Peptide Synthesis. Part 12. 3,4-Dihydro-4-oxo-1,2,3-benzotriazin-3-yl Esters of Fluorenylmethoxycarbonyl Amino Acids as Self-indicating Reagents for Solid Phase Peptide Synthesis

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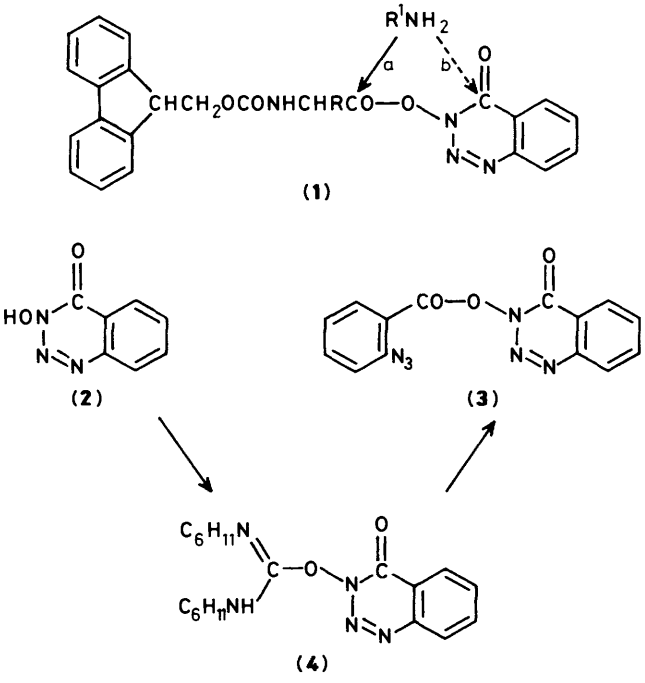
The preparation and characterisation of 3,4-dihydro-4-oxo-1,2,3-benzotriazinyl esters (**1**) of Fmoc-amino acids and their use in solid phase peptide synthesis is described. Ionisation of the liberated hydroxy component (**2**) by the starting resin-bound amine provides a useful colour indicator of the progress of acylation reactions.

Previous papers from this laboratory[1–4] have described the continuous flow Fmoc-polyamide method of solid phase peptide synthesis. In its original form[5] the Fmoc-polyamide, as well as the earlier Boc-polyamide[6] techniques, used mostly symmetrical anhydrides as the activated derivatives in coupling reactions. Although long term stability of Fmoc- and Boc-amino acid anhydrides at low temperature and in the absence of moisture has been claimed,[7] we considered that their high reactivity and the requirement in solid phase synthesis for very pure reagents demanded that the appropriate anhydride should be prepared and isolated immediately prior to each acylation reaction. Similar considerations have evidently applied in the Boc-polystyrene series.§ Therefore to facilitate further mechanisation and ultimately true automation of the continuous flow synthesis technique, we sought alternative reagents which would combine the necessary reactivity and freedom from side reactions, and yet be capable of bulk preparation beforehand, long term storage, and of being easily dispensed in a stable, solid form.

Fmoc-amino acid pentafluorophenyl (Pfp) esters[8] largely met these criteria.[9,10] In recent papers we have described their successful application using both semi-automatic (single residue)[11] and fully automatic equipment.[4] The reactivity of Pfp derivatives is higher than that of most simple activated esters, and under appropriate reaction conditons is usually adequate except for particularly hindered acylations. It is further increased in the presence of the catalyst 1-hydroxybenzotriazole to a level comparable with that of symmetrical anhydrides (see below). On the other hand, the very soluble esters of Fmoc-*O*-t-butylserine and Fmoc-*O*-t-butylthreonine proved difficult to crystallise, and the additional ultraviolet absorption introduced by the pentafluorophenyl chromophore complicated the spectroscopic reaction monitoring techniques[2] which had proved useful with symmetrical anhydrides. In continuation of our search for new reagents useful in solid phase synthesis, we have reported briefly on Fmoc-amino acid esters (**1**) of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine [Dhbt-OH (**2**)].[12–14] The favourable acylating and racemisation-resistant properties of the similar esters of benzyloxycarbonylamino acids were recognised in 1970 by König and Geiger,[15] though no substantial application in peptide synthesis apparently ensued. Probably



this was because of the reported formation of the *o*-azidobenzoic acid ester (**3**) as a by-product of their preparation.[15] The activated derivative (**3**) is itself an effective acylating agent, and if present as a contaminant in amino acid derivatives, causes chain-termination in solid phase synthesis. We now find that Fmoc-amino acid esters (**1**) (Dhbt esters) are easily prepared free of (**3**), and that they possess special properties which make them exceptionally valuable in solid phase peptide synthesis.

Since our original publications,[12–14] Geiger and his colleagues have confirmed their ease of preparation and use in synthesis.[16]

Our early preparative experiments in which the Fmoc-amino acid, triazine derivative (**2**), and dicyclohexylcarbodi-imide were simply mixed in tetrahydrofuran, dioxane, or dimethylformamide (*cf.* ref. 15), confirmed that the desired Dhbt ester was indeed frequently accompanied by small amounts of the azidobenzoate (**3**). The latter could easily be detected by h.p.l.c. and recognised by the characteristic azide absorption at 2 120 cm^{-1} in the infrared spectrum and by comparison with an authentic sample, itself readily prepared by direct reaction between two equivalents of (**2**) and dicyclohexylcarbodi-

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§ As, for example, in the technique utilised in the Applied Biosystems Model 403A Peptide Synthesiser.

Table.

