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N^{α}-Fmoc-Peptide azides: Synthesis, isolation, characterization and utility in the extension of peptide chains

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Syntheses of N^{α}-Fmoc-peptide azides employing acid chloride as well as mixed anhydride methods have been carried out. The resulting Fmoc-peptide azides prepared have been isolated as solids in good yield (75-92 %) and are found to be analytically pure. Further, long shelf life of N^{α}-Fmoc-peptide azides makes them useful in the extension of peptide chains.

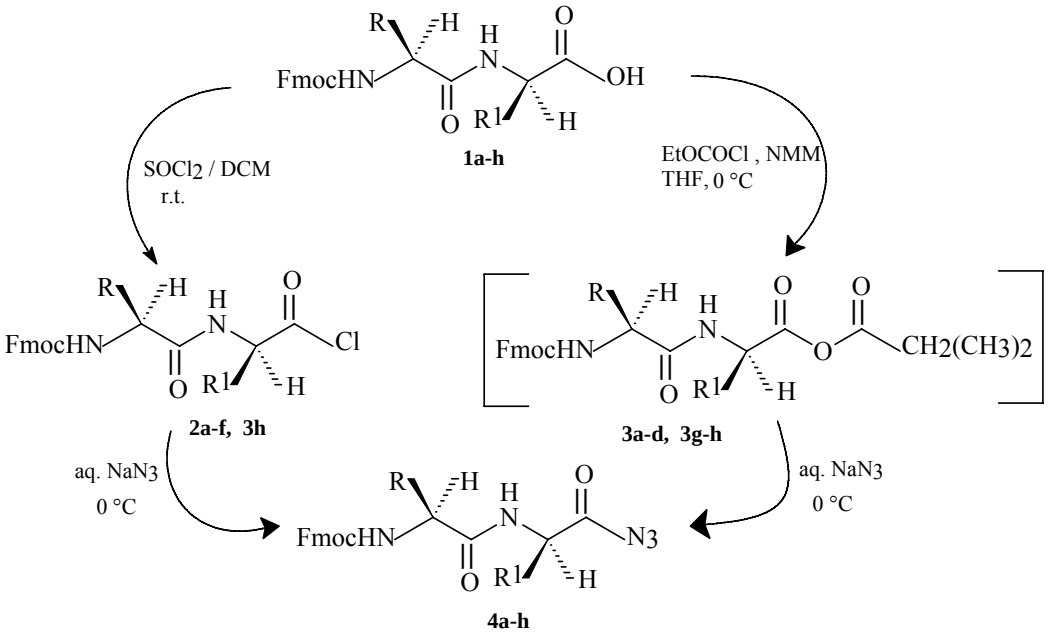
Keywords: Fmoc-peptide acid, peptide acid azide, Fmoc-peptide isocyanate.

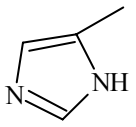
IPC: **Int.Cl.**[7] **C 07 K**

The chemical approaches to the synthesis of peptides and proteins by the azide coupling[1, 2] have turned out to be one of the safest methods of avoiding racemization. The azide procedure has been widely used for the coupling of difficult amino acid residues such as His, Thr, Ser, Trp, etc., and also particularly for the condensation of peptide fragments using both the maximum and minimum protection strategy in the solution phase synthesis of peptides[3]. Yajima's successful synthesis of a 124 residue protein ribonuclease A utilizing about thirty small segments as key intermediates by the azide method following the segment condensation approach in solution is a landmark achievement in the history of chemical synthesis of proteins[4-7]. Similar strategy has been employed in the synthesis of several other polypeptides and proteins[8-13]. Their synthesis mainly involves the generation of N^{α}-Boc- or *Z*-peptide azides as key intermediates.

