formation after coupling the second amino-acid, the phenylalanine residue was added by the reversed coupling procedure.[17] All peptide bonds were formed using *N,N'*-dicyclohexylcarbodi-imide, and asparagine was added using N^{α}-t-butyloxycarbonyl-N^{γ}-xanthydryl-L-asparagine. Completeness of coupling was assessed by the ninhydrin methods; [18] only with the addition of pyroglutamic acid was a repeat coupling necessary. The hexapeptide was cleaved from the resin by hydrogen fluoride, purified by gel filtration, using 0.05M-ammonium hydrogen carbonate as eluant, and reconverted to the free acid by ion-exchange chromatography. The overall yield was low, partly due to losses in columns, but for our purposes purity of product was more important than yield.

The synthesis of the hexapeptide containing 4,5-dehydroleucine proved more troublesome. An initial preparation using a procedure similar to that successful for the dehydroproline peptide gave a product with a good amino-acid analysis, but on h.p.l.c. the elution time was shorter than expected. Unlike the dehydroproline hexapeptide, the material in this case was unaffected by catalytic hydrogenation, even after a prolonged period. It therefore appeared to have lost the alkene linkage, but acidic hydrolysis liberated α-amino-γ-methyl-γ-valerolactone, the normal product formed from 4,5-dehydroleucine under the hydrolysis conditions. The model compound *N*-acetyl-4,5-dehydroleucine is stable to hydrogen fluoride, so it was suspected that addition of trifluoroacetic acid to the isopropenyl group had occurred, by analogy to the known addition of this acid to isobutene.[19] Such an adduct would be expected to hydrolyse under acidic conditions to γ-hydroxyleucine, which spontaneously lactonises. To avoid exposure of the dehydroleucyl peptide to trifluoroacetic acid, the synthesis was repeated as before except that 4,5-dehydroleucine was added as its *N*-fluoren-9-ylmethyloxycarbonyl derivative,[16] and deprotection of the pentapeptide was carried out with piperidine.[20] However, after removal of the resultant hexapeptide from the resin, the same reduction resistant product was obtained. The properties of this product would be shown by the macrocyclic lactone (2), but we have no conclusive proof of its structure. An authentic sample of [4,5-dehydroLeu^2]-LAKH(1—6) subsequently prepared has been shown on treatment with hydrogen fluoride to generate material identical with the suspected macrocyclic lactone.

It was therefore clear that hydrogen fluoride must be avoided in the last stage of the synthesis. Accordingly, the synthesis was redesigned using *N*-fluoren-9-ylmethyloxycarbonyl protection for all the amino-acids, piperidine for all *N*-deprotection and a benzyloxybenzyl ester type of resin.[21] Coupling was carried out using preformed symmetrical anhydrides [22] except in the case of phenylalanine, which was added by the original reversed coupling procedure, and *N*-fluoren-9-ylmethoxycarbonyl asparagine, which was added using *N,N'*-dicyclohexylcarbodi-imide in the presence of 1-hydroxybenzotriazole.[23] The peptide was cleaved from the resin using trifluoroacetic acid in the presence of dioxan; the latter is reported to inhibit the addition of trifluoroacetic acid to isobutene.[24] A very low yield of material having the properties of the desired hexapeptide was obtained.

It was clear that, despite good ninhydrin tests for the completeness of coupling at each stage, little peptide was attached to the resin at the end of the synthesis. This was found to be due to the high susceptibility of dipeptides attached to the benzyloxybenzyl resin towards dioxopiperazine formation. The reversed coupling procedure, effective in the dehydroprolyl peptide synthesis, affords little protection in this case. Coupling the phenylalanine residue as its *o*-nitrophenyl ester [25] did not prevent dioxopiperazine extrusion, so the problem was circumvented by adding *N*-fluoren-9-ylmethyloxycarbonyl-phenylalanyl-threonine to the prolyl-resin, using an *N,N'*-dicyclohexylcarbodi-imide coupling in the presence of 1-hydroxybenzotriazole. The desired hexapeptide was obtained from this modified synthesis in a crude yield of 87%; analytical h.p.l.c. showed it to be essentially a single compound, readily reduced in the presence of a palladium catalyst to material indistinguishable on h.p.l.c. from authentic LAKH(1—6). Purification by preparative h.p.l.c. was carried out to give pure hexapeptide ready for elaboration to the decapeptide.