Compound	Crystallisation solvent	Yield (%)	M.p. (°C)	$[\alpha]_D^{25}$ (in DMF)	Composition	Found (%) (requires) C	H	N
Fmoc-Ala-ODhbt	Dioxane–diethyl ether	91	156—158	−137.0	$C_{25}H_{20}N_4O_5$	66.05 (65.78)	4.65 (4.42)	12.0 (12.27)
Fmoc-Arg(Mtr)-ODhbt	Dichloromethane–light petroleum	84	125—130	−58.8	$C_{38}H_{39}N_7O_8S$	60.2 (60.55)	5.7 (5.22)	12.55 (13.00)
Fmoc-Asn-ODhbt	DMF–diethyl ether	86	129—132	−57.4	$C_{26}H_{21}N_5O_6$	62.4 (62.52)	4.5 (4.24)	13.6 (14.02)
Fmoc-Asp(OBu^t)-ODhbt	Diethyl ether–hexane	87	87—91	−77.7	$C_{30}H_{28}N_4O_7$	64.85 (64.74)	5.1 (5.07)	10.15 (10.06)
Fmoc-Cys(Acm)-ODhbt	DMF–diethyl ether	86	154—156	−103.0	$C_{28}H_{25}N_5O_6S$	60.3 (60.10)	4.55 (4.50)	12.25 (12.51)
Fmoc-Gln-ODhbt	DMF–diethyl ether	75	110—120	−85.3	$C_{27}H_{23}N_5O_6$	62.95 (63.15)	4.6 (4.51)	13.6 (13.64)
Fmoc-Glu(OBu^t)-ODhbt	Diethyl ether–hexane	89	103—107	−99.7	$C_{31}H_{30}N_4O_7$	65.0 (65.25)	5.3 (5.30)	9.8 (9.82)
Fmoc-Gly-ODhbt	Diethyl ether	85	156—159		$C_{24}H_{18}N_4O_5$	64.45 (65.16)	4.4 (4.10)	12.7 (12.66)
Fmoc-His(Boc)-ODhbt	Diethyl ether–light petroleum	91	95—105	−35.8	$C_{44}H_{30}N_6O_7$	63.65 (63.66)	5.25 (4.86)	13.3 (13.50)
Fmoc-Ile-ODhbt	Dioxane–diethyl ether	83	122—125	−111.0	$C_{28}H_{26}N_4O_5$	67.2 (67.44)	5.4 (5.26)	11.1 (11.24)
Fmoc-Leu-ODhbt	Diethyl ether–hexane	80	83—84	−116.0	$C_{28}H_{26}N_4O_5$	67.2 (67.46)	5.5 (5.26)	11.15 (11.24)
Fmoc-Lys(Boc)-ODhbt	Diethyl ether	89	158—162	−85.2	$C_{33}H_{35}N_5O_7$	64.3 (64.59)	5.9 (5.75)	11.35 (11.41)
Fmoc-Met-ODhbt	Diethyl ether	95	136—138	−107.0	$C_{27}H_{24}N_4O_5S$	63.0 (62.78)	4.7 (4.68)	10.85 (10.84)
Fmoc-Phe-ODhbt	Dichloromethane–hexane	94	194—199	−110.9	$C_{31}H_{24}N_4O_5$	70.3 (70.58)	4.55 (4.44)	10.15 (10.29)
Fmoc-Pro-ODhbt	Diethyl ether	94	167—169	−155.5	$C_{27}H_{22}N_4O_5$	67.05 (67.21)	4.85 (4.40)	11.45 (11.61)
Fmoc-Ser(OBu^t)-ODhbt	Diethyl ether–hexane	96	65—75	−29.3	$C_{29}H_{28}N_4O_6$	66.7 (65.90)	5.8 (5.34)	10.15 (10.59)
Fmoc-Thr(OBu^t)-ODhbt	Diethyl ether–hexane	96	65—73	−32.2	$C_{30}H_{30}N_4O_6$	67.1 (66.41)	6.1 (5.57)	9.5 (10.33)
Fmoc-Trp-ODhbt	THF–diethyl ether	88	144—146	−79.6	$C_{33}H_{25}N_5O_5$	69.4 (69.34)	4.4 (4.41)	12.15 (12.25)
Fmoc-Tyr(OBu^t)-ODhbt	Diethyl ether–hexane	97	91—96	−85.3	$C_{31}H_{24}N_4O_6$	69.7 (69.52)	5.5 (5.33)	9.35 (9.27)
Fmoc-Val-ODhbt	Diethyl ether	80	116—118	−125.5	$C_{27}H_{24}N_4O_5$	67.0 (66.90)	5.1 (4.99)	11.4 (11.56)
4-Hydroxymethylphenoxyacetyl-ODhbt	Diethyl ether	94	98—99		$C_{16}H_{13}N_3O_5$	58.65 (58.71)	4.05 (4.00)	12.6 (12.84)
4-Chloromethylphenoxyacetyl-ODhbt	THF–diethyl ether	77	154—155		$C_{16}H_{12}N_3ClO_4$, $\frac{1}{2}$ Et_2O	57.05 (56.70)	4.1 (4.47)	11.2 (10.99)
4-Hydroxymethyl-3-methoxyphenoxy-acetyl-ODhbt	Diethyl ether	74	101—110		$C_{17}H_{16}N_3O_6$	57.00 (56.98)	4.55 (4.50)	11.3 (11.73)

imide.[15] Its formation clearly involves initial activation of the triazine (**2**) by reaction with carbodi-imide,* followed by attack at the ring carbonyl of the product (**4**) by a second molecule of triazine. Thus formation of contaminating azidobenzoate during preparation of Fmoc-amino acid Dhbt esters (**1**) is a consequence of initial competition between the carboxylic acid and triazine (**2**) for carbodi-imide. In agreement, we found that when the preparative reaction was carried out with prior mixing of Fmoc-amino acid and carbodi-imide in tetrahydrofuran at low temperature and with subsequent addition of the triazine formation of azidobenzoate was effectively abolished.

A general procedure for the preparation of Fmoc-amino acid esters (**1**) is given in the Experimental section, together with some illustrative examples. Amino acids with functional side chains which may interact with the activated ester grouping (*e.g.* asparagine, glutamine, methoxytrimethylphenylsulphonyl-arginine) are best prepared at very low temperature (−30 to −50 °C), and some (asparagine, glutamine) require a better solvent (dimethylformamide) than tetrahydrofuran. In the case of Fmoc-asparagine, use of cold dimethylformamide as the solvent effectively suppresses the formation of a β-cyanoalanine derivative which was observed (by t.l.c.) in reactions carried out in dioxane. The Dhbt esters of the peptide–resin linkage agents, 4-hydroxymethylphenoxyacetic acid,[18] 4-chloromethylphenoxyacetic acid,[19] and 4-hydroxymethyl-3-methoxyphenoxyacetic acid,[18] were also prepared. Use of these derivatives in initial resin functionalisation allows this step also to be followed by the new monitoring procedure described below.

Analytical data for a range of Dhbt esters are collected in the Table. They have thus far proved stable to storage in the solid state at low temperature (−30 °C). In solution, the N_G-

* Thus procedures for the preparation of protected amino acid Dhbt esters involving deliberate prior activation of (**2**) are unlikely to be successful. We and others[17] have found this to be the case for the mixed 1,2,2,2-tetrachloroethyl carbonate.

