Fmoc Amino Acid Fluorides: Convenient Reagents for the Solid-Phase Assembly of Peptides Incorporating Sterically Hindered Residues

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Fmoc amino acid fluorides, recently shown to be a new class of rapid-acting acylating agents in peptide synthesis are well suited for the solid-phase synthesis of medium-sized peptides such as ACP(65-74), magainin-II-amide, and h-CRF. The most important advantage of these reagents is their high reactivity in the coupling of sterically hindered amino acid residues, such as α-aminoisobutyric acid (Aib), results which are at least partly due to the small size of the fluoride leaving group. Both h-(Aib$^{32-35}$)-CRF(1-41), bearing four consecutive Aib-residues, and alamethicin acid, neither previously accessible by solid-phase synthesis, were successfully synthesized via acid fluorides using unusually short coupling times. In contrast, attempted syntheses via UNCA's and PyBroP activation, both reported to be well suited for sterically hindered systems, failed to give the desired peptides. These remarkable differences prompted a more detailed comparison of the acid fluorides with symmetric anhydrides, UNCA's, and the PyBroP activation technique. Side products formed during the acylation of hindered amino components by Fmoc-Aib-NCA were identified and their formation rationalized. These side products could have their origin in the demonstrated instability of Fmoc-NCA's in the presence of tertiary bases or in a diversion of the position of attack on the NCA from the more hindered to the less-hindered carbonyl function by a bulky nucleophile. Clearly caution is required when such bases are employed to enhance coupling rates for hindered systems.

Introduction

Ever since Merrifield's original report on solid-phase peptide synthesis,[1] numerous methods of effecting the coupling step have been examined.[2] For many years carbodiimides (e.g. DCC[3,4]) proved to be the most popular activating agents, although the occurrence of certain side reactions led to the incorporation of additives, such as HOBt,[5] during the use of these reagents. An especially effective catalyst is the binary mixture of HOBt/DIEA.[6,7] In addition to the carbodiimide method a number of new acylating agents such as uronium (HBTU[8]) and phosphonium (BOP[9]) salts, preformed anhydrides, active esters, and acid chlorides[10] have been developed in recent years. These methods have been reasonably effective in the case of ordinary peptides although special problems have often arisen due to secondary structure formation[11] or the presence of sterically hindered amino acids. The incorporation of one or more adjacent highly hindered amino acid residues, such as Aib, is often inefficient, although various techniques have been shown to be useful in special cases.[12–14] However, unusually long reaction times or increased reaction temperatures are necessary to perform these couplings.

Fmoc amino acid fluorides, recently shown to be rapidly acting species for peptide synthesis in solution or for the solid-phase synthesis of simple peptides,[15,16] were also expected to be useful for solid-phase syntheses of more complicated, longer peptides and for the coupling of sterically hindered units, such as those which are common among the naturally occuring peptaibols which to date have never been assembled by solid-phase methods. A preliminary report covering a portion of the current work on the coupling of sterically hindered amino acids by means of acid fluorides has appeared.[17]

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(2) Barany, G.; Kneib-Cordonier, N.; Mullen, D. G. *Int. J. Pept. Protein Res.* **1987**, *30*, 705.

(3) Abbreviations: DCC = *N,N'*-dicyclohexylcarbodiimide, HOBt = 1-hydroxybenzotriazole, DIEA = diisopropylethylamine, HBTU = 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, BOP = (benzotriazolyl-*N*^{1}-oxy)tris(dimethylamino)phosphonium hexafluorophosphate, Pfp = pentafluorophenyl, NMM = *N*-methylmorpholine, TOPPIPU = 2-(2-oxo-1,2-dihydro-1-pyridyl)-1,1,3,3-bis(pentamethylene)-uronium tetrafluoroborate, UNCA = urethane-protected amino acid N-carboxy anhydride, PyBroP = bromotris(pyrrolidino)phosphonium hexafluorophosphate, DMAP = 4-(dimethylamino)pyridine.

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Table 1. Conditions for the Stepwise Solid-Phase Synthesis of ACP(65-74), Magainin-II-amide, h-CRF, and h-(Aib$^{32-35}$)-CRF by Means of Fmoc Amino Acid Fluorides[a,b]

	ACP(65-74)	magainin-II-amide	h-CRF	h-(Aib$^{32-35}$)-CRF
resin	TG S PHB 0.24 mmol/g	TG SRAM 0.22 mmol/g	TG SRAM 0.22 mmol/g	TG SRAM 0.22 mmol/g
couplings	single[c] 20 min	double 15 min	double 15 min	double 15 min
coupling concentration	0.3 M in DMF 3 equiv of amino acid derivative	0.3 M in DMF 3 equiv of amino acid derivative	0.3 M in DMF 3 equiv of amino acid derivative	0.3 M in DMF 3 equiv of amino acid derivative
deprotection	20% piperidine/DMF	20% piperidine/DMF	20% piperidine/DMF	20% piperidine/DMF
base	1 equiv of DIEA	1 equiv DIEA	1 equiv DIEA	1 equiv of DIEA
yield of crude products	73%	82%	76%	74%

[a] All syntheses were carried out manually using a simple batch reactor. The peptide resin cleavage was performed for 2 h with TFA, 5% water, 5% phenol, 5% thioanisole, and 2.5% ethanedithiol. [b] For His and Arg, coupling via activation by TOPPIPU and 2 equiv of DIEA was used. [c] In the case of chain assembly on the TG S PHB resin the first amino acid was incorporated by using a double coupling (45 min for each coupling).

