N,O-bisFmoc Derivatives of N-(2-Hydroxy-4-methoxybenzyl)-amino acids: Useful Intermediates in Peptide Synthesis

TONY JOHNSON, MARTIN QUIBELL and ROBERT C. SHEPPARD*

MRC Laboratory of Molecular Biology, Cambridge, UK

Abstract: 2-Hydroxy-4-methoxybenzyl-amino acid residues inhibit interchain association in solid phase peptide synthesis. They are easily introduced through their N,O-bisFmoc derivatives. Preparation of a range of these derivatives is described.

Keywords: 2-Hydroxy-4-methoxybenzyl-amino acids; Hmb-derivatives; peptide synthesis; solid phase synthesis; difficult sequences

We recently described the application of the title compounds 1 in solid phase peptide synthesis [1]. These derivatives constitute a new class of reversibly protected amino acids in which two different protecting substituents are attached to the same α-nitrogen atom. The temporary Fmoc group [2] (for recent reviews see [3, 4]) retains its conventional role of protecting the otherwise basic nitrogen atom from acylation and other side reactions during activation and coupling steps. It is removed at each cycle of

$$\text{Fmoc-N-CHR-CO}_2\text{H}$$

(1)
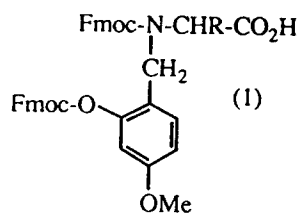
stepwise amino acid addition. The more permanent substituted benzyl group is retained in the growing peptide chain until the final stages of the synthesis. It thus protects the newly formed peptide bond against side reactions requiring deprotonation, e.g. base-catalysed aspartimide formation from preceding asparagine and β-aspartate residues. Note, however, that alternative side reaction paths involving nucleophilic attack by the amide oxygen atom will not necessarily be inhibited by N-substitution.

More importantly, it prevents aggregation through intermolecular hydrogen bonding. In solid phase synthesis, this hydrogen-bonded association is believed to be the cause of the seriously slowed acylation and deprotection rates encountered with so-called 'difficult sequences' [5]. In solution chemistry, hydrogen-bonded association may be the cause of the marked insolubility observed for many free and especially protected peptides. This insolubility has hindered the development of potentially useful solid phase fragment condensation methods [6].

The need for a reversible N-alkyl protecting group for the peptide bond first became apparent to us during a study of aggregation in oligoalanylvaline sequences [7, 8]. This showed, as others had indicated before for other sequences [9], that association was markedly inhibited by the incorporation of proline or sarcosine (*N*-methylglycine) residues into the peptide chain. It put the earlier observations on a more quantitative basis, demonstrating, for example, that proline or sarcosine residues added to a growing oligoalanine chain could inhibit association during stepwise addition of a further six alanines. Thus, only occasional residues in the peptide need to be substituted in order to inhibit association during an entire synthesis. This very favourable result has been confirmed in a number of recent case studies [1].

In this paper we describe exploratory experiments which led to selection of the 2-hydroxy-4-methoxybenzyl substituent as a protecting group for the peptide bond and give preparative details for a number of N-substituted Fmoc-amino acids and their activated derivatives. A preliminary description [10] and symposium account [11] of part of this work have already been given.

Several factors needed to be considered in the design of a useful protecting group (X) for the secondary amide **2**. Complete stability during the repetitive acylation and deprotection conditions of peptide synthesis was required, as well as ease of final cleavage. A base-stable group was needed for use with Fmoc chemistry. Acid lability comparable to that of side-chain *t*-butyl derivatives and the usual *p*-alkoxybenzyl peptide–resin linkage would enable cleavage alongside other protecting groups at the end of the synthesis. A similarly conceived amide protecting group compatible with Boc-benzyl chemistry has recently been described [12]. N-substituted amides **3** rather than the more reactive O-substituted imino ether derivatives **4** were clearly to be preferred, particularly because the former could be simply introduced by way of readily preparable, optically pure N-substituted amino acids.
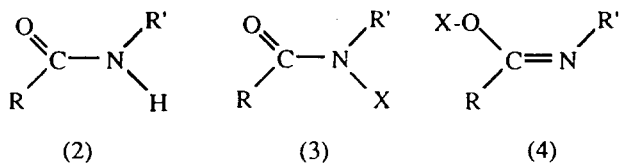
These initial requirements pointed immediately to substituted N-aralkyl derivatives as strong candidates. Acid lability could be varied by appropriate alkyl or alkoxyl substitution in the aromatic ring, though there were foreseeable problems. The long-lived cations produced on acidolysis of alkoxyl-substituted acylbenzylamines may react near quantitatively with, for example, tryptophan residues [13]. Furthermore, there were strong indications in the literature that some N-benzylamino-acid and peptide derivatives may be seriously sterically hindered to

Fmoc-N-CHR-CO$_2$H
|
CH$_2$
(5)

Fmoc-N-CHR-CO$_2$H
|
CH$_2$
(6)

Fmoc-N-CHR-CO$_2$H
|
CH$_2$
(7)
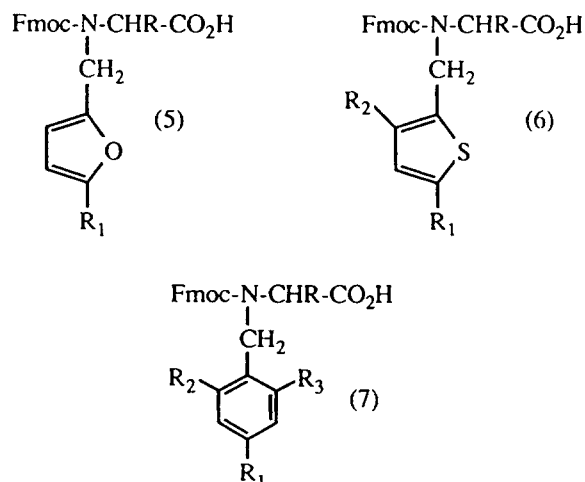
acylation [14, 15]. For these reasons, our initial study was broadened to include Fmoc-derivatives of substituted furfuryl **5** and thienylmethyl derivatives **6**. These are sterically smaller than corresponding benzyl derivatives **7**, lacking one of the *ortho* hydrogens or substituents of the latter. The derivatives **6** of thiophen were expected to have better acid stability than those of furan, and to some degree might also be self-scavenging.

Simple N$_\alpha$-aralkylamino acids were initially prepared following the method used in a different context by Coy and his colleagues [16]. Various methyl and methoxyl-substituted benzaldehydes, furfurals and 2-formylthiophens were combined with the trifluoroacetate salts of glycine or alanine, and the resulting Schiff bases reduced *in situ* with sodium cyanoborohydride (see the Experimental section, method 1). Isolation was facilitated by immediate conversion to the N$_\alpha$-Fmoc derivatives. After purification by chromatography on silica, overall yields were low (10–25%) (Table 1) but workable in this exploratory phase. In his preparative work, Coy noted [16] that 'a small but perhaps acceptable

(2)   (3)   (4)

Table 1. Approximate Times for Complete Cleavage of (A) N-substituted Fmoc-Amino Acids, and (B) N-substituted Phenacetylamino Acids by 95% aq Trifluoroacetic Acid

Compound	R	R_1	R_2	R_3	Yield (%)	Cleavage time A	Cleavage time B
5a	H	H			20	2 h	>7 h
5b	H	Me			22	<2 min	<1 h
5c	H	OMe			25		
6a	H	H	H		25	3 h	
6b	H	Me	H		13	<2 min	<1 h
6c	H	OMe	H		15	<2 min	<2 min
6d	H	H	OMe		10	<2 min	<5 min
7a	H	OMe	OMe		19	<2 min	<15 min
7b	H	OMe	OMe	OMe	25	<2 min	<2 min
5d	Me	Me	H		17	<2 min	
6e	Me	Me	H		14	<2 min	
7c	Me	OMe	OMe	OMe	21	<2 min	

degree of racemisation did indeed occur', but both aldehyde and amine components were asymmetric in his case and it is likely that it was the former which was partly racemised. In the present work, no diastereoisomers could be detected by high-performance liquid chromatography (HPLC) using Marfey's test [17] which was carried out on all the new chiral derivatives prepared.

