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PEPTIDE SYNTHESIS. PART 10. USE OF PENTAFLUOROPHENYL ESTERS OF FLUORENYL METHOXYCARBONYLAMINO ACIDS IN SOLID PHASE PEPTIDE SYNTHESIS.

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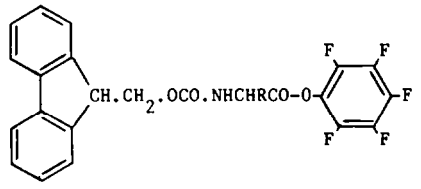
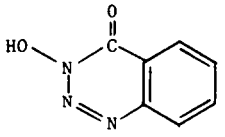
The application of fluorenylmethoxycarbonyl-amino-acid pentafluorophenyl esters to solid phase peptide synthesis under polar reaction conditions is described. Reaction rates are increased in the presence of 1-hydroxybenzotriazole. The technique is illustrated by preparation of decapeptide and dodecapeptide sequences.

Previous papers in this series[1] have described the development and application of the Fmoc-polyamide method of solid phase peptide synthesis. The technique has acquired its name from the adoption[2] of the fluorenylmethoxycarbonyl (Fmoc) group[3] for the protection of α-amino-acids, and from the use of novel polar polydimethylacrylamide solid supports which are well permeated by dipolar aprotic media of the dimethylformamide type.[4] These features contrast with the near exclusive use of t-butoxycarbonyl (Boc) amino-acids and apolar polystyrene resins in the now classical Merrifield solid phase technique. The advantages which we believe derive from these changes have been discussed elsewhere.[5] Until relatively recently, Fmoc-polyamide peptide synthesis used symmetrical anhydrides[4] as activated derivatives for the majority of Fmoc-amino-acids, the exceptions being asparagine, glutamine, and ε-Boc-lysine, for which p-nitrophenyl activated esters were preferred. Nitrophenyl and most other esters are distinctly less reactive than symmetrical anhydrides, and commonly require extended reaction times, even in favourable polar solvents and in the presence of acylation catalysts such as 1-hydroxybenzotriazole. On the other hand, such esters are frequently stable crystalline solids and offer distinct practical advantages. Manual preparation of symmetrical anhydrides immediately prior to each acylation reaction is avoided, and the design of fully automatic peptide synthesising equipment much simplified. For these reasons we sought for some time activated derivatives of Fmoc-amino-acids which would combine the high reactivity and freedom from side reactions characteristic of symmetrical anhydrides, with the crystallinity, ease of preparation, and stability to storage of activated esters. Two series of such esters (1,2) have proved outstanding in Fmoc-polyamide synthesis, and another (3) has properties of special interest. We report now on the use in solid phase synthesis of Fmoc-amino-acid pentafluorophenyl esters (1).[6] Further

publications will describe esters of 3-hydroxy-2,3-dihydro-4-oxo-benzotriazine (2)[7,8] and of 2,5-diphenyl-3,4-dihydroxythiophene dioxide (3).



(1)



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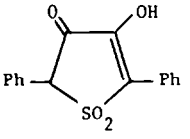

(3)

The high reactivity of protected amino-acid pentafluorophenyl esters as acylating agents has been reported by Kisfaludy and his colleagues.[9] In our experience they are less reactive than symmetrical anhydrides, but share with the latter the necessary low tendency towards side reactions. This is crucial for use in solid phase synthesis, and contrasts, for example, with esters of N-hydroxysuccinimide which we also studied but where the structure of the hydroxy component itself permits alternative reaction paths. In practice, hydroxysuccinimide esters proved unsatisfactory in Fmoc-polyamide solid phase synthesis with evidence of incomplete amino-acid incorporation and byproduct formation. Subsequent to the adoption of Fmoc-amino-acids in solid phase synthesis, Kisfaludy and Schön[10] reported the synthesis of a number of Fmoc-amino-acid pentafluorophenyl esters, and applied them to the conventional solution synthesis of the gastrin C-terminal tetrapeptide. Their list has been extended in a more recent publication by Schön and Kisfaludy,[11] in which a pentafluorophenyl ester derivative was also used for the preparation of Fmoc-amino-acids themselves.