The preparation, properties, stability, handling and coupling conditions of N^{α}-Boc- or *Z*-peptide acid azides are almost similar to the monomeric building blocks Boc-/*Z*-amino acid azides. Recently, the acid azides of urethane protected amino acids have been employed as building blocks for the synthesis of partially modified retro-inverso peptides, peptidomimetics, *gem*-dialkylamines, oligoureas, etc[14-18]. Azides are particularly attractive as activated derivatives because they originate from commonly used carboxyl protecting groups (methyl, ethyl and benzyl) *via* hydrazides. The azides can be prepared by reaction of suitably protected amino acid or peptide hydrazides with nitrous acid or directly from the related carboxylic acids by azide transfer reagents. The most frequently used conditions for azide formation have been elaborated by Rudinger and Honzl[19]. They showed that optimal formation is obtained in anhydrous organic solvent at high acidity with organic nitrile at low temperature. Later on, few more modifications for the preparation of peptide azides were introduced. Among them, the use of tetrabutylammonium nitrate as an auxiliary reagent[21] and diphenylphosphorazide (DPPA) for the preparation of peptide acid azides in the presence of a base were explored[21].

The main side reactions[22] are the formation of isocyanates by the Curtius rearrangement which lead to the undesired ureido bond formation with the amine and the amide formation by the hydrolysis of azide. However, the majority of the known side reactions have been suppressed by carrying out all the three steps involved in azide coupling (hydrazide preparation from Boc-/Z-protected peptide ester, azide formation and coupling) below 0°C (between -20 to -40°C)[23]. Thus, similar to Boc-/Z-amino acid azides[24-27], N^{α}-Boc-/Z-protected peptide azides have neither been isolated nor characterized. Moreover, they have been utilized soon after their generation.



Compd	n	R	R^1	R^2*
4a	2	$CH(CH_3)_2$	$CH_2C_6H_5$	-
4b	2	H	$CH_2C_6H_5$	-
4c	2	CH_3	$CH_2CH(CH_3)_2$	-
4d	2	$CH_2CH_2CONH_2$	$CH(CH_3)CH_2CH_3$	-
4e	2		$CH(CH_3)CH_2CH_3$ -	-
4f	2	CH_2O^tBu	$CH_2C_6H_5$	-
4g	3	C_6H_5	$CH_2C_6H_5$	$CH(CH_3)_2$
4h	3	H	$CH_2C_6H_5$	$CH(CH_3)_2$

Scheme I — Synthesis of N^{α}-Fmoc-peptide azides

Recently, our group reported the preparation of Fmoc-amino acid azides which have been isolated as crystalline solids[18b-c]. In continuation of our interest in the azide method, we here describe the preparation, isolation, properties, stability and utility of N^{α}-Fmoc-protected peptide azides.

It is found that N^{α}-Fmoc-peptide acid azides can be prepared starting from the corresponding N^{α}-Fmoc-peptide acid chlorides[28] by treating the acid chloride in acetonitrile or acetone with NaN_3 in water (**Scheme I**). The TLC analysis revealed that the reaction was complete in about 15 min. The simple work up gave the pure azides in good yield. The IR spectral analysis of the products clearly confirmed that a sharp and strong peak corresponding to the carbonyl stretching characteristic of an azide functionality at around ~2145 cm^{-1} is present in all the peptide azides **4a-f, 4h** obtained. Also, the absence of a peak at around 2250 cm^{-1} corresponding to isocyanate has proved that no Curtius rearrangement is taking place in the given reaction conditions. As it is well known, peptides bearing acid labile side chain protecting groups *t*-butyl and trityl groups cannot be converted to the corresponding acid chlorides employing thionyl chloride. Consequently, for such peptides mixed anhydride method is a preferred alternative. And so, the peptide azides **4a-d, 4g-h** have been prepared by treating the Fmoc-peptide acid with ethyl chloroformate (EtOCOCl) and *N*-methyl morpholine (NMM) at -10 °C for ten minutes and the *in situ* generated mixed anhydride was treated with aqueous sodium azide. The simple work-up resulted in almost pure N^{α}-Fmoc-peptide azides and if necessary they can be purified by crystallization using

