(Fluoren-9-ylmethoxy)carbonyl (Fmoc) amino acid azides: Synthesis, isolation, characterisation, stability and application to synthesis of peptides

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The synthesis of Fmoc amino acid azides starting from the corresponding protected amino acid and sodium azide (NaN_3) by the mixed anhydride method using isobutoxycarbonyl chloride (IBC-Cl) or by the acid chloride method is described. Isolated as crystalline solids, they are stable at room temperature, with a long shelf-life, as well as in aqueous washing operations. They are useful as coupling agents in peptide synthesis.

The acid azide method, introduced by Theodor Curtius nearly a century ago,[1–3] survived the scrutiny of numerous investigators, resisted the challenge of many alternative procedures, and is still practised by peptide chemists.[4] The conventional total synthesis of bovine pancreatic ribonuclease (RNase) A with 124 amino acids was accomplished by successive assembly of 30 peptide fragments, for the most part using the azide procedure, by Haruaki Yajima.[5,6] In recent years, acid azides have been utilised as useful intermediates/building blocks for the synthesis of partially modified retro-inverso peptides and peptidomimetics,[7] *gem*-diaminoalkylamines,[8,9] β-amino acids in a regio- and stereoselective manner, oligoureas,[10] *etc*.

The acid azide method, under carefully controlled conditions, allows racemisation at very low levels and thus it is routinely used in the segment condensation of peptide fragments by the divergent approach.[11] As amino acid and peptide azides with free termini can be made easily, they have been used in the synthesis of polyamino acids[12] and cyclic peptides, respectively.[13]

The acid azide method used by peptide chemists generally involves three consecutive stages, *i.e.*, hydrazine preparation from Boc-/Z-amino acid esters, azide formation, and coupling.[14] Numerous side reactions have been reported to occur in each of the three stages.[15,16] Among them, side reactions that normally occur during the formation of azide are more serious and hence this method is not considered to be a simple one. The majority of the known side reactions have been suppresed by carrying out the entire process below 0 °C (between −20 and −40 °C). Like acid chlorides, acid azides of Boc-/Z-amino acids are known for their instability at room temperature.[8a,17] They undergo Curtius rearrangement leading to formation of the reactive amino acid isocyanates which then produce amines with water and urea derivatives. Consequently Boc-/Z-amino acid azides are not isolated, but react *in situ* immediately after their preparation. Naturally, time was considered as an important factor in processes involving not entirely stable intermediates.

The introduction of the (fluoren-9-ylmethoxy)carbonyl (Fmoc) group by Carpino for N^{α}-protection has ushered in alternative approaches to chemical synthesis of peptides.[18] Its use represents a truly orthogonal scheme in solid-phase peptide synthesis and thus offers many unique opportunities in bio-organic chemistry.[19,20] Its utility for the synthesis of peptide conjugates like glycopeptides, phosphopeptides, peptide-nucleic acids, *etc*. is well documented. A recent application is the isolation of acid chlorides and acid fluorides of Fmoc-amino acids.[21–24] These have been shown to be stable, optically active, and rapid acylating agents. However, the Fmoc group, being a base-labile one, is not stable during amino acid ester hydrazinolysis with prolonged reaction periods, elevated temperatures or with excess of hydrazine. The production of acid azides from acyl chlorides has long been considered as of no interest.[11] Though the acid azide method is paramount as a technique in peptide synthesis, Fmoc-amino acid azides have not been prepared earlier.[4,7,8,11,13,16,19,20,25–27] This paper describes a method to produce stable acid azides.