We adopted the general preparative procedure already described by Kisfaludy and Schön[10] using dioxan or ethyl acetate as solvent. An illustrative procedure for Fmoc-leucine pentafluorophenyl ester is given in the Experimental Part. The derivatives of Fmoc-O-t-butyl-serine and Fmoc-O-t-butyl-threonine were exceptionally soluble and could not be obtained in stable crystalline form. At the time of the present work, the freshly prepared oily serine or threonine derivatives were used satisfactorily in

synthesis. Subsequently the analogous crystalline pentachlorophenyl esters were prepared. These proved to be distinctly less reactive than the pentafluorophenyl derivatives. We would now recommend esters of 3-hydroxy-4-oxodihydrobenzotriazine for these two amino-acids.[7] The rather insoluble Fmoc-amino-acid derivatives of asparagine and glutamine required addition of dimethylformamide (30%) to the dioxan esterification reaction medium, and the resulting pentafluorophenyl esters were the least soluble of those prepared. Complete separation from the sparingly soluble dicyclohexylurea co-product was correspondingly difficult. N_G-methoxytrimethylbenzenesulphonyl-Fmoc-arginine is discussed elsewhere[12] in relation to the analogous hydroxyoxodihydrobenzotriazine derivative. The chemistry relating to Fmoc-His(Boc) and its pentafluorophenyl ester will be the subject of a future publication.[13]

All the foregoing Fmoc-amino-acid pentafluorophenyl esters or the corresponding hydroxybenzotriazine derivatives are now commercially available.*

Application of Fmoc-amino-acid pentafluorophenyl esters in solid phase synthesis is illustrated here by preparations of the test decapeptide sequence (4) (a part sequence of acyl carrier protein), and of the dodecapeptide (5) (an analogue of the C-terminal sequence of penicillinase).

H.Val.Gln.Ala.Ala.Ile.Asp.Tyr.Ile.Asn.Gly.OH

(4)

H.Leu.Ala.Glu.Leu.Gly.Ala.Ser.Leu.Leu.Lys.His.Trp.OH

(5)

The decapeptide (4) has been our standard test case for many years. It contains normal, sterically hindered, and functional amino-acids, but its special significance lies in the particularly difficult synthetic problems it presents arising from internal aggregation phenomena. Its synthesis failed using early Boc-polystyrene solid phase techniques.[14] Most of the problems were overcome immediately by use of the more polar

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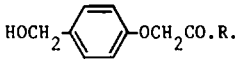
* Manufactured by Cambridge Research Biochemicals Ltd, Button End Industrial Estate, Harston, Cambridge; distributed by LKB Biochrom, Science Park, Milton Road, Cambridge, and by Milligen Division, Millipore (UK) Ltd, 11-15 Peterborough Road, Harrow HA1 2YH.

polyamide/dimethylformamide system,* and excellent syntheses were achieved using a combination of symmetrical anhydride and p-nitrophenyl ester (asparagine, glutamine) activation.[2,4] Even in the polar system, formation of the last peptide bond (valine to glutamine) remained exceptionally slow, and synthesis of (4) therefore constitutes a stringent test for new activated derivatives. An early attempted synthesis in our laboratory using p-nitrophenyl esters exclusively was quite unsatisfactory.

For this first synthesis using pentafluorophenyl esters, our original discontinuous solid phase technique using beaded polydimethylacrylamide gel resin was employed in an investigative manner. Five fold excesses of individual pentafluorophenyl esters (0.75 mmole) were used throughout, and these were dissolved in the minimum volume of solvent Dmf (ca 6 ml, initial concentration ca 0.125 M)) required to give a mobile slurry when added to the drained resin. The resin was initially functionalised with an internal reference norleucine residue and then with the acid-labile linkage agent [6, R=$OC_6H_2Cl_3$(2,4,5)] as previously described.[2,4] Esterification of the first residue (glycine) to the resulting hydroxymethyl resin utilised the established symmetrical anhydride/dimethylaminopyridine procedure,[2] but see below. All the following peptide bond-forming reactions were regularly monitored by ninhydrin and/or trinitrobenzene sulphonic acid colour reactions on withdrawn resin samples. Thus some picture of relative reactivities could be obtained within the limitations of these qualitative tests and the time intervals employed.