Final couplings were carried out on a 100 μmolar scale using *N,N'*-dicyclohexylcarbodi-imide in the presence of 1-hydroxybenzotriazole [26] as in our original synthesis of LAKH. Subsequent purification also paralleled that used in the LAKH synthesis. Although the yield on couplings of this type was known to be modest (*ca.* 40%) and losses also occurred during gel filtration (due in part to the limited water solubility of the peptides, in pure form), our primary aim was to obtain pure peptides, and optimisation of yields was secondary. The methods proven in the LAKH synthesis were therefore used as separation of the desired decapeptides from the original fragments and derivatives was well established.*

The two decapeptides [3,4-dehydroPro^6]- and [4,5-dehydroLeu^2]-LAKH eluted as single peaks on h.p.l.c., clearly separable from each other and also from LAKH. Table 1 shows the details of amino-acid analyses on tetra-, hexa-, and deca-peptides. Good results were obtained for all amino-acids except asparagine. In both the tetra- and deca-peptides, low values were obtained on acid hydrolysis of the derived aspartic acid, in contrast to the hexapeptides. Similar low results have been observed with both natural and synthetic LAKH,[2] suggesting a sequence-dependent phenomenon. The results of enzymic digestion failed to clarify the situation, but on alkaline hydrolysis a better result was obtained. We do not therefore regard the low values obtained as significant.

On bio-assay the decapeptide analogues gave the dose-response curves shown in the Figure. High activity was found for [4,5-dehydroLeu^2]-LAKH, but since standard errors of the mean activity per dose of peptide are of the order ±10%, its activity probably does not significantly exceed that of LAKH itself. Much lower activity was observed for [3,4-dehydroPro^6]-LAKH, and the curve differs significantly in shape from that of LAKH. These results indicate that neither receptor binding nor resistance to enzymic attack are appreciably enhanced by the introduction of alkene linkage in these positions. The proline residue, however, appears to be important in the hormone agonist activity as it is sensitive to such a relatively subtle structural variation.

Experimental

N-t-Butyloxycarbonyl-amino acids were prepared by the Schnabel procedure [14] and physical properties were in accord

* In our experience, recovery of free peptides after h.p.l.c. is normally of the order of 50%. However, in the case of [3,4-dihydroPro^6]-LAKH recovery was markedly lower even allowing for taking a narrow cut of the eluted peak.

Table 1. Amino-acid analyses

Amino-acid	[6]Dhp-LAKH-(1—6) Acidic [c] digest	[2]Dhl-LAKH-(1—6) Acidic digest	BOC-LAKH-(7—10) Acidic digest	LAKH(7—10) Acidic digest	LAKH(7—10) Enzymic digest	[6]Dhp-LAKH-(1—10) Acidic [c] digest	[2]Dhl-LAKH-(1—10) Acidic digest	[2]Dhl-LAKH-(1—10) Basic [a] digest	4,5-H-Dhl-LAKH-(1—10) Acidic digest
Ala								0.16 [b]	
Asn					0.66				
Asp	1.04	0.98	0.72	0.73		1.87	1.84	2.12	1.65
Dhl		0.94					1.00	1.00	0.04
Gln	0.99	0.99				1.03	1.03	1.03	0.98
Gly			1.00	1.00	1.00	0.99	1.08	2.11 [b]	1.01
Leu	1.06					1.02			0.96
Phe	1.00	1.00				1.00	1.00	1.00	1.00
Pro		1.00					1.03	1.05	1.03
Thr	1.00	1.00	1.01	1.02	0.78	2.00	1.99	0.30 [b]	2.04
$ThrNH_2$					0.22				
Trp			0.70	0.68	1.01			106	

[a] 4.2M-NaOH, 110 °C, 22 h in the presence of starch (25 mg). [b] Thr decomposes on alkaline hydrolysis to give Gly and some Ala. [c] Dehydroproline was not quantified because of its low colour yield.

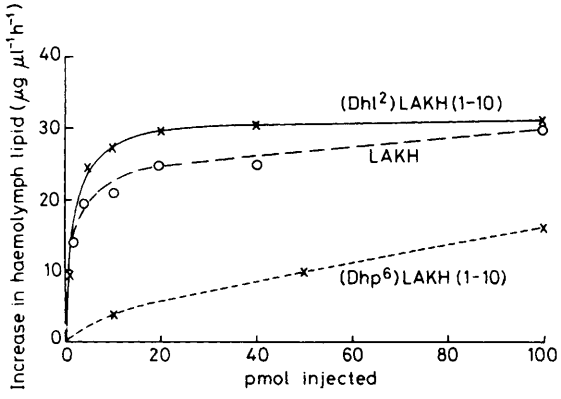


Figure. Dose–response curves for decapeptide analogues

with published values. *N*-Fluoren-9-ylmethyloxycarbonyl-amino-acids were prepared by the method of Chang *et al.*;[16] their physical properties agree with published values with the exception of *N*-fluoren-9-ylmethyloxycarbonyl-phenylalanine m.p. 156—157 °C (lit.,[16] m.p. 181—183 °C). *p*-Benzyloxybenzyl alcohol resin was prepared from *p*-hydroxybenzyl alcohol and chloromethylpoly(styrene–2% divinylbenzene) [0.9 mmol Cl g^{-1}; Pierce & Warriner (U.K.) Ltd., Chester] as described by Wang.[21]

Dimethylformamide (DMF) was purified by shaking over anhydrous copper(II) sulphate overnight, filtering, and fractionally distilling under reduced pressure from calcium hydride. Before use dry nitrogen was bubbled through for 15 min. Dichloromethane, ethyl acetate, and acetonitrile were purified by distillation from calcium hydride. Dioxan and propan-2-ol were distilled from calcium hydride and copper(I) chloride. Triethylamine, piperidine, and dicyclohexylamine were dried over KOH pellets then fractionally distilled. Diethyl ether and light petroleum were dried over sodium wire. Water was distilled from glass.