The initial N_α-Fmoc-glycine and alanine derivatives (**5-7**, R = H or Me; R_1, R_2, R_3 = H, Me or MeO, Table 1) were used to assess relative acid labilities of the N_α-aralkyl substituents. The various derivatives were dissolved in 95% aqueous trifluoroacetic acid, and the time for complete conversion to the parent Fmoc-amino acid estimated using analytical HPLC (Table 1). All the compounds examined were acid-labile (Table 1, column A), with 2-furfuryl (**5a**, R = R_1 = H) and 2-thienylmethyl (**6a**, R = R_1 = H) the slowest (simple unsubstituted benzyl derivatives were not prepared). The 5-methoxyfurfuryl-glycine (**5c**, R = H; R_1 = OMe) derivative decomposed in acid but was exceptional in not regenerating the parent Fmoc-amino acid. Presumably opening of the furan ring intervened in this case. All the remaining compounds were unexpectedly acid-labile with cleavage of the aralkyl substituent complete in less than 2 min. Subsequently we recognised that cleavage rates were particularly sensitive to the environment of the substituted nitrogen atom. Urethane Fmoc-derivatives were exceptionally reactive. Conversely, cleavage from free peptides was inhibited when the substituted nitrogen atom was basic, i.e. N-terminal,

and slowed when close to other readily protonatable centres, e.g. next to the N-terminal residue, or to unprotected histidine etc. This is a complicating factor in the analysis of acylation reactions by HPLC separation of acylated, non-basic substituted nitrogen, and unacylated, basic N-terminal substituted nitrogen atom. To provide data more relevant to that expected for typical peptide environments, a number of cleavage experiments were repeated replacing the Fmoc-compounds **5-7** by simple N_α-phenacetyl derivatives ($PhCH_2CO-NXCH_2CO_2H$). These were non-crystalline and could be characterized by fast atom bombardment mass spectroscopy (FAB-MS) and HPLC. They were much less labile than Fmoc-derivatives, and enabled the more reactive X substituents to be placed in the order of acid lability: 2,4,6-trimethoxybenzyl (Tmb) $\approx$ 5-methoxythienyl-methyl > 2,4-dimethoxybenzyl (Dmb) > 5-methyl-furfuryl $\approx$ 5-methyl-thienylmethyl (Table 1, column B). In 5% trifluoroacetic acid/dichloromethane, the half lives of the first two derivatives were approximately 3 and 6 min respectively.

All five potential protecting substituents were selected for acylation studies. Later the 3-methoxythienylmethyl derivative (**6d**, R = R_1 = H; R_2 = OMe) was shown to be approximately equal to the 5-isomer in acid lability and was included in these studies (see below).

The pentapeptide derivative **8** was selected as the test sequence, and amino acid analysis used to determine coupling yields (see Experimental section). No difficulty was experienced in coupling the

Table 2. Yields in the Single Coupling of Fmoc-Ala-OPfp to H-(X)Gly-Phe-Val-Resin

	Substituent X in Peptide **8**	Yield (%)
a	5-Methylfurfuryl	65
b	5-Methylthienylmethyl	45
c	5-Methoxythienlmethyl	25
d	3-Methoxythienylmethyl	85
e	2,4-Dimethoxybenzyl (Dmb)	80
f	2,4,6-Trimethoxybenzyle (Tmb)	90

N_α-substituted Fmoc-glycine and alanine derivatives (**5-7**) to phenylalanylvalyl-polydimethylacrylamide resin using, for these early experiments, the O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU) coupling reagent. The Fmoc-derivatives were also readily cleaved. Acylation of the liberated secondary amino groups was, however, particularly sluggish. Coupling yields for this step using Fmoc-alanine pentafluorophenyl ester (5 equiv.) for 1 h with the N-substituted glycylphenylalanylvalyl–resin (cf. Experimental section) suspended in the minimum volume of dimethylformamide (DMF) are given in Table 2. Addition of the final leucine residue was unexceptional. Remarkably, the highest yield for acylation of the substituted amino group was initially obtained with the most heavily substituted 2,4,6-trimethoxybenzyl (Tmb) derivative (**8**, X = 2,4,6-trimethoxybenzyl, 90% coupling after 1 h reaction). Yields for the notionally less hindered furfuryl and thienylmethyl derivatives were all significantly lower. In no case did the coupling yield reach the near-quantitative levels required for effective solid phase synthesis, though repeated coupling using either symmetric anhydride (experiments a–c in Table 2) or pentafluorophenyl ester activation (experiments d–f) enabled full incorporation to be achieved in all cases.

$$H-Leu-Ala-(X)Gly-Phe-Val-resin \quad \textbf{8}$$
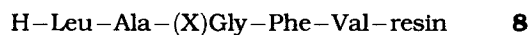
These results confirmed that N-substitution introduced severe steric hindrance, even in the glycine series **8**. Hindrance was very much worse when glycine was replaced by alanine. Negligible reaction occurred between Fmoc-alanine pentafluorophenyl ester and the Tmb-peptide **9** under the above conditions. Results with more powerful activation methods are given in Table 3. In *N,N*-dimethylformamide (DMF) suspension, double coupling with symmetric anhydride was required to obtain quantitative incorporation (experiment 3, Table 3).

$$H-(2,4,6-trimethoxybenzyl)Ala-Phe-Val-Resin$$
$$\textbf{9}$$
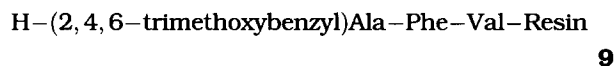
The enhanced rate of acylation of the H-(Tmb)glycylphenylalanylvalyl-resin compared with the expectedly less hindered 2,4-dimethoxybenzyl (Dmb), furan and thiophen derivatives (Table 2) was significant, and suggested that hydrogen-bonded structures such as **10** might be important. Two lines of evidence supported this. The rate of acylation of N-Tmb-tripeptide resin **9** by Fmoc-alanine anhydride was *increased* in the less polar aprotic solvent dichloromethane (DCM) (Table 3, experiments 2 and 7). This solvent dependency is not observed for normal symmetric anhydride coupling reactions. In the very hindered coupling of Fmoc-valine anhydride to H-Ile-Phe-Val-resin, for example, approximately equal acylation rates were observed in DCM and DMF solutions. For activated ester reagents, *decreased* coupling rates are usually observed in less polar solvents [18]. The special effect for N-Tmb derivatives is consistent with enhanced stability of an H-bonded intermediate **10** in aprotic, non-hydrogen-bonding solvents. The generality of acceleration by *ortho*-type substituent was indicated by comparison of 3- and

Table 3. Reaction Yields in the Acylation of H-(Tmb)Ala-Phe-Val-Resin (9) by Fmoc-Ala Under Various Conditions

	Coupling	Solvent	Time (h)	Yield (%)
1	Symm. anhydride	DMF	1	40
2	Symm. anhydride	DMF	3	80
3	Symm. anhydride	DMF	3 + 18	100
4	PyBrop	DMF	3	50
5	PyBrop	DMF	3 + 18	70
6	Symm. anhydride/Dmap (1 equiv.)	DMF	3	25
7	Symm. anhydride	DCM	3	100

(10)
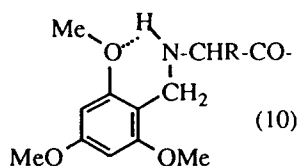
5-methoxythiophens as in **6**, R_1 = H; R_2 = OMe ('*ortho*' methoxyl), and R_1 = OMe; R_2 = H ('*para*' methoxyl). In the glycine series **(8)**, the N-(3-methoxythienylmethyl)–tripeptide resin was acylated at a rate similar to that of the Tmb-derivative (Table 2), and substantially faster than the less hindered N-(5-methoxythienylmethyl)-tripeptide resin. Nevertheless, none of the derivatives thus far examined offered real promise in peptide synthesis because of the generally high levels of hindrance encountered. This problem was largely overcome by simple replacement of the o-methoxy by an o-hydroxy group.

Like the favourable H-bonded arrangement **10**, an o-hydroxy group could participate in the similar structure **11a**, or, more importantly, in the tautomerically equivalent **11b**. In this last arrangement, the secondary amino group is well placed to act as internal base catalyst, facilitating acylation of the relatively unhindered hydroxy function. In turn, the resulting reactive phenyl ester **12** is seemingly well placed to undergo intramolecular acyl transfer to the amino group **12 → 13**, a process which we thought would not be subject to the massive steric hindrance encountered in direct acylation of the secondary amino group.

(11a)

(11b)

Fmoc-NH-CHR'-CO

(12)

Fmoc-NH-CHR'-CO

(13)
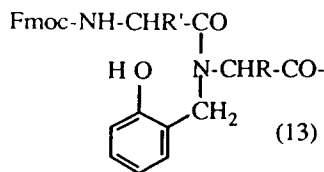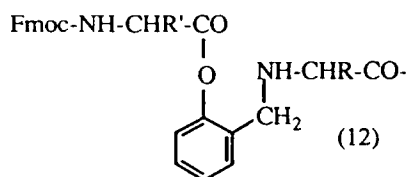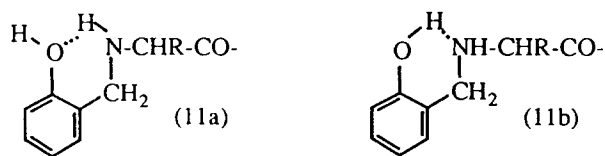
In practice a remarkable rate enhancement was observed when the o-methoxyl group was replaced by hydroxyl. Table 4 lists a number of experiments comparing coupling of activated Fmoc-alanine to the N-(2-methoxybenzyl)- and 2-hydroxybenzyl-derivatives **(14**, R = H and Me.) Quantitative coupling of the 2-hydroxy derivative could be obtained within 1 h using Fmoc-alanine anhydride or within 11 h using the pentafluorophenyl ester. Under the latter conditions, only 2% coupling was obtained with the 2-methoxybenzyl peptide resin.