Table I — Physical constants of N^{α}-Fmoc-peptide azides

Peptide azide	Method	IR cm^{-1}	m.p. °C	Yield (%)	Calcd (Found) (%) C	H	N	^{1}H NMR (δ in $CDCl_3$)
Fmoc-Val-Phe-CON_3	A B	2137	188	89 91	68.09 (68.07	5.71 5.68	13.69 13.67)	0.95(6H, d), 1.85(1H, m), 2.9(2H, d), 3.82(1H, m), 4.2–4.45(4H, m), 5.2(1H, d), 6.3(1H, s), 7.2–7.85(13H, m),
Fmoc-Gly-Phe-CON_3	A B	2142	205-06	90 92	66.51 (66.48	4.93 4.91	14.91 14.86)	2.9(2H, d), 3.25(2H, d), 4.2–4.45(3H, m), 5.2(1H, d), 6.3(1H, s), 7.2–7.8(13H, m)
Fmoc-Ala-Leu-CON_3	A B	2139	176	88 89	64.13 (64.11	6.05 6.02	15.58 15.52)	0.98(6H, d), 1.16(3H, d), 1.4(2H, m), 1.7(1H, m), 3.82(1H, m), 4.2–4.4(4H, m), 5.3(1H, d), 6.35(1H, d), 7.25–7.85 (8H, m)
Fmoc-Gln(Trt)-Ile-CON_3	B	2138	160-62	84	59.74 (59.72	6.26 6.22	17.41 17.38)	0.98(6H, m), 1.19(1H, m), 1.31.75(6H, m), 3.85(1H, m), 4.22(1H, t), 4.44(2H, m), 5.3(1H, s), 6.1-6.35(3H, s), 7.25-7.85(23H, m)
Fmoc-His(Trt)-Ile-CON_3	B	2145	156-59	86	62.90 (62.75	5.67 5.51	19.02 18.79)	0.98(6H, m), 1.19(1H, m), 1.65(2H, m), 2.6(2H, m), 3.85(1H, m), 4.22(1H, t), 4.44(2H, m), 5.3(1H, s), 6.35(1H, s), 7.25–7.85(26H, m)
Fmoc-Ser(^{t}Bu)-Phe-CON_3	B	2135	164-65	79	67.01 (66.92	5.98 5.93	12.60 12.56)	1.2(9H, s), 2.95(2H, d), 3.65(2H, d), 4.0–4.45(5H, m), 5.3(1H, s), 6.35(1H, s), 7.25–7.85(13H, m)
Fmoc-Phg-Phe-Val-CON_3	A	2141		82	68.93 (68.95	5.62 5.58	13.03 12.92)	0.98(6H, d), 1.9(1H, m), 2.9(2H, d), 4.2–4.5(6H, m), 5.3(1H, d), 6.45-6.6 (2H, m), 7.2–7.9(18H, m)
Fmoc-Gly-Phe-Val-CON_3	A B	2138		78 75	65.48 (65.41	5.67 5.61	14.78 14.65)	0.98(6H, d), 1.9(1H, m), 2.9(2H, d), 3.2(2H, d), 4.1–4.5(5H, m), 5.35(1H, d), 6.4(1H, d), 7.25–7.85(13H, m)

A: Employing N^{α}-Fmoc-peptide acid chlorides ; B: Employing *in situ* generated mixed anhydride

dichloromethane (DCM) and petroleum ether to get the pure compounds in almost quantitative yield (**Table I**).

All the N^{α}-Fmoc-peptide azides **4a-h** made are soluble in organic solvents like ethyl acetate, DCM, chloroform, *etc*. The HPLC analysis of the peptide azides confirmed their purity. They can be stored for several weeks at 0°C under anhydrous conditions. The storage for few hours at r.t. led to their decomposition leading to the formation of the corresponding isocyanates which have been characterized by the presence of a peak at around ~2250 cm^{-1} in IR as well as by HPLC (**Figures 1** and **2**) analysis. All the peptide azides **4** obtained have been fully characterized by ^{1}H NMR, ^{13}C NMR and mass spectroscopy.