Amino-acid analyses were performed on a Jeol JLC-5AH Amino Acid Analyser. Peptide resins were hydrolysed with a 1 : 1 (v/v) mixture of 6M-HCl and propionic acid in sealed evacuated tubes at 130 °C for 3 h.[17] Free peptides were hydrolysed with 6M-HCl in sealed evacuated tubes at 110 °C for 18 h. In acidic hydrolyses 4,5-dehydroleucine was determined as α-amino-γ-methyl-γ-valerolactone. Enzyme hydrolyses were carried out by the method of Bennett *et al.*[28] Thin layer chromatographs were run on silica plates (Merck GF 254) in the following solvent systems (all v/v): (A) chloroform–methanol, 9 : 1; (B) butan-1-ol–acetic acid–water, 3 : 1 : 1; (C) propan-2-ol–acetic acid–water, 25 : 10 : 1; (D) chloroform–ethanol, 3 : 1; (E) butan-1-ol–acetic acid–water, 4 : 1 : 5; (F) butan-1-ol–acetic acid–water–piperidine, 1 : 3 : 12 : 10. Gel filtration chromatography was carried out on Pharmacia columns using a Varioperpex 12000 peristaltic pump and an LKB Uvicord II Type 8303A detector monitoring at 254 nm. Reverse phase h.p.l.c. was carried out using a Waters Associates M6000 solvent delivery system, an Altex injection valve (model 905-42) a Cecil variable wavelength u.v. detector (model CE 2012) and a Waters Associates differential refractometer, both linked to a dual channel Rikadenki chart recorder (model DBE-2). All columns used were Spherisorb 5/10 ODS (HPLC Technology Ltd.) and were allowed to equilibrate for at least 3 h before use. The solvent systems commonly used for analytical studies were (v/v) : (1) methanol–water–acetic acid, 50 : 50 : 1, and (2) acetonitrile–water–phosphoric acid, 25 : 75 : 0.1. For a peptide to be classed as chromatographically homogeneous it was required to show homogeneity in all six t.l.c. systems and in both h.p.l.c. systems. Melting points were determined by the capillary tube method with an Electrothermal apparatus and are uncorrected. Optical rotations were determined at room temperature using a Thorn Automation NPL Automatic Polarimeter Type 243 with a 0.1 dm path-length cell.

N-*t-Butyloxycarbonyl*-3,4-*dehydroproline*.—3,4-Dehydro-DL-proline was prepared by the method of Corbella *et al.*,[9] converted into the *N*-t-butyloxycarbonyl derivative by the Schnabel procedure [14] at pH 8.6, and the latter resolved using *R*(+)-α-methyl-*p*-nitrobenzylamine.[6] *N*-t-Butyloxycarbonyl-3,4-dehydro-L-proline (*R*)-methyl-*p*-nitrobenzylammonium salt (0.73 g, 1.93 mmol) was partitioned between 1M-citric acid (2.3 ml) and EtOAc (3 × 3 ml). The combined organic extracts were dried ($MgSO_4$) and the solvent removed under reduced pressure. The residual colourless oil crystallised on trituration with light petroleum (b.p. 40—60 °C) to give the *N*-protected L-amino acid (0.307 g, 75%), m.p. 86—87 °C, $[\alpha]_D^{26}$ −264.0° (*c* 1.36, MeOH) {lit.,[6] m.p. 94—96.5 °C, $[\alpha]_D^{25}$ −272.5° (*c* 1.0, MeOH)} (Found: C, 56.3; H, 7.0; N, 6.5.

Table 2. Sequence of steps used for Merrifield solid-phase synthesis

Reagent [a]	Duration	Operation
1 TFA–CH_2Cl_2, 1 : 1	2 × 2 + 1 × 30 min	Deprotection
2 CH_2Cl_2	3 × 5 min	Wash
3 EtOH	3 × 5 min	Wash
4 $CHCl_3$	3 × 5 min	Wash
5 NEt_3–$CHCl_3$, 1 : 10	2 × 5 + 1 × 10 min	Neutralisation
6 $CHCl_3$	3 × 5 min	Wash
6 CH_2Cl_2	3 × 5 min	Wash
8 DMF [b]	1 × 5 min	Wash
9 BOc-AA-OH–coupling solvent [e]	1 × 2 min	Pre-equilibration [c]
10 DCCI–coupling solvent [e]	1 × 18 h	Coupling [d]
11 DMF [b]	1 × 5 min	Wash
12 CH_2Cl_2	3 × 5 min	Wash
13 EtOH	3 × 5 min	Wash
14 CH_2Cl_2	3 × 5 min	Wash

[a] Solvent volume normally 20 ml. [b] Not included unless coupling solvent is DMF. [c] Boc-AA-OH added in 15 ml coupling solvent. [d] DCCI added in 5 ml coupling solvent. [e] Coupling solvent normally CH_2Cl_2, but DMF for Asn and Glp.