From the foregoing two candidate structures were likely to possess the appropriate degree of acid lability and to acylate relatively easily. 3-Hydroxythienylmethyl derivatives as in **6**, R_1=H, R_2=OH, proved difficult to prepare and were not proceeded with. In any event, such derivatives may exist predominantly in the keto form [19, 20], with unknown effect on acid lability and ease of acylation. In the benzyl series, an additional methoxy group was required to ensure adequate acid lability, and 2-hydroxy-4-methoxybenzyl (Hmb) derivatives were therefore explored fully.

(14)
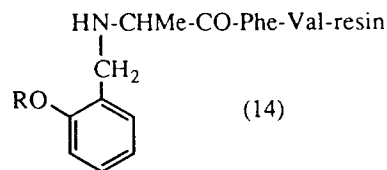
The N-substituted amino acids were easily prepared from 2-hydroxy-4-methoxybenzaldehyde by a variation of the intermediate Schiff base route [21] (see Experimental section, method 2), and converted by Fmoc-Cl to N-mono and then N,O-bisFmoc-substituted amino acids (Table 5). The mono derivatives were shown to be N-substituted by conversion with trifluoroacetic acid to the parent N-Fmoc-amino acids, and further converted to the amino acid for racemization testing. Activated pentafluorophenyl esters of the bisFmoc derivatives were also easily formed (Table 6).

A detailed study was made of the coupling efficiency of a range of Fmoc-amino acid anhydrides and pentafluorophenyl esters with Hmb-substituted peptide–resins, examples of which are collected in Table 7.

Although the steric hindrance experienced was very substantially less than in the earlier 2-methoxy and 2,4-dimethoxy series, coupling reactions were

Table 4   Coupling of Fmoc-Alanine to the N-substituted Tripeptide Derivative 14 (R=H and R=Me). The Analogous 2-hydroxy-4-methoxybenzyl Derivative Behaved Similarly, e.g. Quantitative Acylation by Fmoc-alanine Anhydride Within 15 min in Dichloromethane Solution.

	Coupling method	Solvent	Time	R in **14**	Yield (%)
1	Pfp ester/HOBt	DMF	1 h	H	54
2	Pfp ester/HOBt	DMF	11 h	H	100
3	Pfp ester/HOBt	DMF	11 h	Me	2
4	Symm. anhydr.	DMF	15 min	H	64
5	Symm. anhydr.	DMF	1 h	H	100
6	Symm. anhydr.	DCM	15 min	H	100
7	Symm. anhydr.	DMF	12 h	Me	55

Table 5   Analytical Data for N,O-bisFmoc-N-(2-hydroxy-4-methoxybenzyl) Amino Acids

Amino acid	Yield (%)	M.p.[a] (°C)	% C Found (reqd)	% H Found (reqd)	% N Found (reqd)	FAB-MS m/z, fnd (mw)	$[\alpha]_D^{20}$ ($c=1$, DMF)
Ala	38.5	86–88 (sh); 121–124 (gl)	73.04 (73.53)	5.35 (5.27)	1.87 (2.09)	670.5 (669.7)	–4.1°
Asp(OBu^t)	16	90–93 (sh); 106–110 (gl)	70.07[b] (70.12)	5.60 (5.76)	1.78 (1.78)	770.8 (769.8)	–35.9°
Glu(OBu^t)	11	77–80 (sh); 127–139 (m)	69.87[b] (70.39)	5.81 (5.91)	1.62 (1.75)	785.0 (783.9)	–14.4°
Gly	18.5	80–82 (sh); 90–94 (gl)	72.86 (73.27)	5.09 (5.07)	1.97 (2.14)	656.4 (655.7)	–
Ile	28	83–85 (sh); 102–105 (gl)	73.85 (74.24)	5.78 (5.81)	1.81 (1.97)	712.8 (711.8)	–26.2°
Leu	32	84–86 (sh); 101–104 (gl)	73.80 (74.24)	5.85 (5.81)	1.81 (1.97)	712.8 (712.8)	–17.5°
Lys(Boc)	27.5	77–80 (sh); 106–114 (gl)	67.68[c] (68.20)	6.02 (6.31)	3.00 (3.24)	827.8 (826.9)	–15.4°
Phe	37.5	100–102 (sh); 110–113 (gl)	75.12 (75.69)	5.48 (5.27)	1.74 (1.88)	746.9 (745.8)	–47.7°
Ser(Bu^t)	23.5	81–84 (sh); 92–96 (gl)	71.43[b] (71.13)	5.85 (5.97)	1.66 (1.84)	742.7 (741.8)	–7.2°
Thr(Bu^t)	23	57–61 (sh); 64 (m)	71.16[b] (71.39)	6.15 (6.12)	2.06 (1.81)	756.7 (755.8)	–17.7°
Tyr(Bu^t)	20	85–90 (sh); 94–99 (gl)	72.13[c] (71.73)	5.61 (6.02)	1.64 (1.64)	818.5 (817.9)	–48.6°
Val	19	87–90 (sh); 106–108 (gl)	73.75 (74.01)	5.77 (5.63)	1.83 (2.01)	698.8 (697.8)	–34.0°

[a] sh, shrinks, gl, becomes a clear glass, m, melts.
[b] Monohydrate.
[c] Dihydrate.

still slower than expected. In part this slowness is due to the initial O- rather than N-acylation. Subsequently it became clear, however, that in many cases the rate-limiting step was the O → N rearrangement reaction rather than the initial acylation. Coupling reactions were encountered in which amino acid analysis of the Fmoc-peptide resin indicated quantitative coupling but which, *after cleavage of the Fmoc group*, showed a substantially reduced content of the incoming amino acid residue. If the Fmoc-peptide resin was washed free of acylating reagent and then stored before treatment with piperidine, more of the incoming residue was retained. This behaviour is consistent with incomplete O→N migration in the peptide-resin during the

acylation reaction period. The unrearranged phenolic esters in O-acylated derivatives (and O,N-diacylated derivatives) are cleaved by piperidine. Although rapid rearrangement through the 6-membered cyclic intermediate (**15**) was originally expected, we subsequently discovered literature precedent [22, 23] for hindered migration in a closely related series. Rapid rearrangement only occurs when the N-substituted amino acid is glycine. Molecular modelling indicates that for residues other than glycine, interference between the side chains R_1 and R_2 and between R_2 and the benzylic hydrogen atoms in the cyclic intermediate may be significantly retarding factors in the rearrangement process.

From these acylation studies on various (Hmb)-

Table 6 Analytical Data for *N,O*-bisFmoc-*N*-(2-hydroxy-4-methoxybenzyl) Amino Acid Pentafluoro-phenyl Esters

Amino acid	Yield (%)	M.p.[a] (°C)	% C Found (reqd)	% H Found (reqd)	% N Found (reqd)	FAB-MS m/z, fnd (mw)	$[\alpha]_D^{20}$ (c=1, DMF)
Ala	93	67 (sh); 73–75 (gl)	67.76 (67.54)	4.17 (4.10)	1.32 (1.68)	836.8 (835.8)	−7.8°
Asp(OBu^t)	84	50–53 (sh); 68–71 (gl)	66.61 (66.73)	5.02 (4.52)	2.00 (1.50)	936.7 (935.9)	−21.1°
Glu(OBu^t)	86	55–58 (sh); 70–72 (gl)	66.97 (67.02)	5.09 (4.67)	1.58 (1.47)	950.9 (949.8)	−17.4°
Gly	76	73–75 (gl)	66.95 (67.23)	3.84 (3.92)	1.60 (1.70)	822.8 (821.8)	–
Ile	93	70 (sh); 75–77 (gl)	68.76 (68.40)	4.82 (4.59)	1.90 (1.60)	878.5 (877.9)	−22.8°
Leu	90	68 (sh); 74–76 (gl)	68.56 (68.40)	4.75 (4.59)	1.75 (1.60)	878.6 (877.9)	−16.6°
Lys(Boc)	89	61–62 (sh); 67 (gl)	66.34 (66.53)	5.17 (4.97)	3.12 (2.82)	994.3 (992.9)	−14.6°
Phe	91	67 (sh); 73–75 (gl)	69.87 (69.80)	4.31 (4.20)	1.70 (1.54)	912.4 (911.9)	−31.4°
Ser(Bu^t)	95	63–65 (sh); 69–71 (gl)	67.64 (67.47)	4.70 (4.66)	1.68 (1.54)	908.3 (907.8)	−11.0°
Thr(Bu^t)	67	76 (sh); 81–86 (gl)	67.95 (67.77)	5.02 (4.81)	1.55 (1.52)	922.4 (921.9)	−39.6°
Tyr(Bu^t)	91	64 (sh); 67–68.5 (gl)	69.59 (69.57)	4.65 (4.71)	1.46 (1.42)	985.1 (983.9)	−31.6°
Val	88	68 (sh) 73–76 (gl)	68.24 (68.12)	4.61 (4.43)	1.85 (1.62)	864.6 (863.9)	−21.0°

[a] sh, shrinks; gl, clear glass.