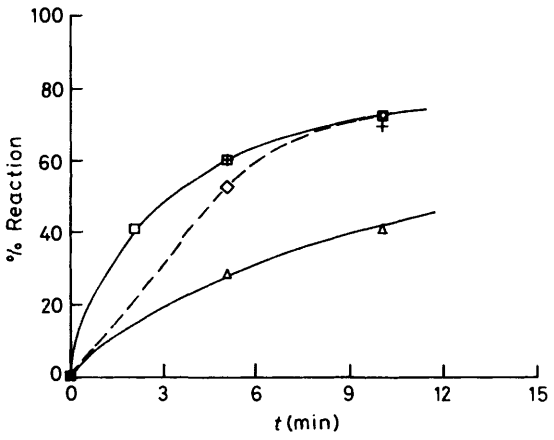

Figure 1. Relative rates of reaction of activated Fmoc-isoleucine derivatives with glycyl resin: +, Dhbt ester; □, symmetric anhydride; △, pentafluorophenyl ester; ◇, pentafluorophenyl ester in the presence of 1-hydroxybenzotriazole. For conditions see the Experimental section

methoxytrimethylphenylsulphonylarginine derivative is slowly converted into its lactam with a half life of *ca.* 10 h in dimethylformamide.[20] It is apparently very much less stable in dichloromethane ($t_{\frac{1}{2}}$ 15 min).[20] The lactam does not interfere with peptide bond formation by the Dhbt ester itself.

The relative reactivity of Fmoc-amino acid Dhbt esters in Fmoc-polyamide solid phase synthesis was estimated using the isoleucine derivative. This was treated at low concentration* with glycyl-resin in dimethylformamide and the isoleucine uptake by the resin measured by amino acid analysis on withdrawn resin samples. The results for Fmoc-Ile-ODhbt, the corresponding symmetric anhydride, Pfp ester, and Pfp ester in the presence of hydroxybenzotriazole catalyst, are shown in Figure 1. The Dhbt ester and symmetric anhydride react at comparable rates under these conditions with $t_{\frac{1}{2}}$ *ca.* one fifth of that of the Pfp ester. After an initial lag period, the Pfp ester is accelerated in the presence of hydroxybenzotriazole to a rate again comparable with that of a symmetric anhydride.

In contrast to the above, Dhbt esters (**1**) were found to be much less effective in 4-dimethylaminopyridine-catalysed ester bond forming reactions than the corresponding pentafluorophenyl esters. Thus, Fmoc-Gly-ODhbt reacted five times more slowly with 4-hydroxymethylphenoxyacetylated polydimethylacrylamide resin in the presence of 4-dimethylaminopyridine than did the corresponding Pfp ester, and overnight reaction was necessary to ensure complete esterification. Thus Dhbt esters are not especially suitable for attachment of the first amino acid in solid phase synthesis.

As expected from the early work of Geiger and his colleagues,[21] the Dhbt esters show excellent optical stability and resistance to racemisation under peptide bond forming conditions. The optical rotation of Fmoc-Asp(OBu^t)-ODhbt in pure dimethylformamide was nearly unchanged over a 3.5 h period. In the presence of 1% ethyldi-isopropylamine, some 20% D-isomer was generated during the same period. In Fmoc-polyamide solid phase synthesis, optical purity was investigated by preparation of the tripeptide Fmoc-L-Ser(Bu^t)-L-Leu-Gly-OH using Fmoc-L-Ser(Bu^t)-ODhbt and the similar leucine derivative. Separation of the diastereoisomeric tripeptides by h.p.l.c. (an authentic mixture was prepared using the DL-leucine derivative) showed that racemisation was less than 0.5% over the two coupling reactions.

The formation of the azidobenzoyl derivative (**3**) during the preparation of Dhbt esters (**1**) raises the possibility of similar attack at the ring carbonyl in (**1**) (arrow b) competing with peptide bond formation (arrow a). If such a side reaction occurred, it would generate a blocked azidobenzoyl peptide. The situation is somewhat analogous to peptide synthesis using unsymmetrical anhydrides where similar attack at two different carbonyl groups is theoretically possible. Although there had been no indications that this occurred with Dhbt esters to a detectable extent, it constitutes such a potentially serious side reaction in solid phase synthesis that we thought it worthy of special investigation.

Essentially the Fmoc-amino acid residue in ester (**1**) would be acting as a leaving group, imparting special reactivity to the ring carbonyl. As a stringent test case we studied the reaction of glycyl-resin with highly purified Fmoc-isoleucine Dhbt ester (**1**; R = isobutyl). Activated isoleucine derivatives are recognised as amongst the most sterically hindered in peptide synthesis, so that attack at the normal carbonyl [(**1**); arrow a] is disfavoured relative to abnormal attack at the ring carbonyl [(**1**); arrow b]. An authentic sample of azidobenzoylglycine was prepared by reaction of the same glycyl-resin with the Dhbt ester (**3**) and cleavage from the resin in the usual manner. In the event, h.p.l.c. separation of products from the reaction of Fmoc-isoleucine Dhbt ester with glycyl resin gave the expected isoleucylglycine together with a later emerging, minor but distinct peak coincident with azidobenzoylglycine. The test is a very sensitive one because the extinction coefficients of the major and minor products differ twenty-fold at 230 nm in favour of the minor component; allowing for this difference, the proportion of azidobenzoylglycine was estimated as less than 0.1%. This is a worst case result and most other couplings should be even more favourable.

From the beginning of our work on Dhbt esters, we observed that a transient bright yellow colour was produced during acylation reactions. In solid phase synthesis the yellow colour developed on the resin support and the solution remained colourless in the absence of dissolved base. When the acylation reaction was complete as judged by the Kaiser ninhydrin test[11] for residual amino groups, the resin had faded to a very pale yellow or cream colour, and when washed returned close to its initial off white shade.

We attributed this colouration to ionisation of the liberated hydroxy component (**2**) by residual resin-bound amino groups, and this was readily confirmed by the similar colours produced when (**2**) alone was treated with a variety of basic reagents. Thus, the Dhbt ester (**1**) provides not only an efficient acylating agent but also a sensitive indicator (**1**) for residual unchanged amine. This is an important observation because it provides, for the first time, a totally non-invasive method for following the progress of solid phase synthesis on the solid phase and while it is actually proceeding. No withdrawal of resin samples is required and no external reagents are added which might adversely affect peptide bond formation.