(6)

The first coupling, Fmoc-asparagine pentafluorphenyl ester to glycyl resin, proceded smoothly without catalysis. It was evidently complete at the time of the first colour tests (25min). The reaction was terminated after 50min. The sterically hindered isoleucine residue reacted more slowly as expected. It was incomplete after 49 min, and at 60 min catalyst 1-hydroxybenzotriazole was added. A very weakly positive test was obtained after a further 45 min and the reaction was terminated after a total reaction period of 3 hours. The

* Interestingly, (4) can now be prepared using dimethylformamide as solvent in polystyrene-based solid phase synthesis,[15] confirming our view[16,17] that inadequate solvation of the peptide chain by less polar media (eg dichloromethane) can be a cause of difficulty in conventional solid phase synthesis.

following residue (O-t-butyl-tyrosine) reacted completely at the time of the first test (25 min). This general pattern of reactivity was followed by the succeeding residues, with the second isoleucine requiring catalysis and an extended reaction period (120 min), whereas t-butyl aspartate and the two alanines were all apparently complete without catalysis at the first colour test (10, 13, and 5 min respectively). The penultimate glutamine gave an anomalous colour test after 125 min with occasional coloured beads in a generally colourless bulk background. Hydroxybenzotriazole was added and the acylation was complete at the next test 35 min later. The total reaction period was 193 min. Incorporation of the final valine residue was far advanced after 25 min but previous experience had indicated sluggish completion of this coupling and the reaction was left overnight without catalyst.

After cleavage of the final fluorenylmethoxycarbonyl protecting group, the completed decapeptide (96%) was detached from the resin using 95% aqueous trifluoroacetic acid in the usual manner. Side chain protecting groups were cleaved simultaneously. The crude decapeptide was characterised by amino-acid analysis and by analytical hplc (Fig. 1). It was of outstanding quality.

The second illustrative synthesis described here is of the dodecapeptide sequence (5) related to the C-terminus of penicillinase. This was carried out using the kieselguhr-supported polydimethylacrylamide resin in the continuous flow variant of the Fmoc-polyamide technique.[18] By this time the versatility of pentafluorophenyl ester derivatives had been further recognised, and they were now used at every stage in the synthesis. This included attachment of the internal reference amino-acid (norleucine) to the amino-polymer, addition of the reversible linkage agent (6, R=OC_6F_5), and transesterification of the first, carboxy-terminal amino-acid (tryptophan) of the sequence to this linkage agent. This last reaction is of special note since it establishes the value of pentafluorophenyl esters in ester-forming reactions. In the presence of catalyst 4-dimethylaminopyridine, attachment of Fmoc-tryptophan was 75% complete after 1 hr, and 92% after overnight reaction. The dipeptide content[2] at this stage was about 1%. Reaction of a small sample of the deprotected tryptophyl-resin with excess Boc-L-leucine p-nitrophenyl ester, Boc group cleavage and resin detachment with trifluoroacetic acid, and separation of diasteroisomeric dipeptides by hplc* gave only a very small peak coincident with separately prepared D-leucyl-L-tryptophan which could not be quantified. Thus racemisation in the base-catalysed transesterification was not serious.[19] Although we have not examined the full range of amino-acid pentafluorophenyl

* This simple procedure for determination of optical purity of the C-terminal amino-acid in solid phase synthesis was developed in our laboratory by Dr. P. Goddard, and will be described in detail in a future publication.

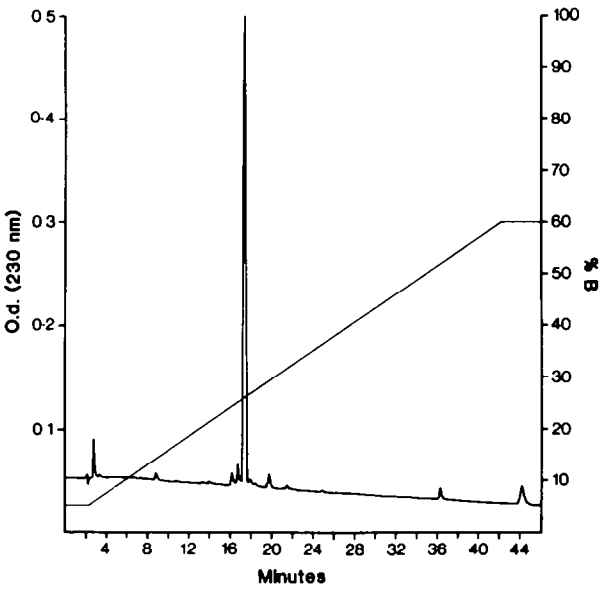


Fig. 1. Analytical h.p.l.c. of total crude decapeptide on Aquapore RP-300. Reservoir A contained 0.1% aq. trifluoroacetic acid; B contained 90% acetonitrile, 10% A. After 2 min elution with 5% B, a linear gradient of 5-60% B was developed over 40 min at a flow rate of 1.5 ml/min.

esters or linkage agents, this procedure seems to have some generality for initiating solid phase synthesis. The generally lower reactivity of these esters compared with previously used symmetric anhydrides does suggest caution, however, particularly with the more sterically hindered amino-acids. In difficult cases we would continue to recommend use of anhydrides for this key step.* The obvious importance of avoiding contamination with water in this particular step (compared with peptide bond-forming reactions) should also be stressed.