Table 7 Typical Reaction Times Required for Complete N-acylation of Terminal (Hmb) Amino Acid Residues by Fmoc Amino Acid Symmetric Anhydrides in Dichloromethane Solution. Fmoc–Amino Acid *N*-carboxy Anhydrides were Subsequently Shown to Behave Similarly

| Acylated by	N-Terminal (Hmb)-amino acid residue			
	Gly	Ala	Phe	Val
Fmoc–Gly	15 min	15 min	30 min	3 h
Fmoc–Ala	15 min	15 min	2 h	5 h
Fmoc–Leu	15 min	1 h	5 h	24 h
Fmoc–Val	30 min	_a	_a	_a

[a] Slow reactions, not reaching completion within 24 h.

(15)
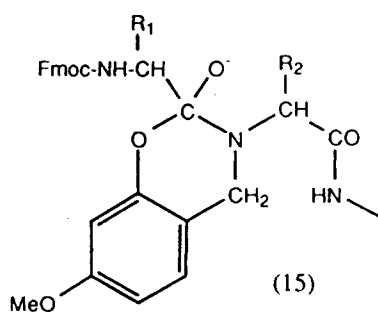
derivatives the following tentative rules regarding the acylation of Hmb-peptide resins are proposed for the coupling of Fmoc-Y-OH to (Hmb)X-peptide resin:

(i) If residue X is glycine then Y may be any residue and may usually be coupled through its penta-fluorophenyl ester in the presence of hydroxybenzotriazole.

(ii) If X is other than glycine then Y should not be a β-branched residue (normally valine, isoleucine or O-*t*-butylthreonine) and requires a more powerful activating method. In our experience, preformed symmetric anhydrides or commercially available urethane N-carboxyanhydrides are suitable.

Acidolysis of the Hmb group might generate reactive carbocations which could alkylate sensitive amino acid residues. These side reactions have previously been noted with other benzyl-based protecting groups, e.g. Tmob, Mbh (used for the side-chain protection of Asn and Gln) [24]. A series of test pentapeptide resins of type **16** were prepared to investigate the seriousness of these alkylation reactions.

H–Leu–Ala–(Hmb)Gly–Y–Val–resin **16**
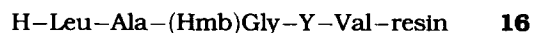
The sensitive residues (Y) investigated were Met, Tyr, His(Trt), Cys(Trt), Cys(Acm), Trp and Trp(Boc). Experience has shown that ethane dithiol (EDT) [13, 25, 26] and silane-based additives [27] have the most efficient scavenging properties. Thus, test peptides **16** were cleaved with trifluoroacetic acid (TFA)/EDT/triethylsilane mixture (94:3:3), except for Trp peptides where triisopropylsilane re-

placed the triethyl derivative and Tyr peptides where phenol replaced EDT. The resultant crude peptides (after gel filtration to remove non-peptidic material) were checked by amino acid analysis and compared (by reversed-phase HPLC, RP-HPLC) to the same pentapeptides synthesized without the use of the Hmb group. Only **16** (Y = Trp) showed any significant side product formation, this showing a UV λ_{max} shift from 280 to 283 nm. This suggested that alkylation of the indole nucleus [13] had occurred. However, **16** (Y = Trp(Boc)) [28] gave very little indication of alkylation and its use minimizes this problem. These results suggest that provided efficient scavenger combinations are employed, little side-chain substitution by the Hmb group is to be expected.

Examples of the value of Fmoc-(Hmb) amino acid derivatives in the synthesis of 'difficult sequences' have been given elsewhere [1]. These include the well-known acyl carrier protein decapeptide sequence [29, 30] which was synthesized with no trace of des-valine-10 failure sequence when alanine-7 was replaced by its Hmb-derivative, and longer peptides from HIV TAT protein, Alzheimer amyloid and other series [1]. As expected, the disruption of interchain hydrogen bonding in the peptide–resin complex is paralleled in free solution. Intermolecular association of free or protected peptides may be reduced by inclusion of Hmb residues resulting in enhanced solubility. The value of Hmb-derivatives in facilitating purification of insoluble peptides has been discussed, and an example from the β-amyloid series described [31]. An as yet unexplored application is in the synthesis of protected peptide fragments for use in solid phase fragment condensation studies [6].

EXPERIMENTAL

Throughout this paper, 'resin' refers to cross-linked and functionalised polydimethylacrylamide supported in macroporous kieselguhr (Pepsin KA). In peptide sequences, attachment to this resin is indicated by the suffix KA.

Analytical HPLC was performed on a Brownlee Aquapore RP-300 C-8 column using a gradient of 10–90% B in A over 25 min, where solvent A = 0.1% aq. TFA and B = acetonitrile containing 10% A. Flow rate = 1.5 ml/min, eluent monitored at 215 nm. Optical rotations were determined using a Perkin-Elmer 241 polarimeter (sodium lamp, 589 nm) at 20 °C. Melting points are uncorrected. Thin layer

chromatography (TLC) samples were run on pre-coated aluminium sheets of silica gel 60 F_{254} (Merck), and column purification performed using Kieselgel 60 (Merck). Mass spectra were obtained on an MS80 RF double focusing spectrometer, with the FAB gun operating at 6 kV krypton atoms and 20 μA discharging current. Samples were dissolved in dimethyl-sulphoxide.

Continuous flow Fmoc-polyamide peptide synthesis methods were used exclusively as described generally by Atherton and Sheppard [32] and references there cited. Fmoc-amino acid activated esters (0.5 mmol vials, Novabiochem, UK), Fmoc-*N*-carboxyanhydrides (Propeptide, Vert Le Petit, France) and Fmoc-amino acid-Pepsyn KA resins (Milligen, UK) were commercial samples. All chiral amino acids used were of the L-configuration. Solid-phase peptide synthesis was performed on an LKB 'Biolynx' automated synthesizer programmed to perform acylation reactions (in DMF) for 45 min, and Fmoc deprotection reactions (in 20% piperidine/DMF v/v) for 10 min. Peptides and peptide–resins were hydrolysed *in vacuo* in 6 N HCl containing a trace of phenol at 110 °C for 96 h. Amino acid analysis utilized a Beckman 7300 analyser.

N-Substituted-*N*-Fmoc–Amino Acids and Derivatives: General Method 1 (Compounds 5–7).

Amino acid trifluoroacetate salt (1 mmol) was dissolved in 1% v/v acetic acid in methanol (10 ml). The appropriate aldehyde (1 mmol) was added, followed by sodium cyanoborohydride (1.05 mmol) as a solid over 1 h. After a further 3 h stirring, the solvents were removed *in vacuo*. The residue was taken into 10% aq. sodium carbonate (10 ml) and washed with ethyl acetate (2 × 10 ml). The aqueous layer was then adjusted to pH 7 with 1 M HCl. Solid sodium carbonate decahydrate (2.2 mmol) was added to the aqueous layer, followed by dioxan (10 ml) and the mixture stirred in an ice-bath. Fluorenymethyl chloroformate (1.05 mmol) in dioxan (5 ml) was added with stirring over 30 min, and the mixture left overnight. The solution and precipitate was washed with ether (2×25 ml), the basic aqueous layer cooled in an ice-bath and rapidly stirred while 5 N aq. hydrochloric acid was added dropwise to ca. pH 3–4. The precipitate was extracted into chloroform (2 × 50 ml), the combined organic extracts dried (sodium sulphate) and evaporated *in vacuo* to give the crude product as an oily solid.

Crude products were purified over silica gel (100 g) packed in methanol–chloroform (15–20% methanol). Samples were loaded in chloroform and eluted with methanol–chloroform mixture. Fractions were then pooled, evaporated *in vacuo*, flashed down from ether and re-precipitated from hexane–toluene. Purified products were then filtered, and dried *in vacuo* at ca. 50 °C overnight. The following compounds were prepared by method 1. Overall yields are calculated from the starting amino acid.

***N*-Fmoc-*N*-(furfuryl)glycine (5a).** Yield 20%, m.p. 99–100°C HPLC (10–90% B gradient over 25 min), R_t 20.32 min (>95%). Found: C, 69.73; H, 5.02; N, 3.73%; $C_{22}H_{19}NO_5$ (377.37) requires C 70.02; H, 5.07; N 3.71%. FAB-MS (+ve m/z) 400.1 [M+Na]$^+$, 378.1 [M+H]$^+$.