We have now extended these initial observations by comparing the colour produced at every stage during a 19-residue continuous-flow solid phase synthesis with that of the conventional ninhydrin[22] and trinitrobenzenesulphonic acid tests.[23] Chemical aspects of this synthesis of the sequence (**6**) are described in detail below. In every step the yellow colour of the column developed immediately the Dhbt ester was introduced and then faded rapidly, usually within 10—20 min. Two residues in the middle of the sequence coupled particularly slowly and required extended acylation periods. This was clearly indicated by the slow fading of the column colour and use of this observation alone would have prevented failure of the synthesis

* Under normal solid phase synthesis reaction conditions, acylation is too fast for ease of analysis.

through premature termination. In experienced hands, the Dhbt resin colouration provides an indicator of completeness of acylation at least as sensitive as the chemical tests on withdrawn resin samples (see the Experimental section). The end point is appreciably more difficult to detect if the recirculating solution also acquires a yellow colour due to traces of residual piperidine. This did not occur in the present case, but care should be taken with the design of column end pieces and connections to avoid liquid hold up, and with the washing of lines and valves after the deprotection step.

We reserve further general discussion of the self-indicating properties of Dhbt esters because we have recently[13] constructed a photometric system which allows quantitative monitoring and feed back control of solid phase synthesis using this principle.* We expect to describe this in detail shortly. However, it should be pointed out now that the reagent (**2**) also provides a potential indicator for monitoring peptide synthesis under a wide range of conditions, not necessarily just those involving Dhbt esters (**1**) as the prime acylating agents and not only in the solid phase techniques. Thus addition of small amounts of the triazine (**2**) to peptide bond-forming reactions utilising preformed esters of 1-hydroxybenzotriazole has provided a useful internal indicator for completion of acylation.[24] The free hydroxybenzotriazole liberated during the reaction apparently does not suppress the ionisation of (**2**) and does not quench the colour prematurely.[24] Other coupling methods (*e.g.* symmetrical anhydride and activated ester) which similarly liberate weakly acidic species during acylation will require checking beforehand. Caution may be necessary in activating circumstances where conversion of (**2**) into (**3**) is possible. In our experience, the hydroxybenzotriazine (**2**) is not a good general additive compared with 1-hydroxybenzotriazole for *in situ* activation and coupling reactions involving dicyclohexylcarbodi-imide, at least in the case of side-chain unprotected arginine-containing peptides.[25] Probably this is due to the formation of *o*-azidobenzoyl-peptide contaminants. Depending as it does on the acid-base relationship of the amino component and hydroxytriazine, the indicating properties of (**2**) may also be solvent dependent. We have not examined its value under the more apolar conditions of the conventional Merrifield synthesis.

H-Val-Gl-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-OH
(**5**)

H-Asp-Asp-Glu-Val-Asp-Val-Asp-Gly-Thr-Val-
Glu-Glu-Asp-Leu-Gly-Lys-Ser-Tyr-Gly-OH
(**6**)

We have already reported[12–14] briefly the solid phase synthesis of a number of peptides using Dhbt esters as the acylating species, particularly of our standard test case, the acyl carrier protein decapeptide sequence 65—74 (**5**). The h.p.l.c. profile of the crude decapeptide has been published[12] and indicated a product of outstanding quality. As a further general illustration of the value of Fmoc-amino acid Dhbt esters, we describe now the recent nonadecapeptide (**6**) synthesis referred to above. The first 17 residues of this sequence are part of the protein endoplasmin.

The sequence was assembled using the continuous flow variant[2,4] of the Fmoc-polyamide technique. The automatic continuous flow synthesizer already described[4] was used in single residue mode, permitting withdrawal of resin samples for ninhydrin and trinitrobenzenesulphonic acid colour tests at operator selected time intervals during the acylation period. Polydimethylacrylamide resin supported in macroporous Keiselguhr,[2,26] previously functionalised with an internal reference norleucine residue and the acid-labile linkage agent (**7**) was used. Cleavage of Fmoc-groups was monitored by u.v. absorption. Full details of the synthesis are given in the Experimental section, and we comment now only on points of special interest.

The assembly using Fmoc-amino acid Dhbt esters proceeded uneventfully for the first 9 residues. Apart from two occasions when single coloured beads were observed in a colourless bulk background (this anomalous behaviour has been observed several times before[9]), negative tests for residual amine were obtained at the first colour tests. These were carried out when the resin column colour had faded to a barely detectable pale yellow or cream shade, between 9 and 17 min after sample application. Acylation reactions were terminated as convenient after the results of the colour tests were available (see the Experimental section). The resin colour faded very much more slowly for the tenth residue (valine), with traces of unchanged amine possibly present after 1 h. Although the molar excess of Dhbt ester was increased from two- to four-fold thereafter, the following residue (*O*-t-butylthreonine) behaved similarly. Deprotection reactions at both steps gave atypical u.v. absorbance profiles characteristic of slow, highly hindered protecting group release.[2] The synthesis reverted to near normal for the next residue (glycine), and although there was the usual general slowing as chain extension proceeded, the synthesis was completed without difficulty. No repeated acylation reactions were required.

It is likely that some form of aggregation of the peptide chains occurred within the resin matrix at the 10—11 residue stage, but that this subsequently dispersed. We have previously drawn attention[27] to the possible significance of long runs of highly hydrophobic (protected) amino acid residues in this connection, as in residues 6—11 in sequence (**6**). However the following sequence 13—19 is similarly constituted, including two more intrinsically hindered valine residues, and did not show this effect. Our general experience now suggests that difficulties in solid phase synthesis are most likely to occur in this medium-length region if a substantial run of apolar side chains is present, but that intervening glycine and probably proline residues which may disrupt secondary structures are beneficial.

Conclusions of this sort may be important in view of attempts to construct 'intelligent' controlling programs for automatic peptide synthesizers which are able to anticipate difficulties. If such programs can be constructed they will have to take into account substantial sequence data rather than just individual or pairs of adjacent amino acid residues.