Three equivalents of Fmoc-amino-acid pentafluorophenyl ester derivatives were used in the chain extensions. Up to residue 8 (glycine), all acylations were complete at the time of the first colour test (20min) apart from serine-6 (60min). At this stage the acylation reactions became noticably slower, and subsequently catalyst hydroxybenzotriazole was added. Coupling of the last

* Experiments now in progress suggest that esters of type 3 may be particularly useful in esterification reactions.

residue was repeated since a marginally positive colour test was initially obtained. The crude peptide was detached from the resin with trifluoroacetic acid in the presence of ethanedithiol t-butyl cation-scavenger. In contrast to the foregoing synthesis, only 42% of the peptide was cleaved from the resin and only 37% of crude peptide was obtained, though this was of good quality (fig. 2) for a sequence containg both tryptophan and histidine. It seems probable that this low yield is due not to incomplete cleavage of the p-alkoxybenzyl ester bond, but to re-addition of the intermediate benzyl cation to the indole nucleus. In agreement, no further peptide was released from the resin on repeated treatment with trifluoroacetic acid. The C-terminal position of the tryptophan residue could favour the intramolecular process depicted in (7). The free peptide was purified by ion-exchange chromatography (72% of the material applied to the column was recovered in the main peak, hplc fig 3) and characterised by amino-acid analysis.

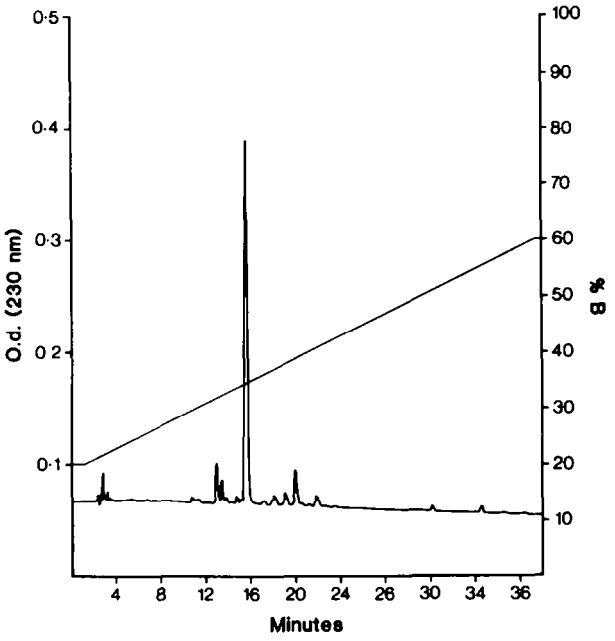


Fig. 2. Analytical h.p.l.c. of total crude dodecapeptide (5). Conditions as fig. 1 except that a linear gradient of 20-60% B was developed over 36 min.

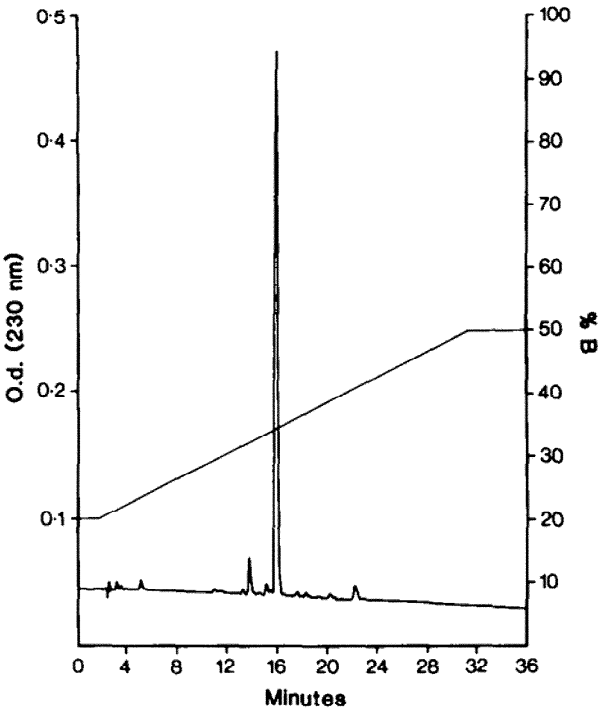
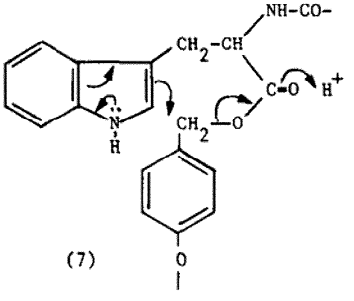


Fig. 3. Analytical h.p.l.c. of dodecapeptide (5) after ion-exchange chromatography. Conditions as fig. 1 except that a linear gradient of 20-50% B was developed over 30 min.