***N*-Fmoc-*N*-(5-methylfurfuryl)glycine (5b).** Yield 22%, m.p. 163–168°C (shrinks 125°C). HPLC (10–90% B), R_t 20.76 min (93.5%). $C_{23}H_{21}NO_5$ = 391.40. FAB-MS (+ve m/z) 414.2 [M+Na]$^+$, 392.1 [M+H]$^+$.

***N*-Fmoc-*N*-(5-methoxyfurfuryl)glycine (5c).** Yield 25%, m.p. 161–170 °C (shrinks 130°C). HPLC (10–90% B), R_t 20.28 min (>90%). $C_{23}H_{21}NO_6$ = 407.40. FAB-MS (+ve m/z) 446.5 [M+K]$^+$, 430.5 [M+Na]$^+$.

***N*-Fmoc-*N*-(2-thienylmethyl)glycine (6a).** Yield 25%, m.p. 170–174°C (shrinks 142°C). HPLC (10–90% B), R_t 20.55 min (95%). $C_{22}H_{19}NO_4S$ = 393.42. FAB-MS (+ve m/z) 394.1 [M+H]$^+$.

***N*-Fmoc-*N*-(5-methyl-2-thienylmethyl)glycine (6b).** Yield 13%, m.p. 139–145°C (shrinks 127°C). HPLC (10–90% B), R_t 21.37 min (>91%). $C_{23}H_{21}NO_4S$ = 407.4. FAB-MS (+ve m/z) 446.2 [M+K]$^+$, 430.0 [M+Na]$^+$.

***N*-Fmoc-*N*-(5-methoxythienyl)glycine (6c).** Yield 5%, m.p. 162–170°C (shrinks 120°C). HPLC (10–90% B), R_t 21.04 min (>90%). $C_{23}H_{21}NO_5S$ = 423.4. FAB-MS (+ve m/z) 462.3 [M+K]$^+$, 446.3 [M+Na]$^+$.

***N*-Fmoc-*N*-(3-methoxythienyl)glycine (6d).** Yield 10%, m.p. 153–158°C (shrinks 91°C). HPLC (10–90% B), R_t 20.88 min (>90%). $C_{23}H_{21}NO_5$ = 423.4. FAB-MS (+ve m/z) 462.3 [M+K]$^+$.

***N*-Fmoc-*N*-(2,4-dimethoxybenzyl)glycine (7a).** Yield 19%, m.p. 155–157°C (shrinks 120 °C), HPLC (10–90% B), R_t 20.73 min (94.5%). $C_{26}H_{25}NO_6$ = 447.5. FAB-MS (+ve m/z) 486.6 [M+K]$^+$, 470.6 [M+Na]$^+$, 448.6 [M+H]$^+$.

***N*-Fmoc-*N*-(2,4,6-trimethoxybenzyl)glycine (7b).** Yield 25%, m.p. 157–159°C (shrinks 143°C). HPLC (10–90% B), R_t 21.30 min (96.6%). $C_{27}H_{27}NO_7$ = 477.5. FAB-MS (+ve m/z) 500.7 [M+Na]$^+$, 478.7 [M+H]$^+$.

***N*-Fmoc-*N*-(5-methylfurfuryl)-L-alanine (5d).** Yield 17%, m.p. 172–176°C (shrinks 117°C). HPLC (10–90% B), R_t 21.33 min (96%). $C_{24}H_{23}NO_5$=405.4. FAB-MS (+ve m/z). 444.4 [M+K]$^+$, 406.1 [M+H]$^+$, $[\alpha]_D$=−17.6 ° (c=1, DMF).

***N*-Fmoc-*N*-(5-methyl-2-thienylmethyl-L-alanine (6e).** Yield 14%, m.p. 139–146°C (shrinks 105°C). HPLC (10–90%), R_t 22.14 min (>95%). $C_{24}H_{23}NO_4S$ = 421.4. FAB-MS (+ve m/z); 460.3 [M+K]$^+$, 444.3 [M+Na]$^+$, 422.3 [M+H]$^+$. $[\alpha]_D$=−17.8 ° (c=1, DMF).

***N*-Fmoc-*N*-(2,4,6-trimethoxybenzyl)-L-alanine (7c).** Yield 21%, m.p. 128–137°C (shrinks 90°C). HPLC (10–90% B), R_t 22.03 min (>98%). $C_{28}H_{29}NO_9$ = 491.5. FAB-MS (+ve m/z) 530.5 [M+K]$^+$, 514.5 [M+Na]$^+$, $[\alpha]_D$=+10.6 ° (c=1, DMF).

***N*-Fmoc-*N*-(2-methoxybenzyl)-L-alanine (7d).** Yield 11%, m.p. 165–170°C (dec) (shrinks 123°C). HPLC (10–90% B), R_t 20.04 min (>99%). Found: C, 69.56; H, 5.89; N, 3.08%. $C_{26}H_{25}NO_5 \cdot H_2O$ (449.36) requires C, 69.47; H, 6.05; N, 3.11%. FAB-MS (+ve m/z) 454.3 [M+Na]$^+$, 432.4 [M+H]$^+$. $[\alpha]_D$ = −5.2 ° (c=1, DMF).

Synthesis of phenylacetyl derivatives. N-phenylacetyl derivatives were prepared from the corresponding purified Fmoc derivatives. The N-aralkylated Fmoc-glycine was treated with 20% piperidine/DMF for 15 min, the solution evaporated *in vacuo*, and the N-aralkylated glycine recovered by trituration with ether. This (0.05 mmol) was dissolved in water (2 ml) containing sodium carbonate decahydrate (2.1 × 0.05 mmol, 30.1 mg) and dioxan (1 ml) added, with stirring in an ice-bath. Phenylacetylchloride (1.05 × 0.05 mmol, 8.1 mg) in dioxan (1 ml) was added over 10 min and stirring continued for 2 h. The mixture was diluted with water (5 ml) and ether (10 ml), and the separated aqueous layer

washed with ether (2 × 10 ml). The basic layer was then acidified with 5 N hydrochloric acid and back-extracted with chloroform (2×10 ml). The combined organic layers were dried (sodium sulphate) and evaporated *in vacuo* to crude oils (~ 10 mg) which were purified on a semi-preparative reverse phase HPLC system, using a 15–40% gradient of B in A over 25 min. The purified phenylacetyl derivatives were analysed by analytical HPLC and FAB-MS, all giving >95% purity and the correct *m/z* top mass.

Acidic cleavage studies. Samples of *ca.* 0.1 mg of the various Fmoc and phenylacetyl derivatives, were dissolved in 95% aq. TFA (500 µl). Equal samples were withdrawn at intervals and quickly evaporated *in vacuo.* The residues were dissolved in 50% aq. acetonitrile and analysed by HPLC for uncleaved derivative.

N-(2-Hydroxybenzyl)Amino Acids and the 4-Methoxyl Analogues: General Method 2

Amino acid (10 mmol) was dissolved in water (10 ml) containing potassium hydroxide (10 mmol) with stirring. A solution of the appropriate aldehyde (salicylaldehyde or 4-methoxysalicylaldehyde, 10 mmol) in ethanol (10 ml) was added, and the bright yellow solution stirred for 30 min at room temperature. Sodium borohydride (10.1 mmol) dissolved in water (5 ml, containing five drops of 1 M NaOH) was then added over 15 min (with effervescence and some colour loss), and stirred for a further 15 min. The mixture was then heated on a water bath at *ca.* 60 °C for 10 min, and cooled. The almost colourless solution was stirred and acidified to pH 4 with concentrated hydrochloric acid to precipitate the product. (If precipitation was slight, the acidified solution was evaporated *in vacuo* to approximately half the volume.) After standing overnight at 4 °C, the precipitate was collected, washed carefully with ice-cold water and 50% aq. methanol (some derivatives show appreciable solubility), and then dried overnight at 80 °C. The following compounds were prepared in this manner.

N-(2-Hydroxy-4-methoxybenzyl)-L-aspartic acid *t*-butyl ester. Yield 63%, m.p. >300°C. HPLC (10–90% B), R_t 12.51 min. Found: C, 57.99; H, 7.05; N, 4.11%. $C_{16}H_{23}NO_6$ (325.3) requires C, 59.06; H, 7.13; N, 4.30%. $[\alpha]_D$ = −13.9°C (*c*, 1 in N aq. sodium

hydroxide).

N-(2-Hydroxybenzyl)-L-alanine. Yield 74%, m.p. >300°C. HPLC (10–90% B), R_t 3.19 min. Found; C, 61.09; H, 6.82; N, 7.17%. $C_{10}H_{13}NO_3$ (195.2) requires C, 61.53; H 6.71; N, 7.17%. $[\alpha]_D$ = −16.9 °C (*c*, 1 in N aq. sodium hydroxide).