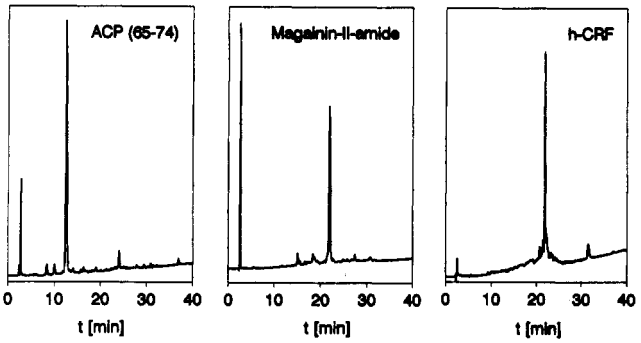

Figure 1. HPLC profiles of crude ACP(65-74), magainin-II-amide, and h-CRF synthesized by means of Fmoc amino acid fluorides.

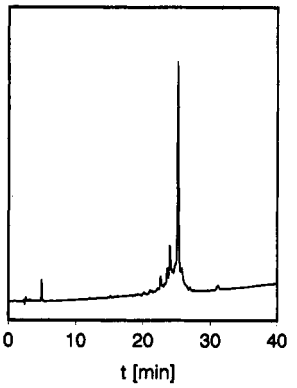

Figure 2. HPLC profile of crude h-(Aib$^{32-35}$)-CRF(1-41) synthesized by means of Fmoc amino acid fluorides.

Results and Discussion

Initially, in order to investigate the general applicability of the Fmoc amino acid fluorides to solid-phase peptide synthesis, three model peptides ACP(65-74) (a decapeptide portion of the acyl carrier protein), magainin-II-amide, reported to be a difficult sequence,[18] and the 41-amino acid peptide h-CRF (human-corticotropin-releasing factor) were synthesized by the fluoride method (for experimental details see Table 1). All three peptides were readily assembled and the purity of the crude products, according to HPLC profiles (Figure 1) and ES-MS data,[19] was similar to that of the same peptides synthesized using the recently introduced coupling agent TOPPIPU.[20,21] Amino acid composition was determined by amino acid analysis.[19]

A more demanding task was the synthesis of the h-CRF analog h-(Aib$^{32-35}$)-CRF(1-41), bearing four consecutive sterically hindered Aib residues (Table 1). Here again, surprisingly, using Fmoc amino acid fluorides the synthesis proceeded without any problems, as indicated clearly by the HPLC profile (Figure 2), ES-MS data, and amino acid analysis.[22]

In order to verify the successful application of Fmoc-amino acid fluorides for the coupling of sterically hindered units, solid-phase assembly of the difficult sequence alamethicin acid (previous syntheses of alamethicin have succeeded only by segment condensation strategies[23]), which contains eight Aib and two proline residues, was examined (Table 2). No difficulties were encountered. This new method is doubly interesting since other recently developed methods of handling peptides bearing multi-Aib units such as Heimgartner's so-called "azirine/oxazolone method"[24] are not applicable to the solid-phase approach.

The synthesis of alamethicin acid was carried out on Tenta Gel S AC,[25,26] in order to avoid cleavage of acid labile Aib–Pro bonds during the final acidic cleavage of the peptide from the resin.[27] The crude alamethicin acid obtained in this way proved to be of remarkable HPLC

(18) Barris, C.; Brass, A.; Robson, B.; Tomalin, G. In *Innovation and Perspectives in Solid Phase Synthesis*; Epton, R., Ed.; SPCC(UK) Ltd.: Birmingham, 1990; p 441.

(19) Characterizing data for ACP(65-74), magainin-II-amide, and h-CRF synthesized by Fmoc amino acid fluorides: ACP(65-74) (H-VQAAIDYING-OH): ES-MS calcd (monoisotopic) 1062.5, found 1063.6 [M + H]$^+$; amino acid analysis, Val 0.94 (1), Glu 1.01 (1), Ala 1.92 (2), Ile 1.83 (2), Asp 2.20 (2), Gly 1.00 (1), Tyr 0.96 (1); magainin-II-amide (H-GIGKFLHGAKKFGKAFVGEIMNS-NH$_2$): ES-MS calcd (monoisotopic) 2464.3, found 2465.0 [M + H]$^+$; amino acid analysis, Asp 0.98 (1), Ser 1.81 (2), Glu 1.05 (1), Gly 4.22 (4), Ala 1.86 (2), Val 1.00 (1), Met 0.98 (1), Ile 1.97 (2), Leu 0.96 (1), Phe 3.09 (3), His 1.03 (1), Lys 4.27 (4); h-CRF (H-SEEPPISLDLTFHLLREVLFMARAE-QLAQQAHSNRKLMFII-NH$_2$): ES-MS calcd (monoisotopic) 4754.5, found 4755.5 [M + H]$^+$; amino acid analysis, Asp 1.98 (2), Thr 0.92 (1), Ser 2.89 (3), Glu 8.96 (9), Ala 3.86 (4), Val 1.00 (1), Met 1.74 (2), Ile 2.94 (3), Leu 6.72 (7), Phe 0.96 (1), His 2.04 (2), Lys 1.06 (1), Arg 3.04 (3), Pro 2.03 (2).