(7)

For this synthesis, the novel histidine derivative Fmoc-His(Boc)-OPfp was used. Each deprotection step was monitored for release of dibenzofulvene-piperidine adduct spectrometrically. No substantial increase was noted as the chain length increased, as would be expected from progressive loss of the im-Boc protecting group and re-acylation of the imidazole ring with each Fmoc-amino-acid in turn. This result contrasts with earlier investigations using symmetric anhydride intermediates. Loss of the im-Boc group may take

place in both deprotection and acylation steps, and in the latter may be related to the reactivity of the particular acylating agent. We expect to discuss the general problem of im-protecting group stability in Fmoc-histidine derivatives in a future publication.[13]

We conclude that pentafluorophenyl esters of Fmoc-amino-acids are useful intermediates in Fmoc-polyamide solid phase peptide synthesis. They are easily prepared, and with the present exception of the t-butyl esters of serine and threonine and the methoxytrimethylbenzenesulphonyl derivative of arginine, are well defined, stable crystalline solids. In polar dimethylformamide solution they have generally adequate reactivity in acylation reactions. In amide bond formation, this reactivity may be further enhanced by addition of hydroxybenzotriazole catalyst. Transesterification catalysed by 4-dimethylaminopyridine offers promise as a particularly simple method for attachment of the first amino-acid residue, at least for unhindered residues.

Use of pentafluorophenyl esters in conjunction with the continuous flow solid phase technique allows particularly simple instrumentation to be developed[20] for sequential addition of successive amino-acid residues. On the other hand, the additional ultra violet absorption introduced by the aryl ester (and particularly by hydroxybenzotriazole or similar catalysts if used) complicates and reduces the value of spectrometric monitoring[18] of the recirculating stream during acylation (but not deprotection) reactions. Recourse to qualitative ninhydrin or trinitrobenzene sulphonic acid tests is still to be commended. The general problem of full automation of peptide synthesis with feedback control is now being addressed through an alternative series of active esters.[8]

EXPERIMENTAL

Fluorenylmethoxycarbonyl-L-leucine Pentafluorophenyl Ester. Fluorenylmethoxycarbonyl-L-leucine (10.6g, 30mmole) was dissolved in dioxan (40ml) and a solution of pentafluorophenol (6.07g, 33mmole) in dioxan (10ml) added. The solution was stirred and cooled in an ice bath and dicyclohexylcarbodiimide (6.81g, 33mmole) in dioxan (15ml) added. The mixture became very viscous and was diluted with further dioxan (55ml). It was stirred for 60min in the ice bath and a further 3h at room temperature. Thin layer chromatography ($CHCl_3$, MeOH, AcOH, 85:10:5) indicated little Fmoc-leucine remaining at this time and after a total of 4.5h. the reaction mixture was filtered and evaporated *in vacuo*. The pentafluorophenyl ester crystallised on addition of n-hexane and was collected after standing overnight, 13.5g (87%), mp 112-113°, $[\alpha]_D$ -24.2° (c,1 in chloroform), (lit.[10] mp 113-116°, $[\alpha]_D$ -25.2°). Tlc revealed the presence of trace amounts of Fmoc-leucine and dicyclohexylurea as the only contaminants.

4-Hydroxymethylphenoxyacetic Acid Pentafluorophenyl Ester. 4-Hydroxymethylphenoxyacetic acid (2.0g,10.98mmol) was dissolved in dioxan (20ml) and pentafluorophenol (2.22g,12.08mmol) added in dioxan (10ml). The mixture was stirred, cooled to ice water temperature and dicyclohexylcarbodiimide (2.49g,12.08mmol) added in dioxan (10ml). After 60 min stirring at ice temperature the cold bath was removed and the mixture allowed to warm to room temperature. After stirring for a total of $1\frac{3}{4}$ hours tlc indicated no unchanged acid remaining. After $2\frac{1}{4}$ hours the reaction mixture was filtered, the dicyclohexylurea washed with dioxan and the total filtrate evaporated under reduced pressure. The resulting yellowish oil was stirred with n-hexane for 3h and the solid obtained fitered and dried under high vacuum giving 3.7g of crude product. This was recrystallised from hexane/ether to give 2.1g (54%) mp.112-113° (Found: C,51.71;H,2.68. $C_{15}H_9O_4F_5$ requires C,51.74;H,2.61%).