Calc. for $C_{10}H_{15}NO_4$: C, 56.3; H, 7.0; N, 6.6%). Application of the method of Manning and Moore [12] showed this product to have an optical purity >99.5%.

N-t-Butyloxycarbonyl-3,4-dehydroproline Esterified Merrifield Resin.—Solid-phase resin (chloromethylated Bio-Beads 8 × 2, 200—400 mesh, 0.9 mmol Cl g^{-1}) (2.00 g, 1.8 mmol) was suspended in EtOAc (15 ml) and *N*-t-butyloxycarbonyl-3,4-dehydroproline (0.40 g, 1.88 mmol) added. The suspension was heated to reflux, NEt_3 (0.24 ml; 1.6 mmol) added, and the mixture heated at reflux for 68 h. The suspension was cooled to 20 °C, and the resin filtered off and washed in succession with DMF (3 × 20 ml), DMF–H_2O (9 : 1, v/v; 3 × 20 ml), DMF (3 × 20 ml), and EtOH (3 × 20 ml), then dried *in vacuo* for 2 h. The fines were removed by decantation using CH_2Cl_2 and the residual material was collected and dried (KOH) *in vacuo* for 24 h (2.15 g). Amino-acid analysis indicated a loading of 0.36 mmol of 3,4-dehydroproline per g of resin.

Pyroglutamyl-leucyl-asparaginyl-phenylalanyl-threonyl-3,4-dehydroproline.—Solid-phase syntheses were carried out in a Kel F cylinder (internal dimensions 1.8 × 12.5 cm) fitted with sintered glass end discs leading to low dead space narrow bore PTFE tubing connections for liquid transfer. Introduction and removal of solvents and solutions was carried out using a water pump vacuum with the cylinder in the vertical position. Resin suspensions were agitated mechanically by rotation of the vessel through 30° above and below the horizontal position (2 second cycle).

N-t-Butyloxycarbonyl-3,4-dehydroproline esterified Merrifield resin (2.00 g, 0.72 mmol amino-acid) was loaded into the solid-phase synthesis vessel and the hexapeptide built up using the sequence of steps shown in Table 2, using 1.8 mmol (2.5 equiv.) of each BOC-amino-acid and 1.94 mmol (2.7 equiv.) of *N,N'*-dicyclohexylcarbodi-imide (DCC). In the phenylalanine coupling, however, the DCC was added before the *N*-protected amino-acid to minimise 2,5-dioxopiperazine formation.[17] The asparagine residue was added as its N^{α}-BOC-N^{α}-xanthydryl derivative [2] to prevent formation of β-cyanoalanine. Coupling reactions were monitored using the ninhydrin test of Kaiser,[18] and all except that of pyroglutamic acid were complete after 18 h. Upon completion of the synthesis, the peptide-resin was dried at 40 °C *in vacuo* for 3 h (2.36 g). Amino-acid analysis: Glu 1.22, Leu 1.02, Asp 0.96, Phe 1.00, Thr 0.85.*

A mixture of the peptide resin and anisole (2 ml) was allowed to stand with liquid hydrogen fluoride (20 ml) at −20 °C for 1 h in a Kel-F vessel. The hydrogen fluoride was removed under reduced pressure and the residue dried over KOH *in vacuo* for 12 h before extraction with Et_2O (5 × 10 ml), redrying *in vacuo*, and extraction with water (5 × 10 ml). Evaporation of the combined aqueous extracts under reduced pressure afforded a fawn solid. This was dissolved in 0.05M-aqueous NH_4HCO_3 (15 ml), and the solution degassed (14 mmHg, 15 min), and then loaded onto a superfine Sephadex G-25 gel filtration column (2.5 cm × 85 cm). The column was eluted with 0.05M-NH_4HCO_3 solution at a flow rate of 35 cm^3 h^{-1} and the u.v. absorbance of the eluate continuously monitored at 254 nm, fractions (9 ml) being collected. Fractions 25—30 were combined and the solvent lyophilised, to afford a white solid (171 mg). This solid was rechromatographed using the same column and solvent and fractions 24—27 (elution volume 216—243 ml) combined and lyophilised, the buffer salt being removed by heating at 60 °C for 15 min *in vacuo*. The resultant solid was dissolved in water (10 ml) and passed down a Dowex AG 50W × 3 ion-exchange column (1 × 10 cm; H^+ form). Lyophilisation of the eluate afforded the hexapeptide as a chromatographically homogeneous white amorphous solid (74 mg, 15%). The amino-acid analysis is given in Table 3. Catalytic hydrogenation (10% Pd/C) of a small sample in DMF gave a product that was chromatographically indistinguishable from a sample of authentic LAKH(1—6) on t.l.c. and h.p.l.c.