In our view, a more promising approach is to make fuller use of real-time quantitative-monitoring devices which provide data about reaction speed and completion as the reactions are actually proceeding. In the synthesis above, the controlling computer recognised the deprotection reactions at residues 10 and 11 as being abnormally slow and possibly incomplete during the monitoring period (6 min). Both were repeated after completion of the normal full 10 min deprotection, though neither then provided evidence for further release of fluorene derivatives. Automatic monitoring of acylation reactions using Dhbt esters is now possible,[13] and we are presently gaining experience on the value of this and of feed-back control in routine and investigative synthesis.

The h.p.l.c. profile of total crude nonadecapeptide (**6**) released from the resin by trifluoroacetic acid is shown in Figure 2(*a*). This is a very satisfactory result for a synthesis of this length and strengthens our confidence that Fmoc-amino acid Dhbt esters are likely to prove particularly valuable intermediates in solid phase synthesis. An excellent amino acid analysis (Experimental section) was obtained for the principal product (peak A) separated by h.p.l.c. [Figure 2(*b*)]. The major contaminant

* Patent applied for.

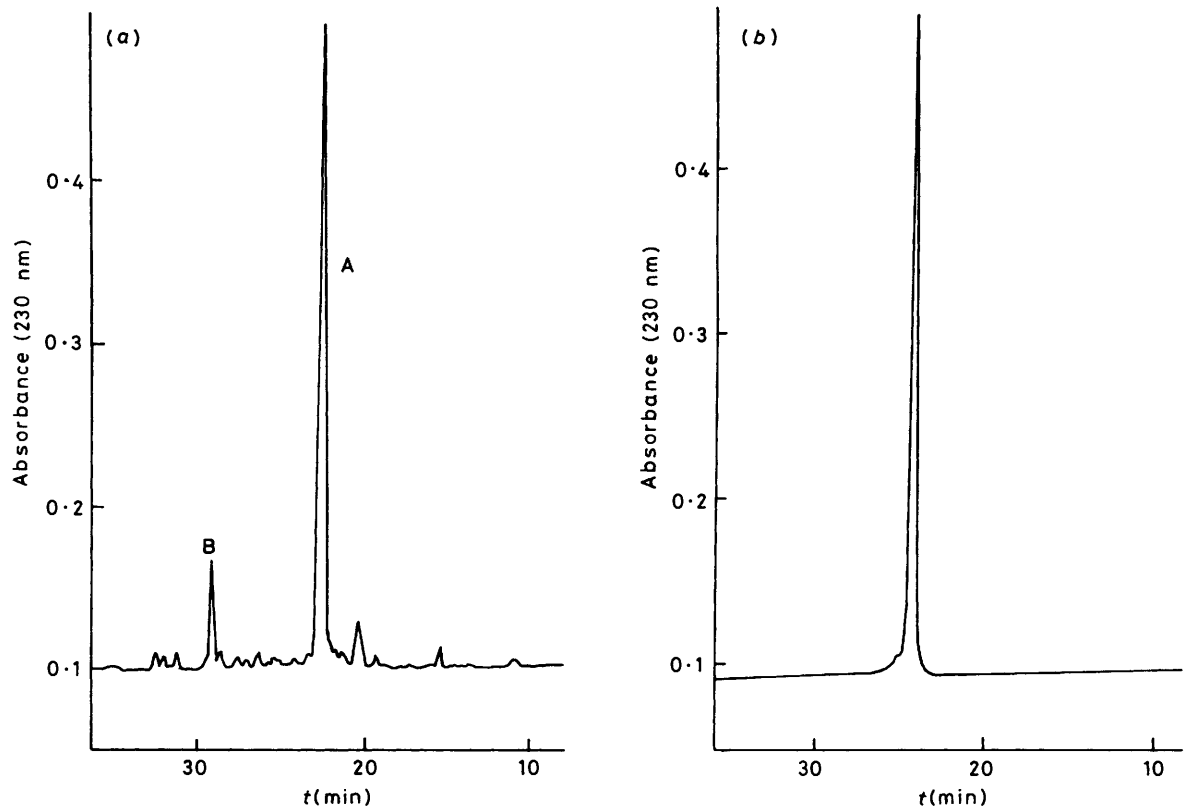

Figure 2. H.p.l.c. of (*a*) total crude product and (*b*) purified product from the synthesis of the nonadecapeptide (**6**). *Conditions:* Aquapore RP300 columns eluted isocratically with 10% buffer B in A (see the Experimental section) for 2 min and then with a linear gradient of 10—40% B developed over 45 min. The effluent was monitored at 230 nm. Different columns were used for the two experiments

(peak B) was also collected, though amino acid analysis failed to identify it as a single product of amino acid omission or insertion, or as a truncated derivative. Notwithstanding its sharp elution on h.p.l.c., peak B probably constitutes a mixture of artefact derivatives.

Experimental

General procedures for the purification of solvents and reagents have been described previously,[2] and were rigorously applied. Dioxane and tetrahydrofuran were passed through columns of basic alumina prior to use. Fmoc-amino acids were purchased from Cambridge Research Biochemicals or from Bachem (Switzerland), and were checked for purity and identity (h.p.l.c. and m.p.) before use. 3,4-Dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH, Fluka) was recrystallised from acetonitrile (1 g/40 ml) or from ethanol–water (1:4; 1 g/5 ml) and the product dried *in vacuo* over phosphorus pentaoxide. Melting points are uncorrected. Microanalyses were generously carried out by the staff of the University Chemical Laboratory, Cambridge.

The Fmoc group was removed from resin samples before they were subjected to hydrolysis with redistilled 6M hydrochloric acid containing phenol (*ca.* 5 mg mol^{-1}) at 110 °C for 18 h. Hydrolysates were analysed on a Beckman Amino Acid Analyser Model 119CL or 7300. High performance liquid chromatography was carried out on analytical Aquapore RP300 (25 cm × 0.4 cm diam.) columns using the following buffer systems: A, 0.1% trifluoracetic acid, pH 2.0; B: 10% buffer A in acetonitrile. All the active ester derivatives were analysed using a linear gradient of 0—100% B in 40 min at a flow rate of 1.5 ml min^{-1}.

4-Hydroxymethylphenoxyacetic acid is abbreviated as HMPA and is treated in formulae as equivalent to an amino acid residue, *e.g.* H-Gly-HMPA-Nle-resin is glycyloxymethylphenoxyacetyl-norleucyl-resin, or 'glycyl resin'.