Solid Phase Synthesis of Acyl Carrier Protein Residues 65-74. Copoly(dimethylacrylamide-bisacryloylethylenediamine-acryloylsarcosine methyl ester)[4] (0.5g, nominal sarcosine loading 0.30mmol/g) was shaken overnight with ethylenediamine (16ml) on a bench top shaker, and then washed with DMF (15x1min), 10% diisopropylethylamine in DMF (3x1min) and DMF (5x1min). The amino resin was acylated with the symmetrical anhydride prepared[18] from Fmoc-norleucine (0.7mmol) and dissolved in DMF (7ml). Ninhydrin[21] and trinitrobenzenesulphonic acid[22] tests after 50min indicated complete reaction and after 70min shaking the acylation was terminated by washing with DMF(5x1min). The Fmoc-norleucyl-resin was deprotected by treatment with 20% piperidine in DMF (1x3min and 1x7min) followed by washing with DMF(10x1m).

The deprotected resin was reacted with a mixture of 4-hydroxymethylphenoxyacetic acid 2,4,5-trichlorophenyl ester (0.75mmol) and 1-hydroxybenzotriazole (0.75mmol) in DMF (6ml). Ninhydrin[21] and trinitrobenzene sulphonic acid[22] colour reactions indicated complete reaction at 55min and the acylation was terminated at 70min. The resin was washed with DMF (10x1min), 10% diisopropylethylamine in DMF (3x1min) and DMF (5x1min) before esterification with the symmetrical anhydride of Fmoc-glycine (1.2mmol) in the presence of 4-dimethylaminopyridine (0.15mmol) in DMF (ca 7ml). After 15 min the resin was washed with DMF (10x1min) and acetylated with acetic anhydride (0.75mmol) and 4-dimethylaminopyridine (0.15mmol) in DMF (6ml) for 40min. Acid hydrolysis and amino-acid analysis of a resin sample deprotected and washed as above gave Gly:Nle 0.72.

The decapeptide sequence was assembled using the appropriate Fmoc-amino-acid pentafluorophenyl esters (0.75mmol) dissolved in DMF (6ml) in the following standard solid phase deprotection and acylation protocol: DMF washes (5x1min); treatment with 20% piperidine in DMF (1x3min and 1x7min); DMF

washes (10x1m); acylation reaction; DMF washes (5x1min). Colour tests were carried out during each acylation step: Asn(2) - negative 25min, acylation terminated at 50min; Ile(3) - faintly positive 49min, 1-hydroxybenzotriazole (0.75mmol) added at 60min and the reaction was terminated after 180min; Tyr(Bu^t)(4), negative 25min, acylation terminated at 60min; Asp(OBu^t)(5) - negative 10min, terminated after 50min; Ile(6) - still positive at 34min, 1-hydroxybenzotriazole (0.75mmol) added after 43min and acylation terminated after 120min; Ala(7) - negative 13min, terminated after 54min; Ala(8) - negative 5min, acylation terminated after 79min; Gln(9) - isolated coloured beads at 125min; after 140min 1-hydroxybenzotriazole (0.75mmol) added and the reaction terminated after 193min; Val(10) - after 25min the ninhydrin test was negative but a slight colouration was observed with the trinitrobenzene-sulphonic acid test; the reaction was left overnight (17h).

The final Fmoc-peptide-resin was washed with DMF (5x1m) and half the resin removed. The remainder was deprotected in the usual manner and then washed on a sintered funnel with t-amyl alcohol, acetic acid t-amyl alcohol and ether before drying in high vacuum to yield 0.231g of peptide resin (Found: 0.173mmol Gly/g; Nle, 1.39 (1); Gly, 1.00 (1); Asx, 1.89 (2); Ile, 1.75 (2); Tyr, 0.77 (1); Ala, 1.88 (2); Gly, 0.98 (1); Val, 0.98 (1)).