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(22) ES-MS and amino acid analysis for h-(Aib$^{32-35}$)-CRF(1-41) synthesized by means of fluorides: ES-MS calcd (monoisotopic) 4601.0, found 4601.0 [M + H]$^+$; amino acid analysis, Asp 1.00 (1), Thr 0.92 (1), Ser 1.82 (2), Glu 8.89 (9), Ala 3.99 (4), Aib 4.31 (4), Val 1.00 (1), Met 1.96 (2), Ile 2.82 (3), Leu 6.90 (7), Phe 0.99 (1), His 1.02 (1), Arg 2.03 (2), Lys 1.09 (1), Pro 2.09 (2).

(23) Balasubramanian, T. M.; Kendrick, N. C. E.; Taylor, M.; Marshall, G. R.; Hall, J. E.; Vodyanoy, I.; Reusser, F. *J. Am. Chem. Soc.* **1981**, *103*, 6127; Nagaraj, R.; Balaram, P. *Tetrahedron* **1981**, *37*, 1263. Schmitt, H.; Jung, G. *Liebigs. Ann. Chem.* **1985**, 321. Gisin, B. F.; Davis, D. G.; Borowska, Z. K.; Hall, J. E.; Kobayashi, S. *J. Am. Chem. Soc.* **1981**, *103*, 6373.

(24) Heimgartner, H. *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 238.

(25) The utilization of the acid-labile linker allowed cleavage of the peptide from the solid support by means of a mixture consisting of 2% triisopropylsilane, 5% phenol, and 5% H_2O in 50% TFA/DCM[26] for 30 min.

(26) Sole, N. A.; Barany, G. *J. Org. Chem.* **1992**, *57*, 5399.

(27) Schmitt, H.; Jung, G. Liebigs *Ann. Chem.* **1985**, 321.

Table 2. Conditions for the Solid-Phase Synthesis of Alamethicin Acid by Means of Fluorides, UNCA's, or Activation by PyBroP

coupling method[a]	Fmoc-AA-F	Fmoc-AA-NCA[b]	PyBroP[d]
resin	TG S AC (0.24 mmol/g)	TG S AC (0.24 mmol/g)	TG S AC (0.24 mmol/g)
equiv of activated amino acid derivative	3	3	3
coupling concentration	0.3 M in DMF	0.3 M in DMF	0.3 M in DMF
coupling conditions	single (15 min) 20 °C	double (2 × 30 min) 50 °C	double (2 × 30 min) 20 °C
base	1 equiv of DIEA[c]	no base added	2 equiv of DIEA[c]
deprotection conditions	20% piperidine/DMF, 15 min	20% piperidine/DMF, 15 min	20% piperidine/DMF, 15 min

[a] The first amino acid was coupled to the resin in all cases by means of the acid fluoride (2 × 45 min) in DCM in the presence of DIEA. [b] Fmoc-Pro-OH was incorporated via PyBroP activation. [c] The amount of amine used is related to the amino acid. [d] Following the proposed coupling mechanism for PyBroP[14] 6 equiv of amino acid and PyBroP were preactivated for 3 min to form a 0.3 M solution of symmetric anhydride (3 equiv) and coupled at 20 °C with 1.8 equiv of DIEA and 0.2 equiv of DMAP. The yield of crude alamethicin acid obtained by synthesis via Fmoc amino acid fluorides was 84%.

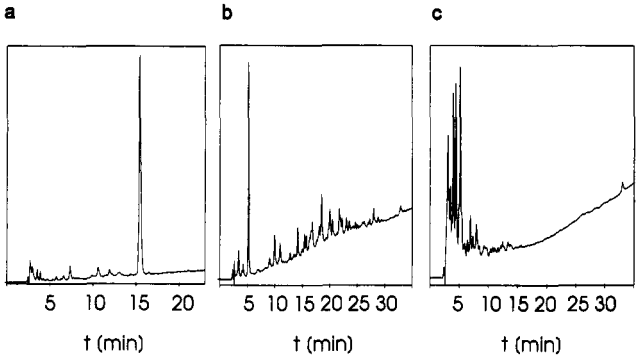


Figure 3. HPLC profiles of crude alamethicin acid assembled by means of fluorides (a), UNCA's (b), or PyBroP activation (c).

purity (Figure 3a). The result was confirmed by ES-MS and amino acid analysis.[28] Amino acid analysis via DCC-catalyzed hydrolysis and examination of the appropriate derivatized amino acid esters on a chiral GC column showed that no amino acid exhibited a D-content greater than 0.25%.[29]

The success of these syntheses prompted a systematic comparison of the fluoride technique with methods previously reported to be exceptionally well suited for the incorporation of sterically hindered amino acid residues. The synthesis of alamethicin acid was repeated using either UNCA's or PyBroP activation (Table 2). Both of these methods were recently shown to be useful techniques for sterically hindered systems. Monitoring of the coupling yields (Figure 4) clearly demonstrates the difficulties which were encountered, especially for coupling of Aib residues to ordinary amino acid residues or acylation onto Aib units. As made clear by the HPLC profiles of the final crude products, no significant amount of alamethicin acid was obtained in the case of syntheses carried out via UNCA's or PyBroP activation (Figure 3b,c).