N-(2-Hydroxy-4-methoxybenzyl)-L-alanine. Yield 72%, m.p. >300°C. HPLC (10–90% B), R_t 4.69 min. Found: C, 59.54; H, 6.47; N, 5.78%. $C_{11}H_{15}NO_4$ (225.23) requires C, 58.66; H, 6.71; N, 6.22. $[\alpha]_D$ = −13.0 °C (*c*, 1 in N aq. sodium hydroxide).

N-(2-Hydroxy-4-methoxybenzyl)-L-glutamic acid *t*-butyl ester. Yield 40%, m.p. >300°C. HPLC (10–90% B), R_t 13.10 min.

N-(2-Hydroxy-4-methoxybenzyl)-glycine. Yield 60%. HPLC (10–90%), R_t 4.04 min. Found: C, 57.66; H, 6.18; N, 5.91%. $C_{10}H_{13}NO_4$ (211.20) requires C, 56.86; H, 6.20; N, 6.63%.

N-(2-Hydroxy-4-methoxybenzyl)-L-isoleucine. Yield 59%, m.p. >300°C. HPLC (10–90% B), R_t 9.85 min. Found: C, 62.38; H, 7.73; N, 4.72%. $C_{14}H_{21}NO_4$ (267.31) requires C, 62.90; H, 7.92; N, 5.24%.

N-(2-Hydroxy-4-methoxybenzyl)-L-leucine. Yield 66%, m.p. >300°C. HPLC (10–90% B), R_t 10.44 min. Found: C, 61.79; H, 7.76; N, 4.83%. $C_{14}H_{21}NO_4$ (267.31) requires C, 62.90; H, 7.92; N, 5.24%. $[\alpha]_D$ = −4.2 °C (*c*, 1 in N aq. sodium hydroxide).

N-(2-Hydroxy-4-methoxybenzyl)-(*N*ᵉ-*t*-butoxycarbonyl)-L-lysine. Yield 63%, m.p. >300°C. HPLC (10–90% B), R_t 13.61 min.

N-(2-Hydroxy-4-methoxybenzyl)-L-phenylalanine. Yield 63%, m.p. >300°C. HPLC (10–90% B), R_t 11.56 min. Found C, 66.56; H, 6.18; N, 4.26%. $C_{17}H_{19}NO_4$ (301.3) requires C, 67.76; H, 6.36; N, 4.65%.

N-(2-Hydroxy-4-methoxybenzyl)-O-*t*-butyl-L-serine. Yield 60%, m.p. >300 °C. HPLC (10–90% B), R_t 12.23 min. Found: C, 57.87; H, 7.56; N, 4.00%. $C_{15}H_{23}NO_5 \cdot H_2O$ (319.3) requires C, 57.13; H, 7.99; N, 4.44%. $[\alpha]_D$ = −7.1 °C (*c*, 1 in N aq. sodium

hydroxide).

N-(2-Hydroxy-4-methoxybenzyl)-O-t-butyl-L-threo-nine. Yield 45%, m.p. >300°C. HPLC: (10–90% B), R_t 12.11 min.

N-(2-Hydroxy-4-methoxybenzyl)-O-t-butyl-L-tyro-sine. Yield 71%, m.p. >300°C. HPLC (10–90% B), R_t 15.34 min. Found: C, 66.09; H, 7.07; N, 3.36%. $C_{21}H_{27}NO_5 \cdot 1/2H_2O$ (382.4) requires C, 65.96; H, 7.38; N, 3.66%. $[\alpha]_D = +8.3$ ° (c, 1 in N aq. sodium hydroxide).

N-(2-Hydroxy-4-methoxybenzyl)-L-valine. Yield 62%, m.p. >300 °C. HPLC (10–90% B), R_t 6.95 min.

Mono and bisFmoc Derivatives of Hmb–Amino Acids

The N-substituted amino acid (5 mmol) was added to an ice-cold stirred solution of sodium carbonate decahydrate (16.0 mmol, 3.2-fold excess) in water (50 ml), and dioxan (30–50 ml) added to complete solution. A solution of fluorenylmethyl chloroformate (10.2 mmol, 2.1-fold excess) in dioxan (10 ml) was then added over 1 h. An aliquot (5 ml) of the reaction mixture was withdrawn, and washed with diethyl ether (2 × 10 ml). The basic aqueous layer was acidified with 5 N hydrochloric acid to pH 4, and washed with chloroform (2 × 10 ml). The combined chloroform layers were dried (sodium sulphate) and evaporated *in vacuo* to give the crude mono-Fmoc derivative. These samples were then converted back to the base amino acid by reaction with 95% aqueous trifluoroacetic acid (2 ml) for 15 min and then with 20% piperidine in DMF (2 ml, 15 min) for optical purity determination by Marfey's method [17].

The remainder of the reaction mixture was left to stir for a further 1–2 h after fluorenylmethyl chloroformate addition, the originally clear solution thickening with formation of a precipitate. The mixture was then extracted with diethyl ether (3 × 100 ml) and the combined etheral solutions (containing the sodium salt of the bis–Fmoc derivatives) washed with 0.5 N hydrochloric acid (3 × 100 ml) and dried (sodium sulphate). Removal of the solvents *in vacuo* gave the crude bis-Fmoc derivatives as oils which were dried *in vacuo* overnight. The crude products were purified by chromatography over silica gel (packed in chloroform). Samples were loaded in diethyl ether, and eluted with chloroform–methanol mixtures (between 4–8%

methanol). Appropriate fractions were pooled and evaporated *in vacuo*. Samples were recrystallised from ethyl acetate-hexane and dried *in vacuo* overnight at 35 °C.

Fmoc-derivatives *N*-Hmb-amino acids prepared by this method are listed in Table 5. Yields are calculated from the starting N-substituted amino acid. The following compound was also prepared.

N,O-bisFmoc-N-(2-Hydroxybenzyl)-L-alanine. Yield 32%, m.p. 165–172°C (shrinks 120–121°C). HPLC (10–90% B), R_t 25.42 min (>95%). TLC (silica), 10% methanol–chloroform, $R_f = 0.70$, Found: C, 72.77; H, 4.90; N, 2.01%. $C_{40}H_{33}NO_7 \cdot H_2O$ (657.66) require C, 73.05; H, 5.36; N, 2.13%. FAB-MS (+ve m/z); 662.5 $[M+Na]^+$, 640.4 $[M+H]^+ \cdot [\alpha]_D = -2.3$ ° (c = 1, DMF).

Racemization Tests

The N-aralkylated Fmoc–amino acids (0.02 mmol) were treated with neat TFA (2 ml) for 15 min, and the coloured solution evaporated *in vacuo*. The residues were treated with 20% piperidine in DMF (2 ml) for 15 min, the solution evaporated *in vacuo*, titrated with ether (3 × 5 ml) and dried *in vacuo*. The regenerated amino acids were dissolved in 50% aqueous acetone (400 μl), and part (100 μl) added to a solution (200 μl) containing 0.3 mg/ml of Marfey's reagent [17] in acetone and 0.5 N aq. sodium carbonate (40 μl). The mixture was kept at 37 °C for 60 min, cooled and 1 N hydrochloric acid (20 μl) then added. Samples (5 μl) were analysed by HPLC using a gradient of 10–40% B in A over 45 min, 2.0 ml/min flow rate and detection at 340 nm. The initial DL amino acids treated similarly gave three peaks corresponding to excess hydrolysed reagent ($R_t=15$ min), the L and later eluting D-derivatives (separations 2–5 min) [17]. The regenerated L-derivatives gave very small peaks corresponding to the D-isomer, in all cases estimated to be <0.5% of the unracemized L-compound.

General Synthesis of Pentafluorophenyl Esters

The *N,O*-bis-Fmoc-*N*-(2-hydroxy-4-methoxybenzyl)-amino acid (10.0 mmol) was dissolved in dry tetrahydrofuran (30 ml) with stirring and cooled in ice. Pentafluorophenol (11 mmol) dissolved in dry tetrahydrofuran (10 ml) was added, followed by dicyclohexylcarbodiimide (10.5 mmol) in the same solvent (10 ml). The mixture was stirred overnight, filtered,

and the residue washed with tetrahydrofuran (2 × 10 ml). The filtrate was evaporated to give an oily solid. The crude oil was chromatographed on silica gel packed and eluted with chloroform. Appropriate fractions were pooled and evaporated *in vacuo*, the solid ester was titrated with cold hexane (3 × 10 ml) and then dried *in vacuo* to give the purified pentafluorophenyl ester. Compounds prepared by this general method are listed in Table 6.

Solid Phase Peptide Synthesis: General

All syntheses utilised commercial Fmoc-Val-Pepsyn KA resin (0.08 meq/g), and all couplings not involving an Hmb substituted residue were performed on the 'Biolynx' synthesiser using standard 0.5 mmol scale coupling protocols. Amino acid analyses are of peptide–resins.