Results and discussion

Our work on the development of new procedures for the utility of Fmoc-amino acid chlorides led us to attempt the preparation of the corresponding acid azides. The reaction of an Fmoc-amino acid chloride with sodium azide was found to be smooth and rapid (Scheme 1). The course of the reaction was followed by TLC and was found to complete in *ca.* 15 min. The resulting Fmoc-amino acid azide separates as a solid almost quantitatively. In general, there was no need to subject it to any purification procedure. In some of the crops, Fmoc-amino acid chlorides were found to contain 1–2% of unchanged Fmoc-amino acid. In such cases, Fmoc-amino acid azides were dissolved in a suitable solvent, washed with 5% aq. $NaHCO_3$, and reprecipitated. A list of compounds made, along with their physical constants, is given in Table 1. The purity of the compounds as checked by HPLC is satisfactory (>98%). Unlike Boc-/Z-amino acid azides, all the Fmoc-amino acid azides were obtained as crystalline solids. They are soluble in solvents like CH_2Cl_2, $CHCl_3$, THF, acetonitrile, *etc.* They were further characterised by IR spectrometry. Their IR spectra contain a sharp, characteristic carbonyl-stretching vibrational frequency in the region 2138–2148 cm^{-1}. Not even traces of the corresponding isocyanate impurities were present in the azides. This was inferred by the absence of a peak in the region 2252–2260 cm^{-1} characteristic of isocyanates. In order to confirm this further, Fmoc-Phe-N_3, under appropriate conditions, was converted into Fmoc-Phe-N=C=O which was isolated as a solid and fully characterised. Similar to the active esters of Fmoc-amino acids, these azides are also stable and can be isolated easily and stored for long periods. The IR spectrum of Fmoc–Phe–N_3 recorded after a long period showed no changes in its characteristics.

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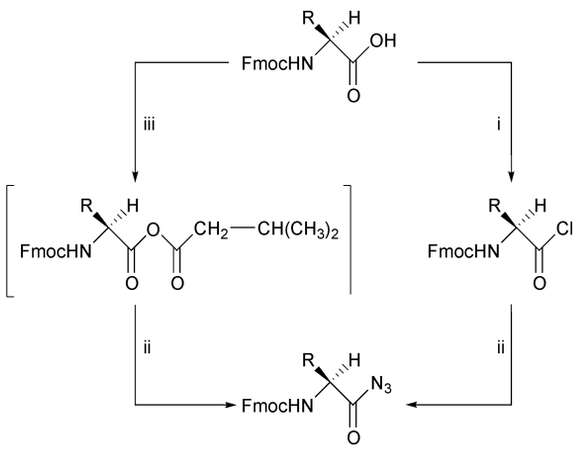

Scheme 1 *Reagents and conditions*: i) CH_2Cl_2, $SOCl_2$, rt; ii) aq. NaN_3, 0 °C; iii) THF, IBC-Cl, NMM, 0 °C.

The acid chloride derivatives of bifunctional amino acids bearing a *t*-butyl group are difficult to make and are unstable. Consequently the corresponding amino acid azides were prepared by the mixed anhydride method using isobutoxycarbonyl chloride (IBC-Cl). In this method, the species Fmoc-NH-CHR-CO-O-CO-O-$CH_2CH(CH_3)_2$, generated *in situ*, was treated with NaN_3. After purification, the corresponding azides were all solids isolated in 68–93% yield (Table 2). In trial experiments, the properties of Fmoc-Phe-N_3 prepared by the azide method were found to be identical with those of the same compound made by the anhydride method.

Encouraged by the above results, we extended similar reaction conditions to the acid chloride method also. The reaction mixture containing an Fmoc-amino acid chloride in CH_2Cl_2, after confirmation of the complete conversion of acid into its acid chloride, was treated directly with NaN_3. Here also, Fmoc-amino acid azides were obtained in good yield with high purity. Thus, there is no need to isolate the acid chloride derivatives. They can be directly converted into the corresponding acid azide.

By employing the amino acid azides as coupling agents, we have accomplished the synthesis of a few model peptides (Table 3). All the peptides obtained were pure solids and were fully characterised. In Boc and Z chemistry, it is essential to maintain low temperatures (−10 to −30 °C) during coupling. Consequently, appropriate time has been allowed for completion of the reaction, which may require several days in specific cases. On the other hand, in the present studies, the coupling of Fmoc-amino acid azides was carried out at room temperature and the reaction was found to be complete in *ca.* 16–18 h. The coupling, as determined by H^1 NMR spectroscopy of the diastereomeric dipeptides Fmoc-L- (or D)-phenylglycyl-L-phenylalanyl methyl esters made by the present method, was found to be free from racemisation.[28]

In conclusion, the easy access, low cost, and long shelf life of Fmoc-amino acid azides make them useful in peptide synthesis. There is no need to use them immediately as and when prepared. Acceleration of the azide-coupling process can be achieved, to some extent, by carrying out the reaction at room temperature.