General Procedure for the Preparation of Dhbt Esters.—*Example A: Fmoc-Gly-ODhbt.* Fmoc-Gly-OH (1.78 g, 6 mmol) was dissolved in THF (20 ml) and the solution cooled to −15 °C. Dicyclohexylcarbodi-imide (DCCI) (1.24 g, 6 mmol) was added and the mixture was stirred at −15 °C for 5 min. Solid Dhbt-OH (0.978 g, 6 mmol) was added and the mixture was stirred at −10 °C for 30 min, then at 0 °C for 4 h, and then allowed to stand overnight* at 0 °C. Precipitated dicyclohexylurea was removed by filtration and the filtrate was concentrated under reduced pressure. The resulting syrup was crystallised from diethyl ether and dried over phosphorus pentaoxide to give *the triazinyl ester* (2.48 g, 93%), m.p. 156—159 °C.

Example B: Fmoc-Lys(Boc)-ODhbt. Fmoc-Lys(Boc)-OH (11.68 g, 25 mmol) was dissolved in dry THF (60 ml) and cooled to −15 °C. DCCI (5.15 g, 25 mmol) dissolved in THF (6 ml) was added and after 5 min at −15 °C, a solid Dhbt-OH (4.0 g, 25 mmol) was rinsed in with THF (5 ml). The mixture was stirred at −15 °C for 20 min and then at 4 °C (cold room) for 3.5 h. The mixture was filtered and the solvent was removed under reduced pressure. The residue was dissolved in ether (100 ml) and hexane (100 ml) was added and the solution stored at 4 °C overnight. The white solid was collected by filtration, washed with light petroleum (b.p. 40—60 °C) and dried (silica) *in vacuo* to give the *ester* (14.5 g, 95%, m.p. 157—159 °C).

* Shorter reaction periods as in example B are now known to be adequate.

Fmoc-Asn-ODhbt. Fmoc-Asn-OH (3.9 g, 11 mol) was dissolved in DMF (43 ml) with gentle warming and the solution was then cooled to −50 °C. Dhbt-OH (1.97 g, 12 mmol), dissolved in DMF (7 ml) was added, followed by solid DCCI (2.25 g, 10.9 mmol) and the mixture was allowed to warm to 0 °C over a period of 1 h. The mixture was stirred at 0 °C for 1 h and left in the refrigerator overnight. The precipitate was removed by filtration and the DMF was evaporated at 0.05 mmHg and 15 °C until the product started to crystallise. Dry diethyl ether (125 ml) was added and stirring was continued for 1 h at 0 °C. The product was collected, washed with diethyl ether and hexane, and dried *in vacuo* to give the crude ester (5.76 g). This material (5.00 g) was dissolved in dry DMF (25 ml) and filtered to remove residual dicyclohexylurea. Dry diethyl ether (300 ml) was added and the mixture was left overnight in the refrigerator to crystallise. The product was filtered off, washed with diethyl ether and dried to afford the *triazinyl ester* (4.07 g, 86%), m.p. 129—132 °C. The analogous glutamine derivative was prepared in a similar way.

Fmoc-Arg(Mtr)-ODhbt. Fmoc-Arg(Mtr)-OH (4.87 g, 8 mmol) was dissolved in dry THF (10 ml) and the solution cooled to −30 °C. DCCI (1.64 g, 8 mmol) dissolved in THF (5 ml) was added and the mixture stirred for 5 min at −30 °C. Solid Dhbt-OH (1.30 g, 8 mmol) was added and washed in with THF (5 ml). The yellow suspension was stirred vigorously at −25 °C for 1 h and then at −10 to −5 °C (ice–salt bath) for 4 h. The reaction mixture was filtered rapidly and evaporated at 25—30 °C under high vacuum. Diethyl ether (150 ml) was added and the mixture kept overnight at −15 °C. The yellowish solid was collected, washed with diethyl ether, and dried (silica) to give the ester (6 g, 99.5%, m.p. 116—121 °C), shown by h.p.l.c. to be completely free of azide and lactam contaminants. An earlier preparation was carried out similarly except that the reaction temperature was higher (−15 °C for 2.5 h and then overnight at 0 °C). The crude product was dissolved in dichloromethane (5 ml) and added dropwise to light petroleum with stirring. The mixture was stirred at 0 °C for 2 h and the product was filtered and dried *in vacuo* to afford Fmoc-Arg(Mtr)-ODhbt (1.90 g, 84%), m.p. 125—130 °C; $[\alpha]_D^{25}$ −58.8 (*c*, 1 in DMF). Other preparations carried out at this temperature usually contained variable amounts of lactam by-product (h.p.l.c.).

Fmoc-Cys(Acm)-ODhbt. The general procedure was followed except that Fmoc-Cys(Acm)-OH was dissolved in DMF (10 ml) prior to the addition of THF. Crystallisation of the crude product from DMF and diethyl ether afforded the *triazinyl ester* (86%) m.p. 154—156 °C; $[\alpha]_D$ −103.0° (*c*, 1 in DMF).

Preparation of Fmoc-His(Boc)-ODhbt. Fmoc-His(Boc)-OH* (1.43 g, 3.00 mmol) was dissolved in the THF (3 ml), cooled to −15 °C and a solution of DCCI (618 mg, 3.00 mmol) in THF (2 ml) was added with stirring. After 4 min, solid Dhbt-OH (489 mg, 3.00 ml) was added and stirring was continued for 3.5 h at −15 °C and then at 0 °C for 4 h. The mixture was filtered and the filtrate was concentrated under reduced pressure. The resulting oil was dissolved in a mixture of THF and diethyl ether (1:4; 20 ml), filtered, and the filtrate was added dropwise to stirred light petroleum (b.p. 80—100 °C, 100 ml). After 3 h at 0 °C the solid product was collected and dried *in vacuo* over phosphorus pentaoxide, to give Fmoc-His(Boc)-ODhbt (1.70 g, 91% yield), m.p. 95—105 °C; $[\alpha]_D^{25}$ −35.8 (*c*, 1 in DMF).