The amino-resin (0.062g, 10.73μmol) was treated with 95% aqueous trifluoroacetic acid (15ml) for 110min, filtered and then washed thoroughly with further aqueous trifluoroacetic acid. The combined filtrate and washings were evaporated, the residue triturated several times with ether and then dried over sodium hydroxide pellets for 5h in vacuo. The yield determined by amino-acid analysis was 9.43 umoles (91%) (Found: Gly, 1.00(1); Asx, 1.89(2); Ile, 1.80(2); Thr, 0.93(1); Ala, 1.90(2); Gly, 0.98(2); Val, 0.97(1)). The residual resin was washed with t-amyl alcohol, DMF, 10% diisopropylethylamine in DMF, DMF, and ether. (Found: Gly/Nle 0.027; cleavage yield 96.2%.)Reverse phase hplc of the crude decapeptide gave the profile shown in Fig. 1.

Synthesis of H.Leu.Ala.Glu.Leu.Gly.Ala.Ser.Leu.Leu.Lys.His.Trp.OH. For a general description of the continuous flow synthesis technique, see ref 18.

(i) Resin preparation and esterification of the first residue. The kieselguhr supported polydimethylacrylamide resin (2.5g, nominal sarcosine content 0.11mmol/g) was treated with ethylenediamine (15ml) overnight with gentle shaking for 30 sec every 10min and then filtered and washed with DMF. The resin was poured into the reaction column as a slurry and washed thoroughly by flowing DMF (3ml/min) for 60min. Fmoc.Nle.OPfp (0.39g, 0.75mmol in Dmf, 2.5ml) was added automatically in DMF and recirculated for 37min. A resin

sample gave negative ninhydrin and trinitrobenzene sulphonic acid colour tests at this stage. After 47min the resin was washed (DMF,6.5min) and then deprotected with 20% piperidine in DMF (9min). After further washing (DMF, 12min), the pentafluorophenyl ester of 4-hydroxymethylphenoxyacetic acid (0.26g, 0.75mmol in Dmf, 2.5ml) was added and recirculated for 20min (negative colour tests). After 40 min the resin was washed (Dmf, 13min) and Fmoc.Trp.OPfp (0.74g, 1.25mmol in Dmf, 2.5ml) added as above. After brief recirculation, 4-dimethylaminopyridine (31mg,0.25mmol) dissolved in the minimum volume of DMF was added manually to the top of the column and recirculation continued for 60min. After removal of a resin sample, the mixture was left recirculating overnight (16.5h), and a second sample removed. The resin was washed (DMF, 6.5min), deprotected (20% piperidine, 9min), and then acetylated by recirculating acetic anhydride (0.75mmol) in the presence of 4-dimethylaminopyridine (31mg,0.25mmol) for 15min. The resin samples removed after 60min and 16.5h reaction were separately washed on a sintered funnel with DMF and 20% piperidine in DMF, and then treated with 20% piperidine in DMF (1x3m and 1x7m periods) followed by washing with DMF, t-amyl alcohol, acetic acid, t-amyl alcohol, DMF, 10% diisopropylethylamine in DMF, DMF, ether and dried in high vacuum.

(ii) Rate of Ester Bond Formation. The resin sample taken after 60min acylation (90mg) was treated with a solution of Boc-Leu-ONp (13.8mg,39μmols) in DMF (0.3ml) for 90min (negative colour tests). The resin was then washed with DMF, t-amyl alcohol, acetic acid, t-amyl alcohol, DMF, 10% diisopropyl-ethylamine in DMF, DMF ether and dried in a high vacuum. (Found: Leu/Nle 0.75.) This is to be compared with a resin sample taken after addition of the histidine residue to the main bulk of resin (see later) (found: His/Nle 0.92).

(iii) Degree of Dipeptide Formation During Esterification. The bulk Fmoc-tryptophyl- resin was deprotected and washed in the usual manner. A sample (15mg) was acylated with Boc.Trp-ONp (16.6mg, 39μmol) in DMF (0.3ml) in the presence of 1-hydroxybenzotriazole (5.8mg,39μmol) to obtain an authentic tryptophyltryptophan standard. After 60min (negative colour tests), the resin was washed, deprotected, washed, and dried as above. A portion of this resin (13.5mg) and a sample of the precursor H.Trp-resin (13.5mg) were then treated separately with a mixture of trifluoroacetic acid, anisole, ethanedithiol (30:1:0.3 v/v/v) for 90min. The resin samples were then filtered and washed with the same mixture. The total filtrate was evaporated at reduced pressure, the residue triturated several times with ether, dissolved in water (0.5ml) and this solution washed several times with ether. Hplc (Aquapore 300 C_{18} column, pump A 0.1% trifluoroacetic acid in water; pump B 90% acetonitrile in 10%, linear gradient 10 to 50%B over 20min)of both samples together with a tryptophan standard showed the presence of 1.2% dipeptide in the 16.5h esterification resin.