Experimental

Solvents were distilled and dried prior to use. Mps were determined using a Leitz-Wetzlar melting-point apparatus and are uncorrected. IR spectra were recorded on a Nicolet model Impact 400D FT-IR spectrometer (KBr pellets, 3 cm^{-1} resolution). Optical rotations were measured with an automatic AA-10 polarimeter (Optical Activity, UK). $[a]_D$-Values are given in units of 10^{-1} deg cm^2 g^{-1}. ^1H NMR spectra were recorded on a Bruker AMX 400 MHz spectrometer using $SiMe_4$ as internal standard. Mass spectra were recorded on a JEOL MS-DX 303 spectrometer operating at 70 eV and fitted with a built-in inlet system. Elemental analyses were done using a Perkin-Elmer Analyser and the samples were vacuum dried for 24 h before analysis. Analytical RP-HPLC was performed with a Waters LC-3000 system using a C-18 Bondapak column (3.9 × 300 mm, 10 μ, spherical) using as eluent an acetonitrile–0.1% TFA in water system (65 : 35, isocratic; flow rate 0.7 mL min^{-1}. Monitoring at 220 nm). Products were dried over P_2O_5.

TLC analysis was carried on precoated silica gel G plates using solvent systems (A) ethyl acetate–hexane (35 : 65, v/v), (B) $CHCl_3$–methanol–acetic acid (40 : 2 : 1, v/v/v) and (C) $CHCl_3$–methanol (9 : 1, v/v), and R_f-values are designated as R_f A, R_f B, and R_f C, respectively. Sodium azide was purchased from Lancaster Synthesis Ltd., England. Fmoc-amino acids and their acid chlorides were prepared using the reported procedures.[22,29] The general procedure for the synthesis of Fmoc-amino acid azides is given below.

Employing Fmoc-amino acid chlorides

(a) Without isolation of acid chlorides. A solution of an Fmoc-amino acid (1 mmol) in CH_2Cl_2 (5 mL) was treated with thionyl dichloride (1.19 g, 10 mmol) and the mixture was stirred for 24 h at rt under N_2 atmosphere. After completion of the reaction, the mixture was cooled in an ice-bath and treated with aq. NaN_3 (0.098 g, 1.5 mmol in 1 mL). The reaction mixture was stirred for *ca.* 15 min. The separated solid was filtered off, washed with water, and dried. When the solid was not separated, additional CH_2Cl_2 (20 mL) was added, and the solution was washed with water (3 × 10 mL) and dried (anhydrous Na_2SO_4). Evaporation of solvent *in vacuo* and recrystallisation using CH_2Cl_2–*n*-hexane gave cystalline Fmoc-amino acid azides.

(b) With isolation of acid chlorides. To an ice-cold solution of an Fmoc-amino acid chloride (1 mmol) in acetone (3 mL) was added aq. NaN_3 (0.098 g, 1.5 mmol in 1 mL). The mixture was stirred for *ca.* 15 min. The separated solid was filtered off, washed with water, and dried.

By the mixed anhydride method

To an ice-cold solution of Fmoc-amino acid (1 mmol) in dry THF (5 mL) were added IBC-Cl (0.135 mL, 1 mmol) and *N*-methylmorpholine (NMM) (0.11 mL, 1 mmol) and the mixture was stirred at −20 °C for 20 min. The resulting reaction mixture was treated with aq. NaN_3 (0.098 g, 1.5 mmol in 1 mL) and the mixture was stirred for another 30 min. After completion of the reaction, the organic layer was evaporated and the residue was dissolved in CH_2Cl_2 (20 mL). It was washed successively and thrice with 10 mL portions of 5% HCl, 5% aq. $NaHCO_3$, and water, and dried over anhydrous Na_2SO_4. Evaporation of solvent *in vacuo* and recrystallisation of the resulting residue using a suitable solvent yielded the azide as a crystalline solid.

General procedure for coupling

To a stirred solution of an Fmoc-amino acid azide (5 mmol) in CH_2Cl_2 (20 mL) was added a solution of free amino acid ester (5.2 mmol) in CH_2Cl_2 (10 mL) and the solution was stirred for 18 h at room temperature. During this period, the pH was being maintained at 7.5 with periodic additions of collidine. After completion of the reaction, the mixture was washed successively with 1 M HCl (3 × 10 mL), 10% aq. $NaHCO_3$ (3 × 10 mL), and water (3 × 10 mL), dried over anhydrous Na_2SO_4, and evaporated. The product was recrystallised using CH_2Cl_2–*n*-hexane.