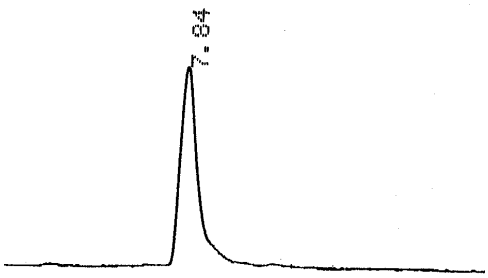
Further, in order to confirm the absence of Fmoc-peptide isocyanate contaminants in Fmoc-peptide azides, in a model experiment the Fmoc-Val-Phe-CON_3 was converted to its isocyanate **5a** by heating at about 60°C for 30 min in toluene (**Scheme II**). The resulting product was isolated and characterized.

The N^{α}-Fmoc protected peptide azides are used as coupling agents in the extension of the peptide chain (**Scheme III**) by converting the dipeptide azides to tripeptides and tripeptide azides to tetrapeptides, which have been isolated as pure solids in good yield and purity.

In conclusion, the N^{α}-Fmoc protected peptide azides can be prepared easily and isolated as pure solids in good yield. They can be used as and when required due to their long shelf life at 0°C under anhydrous condition. Their purity can be checked by HPLC analysis before use. Their utility in the extension of the peptide chains from N $\longrightarrow$ C terminal has been demonstrated.

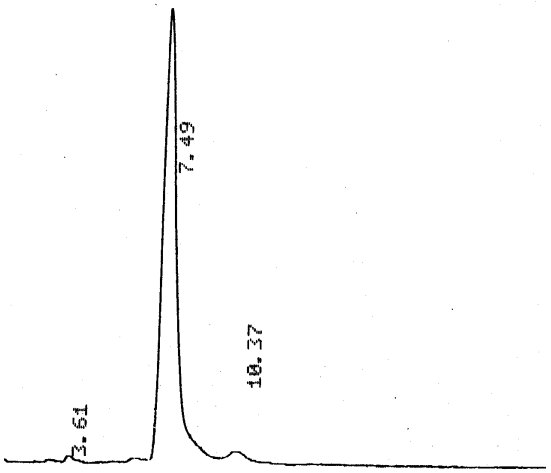
Experimental Section

Melting points were determined using capillary method and are uncorrected. All the amino acids used, except Gly, are of L-configuration. IR spectra were recorded on a Nicolet model impact 400D FT-IR spectrometer (KBr pellets, 3 cm^{-1} resolution). TLC analysis was carried out on precoated silica gel G



Waters C-18 bondapak (3.9 x 300 mm) column; flow rate 1mL/min; eluent : acetonitrile : water (60 : 40; isocratic; monitoring at 254 nm)

Figure 1 — Analytical RP HPLC of **4a**



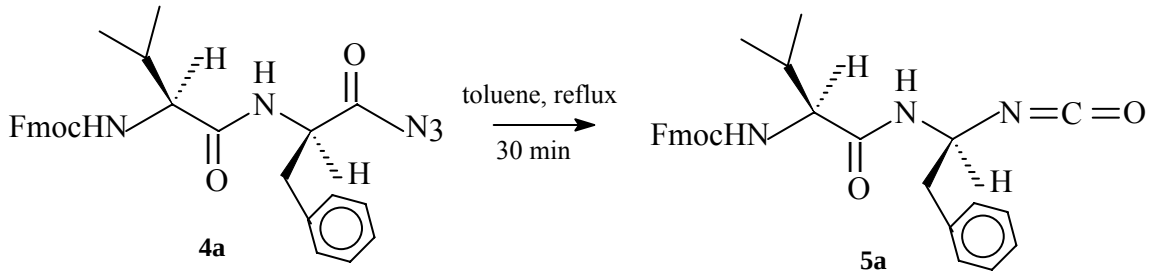
Waters C-18 bondapak (3.9 x 300 mm) column; flow rate 1mL/min; eluent : acetonitrile : water (60 : 40; isocratic; monitoring at 254 nm)