iv) Degree of Racemisation. A sample (20mg) of the foregoing deprotected tryptophyl-resin (16.5h esterification) was reacted with Boc-Leu-ONp (13.8mg,39μmols) in the presence of 1-hydroxybenzotriazole (5.8mg,39μmols) in DMF (0.3ml). After 1.75h (negative colour tests) the resin was washed and the dipeptide leucyltryptophan cleaved as described above for tryptophyltryptophan. A separate sample of trytophyl-resin (20mg) was acylated in Dmf (0.5ml) with the symmetrical anhydride prepared from Boc-DL-leucine (36.2mg 156μmols) and dicyclohexylcardbodiimide (16.0mg,78μmol) in dichloromethane. After 60min (negative colour tests) the resin was washed and cleaved as above. The peptides were triturated several times with ether, dissolved in water (0.5ml) and washed with ether. Both samples were examined by hplc under the foregoing conditions. The L-L dipeptide (eluting at 29% solvent B) showed only trace contamination with L-D dipeptide (eluting at 35.5% B) which could not be quantified.

(v) Completion of the dodecapeptide assembly. The remaining amino-acids were added to the tryptophyl resin by the following general procedure: Flow DMF (2min), Fmoc-amino-acid-OPfp ester (0.75mmol) applied automatically in DMF (2.5ml) and recirculated, flow DMF (6.5m), flow 20% piperidine in DMF (9m), flow DMF (22m). O-t-Butyl or N-Boc derivatives were used for side chain protection where required, including the histidine residue. Apart from Ser(Bu^t)-6, negative colour tests were obtained after 20min in the first eight cycles, and these reactions were terminated after 40min. Fmoc-Ser(Bu^t)-OPfp was reacted for 60min. 1-Hydroxybenzotriazole (0.75mmol) was added to all subsequent couplings. Acylation times were Leu-9, 80min; Glu(OBu^t)-10, 68min; Ala-11, 40min. A questionably positive ninhydrin test was obtained after 120min reaction for the final leucine. The column was washed and the acylation repeated for 36min. The final peptide resin was deprotected and washed as above, transferred to a sintered funnel and further washed with DMF, t-amyl alcohol, acetic acid, t-amyl alcohol, ether and dried in high vacuum. Yield 2.65g Found:0.058mmol Gly/g; Nle, 1.11(1); His, 0.97(1); Lys, 1.04(1); Leu, 4.02(4); Ser, 0.78(1); Ala, 1.97(2); Gly, 1.00(1); Glu, 0.94(1)).

(vi) Cleavage and Isolation. The deprotected peptide resin (0.654g, 37.93μmol) was treated with trifluoroacetic acid/anisole/ethanedithiol (15ml/0.5ml/0.15ml) for 90min at room temperature. The resin was filtered, washed thoroughly with trifluoroacetic acid and the total filtrate evaporated under reduced pressure. The peptide was dissolved in water (30ml) and washed three times with ether. The ether layer was back extracted with water (20ml) and the combined aqueous phases freeze dried to yield 13.9μmols, 36.6% of peptide (Found: His, 1.00(1); Lys, 1.07(1); Leu, 4.04(4); Ser, 0.80(1); Ala, 1.98(2); Gly, 1.00(1); Glu, 1.00(1)). Reverse phase hplc gave the profile shown in Fig 2. The residual resin was washed

with t-amyl alcohol, DMF, 10% diisopropylethylamine in DMF, DMF, ether and dried in high vacuum. Found: Gly/Nle 0.53, 58.8% cleavage. Retreatment of this peptide resin with trifluoroacetic acid containing scavengers failed to release more peptide.

(vii) Purification. The crude dodecapeptide (13.5umols) was chromatographed on a freshly poured column (14 x 1cm diam) of Whatman CM-52 ion exchange resin which was eluted with a linear gradient of 0.01-0.05M ammonium acetate, pH 4.25-6.25. The reservoirs contained 240ml of each buffer. The eluent was monitored at 278nm and fractions (4ml) collected every 6min. the major peak comprising fractions 72-78 was collected and freeze dried, yield 9.75umols, 72% recovery (found: His 0.99 (1); Lys 0.98 (1); Leu 3.99 (4); Ser 0.79 (1); Ala 1.94 82); GLY 1.00 (1); Glu 1.04 (1). The hplc profile is shown in fig 3.

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