In view of marked differences among the various coupling procedures examined, it was of interest to determine whether Fmoc amino acid fluorides would also show higher reactivity toward nonhindered systems. For these studies the comparisons were extended to include Fmoc-Aib symmetric anhydride. As shown in Table 3, using a Tenta Gel S RAM resin, it was found that all four coupling procedures studied gave satisfactory results. On the other hand, for acylation of a sterically hindered Aib-Tenta Gel S RAM resin, only the acid fluoride provided for coupling at an acceptable rate.

In addition, the same four methods were compared with regard to the incorporation of four adjacent Aib residues to give the model peptide h-(Aib$^{32-35}$)-CRF(32-41) (Table 4). In confirmation of the results obtained for the acylation onto the amino resin, incorporation of the first Aib residue proceeded rapidly by all methods, although quantitative reaction occurred only for the fluoride. In marked contrast, for the following three Aib couplings drastic differences were encountered between the fluoride and the three other coupling methods.[17] The peptides were cleaved from the solid support by means of "Solution K"[30] and analyzed by HPLC (Figure 5) and ES-MS.[31] The data demonstrate that only the acid fluoride derivative led to the desired decapeptide with four Aib-residues fully incorporated, whereas all three of the other techniques gave the heptapeptide bearing only a single Aib-unit as the major product. These results are inconsistent with the conclusions of Goodman et al.[12] who reported that acid fluorides react sluggishly in the case of sterically hindered systems. Discrepancies between the two studies may be due to the choice of two-phase coupling systems in the earlier work.

Interestingly, unexpected side products were observed in the case of acylation via Fmoc-Aib-NCA (Figure 5a). ES-MS data imply the formation of additional products with masses of 44 units greater than that of the corresponding peptide with 1–3 incorporated Aib-residues. In order to characterize the nature of these materials the side products were isolated by preparative HPLC and analyzed by ES-MS. In addition, the side product which corresponded to the peptide having two Aib residues incorporated (peak 2[a]) was compared with the matched peptide (peak 2) by means of ^{13}C-NMR. The appearance of an additional carbon for the side product (DMSO-d_6, δ (ppm) 157.13) which matches the carbonyl carbon in urea structures (dicyclohexyl urea in DMSO-d_6 δ (ppm) 156.54) taken together with the detected mass of the side product, suggests that it arises via attack of the amino component on the 2-carbonyl group of Fmoc-Aib-NCA (so called "wrong" ring opening) or by attack at the same carbonyl subsequent to loss of the Fmoc function and ring

(28) Characterizing data for alamethicin acid assembled by fluorides: ES-MS calcd 1977.1 (monoisotopic), found 1011.9 $[M + 2Na]^{2+}$; amino acid analysis, Glu 3.04 (3), Gly 1.06 (1), Ala 1.97 (2), Aib 7.83 (8), Val 2.10 (2), Leu 1.00 (1), Phe 1.00 (1).

(29) The method of Kusumoto (Kusumoto, S.; Matsukura, M.; Shiba, T. *Biopolymers* **1981**, *20*, 1869). The extent of D-amino acid found for alamethicin acid synthesized via Fmoc-amino acid fluorides was as follows: Ala 0.25% (±0.03%), Val 0.06% (±0.01%), Leu 0.1% (±0.05%), Pro 0% (±0.05%), Glu 0.15% (±0.15%), Phe 0.12% (±0.15%).

(30) King, D. S.; Fields, C. G.; Fields, G. B. *Int. J. Pept. Protein Res.* **1990**, *36*, 255.

(31) HPLC conditions: Polyencap A300 column, 250 × 4 mm i.d., Bischoff Analysentechnik GmbH, Germany. Mobile phase A: 0.1% TFA in water, B: 0.1% TFA in 50% ACN/50% water, linear gradient 5–95% B in 40 min, 1 mL/min, 220 nm; ES-MS data calcd (monoisotopic) h-(Aib^{35})-CRF(35-41) 829.5, h-(Aib$^{34-35}$)-CRF(34-41) 914.5, h-(Aib$^{33-35}$)-CRF(33-41) 999.7, h-(Aib$^{32-35}$)-CRF(32-41) 1084.7, found $[M + H]^+$ (a) (1) 830.7, (1[a]) 874.4, (2) 915.9, (2[a]) 959.8, (3) 1000.9, (3[a]) 1044.8, (b) (4) 1086.0, (c) (1) 829.6, (2) 914.7 (3) 999.7, (c) (1) 830.6, (2) 915.7, (3) 1000.9.