N-t-Butyloxycarbonyl-4,5-dehydroleucine.—4,5-Dehydro-L-leucine [13] (2.00 g, 15.5 mmol) and magnesium oxide (1.24 g, 31.0 mmol) were suspended in dioxan–water (20 ml; 1 : 1 v/v), and to the stirred suspension was added t-butyl azidoformate (4.44 g, 31.0 mmol) dropwise during 5 min. After being stirred for 2 h at 45—50 °C, the mixture was cooled, poured into water (70 ml) and the resulting solution washed with EtOAc (3 × 30 ml). The combined organic extracts were back extracted with water (30 ml) and 1M-$NaHCO_3$ (30 ml). The combined aqueous phases were cooled in an ice-bath and acidified to pH 4.5 with 2M-aqueous citric acid before extraction with EtOAc (3 × 20 ml). The combined EtOAc extracts were washed successively with water (1 × 30 ml), brine (1 × 30 ml), and dried (Na_2SO_4). Evaporation of the solvent gave a colourless oil (3.40 g, 14.9 mmol, 96%). To a solution of this oil in Et_2O (20 ml) was added dicyclohexylamine (2.70 g, 14.9 mmol) in Et_2O (20 ml) with stirring. Evaporation of the solvent left a white solid, which crystallised from EtOAc to give the pure dicyclohexylammonium salt as needles (4.18 g, 69%), m.p. 149—150 °C, $[\alpha]_D^{22}$ +11.9° (*c* 1.0, MeOH) (Found: C, 67.4; H, 10.3; N, 6.9%. $C_{23}H_{42}N_2O_4$ requires C, 67.3; H, 10.2; N, 6.8%). A portion of this salt (1.00 g, 2.44 mmol) was partitioned between EtOAc (10 ml) and 5% aqueous $KHSO_4$. After stirring of the mixture for 15 min the layers were separated and the aqueous phase washed with EtOAc (2 × 5 ml). The combined organic extracts were washed with water (3 × 5 ml) and dried ($MgSO_4$). Evaporation of the solvent under reduced pressure gave the protected amino-acid as a colourless oil, which could not be crystallised. After drying 48 h *in vacuo* over P_2O_5, this oily product (0.56 g, 100%), $[\alpha]_D^{26}$ −19.0° (*c* 1.4, HOAc), was shown by the method of Manning and Moore [12] to have an optical purity of ≥99.5%.

* The colour yield of 3,4-dehydroproline is so low (*ca.* 10% of that of proline) that quantification is very inaccurate. No values are therefore given in this paper.

N-*Fluoren*-9-*ylmethyloxycarbonyl*-4,5-*dehydroleucine*.—To a solution of 4,5-dehydro-L-leucine [13] (3.23 g, 25 mmol) in 10% aqueous Na_2CO_3 (66 ml) and dioxan (36 ml) was added a solution of fluoren-9-ylmethyl chloroformate [16] (6.47 g, 25 mmol) in dioxan (54 ml) with stirring at 0 °C. After the addition was completed (20 min) the reaction mixture was stirred for 2 h at 20 °C then poured into water (1 400 ml). The resulting solution was washed with Et_2O (2 × 180 ml), cooled in ice, and acidified to Congo Red with 5% aqueous $KHSO_4$. The precipitated oil was extracted into EtOAc (3 × 180 ml) and the combined extracts dried ($MgSO_4$), filtered, and evaporated under reduced pressure. The residual colourless oil (10.4 g) crystallised on trituration with light petroleum (b.p. 40—60 °C). Recrystallisation from CH_2Cl_2–light petroleum (b.p. 40—60 °C) (30—50 ml) gave the pure *N*-protected amino acid (7.87 g, 90%), m.p. 130—131 °C, $[\alpha]_D^{25}$ —18.7° (*c* 1.0; DMF), R_F 0.84 (B) (Found: C, 71.7; H, 6.0; N, 3.9. $C_{21}H_{21}NO_4$ requires C, 71.8; H, 6.0; N, 4.0%). This material was found to have an optical purity of ≥99.5% by the method of Manning and Moore.[12]

t-Butyl-N-*fluoren*-9-*ylmethyloxycarbonyl-phenylalanyl*-O-*t-butyl-threonine*.—To a solution of t-butyl *O*-t-butylthreoninate [29] (1.18 g, 5.1 mmol) in CH_2Cl_2 (10 ml) was added *N*,*N*′-di-cyclohexylcarbodi-imide (DCC) (1.66 g, 5.6 mmol) followed by *N*-fluoren-9-ylmethyloxycarbonyl-L-phenylalanine (1.98 g, 5.1 mmol) [16] in CH_2Cl_2 (7 ml) and DMF (3 ml) dropwise with stirring during 15 min. The mixture was stirred at 20 °C for 18 h, then acetic acid (1 ml) was added; the precipitated urea was removed after a few minutes, and the filtrate evaporated under reduced pressure. The residual solid was dissolved in CH_2Cl_2 (25 ml) and the solution then filtered, washed with 1M-aqueous HCl (25 ml) and 5% aqueous $NaHCO_3$ (25 ml), and dried ($MgSO_4$). Removal of the solvent under reduced pressure gave the protected dipeptide acid as a white solid foam (3.05 g, 99%).