Figure 2 — Analytical RP HPLC of **5a**

plates using the solvent systems ethyl acetate: *n*-hexane (35 : 65, v/v) and chloroform : methanol : acetic acid (40 : 2 : 1, v/v/v). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX 400 MHz spectrometer. Mass spectra were recorded on a MALDI-TOF (KRATOS). Amino acids were purchased from Sigma Aldrich Co., U.S.A. The N^{α}-Fmoc-peptide acids have been prepared employing N^{α}-Fmoc-amino acid and *N*,*O*-bis(trimethyl silyl) derivatives of amino acids following the reported procedure[29]. They are then converted to their acid chlorides using thionyl chloride[28] which were isolated and characterized before use.

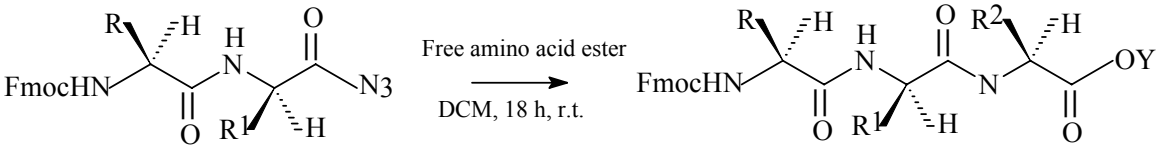
General procedure for the preparation of N^{α}-Fmoc protected peptide azides: Method A — Employing N^{α}-Fmoc-peptide acid chlorides. To a cold solution of N^{α}-Fmoc-peptide acid chloride (1 mmole) in acetonitrile (5 mL) was added aqueous sodium azide (1.5 mmole in 1 mL of water). The resulting reaction mixture was stirred for 15 min at -20°C. The separated solid was filtered, washed with cold water. The resulting solid was dissolved in DCM (5 mL), dried over anhydrous Na_2SO_4 and crystallized using petroleum ether. The peptide azides **2a-h** were obtained almost in quantitative yield (75-92 %) as analytically pure compounds.

Method B — Employing *in situ* generated mixed anhydrides. N^{α}-Fmoc-peptide acid (1 mmole) was taken in dry THF and cooled in an ice-salt-bath. EtOCOCl (1 mmole) and NMM (1 mmole) were added to the above cooled solution and stirred for 10 min. while maintaining the temperature at -10°C. The resulting reaction mixture was treated with aqueous



4a **5a**

Scheme II



4 **6**

Scheme III

sodium azide (1.5 mmoles in 1 mL of water) at the same temperature. The reaction, as monitored by TLC as well as IR analysis, was complete in about 15 min. The THF solution was evaporated under high vacuum at r.t. and the residue was taken in DCM (25 mL). The DCM layer was washed with cold water (2 × 10 mL), dried over anhydrous sodium sulphate and concentrated the half of the volume and petroleum ether (10 mL) was added to obtain pure N^{α}-Fmoc protected peptide azide as a crystalline solids (78-90%).

Fmoc-Val-Phe-NCO 5a. Fmoc-Val-Phe-CON_3 (0.477 g, 1 mmole) was taken in toluene (5 mL) and the solution was heated to about 60°C for 30 min. The conversion of azide to isocyanate was monitored by TLC as well as IR spectroscopy. After completion of the reaction, the solvent was removed under vacuum and the residue was crystallized from petroleum ether. yield 0.46 g, 92 %; m.p. 170-72 °C; IR; 2256 cm^{-1}.

General procedure for coupling. A solution of an N^{α}-Fmoc-peptide azide (2 mmoles) in DCM (10 mL) was added to a clear solution of free amino acid ester (2.1 mmoles) in DCM (5 mL). The resulting reaction mixture was stirred for 18 hr at room temperature. The *p*H was maintained at 7.5 with periodic additions of collidine. After completion of the reaction, the mixture was washed with 1.5 M HCl (3 × 10 mL), 10 % aq. $NaHCO_3$ (3 × 10 mL), and water (3 × 10 mL) and dried over anhydrous Na_2SO_4. The solvent was evaporated and the product was recrystallized using petroleum ether.