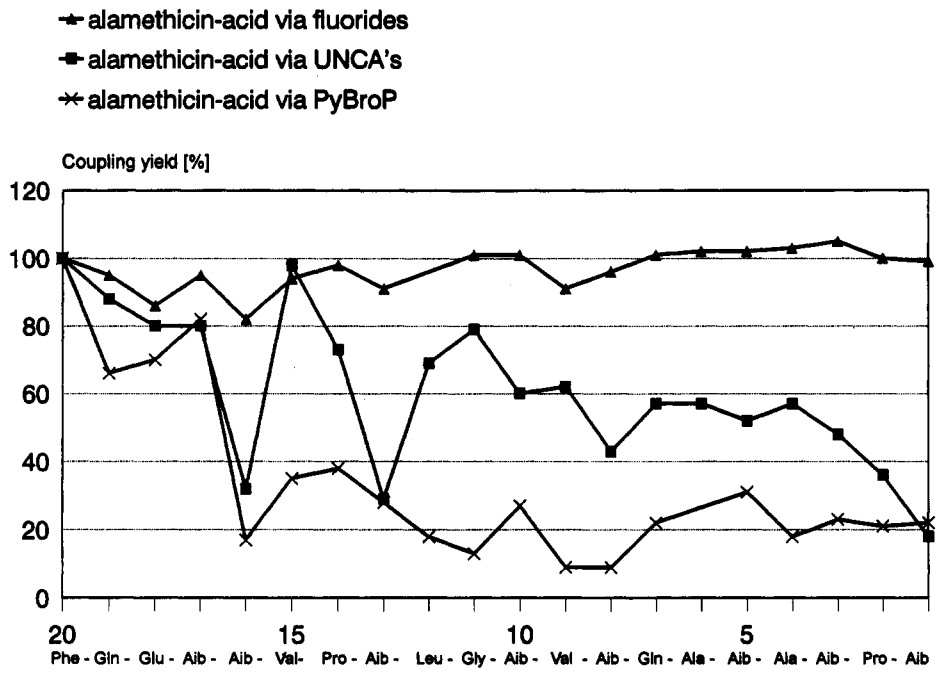


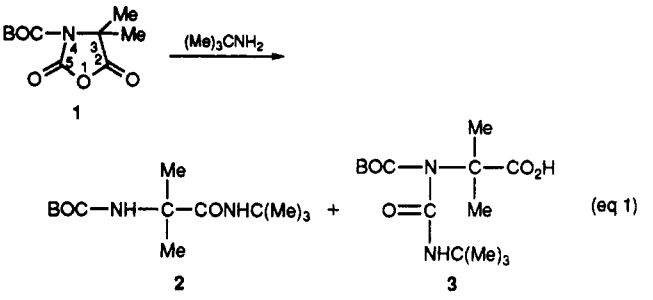
Figure 4. Coupling yields for stepwise solid-phase synthesis of alamethicin acid by means of acid fluorides, UNCA's, and PyBroP activation.

Table 3. Loading of Fmoc-Aib-OH onto TG S RAM-Resin and Aib-TG S RAM-Resin by Different Methods

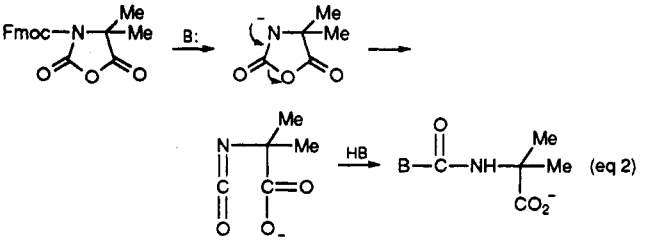
	Fmoc-Aib derivative/activation	
	TG S RAM 15-min coupling yield,[d] %	Aib-TG S RAM 15-min coupling yield,[d] %
Fmoc-Aib-OH/PyBroP[a]	89	15
Fmoc-Aib-F[b]	96	55
Fmoc-Aib-NCA[c] (50 °C)	91	6
Fmoc-Aib-symmetric anhydride[b]	87	9

[a] Fmoc-AA-OH and PyBroP were used at concentrations of 0.6 M in DMF to form a 0.3 M solution of symmetric anhydride. [b] 3 equiv of amino acid derivative were coupled at 20 °C in a 0.2 M solution of DMF with 1 equiv of DIEA. [c] 3 equiv of Fmoc-Aib-NCA were coupled at 50 °C in a 0.2 M solution of DMF without base. [d] The coupling yield was determined by UV analysis of the Fmoc-deprotection step (20% piperidine/DMF, 15 min).

opening to an isocyanate.[32] Such reactions have already been described for unprotected Aib-NCA[32] in the case of reaction with sterically hindered amines. In order to study the same process in a simpler model and to verify the "wrong" ring opening of UNCA's for sterically hindered systems, Fmoc-Aib-NCA and BOC-Aib-NCA were treated with *tert*-butylamine. In case of Fmoc-Aib-NCA after 14 h a variety of products were detected by HPLC and ES-MS, some of which could be rationalized as arising from initial loss of the Fmoc group in the presence of the basic amine. Using BOC-Aib-NCA under the same conditions only two main products could be detected after 14 h (HPLC ratio: 1:1), in addition to small amounts of unreacted BOC-Aib-NCA, namely BOC-Aib-*tert*-butylamide (2) and a product, assigned structure 3, with a mass of 44 units greater than that of BOC-Aib-*tert*-butylamide. The products were isolated by preparative HPLC and examined by ^{1}H-NMR and ^{13}C-NMR spectroscopy, and high resolution mass spectroscopy. As previously observed for the side product derived from the model peptide, the additional carbon atom of the urea unit was visible in the ^{13}C-NMR spectrum of compound 3. It is probable that increased steric hindrance in both reactants is sufficient to overcome electronic factors normally favoring reaction at the 2-carbonyl of the BOC-Aib derivative 1 (eq 1). The



Fmoc case may be special since deblocking could be followed by the same ring opening process observed by Kopple[32] for the analogous unsubstituted Aib derivative (eq 2). Precise mechanistic details for these reactions must await further studies.