N$^{\alpha}$-*Fluoren*-9-*ylmethyloxycarbonyl-phenylalanyl-threonine*.—t-Butyl *N*$^{\alpha}$-phenylalanyl-*O*-t-butyl-threoninate (5.11 g, 8.5 mmol) was dissolved in a 3M-solution of HCl in EtOAc (35 ml), and kept at 20 °C for 2 h; the solvent was evaporated under reduced pressure. The residual oil was dissolved in a mixture of CH_2Cl_2 (35 ml) and EtOAc (35 ml) and the solution extracted with saturated aqueous $NaHCO_3$ (2 × 35 ml). The combined aqueous extracts were washed with EtOAc (1 × 25 ml), and then the combined organic phases were dried ($MgSO_4$), filtered, and the solvent removed under reduced pressure to afford a colourless oil that solidified on trituration with diethyl ether (10 ml). This solid was dissolved in warm water (100 ml), and the solution cooled to 0 °C; it was then acidified to pH 2 with 2M-HCl. The mixture was extracted with diethyl ether (4 × 50 ml), and the extracts combined and dried ($MgSO_4$); after filtration the solvent was removed under reduced pressure to afford the *N*-protected dipeptide as a colourless oil (2.81 g, 68%). This product was purified as its dicyclohexylammonium salt, which was recrystallised from EtOAc, and had m.p. 115—117 °C. Partition between EtOAc and 5% aqueous $KHSO_4$ regenerated the dipeptide acid which was obtained, after drying of the organic layer ($MgSO_4$) and evaporation, as a white foam $[\alpha]_D^{26}$ —11.7° (*c* 1.0, DMF), R_F 0.73 (B) (Found: C, 66.8; H, 5.9; N, 5.4; $C_{28}H_{28}N_2O_6 \cdot H_2O$ requires C, 66.4; H, 6.0; N, 5.5%).

N-*Fluoren*-9-*ylmethyloxycarbonyl-proline*-p-*benzyloxybenzyl Ester Resin*.—*p*-Benzyloxybenzyl alcohol resin (2% cross-linked) [21] (5.50 g) was washed with CH_2Cl_2 (2 × 20 ml) and suspended in DMF–CH_2Cl_2 (4 : 1, v/v; 100 ml). *N*-Fluoren-9-ylmethyloxycarbonyl-proline (2.02 g, 6.0 mmol), DCC (1.30 g, 6.3 mmol), and 4-dimethylaminopyridine (0.70 g, 6.0 mmol) were added to the stirred suspension at 0 °C. The resultant mixture was stirred vigorously for 30 min at 0 °C and then for 5 h at 20 °C. The mixture was chilled and treated with benzoyl chloride (2.2 ml) and pyridine (2 ml) and then stirred for 30 min at 0 °C and 1 h at 20 °C. After filtration the resin was thoroughly washed in succession with CH_2Cl_2 (2 × 50 ml), DMF (2 × 50 ml), propan-2-ol (2 × 50 ml), and diethyl ether (3 × 50 ml); it was then dried (KOH) at 50 °C *in vacuo* for 24 h, to give the polymer-bound amino-acid as a pale yellow resin (5.50 g). Amino-acid analysis indicated a loading of 0.45 mmol of proline per g of resin.

Table 3. Sequence of steps used for Fmoc solid-phase synthesis

Reagent [a]	Duration	Operation
1 CH_2Cl_2	3 × 2 min	Wash
2 Piperidine–CH_2Cl_2 (1 : 1 v/v)	1 × 5 + 1 × 25 min	Deprotection
3 CH_2Cl_2	2 × 2 min	Wash
4 DMF	2 × 2 min	Wash
5 H_2O–Dioxan (1 : 2 v/v)	2 × 5 min	See note *b*
6 DMF	3 × 2 min	Wash
7 CH_2Cl_2	3 × 2 min	Wash
8 DMF [c]	2 × 2 min	Wash
9 Fmoc-AA-Symmetrical Anhydride in CH_2Cl_2 (v/v) [d]	1 × 30 min	Coupling
10 DMF [c]	2 × 2 min	Wash
11 CH_2Cl_2	3 × 2 min	Wash
12 DMF	3 × 2 min	Wash
13 Pr^{i}OH	3 × 2 min	Wash
14 CH_2Cl_2	3 × 2 min	Wash

[a] Solvent volume 12.5 ml per g of resin. [b] Facilitates decomposition of carbamate. [c] Used only when coupling solvent is DMF. [d] After 15 min 0.5M-di-isopropylethylamine in CH_2Cl_2 (1 ml per g of resin) added to counteract any possible protonation of the α-amino-group by residual acid generated from the anhydride during coupling.