4-Hydroxymethylphenoxyacetic acid Dhbt ester (HMPA-ODhbt). 4-Hydroxymethylphenoxyacetic acid (6.0 g, 32.9 mmol) was dissolved in THF (60 ml) and the solution was cooled to −15 °C. DCCI (6.79 g, 32.9 mmol) in THF (10 ml) was added with stirring. After 2 min, Dhbt-OH (5.37 g, 32.9 mmol) was added and the mixture was stirred at −15 °C for 30 min. After 16 h at 20 °C the precipitate was removed by filtration and the filtrate was concentrated under reduced pressure. The residual syrup was stirred with diethyl ether to afford *3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl 4-hydroxymethylphenoxyacetate* (10.1 g, 94%), m.p. 98—99 °C; $\nu_{max.}$ 1 823 (C=O). No absorption at 2 100 cm^{-1} could be observed indicating the absence of *o*-azidobenzoic acid Dhbt ester. 3,4-Dihydro-4-oxo-1,2,3-benzotriazin-3-yl 4-chloromethylphenoxyacetate and 3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl 4-hydroxymethyl-3-methoxyphenoxyacetate were prepared by the same procedure (Table).

Comparison of Acylation Rates for Fmoc-Isoleucine Derivatives.—The reaction between Fmoc-Ile-X (0.022 mmol, X = OPfp, ODhbt and Fmoc-Ile-O) and glycyl HMPA-Nle-resin (50 mg, 0.0055 mol) in stirred DMF (2 ml) was followed by removing resin aliquots (*ca.* 5 mg) at 2, 5, and 20 min intervals which were quenched by being washed several times with DMF (2 ml). The Pfp-ester reaction was carried out with and without the addition of HOBt catalyst (3 mg, 0.02 mmol). The resin aliquots were further washed with DMF and then treated with 20% piperidine–DMF. After successive washings with DMF, 2-methylbutan-2-ol, acetic acid, 2-methylbutan-2-ol, DMF and diethyl ether the samples were dried *in vacuo* and hydrolysed with 6M hydrochloric acid (110 °C/18 h) to give the Ile:Gly ratio by amino acid analysis. The results are presented in Figure 1.

Azidobenzoylglycine.—Fmoc-Gly-HMPA-Nle-resin (0.5 g) was deprotected by treatment with piperidine in DMF (20%, 2 × 5 min) on a sintered glass filter funnel, and then washed well with DMF (6 × 5 ml) and ether (2 × 5 ml). The dried resin was transferred to a round-bottomed flask, a solution of azidobenzoyl Dhbt ester[15] (80 mg) in DMF (2 ml) was added, and the mixture was kept for 3 h at room temperature. Ninhydrin and trinitrobenzenesulphonic acid tests for residual amine were negative. The resin was washed well with DMF (10 × 5 ml) and ether (3 × 5 ml), and dried *in vacuo*. Part of this (0.2 g) was cleaved with trifluoroacetic acid (95%; 90 min) the resin was filtered and washed with further trifluoroacetic acid, and the combined filtrates were evaporated. On h.p.l.c., the azidobenzoyl derivative eluted at 13.8 min.

Investigation of the Reaction between Fmoc-isoleucine Dhbt Ester and Glycyl Resin.—Fmoc-Gly-HMPA-Nle-resin (0.55 g) was deprotected with piperidine (20%; 2 min) and then washed with DMF (20 min) in a semi-automatic flow synthesizer.[2] The resin was treated with Fmoc-isoleucine Dhbt ester (0.15 g, 6 equiv., no contaminating azidobenzoyl Dhbt ester detected by h.p.l.c.) for 2 h (negative colour tests for residual amine), and then washed and deprotected in the usual manner.[2] Part (0.2 g) of the washed and dried isoleucylglycyl resin was cleaved with 95% trifluoroacetic acid and analysed by h.p.l.c. as in the foregoing experiment. Isoleucylglycine eluted at 10.6 min (integrated area, 85 017) and azidobenzoylglycine at 13.8 min (area, 1 701). In separate experiments, the ratio of extinction coefficients of 2-azidobenzoylglycine and isoleucylglycine at 230 nm was shown to be *ca.* 20:1.

Racemisation during Coupling of Fmoc-L-Ser(Bu^t)-ODhbt.—Fmoc-L-Ser(Bu^t)-ODhbt (26 mg, 5 equiv.) was treated with H-L-Leu-Gly-HMPA-Nle-resin (100 mg, 0.011 mmol; similarly prepared using Fmoc-L-Leu-ODhbt) in DMF (0.2 ml). The resin was washed as described above and dried *in vacuo*. A similar

* Preparative details for this compound may be obtained from Dr. J. D. Richards (Cambridge Research Biochemicals Ltd, Button End Industrial Estate, Harston, Cambridge) prior to publication.

experiment was carried out using H-DL-Leu-Gly-HMPA-Nle-resin. The protected peptides were cleaved from the resin by treatment with 95% aqueous trifluoroacetic acid (2 ml) for 1.5 h. The resulting tripeptides were analysed by h.p.l.c., using a linear gradient of 0—50% buffer B over 40 min. Fmoc-L-Ser-Leu-Gly-OH eluted at 34.7 min and the diastereoisomeric Fmoc-L-Ser-D-Leu-Gly-OH and its enantiomer at 34.0 min. The content of DL peptide in the LL preparation was estimated as <0.5% from peak height.

Transesterification 4-Hydroxymethylphenoxyacetyl-Nle-resin with Dhbt Esters.—Fmoc-Gly-ODhbt (70 mg, 0.158 mmol) was treated with H-HMPA-Nle-resin (300 mg, 0.032 mmol of hydroxy groups) by recirculation in DMF (2 ml) containing 4-dimethylaminopyridine (DMAP; 6 mg, 0.05 mmol). Resin samples were taken after 2, 5, 10, 20, 30, and 55 min, and after 24 h. These were washed, deprotected with 20% piperidine in DMF, and washed carefully as described above. The samples were dried and hydrolysed and the glycine incorporation was determined by amino acid analysis (Found: Gly : Nle: 55 min, 0.2; 24 h, 1.1). In the absence of dimethylaminopyridine, the Gly:Nle ratio was only 0.05 after 24 h.

Solid Phase Synthesis of the Nonadecapeptide (**6**).—Kieselguhr-supported polydimethylacrylamide methyl ester resin[2,26] (*ca.* 2.5 g, 0.27 mequiv.) was treated in turn with excess ethylene diamine (overnight), Fmoc-Nle-ODhbt (498 mg, 1 mmol; flow reactor,[2] ninhydrin test negative at 17 min; total reaction time 27 min), 20% piperidine in DMF (10 min, as below), and then pentafluorophenyl 4-hydroxymethylphenoxyacetate (174 mg, 0.5 mmol) in the presence of 1-hydroxybenzotriazole (68 mg, 0.5 mmol) for 1 h (negative ninhydrin test), to generate the functionalised resin in essentially the usual manner.[2]

Fmoc-glycine pentafluorophenyl ester (649 mg, 1.4 mmol) was transesterified to the resin in the presence of 4-dimethylaminopyridine (34.2 mg, 0.28 mmol) for 1 h,[11] and the reaction was repeated. Any residual hydroxy groups were then similarly acetylated using acetic anhydride (5 μl) and dimethylaminopyridine (34 mg). After thorough washing of the resin, the Fmoc group was cleaved with 20% piperidine in DMF as below.