In order to determine whether α-ureido structures, such as 3, were also formed in the case of less-hindered systems, an elongation of h-CRF(36-41) was performed using Fmoc-Ala-NCA instead of Fmoc-Aib-NCA so as to give h-(Ala$^{32-35}$)-CRF(32-41). Under the same conditions used previously a single major peak was detected by HPLC

(32) Kopple, K. D. *J. Am. Chem. Soc.* **1957**, *79*, 6442–6445. Kopple, K. D. *J. Am. Chem. Soc.* **1957**, *79*, 662.

Table 4. Conditions for the Stepwise Elongation of h-CRF(36-41) by Four Aib-Units

	Fmoc-Aib derivative activation			
	Fmoc-Aib-OH/PyBroP[a]	Fmoc-Aib-F	Fmoc-Aib-NCA	Fmoc-Aib symmetric anhydride
coupling times	15 min (3-min preactivation)	15 min	15 min	15 min
coupling concentration	0.3 M in DMF	0.2 M in DMF	0.2 M in DMF	0.2 M in DMF
equivalents of amino acid	3	3	3	3
temperature	20 °C	20 °C	50 °C	20 °C
base	1.8 equiv of DIEA, 0.2 equiv of DMAP	1 equiv of DIEA	no base added	1 equiv of DIEA
deprotection conditions	15 min, 20% piperidine/DMF	15 min, 20% piperidine/DMF	15 min, 20% piperidine/DMF	15 min, 20% piperidine/DMF

[a] Fmoc-AA-OH and PyBroP were used at concentrations of 0.6 M in DMF to form a 0.3 M (3 equiv) solution of symmetric anhydride.

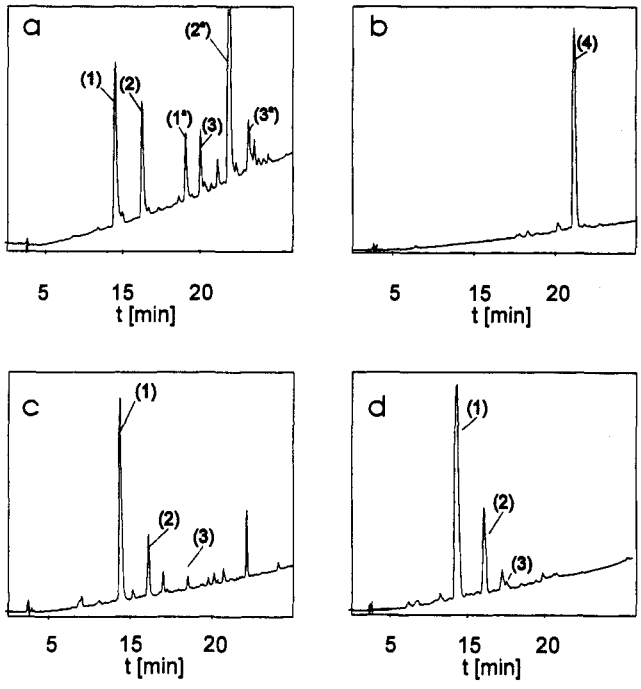


Figure 5. HPLC profiles of crude products from the stepwise elongation of h-CRF(36-41) by four Aib-residues using (a) Fmoc-Aib-NCA, (b) Fmoc-Aib-F, (c) Fmoc-Aib symmetric anhydride, (d) Fmoc-Aib-OH/PyBroP. Peaks 1–4 show the products with 1, 2, 3, and 4 Aib-residues incorporated. Peaks 1*–3* show side products obtained in the case of Fmoc-Aib-NCA with masses of +44 relative to the corresponding peptide with 1, 2, and 3 Aib-units incorporated.

(Figure 6) and its characterization by ES-MS[33] showed it to be the desired product. The lack of any side products for the coupling of adjacent Ala-residues by means of Fmoc-Ala-NCA confirms that formation of the urea derivative in the case of Fmoc-Aib-NCA is related to the steric hindrance of the α,α-dialkyl side chain.

Finally, the expectation that prolonged coupling times may be needed to effect the incorporation of Aib[12-14] or even more highly hindered units into peptides or onto resins, and the observed significant loss of the Fmoc group of Fmoc-Aib-NCA in the presence of tert-butyl amine, has raised the question of the stability of the Fmoc-group of various activated species toward the bases which are routinely used in SPPS. In order to examine the question of premature Fmoc deblocking the release of dibenzofulvene from Fmoc-Aib-OH, Fmoc-Aib-F, Fmoc-Aib-NCA, and Fmoc-Aib symmetric anhydride was examined under conditions normally used for chain assembly (0.2 M solution, in DMF, 1 equiv of DIEA or 0.1 equiv of NMM,

(33) ES-MS data of crude h-(Ala[32-35])-CRF(36-41) (Ala incorporated by means of Fmoc-Ala-NCA): calcd 1028.6 (monoisotopic), found 515.4 $[M + 2H]^{2+}$, 1029.7 $[M + H]^{+}$.