Fmoc-Phe-N=C=O

Fmoc-Phe-N_3 (0.41 g, 1 mmol) was dissolved in toluene (5 mL), and the solution was refluxed for 1 h. The solvent was removed

Table 1 Physical constants of Fmoc-amino acid azides[a]

Entry	Fmoc-amino acid azide	Yield (%)	Mp (°C)	R_f-Value		$[\alpha]_D^{25}$ (c 1, $CHCl_3$)	IR (ν_{max}/cm^{-1})	Elemental analysis Calc. (Found)			^{1}H NMR
				R_fA	R_fB			C	H	N	
1	Gly	92	132	0.61	0.69		2148	63.34 (63.01)	4.37 (4.19)	17.38 (17.03)	δ 3.3 (2H, d), 4.2 (1H, t), 4.5 (2H, d), 5.5 (1H, br), 7.2–7.8 (8H, m)
2	Ala	93	162	0.62	0.68	−16	2148	64.27 (63.09)	4.78 (4.61)	16.66 (16.52)	δ 1.4 (3H, d), 3.85 (1H, m), 4.2 (1H, t), 4.4 (2H, d), 5.3 (1H, s), 7.2–7.8 (8H, m)
3	Leu	86	122–123	0.76	0.68	+7	2138	66.65 (66.20)	5.85 (5.70)	14.80 (14.61)	δ 0.92 (6H, d), 1.6 (3H, m), 3.8 (1H, m), 4.1 (1H, t), 4.3 (2H, d), 4.85 (1H, br), 7.2–7.8 (8H, m)
4	Val	88	168	0.62	0.7	−10	2138	65.92 (65.66)	5.52 (5.38)	15.37 (15.19)	δ 0.95 (6H, m), 2.1 (1H, m), 3.85 (1H, t), 4.1 (2H, d), 4.29 (1H, br), 7.2–7.8 (8H, m)
5	Ile	78	152	0.73	0.66	+14	2143	66.65 (66.28)	5.85 (5.56)	14.80 (14.67)	δ 0.93 (6H, d), 1.9 (1H, m), 4.1 (1H, t), 4.3 (2H, d), 5.01 (1H, s), 7.2–7.8 (8H, m)
6	Pro	90	74	0.64	0.71	+16	2143	66.10 (66.28)	4.98 (4.76)	15.42 (15.21)	δ 2.05 (4H, m), 3.5 (2H, m), 4.1 (1H, t), 4.25 (2H, d), 7.2–7.8 (8H, m)
7	Phe	96	175	0.61	0.75	+3	2143	69.88 (69.59)	4.88 (4.68)	13.58 (13.39)	δ 2.52 (2H, m), 3.1 (2H, d), 4.1 (1H, t), 4.25 (2H, d), 5.09 (1H, br), 7.2–7.8 (13H, m)
8	Phg	89	125–126	0.63	0.76	−11	2148	69.33 (69.01)	4.54 (4.32)	14.06 (13.90)	δ 4.1 (1H, t), 4.25 (2H, d), 5.85 (1H, br), 7.2–7.8 (13H, m)
9	Phg[b]	88	127	0.63	0.76	+11	2147	69.33 (69.08)	4.54 (4.41)	14.06 (13.95)	δ 4.25 (1H, t), 4.25 (2H, d), 5.85 (1H, br), 7.3–7.8 (13H, m)
10	Met	82	172	0.72	0.76	−11	2138	65.92 (65.56)	5.52 (5.34)	15.38 (15.19)	δ 2.0 (2H, m), 2.1 (3H, s), 2.45 (2H, m), 4.25 (1H, t), 4.4 (2H, d), 5.1 (1H, br), 7.2–7.8 (8H, m)

[a] All the Fmoc-N_3 prepared using Fmoc-amino acids; all the compounds except Gly, unless specified, had L-configuration. [b] D-Configuration.