Pyroglutamyl-4,5-*dehydro-leucylasparaginyl-phenylalanyl-threonyl-proline*.—The apparatus used in this synthesis was identical to that described earlier. The cycle of operations used is given in Table 3. All coupling reactions were monitored by the ninhydrin test of Kaiser.[18] *N*-Fluoren-9-ylmethyl-oxycarbonyl-proline-*p*-benzyloxybenzyl ester resin (2.00 g, 0.90 mmol) was placed in the reaction vessel and deprotected by steps 1—7. A solution of *N*-fluoren-9-ylmethyloxycarbonyl-phenylalanyl-threonine (1.19 g, 2.44 mmol) in CH_2Cl_2 (20 ml) and DMF (5 ml) was treated with 1-hydroxybenzotriazole (0.398 g, 2.6 mmol) and DCC (0.537 g, 2.6 mmol) at 0 °C for 1 h and then at 20 °C for 1 h. The mixture was filtered, the filtrate added to the reaction vessel, and the coupling allowed to proceed at 20 °C for 20 h, with di-isopropylethylamine in CH_2Cl_2 (2 ml, 0.5 m) being added before the last hour. The protected tripeptide ester resin was washed, steps 11—14 (Table 2), and used directly for subsequent coupling cycles. Preformed symmetrical anhydrides were prepared from *N*-fluoren-9-ylmethyloxycarbonyl-amino acids (5.4 mmol, 6 equiv.) in CH_2Cl_2–DMF (1 : 3 v/v, 10 ml) and DCC (0.557 g, 2.7 mmol, 3 equiv.) in CH_2Cl_2 (5 ml) at 0 °C. After 30 min the precipitated urea was filtered off and washed with CH_2Cl_2 (10 ml). The combined filtrates were used directly in the coupling except in the addition of asparagine. In this case DCC (2.7 mmol, 3 equiv.) and 1-hydroxybenzotriazole (2.7 mmol, 3 equiv.) were dissolved in DMF (20 ml) and stirred at 0 °C for 10 min. N$^{\alpha}$-Fluoren-9-ylmethyloxycarbonyl-aspar-

agine (2.7 mmol, 3 equiv.) was added and stirring continued at 0 °C for 10 min. The mixture was filtered, the residue washed with DMF (5 ml), and the combined filtrates used directly for a coupling reaction of 1 h duration. On completion of the synthesis the resin was dried (KOH) *in vacuo* for 12 h to afford a pale brown resin (2.415 g, 92%).

The hexapeptide resin was treated with a solution of trifluoroacetic acid in CH_2Cl_2 (20 ml; 1 : 1, v/v) containing dioxan (2 ml) and kept at 20 °C for 1 h. After filtration, the solvent was evaporated to a small volume (*ca.* 1 ml) and dry diethyl ether (10 ml) was added, causing the precipitation of a white solid. Removal of the solvent under reduced pressure gave an off-white solid (0.55 g, 87% based on Fmoc-Pro-resin). This material was purified by reverse-phase h.p.l.c., 10 mm × 25 cm column, solvent system 1, to afford the pure hexapeptide (0.253 g; 40% based on Fmoc-Pro-resin). The amino-acid analysis is given in Table 3. This material was chromatographically homogeneous. Catalytic hydrogenation (10% Pd/C) of a small sample in DMF gave a product that was chromatographically indistinguishable from a sample of authentic LAKH(1—6).

N-*Benzyloxycarbonylglycyl-threonine Amide*.—This compound was prepared as previously reported [2] as a white crystalline solid (86%), m.p. 181—181.5 °C $[\alpha]_D^{25}$ +13.3° (*c* 1.0, DMF) {lit.,[2] m.p. 175—177 °C, $[\alpha]_D^{25}$ +16.5° (*c* 1.92, DMF)} R_F 0.64 (B) (Found: C, 54.1; H, 6.2; N, 13.5. Calc. for $C_{14}H_{19}N_3O_5$: C, 54.5; H, 6.2; N, 13.6%).

N-*t-Butyloxycarbonyl-tryptophanylglycyl-threonine Amide*.—This compound was prepared as previously reported [2] as a white microcrystalline solid (60%), m.p. 151—153 °C {lit.,[2] m.p. 149—151 °C}, R_F 0.66 (B) (Found: C, 55.2; H, 6.7; N, 14.8. Calc. for $C_{22}H_{31}N_5O_6$: C, 55.1; H, 6.8; N, 14.8%).