Fmoc-Val-Ala-Leu-OMe 6a: m.p. 132-35°C; $[\alpha]_D^{25}$ –16.2 (*c* = 1, $CHCl_3$): ^{1}H NMR ($CDCl_3$: δ 0.93 - 1.0 (12H, m), 1.16 (3H, d), 1.35 (2H, m), 1.63 (1H, m), 1.85 (1H, m), 3.65-3.85 (6H, m), 4.2 - 4.45 (3H, m), 5.0 (1H, d), 6.45-6.65 (2H, d), 7.25-7.8 (8H, m); m/z observed: 537.62.

Fmoc-Ala-Leu-Pro-OMe 6b: m.p. 167-69°C; $[\alpha]_D^{25}$ –22.0 (*c* = 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 0.95 (6H, d), 1.16 (3H, d), 1.21-2.1 (7H, m), 3.2-3.65 (6H, m), 3.77-3.85 (2H, m), 3.95 (1H, m), 4.2-4.45 (3H, m), 5.05 (1H, d), 6.4-6.6 (2H, m), 7.3-7.85 (8H, m); m/z observed: 536.61.

Fmoc-Leu-Ala-Gly-OMe 6c: m.p. 92-94°C; $[\alpha]_D^{25}$ +55.4 (*c* = 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 0.95 (6H, d), 1.16 (3H, d), 1.35 (2H, m), 1.6 (1H, m), 3.2 (2H, d), 3.6 (3H, s), 3.75-3.85 (2H, m), 4.2-4.45 (3H, m), 5.05 (1H, d), 6.48-6.65 (2H, d), 7.3-7.85 (8H, m); m/z observed: 495.544.

Fmoc-Gly-Phe-Leu-OBzl 6d: m.p. 131-32°C; $[\alpha]_D^{25}$ –11.3 (*c* = 1, DMF); ^{1}H NMR ($CDCl_3$): δ 0.95 (6H, d), 1.35 (2H, m), 1.65 (1H, m), 2.85 (2H, d), 3.18 (2H, d), 3.75 (1H, m), 4.2-4.45 (4H, m), 5.1 (1H, d), 6.5-6.65 (2H, m), 7.3-7.85 (13H, m); m/z observed: 647.739.

Fmoc-Ala-Gly-Val-OBzl 6e: m.p. 140-41°C; $[\alpha]_D^{25}$ –60.4 (*c* = 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ, 0.95 (6H, d), 1.16 (3H, d), 1.85 (1H, m), 2.85 (2H, d), 3.2 (2H, d), 3.8-3.85 (2H, m), 4.2-4.45 (3H, m), 5.15 (1H, d), 6.45-6.65 (2H, m), 7.3-7.85 (13H, m); m/z observed: 557.615.

Fmoc-Leu-Ala-Gly-Val-OBzl 6f: m.p. 130-33°C; $[\alpha]_D^{25}$ –40.1 (*c* = 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 0.95-1.0 (12H, m), 1.15 (3H, d), 1.35 - 1.85 (4H, m), 2.85 (2H, d), 3.15 (2H, d), 3.75-3.85 (3H, m), 4.2-4.45 (3H, m), 5.05 (1H, d), 6.48-6.65 (2H, m), 7.25-7.85 (13H, m); m/z observed: 670.77.

Fmoc-Gly-Gly-Phe-Leu-OBzl 6g: m.p. 163-34°C; $[\alpha]_D^{25}$ –8.2 (*c* = 1, DMF); ^{1}H NMR ($CDCl_3$): δ 0.95 (6H, d), 1.33 (2H, m), 1.65 (1H, m), 2.85 (2H, d), 3.2 (4H, m), 3.75 (1H, m), 4.2-4.45 (3H, m), 5.1 (1H, d), 6.4-6.6 (2H, m), 7.3-7.85 (13H, m); m/z observed: 704.787.

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