Table 2 Physical constants of Fmoc-amino acid azides

Entry	Fmoc-amino acid azide	Yield (%)	Mp (°C)	R_f-Value		$[\alpha]_D^{25}$ (c 1, $CHCl_3$)	IR (ν_{max}/cm^{-1})	Elemental analysis Calc. (Found)			^{1}H NMR
				R_fA	R_fB			C	H	N	
1	Lys(Z)	82	162	0.81	0.53	−29	2143	66.03 (65.91)	5.53 (5.39)	13.27 (13.16)	δ 3.05 (2H, m), 4.01 (1H, t), 4.28 (2H, d), 5.25 (1H, s), 7.2–7.8 (13H, m)
2	Asp(OBu^t)	93	105	0.80	0.83	−28	2143	63.30 (63.01)	5.53 (5.28)	12.83 (12.49)	δ 1.43 (9H, s), 2.85 (2H, m), 4.1 (1H, t), 4.42 (2H, d), 5.01 (1H, s), 7.2–7.8 (8H, m)
3	Glu(OBu^t)	89	168–170	0.79	0.81	+6	2142	63.99 (63.59)	5.81 (5.69)	12.43 (12.12)	δ 1.45 (9H, s), 1.9–2.4 (4H, m), 3.9 (1H, t), 4.01 (2H, d), 5.2 (1H, s), 7.2–7.8 (8H, m)
4	Tyr(Bu^t)	83	173	0.79	0.8	−5	2148	68.63 (68.41)	5.96 (5.73)	11.85 (11.69)	δ 1.3 (9H, s), 3.0 (2H, d), 3.9 (1H, t), 4.2 (2H, d), 5.9 (1H, br), 7.2–7.8 (12H, m)
5	Ser(Bu^t)	91	178	0.81	0.79	−29	2138	64.71 (64.58)	5.91 (5.78)	13.71 (13.66)	δ 1.2 (9H, s), 3.75 (2H, m), 4.01 (1H, t), 4.28 (2H, d), 5.01 (1H, br), 7.2–7.8 (8H, m)
6	Cys(Me)	76	138–139	0.78	0.69	−19	2143	59.67 (59.42)	4.73 (4.50)	14.65 (14.49)	δ 3.2 (2H, d), 4.01 (1H, t), 4.28 (2H, d), 5.25 (1H, s), 7.2–7.8 (8H, m)
7	Lys(Pht)	68	158	0.83	0.63	−16	2138	65.74 (65.59)	4.92 (4.61)	13.69 (13.58)	δ 3.05 (2H, m), 3.95 (1H, t), 4.3 (2H, d), 5.6 (1H, br), 7.2–7.8 (12H, m)

Table 3 Physical constants of Fmoc-dipeptide esters[a]

Entry	Dipeptide	Yield (%)	Mp (°C)	$[a]_D^{25}$ Observed	$[a]_D^{25}$ Reported[23,28,30]
1	Fmoc-Phg[b]-Phe-OMe	82	195–196	+24.00 (*c* 0.5, DMF)	+22.2 (*c* 0.5, DMF)
2	Fmoc-D-Phg[b]-Phe-OMe	79	192–194	−24.0 (*c* 0.5, DMF)	−21.6 (*c* 0.5, DMF)
3	Fmoc-Ala-Ala-OMe	78	195–196	−26.2 (*c* 1, $CHCl_3$)	−28 (*c* 1.35, AcOH)
4	Fmoc-Pro-Pro-OMe	77	118–120	+40.5 (*c* 1, $CHCl_3$)	+40.1 (*c* 1, $CHCl_3$)
5	Fmoc-Gly-Val-OMe	84	97–99	+19.0 (*c* 1, $CHCl_3$)	+18.6 (*c* 1, $CHCl_3$)
6	Fmoc-Phe-Leu-OMe	88	163–165	−21.4 (*c* 1, $CHCl_3$)	−21.6 (*c* 1, $CHCl_3$)

[a] All the peptides gave satisfactory ^{1}H NMR analysis. [b] Phg is 2-amino-2-phenylacetic acid (phenylglycine).

in vacuo and the residue was recrystallised from CH_2Cl_2–*n*-hexane to give the solid title compound (0.275 g, 73%); mp 122–123 °C; IR ν_{max} 2252 cm^{-1}; R_f B, 0.87; $[a]_D^{25}$ +10 (*c* 1, $CHCl_3$).

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