Studies Using the Pentapeptide Sequence (8)

(i) Preparation of N-Substituted Tripeptide Resins H-(X)Gly-Phe-Val-Pepsyn KA (17).
Individual samples of Fmoc-Val-Pepsyn KA resin (500 mg) was extended to the tripeptide stage on the Biolynx by standard deprotection and coupling reactions with Fmoc-Phe-OPfp and then with the N-substituted glycine derivatives [(a) **5**, R = H, R_1 = Me; (b) **6**, R,R_2 = H, R_1 = Me; (c) **6**, R,R_2 = H, R_1 = OMe; (d) **6**, R,R_1 = H, R_2 = OMe; (e) **7**, R,R_3 = H, R_1,R_2 = OMe; and (f) **7**, R = H, R_1,R_2,R_3 = OMe] (0.5 mmol) using TBTU (0.49 mmol), 1-hydroxybenzotriazole (0.5 mmol) and in a separate vial, diisopropylethylamine (0.99 mmol) as coupling agent. The N,O-protected glycine derivatives were pre-activated for 2 min, added to the individual resins, and acylation continued for 45 min.

(ii) Coupling Studies.
Individual samples (50 mg) of the deprotected, washed and dried tripeptide resins were swollen in DMF (50 µl) and reacted for 1 hr with Fmoc-Ala-OPfp (8.25 mg, 5 eq.) and HOBt (3.8 mg, 5 eq) (pre-mixed in DMF (50 µl), 2 min). The peptide resins **17a–f** were then washed with DMF and ether, and samples (25 mg) treated with 20% piperidine in DMF for 3 and 7 min. The deprotected resin samples (**18a–f**, 2 mg) were washed as above, dried briefly *in vacuo*, and then hydrolysed and analysed for amino acid content. Found: (**18a**, X = 5-methylfurfuryl) Ala, 0.64; Gly, 0.96; Phe, 1.03; Val, 0.99; Nle, 1.01; (**18b**, X = 5-methylthienyl) Ala, 0.43; Gly, 1.02; Phe, 1.01;

Val, 0.98; Nle, 1.00; (**18c**, X = 5-methoxythienyl) Ala, 0.24; Gly, 0.94; Phe, 0.97; Val, 1.00; Nle, 0.97; (**18d**, X = 3-methoxythienyl) Ala, 0.83; Gly, 0.96; Phe, 0.96; Val, 1.00; Nle, 1.04; (**18e**, X = 2,4-dimethoxybenzyl) Ala, 0.78; Gly, 1.01; Phe, 0.96; Val, 0.98; Nle, 1.00; (**18f**, X = 2,4,6-trimethoxybenzyl) Ala, 0.87; Gly, 0.98; Phe, 0.96; Val, 1.00; Nle, 0.97.

The Fmoc-resins **17a–c** (25 mg) were then re-acylated for 1 h in DMF with (Fmoc-Ala)$_2$O (5 eq.) (prepared from Fmoc-Ala-OH (1.0 mmol) dissolved in dichloromethane (5 ml, containing 0.5 ml DMF), and stirred in an ice-bath while diisopropylcarbodiimide (0.49 mmol) dissolved in dichloromethane (1 ml) was added and for a further 30 min). The solvent was then removed *in vacuo* and the residue taken into DMF (5 ml). Resin was acylated with 125 µl of this solution. The remaining resins **17d–f** (25 mg) were re-acylated with 5 eq. of Fmoc-alanine pentafluorophenyl ester as above. All the resins were now ninhydrin negative, and amino acid analysis indicated quantitative incorporation of alanine in all cases.

(iii) Completion of Synthesis of Pentapeptides (8).
Each of the above fully acylated resin forms was extended on the Biolynx synthesiser under standard conditions using Fmoc-Leu-OPfp. Deprotected, washed and dried peptide–resins were analysed for amino acid content, and samples (50 mg) cleaved with 95% (v/w) TFA–phenol (1 ml) for 90 min. The cleaved resins were filtered, washed with TFA (3×1 ml) and the combined filtrates evaporated *in vacuo*. The residues were taken into 0.1% aq. TFA (1 ml) and extracted with diethyl ether (6×1 ml). Samples (25 µl) of the aqueous solution were analysed by HPLC. Found: (**8a**, X=5-methylfurfuryl) Leu, 0.8; Ala, 0.97; Gly, 1.05; Phe, 0.97; Val, 1.00; Nle, 0.99; HPLC, R_t=11.95 min (>90%). (**8b**, X=5-methylthienyl) Leu, 0.95; Ala, 0.94; Gly, 1.04; Phe, 0.99; Val, 1.00; Nle, 0.98; HPLC, R_t=12.01 min (>95%). (**8c**, X = 5-methoxythienyl) Leu, 0.93; Ala, 0.91; Gly, 1.03; Phe, 0.98; Val, 1.00; Nle, 0.91; HPLC, R_t=11.80 min (GT95%). (**8d**, X = 3-methoxythienyl) Leu, 0.97; Ala, 0.97; Gly, 0.98; Phe, 0.96; Val, 1.00; Nle, 1.01; HPLC, R_t = 11.95 min (>95%). (**8e**, X=2,4-dimethoxybenzyl) Leu, 0.94; Ala, 0.92; Gly, 1.04; Phe, 1.02; Val, 1.00; Nle, 1.02; HPLC, R_t = 11.91 min (>95%). (**8f**, X = 2,4,6-trimethoxybenzyl) Leu, 0.95; Ala, 0.98; Gly, 1.01; Phe, 0.99; Val, 1.00; Nle, 0.98; HPLC, R_t = 11.95 min (>90%).

Preparation and Coupling Studies on Tripeptide (9)

Prepared as described in (i) above but using Fmoc-*N*-(Tmb)alanine-OH (**7**, R = Me, R₁,R₂,R₃ = OMe)). Resin **9** (50 mg) was coupled with (Fmoc–Ala)₂O (10 eq) (prepared as in (ii) above), or with Fmoc-Ala-OH (16.5 mg, 10 eq), PyBrop (23.3 mg, 9.95 eq) and diisopropyethylamine (12.5 mg, 19.5 eq) (pre-mixed in DMF (150 µl) for 2 min), for various times (Table 3). The peptide resin samples (**9**) were then deprotected and analysed for amino acid content as detailed. (1) Anhydride in DMF, 1 h, Ala, 1.39; Phe, 0.98; Val, 1.00; Nle, 1.01; (2) anhydride in DMF, 3 hr, Ala, 1.78; Phe, 0.98; Val, 1.00; Nle, 0.98; (3) anhydride in DMF, 3 and 18 hr, Ala, 2.03; Phe, 0.92; Val, 1.00; Nle, 0.99; (4) PyBroP in DMF, 3 hr, Ala, 1.52; Phe, 0.95; Val, 1.00; Nle, 0.96; (5) PyBroP in DMF, 3 and 18 hr, Ala, 1.67; Phe, 1.00; Val, 1.00; Nle, 1.00; (6) anhydride in DMF with 1 eq. dimethylaminopyridine, 3 hr, Ala, 1.25; Phe, 0.98; Val, 1.00; Nle, 0.98; (7) anhydride in DCM, 3 hr, Ala, 1.97; Phe, 0.98; Val, 1.00; Nle, 0.98.

Preparation and Coupling Studies on Tripeptides (14)

The 2-methoxybenzyl tripeptides were prepared as detailed in (i) above but using Fmoc-*N*-(2-methoxybenzyl)alanine (**7**, R = Me, R₁ = OMe, R₂, R₃ = H). The 2-hydroxybenzyl tripeptides were prepared from the anhydride of *N*,*O*-bis-Fmoc-*N*-(2-hydroxybenzyl)alanine (**7**, R = Me, R₁, R₃ = H, R₂ = OH, 10 eq.) formed by activation with diisopropyl-carbodiimide (4.9 eq.) as in (ii) above. Resins were then acylated with the pentafluorophenyl ester or symmetric anhydride of Fmoc-alanine as in (ii) above. *N*-(2-hydroxybenzyl)Ala-Phe-Val-KA (**14**, R = H), coupled with Fmoc–Ala–OPfp ester in DMF, found (1 hr acylation) Ala, 0.54; Phe, 1.00; Val, 1.00; Nle, 1.00; 11 hr acylation Ala, 1.06; Phe, 1.03; Val, 1.00; Nle, 1.00. N-(2-Methoxybenzyl)Ala-Phe-Val-KA (**14**, R = Me) coupled with Fmoc-Ala-OPfp in DMF (11 h acylation) found Ala, 0.02; Phe, 1.03; Val, 1.00; Nle, 0.98. *N*-(2-Hydroxybenzyl)Ala-Phe-Val-KA (**14**, R = H) coupled with the symmetric anhydride of Fmoc–alanine in DMF. Found: (15 min acylation) Ala, 0.64; Phe, 0.99; Val, 1.00; Nle, 1.04; (1 hr acylation) Ala, 1.10; Phe, 1.00; Val, 1.00; Nle, 1.02; (15 min acylation in dichloromethane) Ala, 1.02; Phe, 1.01; Val, 0.98; Nle, 0.99. *N*-(2-Methoxybenzyl)Ala-Phe-Val-KA (**14**, R = Me) coupled with the symmetric anhydride of Fmoc–alanine in DMF, 12 h, found Ala,

0.55; Phe, 0.99; Val, 1.00; Nle, 1.02. Note that *N*-2-methoxy and *N*-2-hydroxybenzyl-alanyl peptide-derivatives do not generate alanine on acid hydrolysis.