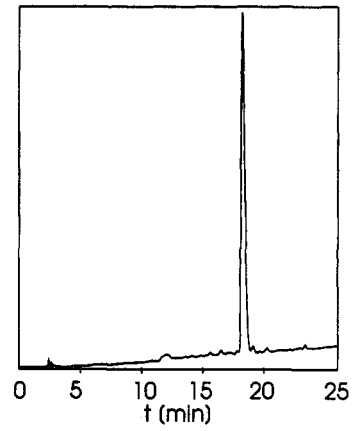


Figure 6. HPLC profile of crude h-(Ala[32-35])-CRF(36-41) (Ala incorporated by means of Fmoc-Ala-NCA).

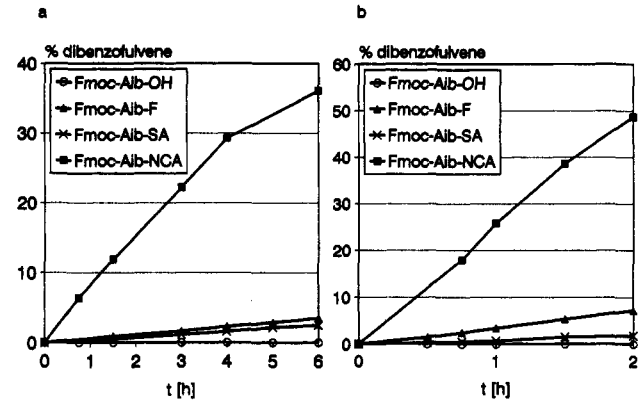


Figure 7. Formation of dibenzofulvene from different Fmoc-Aib species in the presence of 0.1 equiv of NMM in DMF (a) or 1 equiv of DIEA in DMF (b) (concentration 0.2 M, ambient temperature). Fmoc-Aib-SA = Fmoc-Aib symmetric anhydride.

respectively) (Figure 7). Dibenzofulvene was detected qualitatively by ES-MS[34] and measured quantitatively by HPLC.[35] Remarkably, nearly 50% of the theoretical amount of dibenzofulvene was released in the case of Fmoc-Aib-NCA in the presence of DIEA over a period of 2 h. On the other hand only slow degradation occurred in the case of Fmoc-Aib-F and Fmoc-Aib symmetric anhydride. A catalytic amount of *N*-methylmorpholine was less destructive, with approximately 15% of the Fmoc group being lost from Fmoc-Aib-NCA over a period of 2 h. These unexpected results suggest that long coupling times should be avoided, especially in the case of Fmoc-protected NCA's.

(34) ES-MS dibenzofulvene: calcd 178.22 [monoisotopic], found 178.4 $[M + H]^{+}$.

(35) HPLC analysis for dibenzofulvene formation was carried out on a Polyencap column (A300, 250 × 4 mm i.d.), Bischoff Analysentechnik GmbH, Germany, Mobile phase 54.9% ACN/45% water/0.1% TFA, 1 mL/min, 301 nm.

Thus contrary to recent recommendations regarding the use of 1 equiv of DIEA for the coupling of BOC-NCA's,[36] addition of base should be avoided in the case of Fmoc-NCA's.

Conclusion

Fmoc amino acid fluorides have been shown to be excellently suited for the rapid solid-phase peptide synthesis of medium-sized peptides. The most impressive property of the Fmoc amino acid fluorides is their ability to couple sterically hindered α,α-dialkylamino acids, such as Aib, to similarly hindered amino acids. Fmoc-Aib-F is a readily available, highly active acylating agent in which the Fmoc function is relatively stable toward the bases routinely used in SPPS, such as DIEA or NMM. Unique examples of the success of using this reagent are the assemblies of h-(Aib$^{32-35}$)-CRF(1-41) and alamethicin acid, a peptide which is rich in hindered Aib units and which has not previously been synthesized by the solid-phase approach.

Experimental Section

General. Analytical HPLC characterization of all synthesized peptides was carried out on a Polyencap A300 column, 250 × 4 mm i.d., Bischoff Analysentechnik GmbH, Germany, with a Bischoff HPLC system incorporating a Lambda 1000 detector, pumps, central processor, and Knauer mixing chamber. Preparative isolation of peptides was carried out by HPLC on a 10-μm Polyencap A300 preparative column from the same company using a Shimadzu LC-8A system including LC-8A pumps, SCL-8A system controller, SPD-6A UV detector operated at 220 nm, injector, and C-R4A chromatopac recording unit. Peptides were eluted using a linear gradient. Eluant A: 0.1% TFA in water; eluant B: 0.1% TFA in 80% ACN/20% water (v/v).

ES-MS was performed on a TSQ 700, Finnigan MAT (sample flow 1 μL/min methanol/water (1:1), 3–5 kV, 80 °C). IR-spectra (KBr) of fluorides were recorded on a Specord 71 IR (VEB Carl Zeiss) instrument. ^{1}H-NMR and ^{13}C-NMR determinations were carried out on a Varian Gemini 200 instrument. Coupling yields were determined by UV measurements to monitor the Fmoc deprotection step on a LKB Ultrospec II device at 301 nm.

Racemization tests by the GC-MS technique were performed on a GC-Quadrupol-MS (Fisons Instruments, TRIO 1000) using a chiral Chirasil-Val column, 50 m, 0.25 μm i.d. (Machery Nagel). Hydrolysis was carried out in 6 N DCl/D_2O for 24 h (110 °C) and the hydrolysate was converted into the *N*-(trifluoroacetyl)amino acid isopropyl ester. High resolution mass spectroscopy was carried out on a VG AutoSpec EQ (VG Instruments, Wiesbaden, Germany).