N-*t-Butyloxycarbonyl-asparaginyl-tryptophanyl-glycyl-threonine Amide*.—*N*-t-Butyloxycarbonyl-tryptophanyl-glycyl-threonine amide (1.00 g, 2.17 mmol) was stirred with a 2M-solution of HCl in EtOAc (15 ml) for 1 h at room temperature. After removal of the solvent, the residual solid was reprecipitated from EtOH with dry Et_2O, to afford the tripeptide amide hydrochloride as a white amorphous solid (0.68 g, 70%), m.p. 185—187 °C (decomp.) R_F 0.5 (F). To a solution of this product (0.55 g, 1.39 mmol) in DMF (15 ml) was added NEt_3 (0.56 g, 5.56 mmol) and *N*-t-butyloxycarbonylasparagine 2,4,5-trichlorophenyl ester (0.57 g, 1.39 mmol). The mixture was stirred at room temperature for 44 h and then filtered. The filtrate was evaporated under reduced pressure, to afford a yellow oil which on trituration with dry Et_2O gave a white solid. After recrystallisation from EtOH–Et_2O (2 : 5, v/v), further purification was effected by reverse-phase h.p.l.c. (using a 10 mm × 25 cm column, and solvent system 1), to give the tetrapeptide as a chromatographically homogeneous white solid (0.258 g, 32%), m.p. 201—202 °C (decomp.), $[\alpha]_D^{25}$ −9.99° (*c* 1.0, DMF). The amino-acid analyses both before and after *N*-deprotection are shown in Table 3.

Pyroglutamyl-leucyl-asparaginyl-phenylalanyl-threonyl-3,4-dehydro-prolyl-asparaginyl-trypto-phanylglycyl-threonine Amide.—*N*-t-Butyloxycarbonyl-asparaginyl-tryptophanyl-glycyl-threonine amide (58.5 mg, 101.9 μmol) was stirred with 3M-HCl in EtOAC (2 ml) for 90 min at 20 °C. The solvent was removed under reduced pressure and the resultant white solid stirred with a 1M-NEt_3 in EtOAc (2 ml) for 5 min. The solvent was removed under reduced pressure to afford a white solid, R_F 0.45 (F). To a solution of this deprotected tetrapeptide amide in DMF (1.0 ml) was added pyroglutamyl-leucyl-asparaginyl-phenylalanyl-threonyl-3,4-dehydro-proline (71.2 mg, 101.8 μmol) and 1-hydroxybenzotriazole (312 mg, 2 036 μmol). This solution was cooled to −5 °C and a solution of DCC (420 mg, 2 036 μmol) in DMF (0.4 ml) added. The mixture was allowed to warm slowly to 20 °C and was then stirred for 20 h at this temperature. The solvent was removed under reduced pressure to afford a sticky brown solid which was partitioned between 0.05M-aqueous NH_4HCO_3 (10 ml) and Et_2O (10 ml). After 30 min the mixture was filtered, the ethereal phase discarded, and the aqueous phase again extracted with Et_2O (10 ml). After separation of the ethereal phase, the aqueous solution was thoroughly degassed and loaded onto a superfine Sephadex G.25 gel filtration column (2.5 cm × 85 cm) and the column eluted with 0.05M-aqueous NH_4HCO_3 at a flow rate of 35 $cm^3\ h^{-1}$. The u.v. absorbance of the eluate was continuously monitored at 254 nm. The fractions 42—46 (elution volume 378—414 ml) was combined and the solvent lyophilised. The buffer salt was removed by heating at 60 °C *in vacuo* for 15 min, to afford a white amorphous solid (40 mg, 34%). Purification was effected by reverse-phase h.p.l.c. [using a 10 mm × 25 cm column with CH_3CN–water (28 : 72 v/v) as eluant at a flow rate of 3.0 $cm^3\ min^{-1}$ and monitoring at 210 nm] to give the pure decapeptide as a chromatographically homogeneous white solid (7.0 mg, 6%). The amino-acid analysis is given in Table 3. Catalytic hydrogenation (10% Pd/C) of a small sample in DMF gave a product that was chromatographically indistinguishable from a sample of authentic LAKH(1—10).

Pyroglutamyl-4,5-dehydroleucyl-asparaginyl-phenylalanyl-threonyl-prolyl-asparaginyl-tryptophanylglycyl-threonine Amide.—This dehydrodecapeptide was prepared on a 94 μmolar scale in a manner identical to the above using the same *C*-terminal tetrapeptide amide and the dehydroleucine containing *N*-terminal hexapeptide. After gel filtration chromatography fractions 47—52 (elution volume 423—468 ml) were combined, lyophilised, and debuffered to afford a white amorphous solid (28 mg, 26%) which was purified by reverse phase h.p.l.c. as described above. This gave the chromatographically homogeneous decapeptide as a white solid (12 mg, 10%). The amino-acid analysis is given in Table 3. Catalytic hydrogenation (10% Pd/C) of a small sample in DMF gave a product that was chromatographically indistinguishable from a sample of authentic LAKH(1—10). Amino-acid analysis (Table 3) showed only a trace of 3,4-dehydroleucine remaining.

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