The following sequence of operations in the semi-automatic continuous flow peptide synthesizer[11] was used for sequential addition of 18 amino acid residues: flow DMF (2 min); select sample chamber containing solid Fmoc-amino acid Dhbt ester (2 or 4 fold excess—see text); fill chamber (DMF; 0.7 min); agitate (nitrogen; 0.5 min); fill [DMF (displace nitrogen); 0.2 min]; introduce Dhbt ester solution from sample chamber (0.92 min); wash sample onto column (DMF; 0.5 min); recirculate (999 min max.; terminate manually: see below); flow DMF (6.5 min); recirculate (rinse valve; 0.5 min); flow DMF (10 min); fill (DMF; 0.2 min); agitate (nitrogen; 0.2 min) (rinse and empty sample chamber lines); automatic pause in recirculate mode and operator call before proceeding to deprotection. Flow 20% piperidine (0.5 min); collection of u.v. absorbance data (90 readings, 4 s intervals, wavelength 312 μm; 1 mm flow cell), start; flow 20% piperidine (9 min); flow DMF (10 min); recirculate (rinse valve; 0.5 min); flow DMF (15 min); cycle repeats.

Resin samples (*ca.* 20 beads) were removed from the reactor column during the main acylation period when the colour of the column was judged visually to have returned almost to its pre-acylation colour (see text). These were then washed thoroughly on a small sintered glass filter (DMF, t-pentyl alcohol, acetic acid, t-pentyl alcohol, DMF, and ether), and subjected to the ninhydrin[22] (nin) and trinitrobenzenesulphonic acid[23] (tnbsa) colour tests. Larger resin samples were also removed at intervals for subsequent amino acid analysis (see below). Acylations were terminated manually as convenient, usually shortly after negative colour tests were obtained: Tyr-2, 30 min (10 min, nin: 1 blue bead; tnbsa −ve; 19 min; nin −ve); Ser-3, 25 min (11 min; nin −ve, tnbsa −ve); Lys-4, 30 min (17 min; nin −ve, tnbsa −ve); Gly-5, 21 min (9 min; nin −ve, tnbsa −ve); Leu-6, 32 min (9 min; nin; 1 blue bead; tnbsa −ve; 19 min, nin −ve); Asp-7, 31 min (15 min; nin −ve, tnbsa −ve); Glu-8, 32 min (13 min; nin −ve, tnbsa −ve); Glu-9, 32 min (16 min; nin −ve, tnbsa −ve); Val-10, 150 min (62 min; nin and tnbsa: v. weakly +ve; 74 min, column still fading, nin −ve, tnbsa v. weakly +ve; 135 min; nin −ve, tnbsa −ve); Thr-11, 146 min (92 min; nin −ve, tnbsa −ve); Gly-12, 45 min (13 min; nin −ve, tnbsa −ve); Asp-13, 52 min (18 min; nin: 1 blue bead; tnbsa −ve; 34 min, nin −ve, tnbsa −ve); Val-14, 70 min (44 min; nin −ve, tnbsa −ve); Asp-15, 60 min (28 min; nin −ve, tnbsa: 2 coloured beads; 40 min, nin −ve, tnbsa −ve); Val-16, 51 min (33 min; nin −ve, tnbsa −ve); Glu-17, 55 min (41 min; nin −ve, tnbsa −ve); Asp-18, 80 min (66 min; nin −ve, tnbsa −ve); Asp-19, 57 min (49 min; nin −ve, tnbsa −ve).

Intermediate peptide resins (the last amino acid attached is indicated) gave the following amino acid analyses after deprotection and hydrolysis, Tyr-2: Nle, 1.00; Gly, 0.96; Tyr, 0.93. Gly-5: Gly, 2.16; Tyr, 1.04; Ser, 0.92; Lys, 1.00. Glu-8; Gly, 2.17; Tyr, 1.03; Ser, 0.94; Lys, 1.00; Leu, 0.95; Asp, 0.98; Glu, 0.98. Gly-12: Gly, 3.07; Tyr, 1.04; Ser, 0.96; Lys, 1.00; Leu, 0.98; Asp, 1.00; Glu, 1.98; Val, 0.96; Thr, 0.90. Asp-13: Gly, 3.07; Tyr, 1.03; Ser, 0.97; Lys, 1.00; Leu, 0.97; Asp, 1.92; Glu, 2.00; Val. 0.96; Thr, 0.91. Val-14: Gly, 3.05; Tyr, 1.04; Ser, 0.94; Lys, 1.00; Leu, 0.97; Asp, 1.87; Glu, 1.96; Val, 1.82; Thr, 0.90. Asp-15: Gly, 3.05; Tyr, 0.99; Ser, 0.94; Lys, 1.00; Leu, 0.97; Asp, 2.73; Glu, 1.95; Val, 1.82; Thr, 0.90. Asp-19: Gly, 2.92; Tyr, 0.95; Ser, 0.92; Lys, 1.00; Leu, 0.94; Asp, 4.43; Glu, 2.80; Val, 2.66; Thr, 0.88.

A sample of the final deprotected peptide resin (102 mg) was cleaved with 95% aqueous trifluoroacetic acid for 80 min, the resin was washed, and the combined filtrate evaporated (Found: Gly, 2.98; Tyr, 0.98; Ser, 0.92; Lys, 1.00; Leu, 0.96; Asp, 4.42; Glu, 2.82; Val, 2.73; Thr, 0.91; cleavage yield *ca.* 95%). The h.p.l.c. profile shown in Figure 3(*a*) was obtained. The main peak [A, Figure 2(*a*)] was collected (Found: Gly, 3.00; Tyr, 0.97; Ser, 0.94; Lys, 1.00; Leu, 0.99; Asp, 4.98; Glu, 3.00; Val, 2.98; Thr 0.97); h.p.l.c. Figure 2(*b*).

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