Amino Acid Derivatives and Activating Reagents. Fmoc amino acid fluorides were prepared by using a standard procedure described by Carpino et al.[15] and characterized by IR (KBr): yield of Fmoc-Aib-F, 74%; IR 1840 cm^{-1}; ^{1}H-NMR (ppm, $CDCl_3$) δ 1.55 (s, 6, $CH(CH_3)_2$), 4.20 (t, 1, OCH_2CH), 4.46 (d, 2, OCH_2-CH), 5.05, (s, 1, NH), 7.25–7.77 (m, 8, aromatic protons); mp 118–120 °C. Anal. Calcd for $C_{19}H_{18}FNO_3$: C, 69.71%; H, 5.54%, N, 4.28. Found: C, 69.37%; H, 5.54%; N, 4.32%.

(36) Xue, C. B.; Naider, F. *J. Org. Chem.* **1993**, *58*, 350.

Fmoc-Aib symmetric anhydride was synthesized by stirring 6 mmol of Fmoc-Aib-OH with 4.4 mmol of dicyclohexylcarbodiimide in 120 mL of ethyl acetate for 2.5 h at ambient temperature. The mixture was cooled to about −20 °C for 2 h and then filtered in the cold to remove dicyclohexylurea. The filtrate was evaporated under reduced pressure, the oil dissolved in DCM (distilled over P_2O_5 and Na_2CO_3, respectively), and the solution again evaporated in vacuum until it became cloudy. After the solution was allowed to stand in the refrigerator overnight, the Fmoc-Aib symmetric anhydride was filtered, washed with hexane, and dried under vacuum. The anhydride was obtained in a yield of 69% as a white solid: mp 92–94 °C; ^{1}H-NMR (ppm, $CDCl_3$) 1.35 (s, 12, 2 × C(CH_3)$_2$), 4.18 (t, 2, 2 × OCH_2CH), 4.29, (d, 4, 2 × OCH_2CH), 5.75 (s, 2, 2 × NH), 7.26–7.89 (m, 16, aromatic protons); high resolution mass data (FAB MS) calcd 765.1573 [M + Cs]$^+$, found 765.153 [M + Cs]$^+$.

UNCA's were provided by Dr. A. Loffet, Propeptide, Vert le Petit, France. PyBroP was purchased from NovaBiochem, Bad Soden/Ts., Germany. Tenta Gel resins were obtained from Rapp Polymere, Tübingen, Germany.

Synthesis of h-CRF(36-41). h-CRF(36-41) was synthesized on a Tenta Gel support (S RAM, 0.24 mmol/g) using a MilliGen 9050 peptide synthesizer by means of Fmoc amino acids and TOPPIPU as the activating agent. All amino acids were double-coupled (2 × 30 min) with 4.5 equiv at a concentration of 0.3 M in DMF. The Fmoc-group was cleaved by 20% piperidine/DMF for 7 min. The resulting supported hexapeptide was used to examine its further extension by four Aib units (see Table 4 and Figure 5).

Reaction of Fmoc-Aib-NCA or BOC-Aib-NCA with *tert*-Butylamine. UNCA's (3 mmol) were allowed to react with *tert*-butylamine (3 mmol) in a 0.2 M solution of DCM for 14 h at ambient temperature. The DCM was evaporated and the crude products analyzed by HPLC and ES-MS. In the case of BOC-Aib-NCA, compounds **2** (131 mg, 17%) and **3** (181 mg, 20%) were obtained by preparative HPLC BOC-NH-C(CH_3)$_2$-CONHt-Bu (**2**): ^{1}H-NMR (DMSO-d_6) δ 1.22 (s, 9, NC(CH_3)$_3$), 1.25 (s, 6, C(CH_3)$_2$), 1.38 (s, 9, OC(CH_3)$_3$), 6.61 (s, 1, OCONH), 6.73 (s, 1, C(CH_3)$_3$NH); ^{13}C-NMR (DMSO-d_6) δ 25.13 (C(CH_3)$_2$) 28.09, 28.30 (2 × C(CH_3)$_3$), 49.76, 56.05, 78.07 (3 × CCH_3), 154.16, 173.82 (2 × CO); BOC-N(C(CH_3)$_2$COOH)(CONHtBu) (**3**): ^{1}H-NMR (DMSO-d_6) δ 1.27 (s, 9, NC(CH_3)$_3$), 1.37 (s, 9, OC(CH_3)$_3$), 1.40 (s, 6, C(CH_3)$_2$), 7.61 (s, 1, C(CH_3)$_3$NH); ^{13}C-NMR (DMSO-d_6) δ 23.56 C(CH_3)$_2$; 27.82, 27.87 (2 × C(CH_3)$_3$); 50.51, 59.44, 80.48 (3 × CCH_3); 151.60, 152.20, 174.89 (3 × CO); high resolution mass data (FAB MS) BOC-NHC(CH_3)$_2$CONHtBu (**2**) calcd 259.2022 [M + H]$^+$, found 259.2019 [M + H]$^+$; BOC-N(C(CH_3)$_2$COOH)(CONHtBu) (**3**) calcd 303.1920 [M + H]$^+$, found 303.1903 [M + H]$^+$.

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