Preparation and Coupling Studies on Tripeptides (Hmb)X-Phe-Val-KA

The tripeptide–resins were prepared by standard methods on the Biolynx synthesiser using *N*,*O*-bis-Fmoc-*N*-(2-hydroxy-4-methoxybenzyl amino acid pentafluorophenyl esters with HOBt catalysis. Deprotection of the terminal bisFmoc amino acid residues gave an absorbance twice that of the previous phenylalanine residue. Tripeptide–resins (50 mg) were acylated with 10 eq. of Fmoc-amino acid-*N*-carboxyanhydride in a minimum of dichloromethane and at various time (Table 7) samples (10 mg) of resin removed, washed and deprotected with 20% piperidine–DMF in the usual manner. Resins were then analysed for amino acid content. Fmoc-Gly-(Hmb)Gly-Phe-Val-KA, found (30 min acylation) Gly, 1.98; Phe, 0.98; Val, 1.00; Nle, 1.01. Fmoc-Ala-(Hmb)Gly-Phe-Val-KA (15 min acylation), Ala, 0.96; Gly, 0.92; Phe, 1.00; Val, 0.97; Nle, 1.00. Fmoc-Leu-(Hmb)Gly-Phe-Val-KA (15 min acylation), Leu, 0.96; Gly, 1.02; Phe, 1.00; Val, 1.00; Nle, 0.98. Fmoc-Val-(Hmb)Gly-Phe-Val-KA (15 min acylation), Gly, 1.00; Phe, 0.99; Val, 1.97; Nle, 1.03. Fmoc-Gly-(Hmb)Ala-Phe-Val-KA (15 min acylation), Ala, 0.98; Gly, 0.98; Phe, 0.98; Val, 1.00; Nle, 1.01. Fmoc-Ala-(Hmb)Ala-Phe-Val-KA, (15 min acylation) Ala, 1.96; Phe, 1.00; Val, 0.97; Nle, 1.00. Fmoc-Leu-(Hmb)Ala-Phe-Val-KA (15 min acylation), Leu, 0.72; Ala, 1.00; Phe, 0.98; Val, 1.00; Nle, 0.98; (1 hr acylation) Leu, 0.97; Ala, 1.02; Phe, 0.98; Val, 1.00; Nle, 1.03. Fmoc-Val-(Hmb)Ala-Phe-Val-KA, (15 hr acylation) Ala, 0.95; Phe, 1.00; Val, 1.74; Nle, 1.03. Fmoc-Gly-(Hmb)Phe-Phe-Val-KA (30 min acylation), Gly, 0.96; Phe, 1.98; Val, 1.02; Nle, 1.01. Fmoc-Ala-(Hmb)Phe-Phe-Val-KA (15 min acylation), Ala, 0.49; Phe, 2.02; Val, 0.98; Nle, 1.00. (2 hr acylation), Ala, 0.98; Phe, 1.99; Val, 1.01; Nle, 0.99. Fmoc-Leu-(Hmb)Phe-Phe-Val-KA (15 min acylation), Leu, 0.30; Phe, 2.00; Val, 1.00; Nle, 0.98; (5 hr acylation, Leu 1.01; Phe, 1.99,; Val, 0.96; Nle, 1.00. Fmoc-Gly-(Hmb)Val-Phe-Val-KA (30 min acylation) Gly, 0.96; Phe, 0.98; Val, 2.02; Nle, 1.01. Fmoc-Ala-(Hmb)Val-Phe-Val-KA (15 min acylation), Ala, 0.49; Phe, 1.02; Val, 1.98; Nle, 1.00. Fmoc-Ala(Hmb)Val-Phe-Val-KA (5 h acylation), Ala, 0.98; Phe, 0.99; Val, 2.01; Nle, 0.99. Fmoc-Leu-(Hmb)Val-Phe-Val-KA (15 min acylation), Leu, 0.30;

Phe, 1.00; Val, 2.00; Nle, 0.98. Fmoc-Leu-(Hmb)Val-Phe-Val-KA (24 h acylation), Leu, 1.01; Phe, 0.99; Val, 1.96; Nle, 1.00.

Preparation and Cleavage Studies on Pentapeptide-Resins H-Leu-Ala-(Hmb)Gly-Y-Val–resin (16, Y = Met, TyrBu^t), His(Trt), Cys(Trt), Cys(Acm), Trp and Trp(Boc).

The pentapeptide sequences were assembled on the Biolynx synthesiser. N,O-bisFmoc-N-(2-hydroxy-4-methoxybenzyl)glycine was incorporated using its pentafluorophenyl ester with HOBt catalysis for 45 min. Fmoc–Ala–OPfp was double-coupled to the terminal (Hmb) tripeptide–resin for 2 h. The analogous sequences (19) lacking the Hmb on the glycine residue were also prepared using entirely standard procedures (single 45 min couplings throughout). Resin bound peptides were analysed for amino acid content and samples (20 mg) cleaved for 90 min with (a) TFA–EDT–Et$_3$SiH (94:3:3 v/v/v, 1 ml), (b) TFA–EDT–iPr$_3$SiH (94:3:3 v/v/v, 1 ml) or (c) TFA–PhOH–Et$_3$SiH (94:3:3 v/v/v, 1 ml). Cleaved solutions were sparged with nitrogen, extracted with cold diethyl ether (2 × 25 ml), centrifuging after each extraction. The residues were dissolved in 0.1% TFA (1 ml) and eluted through a (2.5 cm) Pharmacia NAP-10 gel column with 0.1% TFA. The first 1 ml of eluate was discarded, then 2 ml collected and analysed by HPLC. **19**, Y = Met, found Leu, 1.01; Ala, 1.02; Gly, 1.03; Met, 0.94; Val, 1.00: Nle, 1.19; HPLC, R_t = 9.9 min (>95%). **16**, Y = Met, found Leu, 1.01; Ala, 1.03; Gly, 1.05; Met, 0.90; Val, 1.00; Nle, 1.21; HPLC, R_t=9.95 min (>95%). **19**, Y=Tyr(Bu^t), found Leu, 1.01; Ala, 1.01; Gly, 1.03; Tyr, 0.98; Val, 0.98; Nle, 1.03; HPLC, R_t = 8.7 min (>97%). **16**, Y = Tyr(Bu^t), found Leu, 0.98; Ala, 0.97; Gly, 1.04; Tyr 1.00; Val, 1.01; Nle, 1.06; HPLC, R_t = 8.7 min (>95%). **19**, Y = His(Trt), found Leu, 1.05; Ala, 1.04; Gly, 1.04; His, 0.94; Val, 0.94; Nle, 1.05; HPLC, R_t = 5.6 min (>98%). **16**, Y = His(Trt), found Leu, 1.04; Ala, 1.03; Gly, 1.06; His, 0.93; Val, 0.94; Nle, 1.06; HPLC, R_t = 5.6 min (>95%). **19**, Y = Cys(Trt), found Leu, 0.99; Ala, 0.99; Gly, 1.00; Val, 1.01; Nle, 1.03; HPLC, R_t=9.4 min (90%), 12.8 min (8%). 16, Y = Cys(Trt), found Leu, 0.95; Ala, 0.94; Gly, 0.99; Val, 1.02; Nle, 1.03; HPLC, R_t = 9.5 min (88%), 13.0 min) 11%). **19**, Y = Cys(Acm), found Leu, 1.00; Ala, 1.00; Gly, 1.01; Val, 0.99; Nle, 1.01; HPLC, R_t = 8.9 min (>98%). **16**,

Y = Cys(Acm), found Leu, 0.97; Ala, 0.97; Gly, 1.03; Val, 1.03; Nle, 1.05; HPLC, R_t = 8.9 min (>95%). **19**, Y = Trp, found Leu, 1.01; Ala, 1.00; Gly, 1.01; Val, 1.01; Nle, 0.97; HPLC, R_t = 12.8 min (85%), 14.3 min (10%). **16**, Y = Trp, Leu, 1.00; Ala, 0.98; Gly, 1.01; Val, 1.01; Nle, 1.00; HPLC, R_t=12.8 min (75%), 15.4 min (20%). **19**, Y=Trp(Boc), standard: Leu, 1.01; Ala, 0.98; Gly, 1.01; Val, 1.00; Nle, 1.14; HPLC, R_t = 12.8 min (>97%). **16**, Y=Trp(Boc) Leu, 0.97; Ala, 0.97; Gly, 0.96; Val, 1.10; Nle, 1.14; HPLC, R_t = 12.8 min (>97%).

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