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"One-Pot" Preparation of *N*-Carbamate Protected Amino Acids via the Azide

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Abstract:

A convenient and efficient method for the preparation of fluorenylmethyloxycarbonyl (Fmoc) and allyloxycarbonyl (Alloc) amino acids is proposed. This method is particularly attractive due to the fact that the reaction sequence Fmoc/Alloc-chloride to Fmoc/Alloc-azide to Fmoc/Alloc-amino acid can readily be carried out in one pot. A further advantage is the minimization of byproducts, which are easily removed during the workup. Most important, this strategy minimizes the formation of dipeptides that are difficult to remove by crystallization. Thus, Fmoc and Alloc amino acids are obtained in high yield (60−90%) and purity as evidenced by thin-layer chromatography, reversed-phase high-performance liquid chromatography, mass spectrometry, and nuclear magnetic resonance.[1]

Introduction

Nowadays synthetic peptides such as T20,[2] R15K (V3 peptide from HIV-1 IIIB gp120),[3] Somatostatin,[4] and Oxytocin[5] are widely recognized as potential pharmaceutical agents.[6] The ultimate purity of any peptide, whether prepared by solution or solid-phase methodology, depends heavily on the purity of the initial starting materials. The most commonly utilized amino acid derivatives for both solution and solid-phase procedures are the *N*-urethane blocked amino acids, in particular Z (benzyloxycarbonyl), Boc (*tert*-butoxycarbonyl), Fmoc (fluorenylmethyloxycarbonyl), and Alloc (allyloxycarbonyl).[7,8] Therefore, there is a growing demand for the preparation of those with a high level of purity.

In the last two decades, the use of the Fmoc and Alloc groups has evidenced an important increase as an alternative to Boc chemistry for the synthesis of peptides. However, their attachment through powerful chloroformate reagents under Schotten−Baumann conditions can lead to the formation of protected dipeptides as side products.[9] In most cases the identified protected dipeptide impurities for Fmoc represent 1−5% of the total. As an example, even when the relatively hindered Alloc-Val-OH was prepared, 14% of the corresponding dipeptide was obtained.[8] This high incidence of protected dipeptide can lead to the insertion of an extra amino acid in the final peptide synthesis, which cannot be tolerated for use in therapeutic applications for some kinds of drug. Several approaches based on the use of different activated formates have been proposed to minimize this problematic side reaction. For instance, azides,[9−11] mixed carbonates such as the succinimidyl,[12−15] the 1,2,2,2-tetrachloroethyl,[16,17] and the 5-norbornene-2,3-dicarboximido,[18] and the symmetrical pyrocarbonates.[19] Although, the succinimidyl carbonate is recognized as the method of choice, it is not practical when protected amino acids are prepared, because the *N*-hydroxysuccinimide formed can contaminate the final product. Alternatively, the Bolin method, which involves the in situ preparation of the trimethylsilyl amino acid derivatives and their subsequent reaction with the chloroformate, is tedious and impractical for large scale.[20]

This paper describes a new protocol for preparing Fmoc and Alloc amino acids using sodium azide as reagent and free amino acids. The syntheses of *N*-protected amino acids are carried out in one pot removing the byproducts during the workup, so it is not necessary to use a crystallization step. Moreover, it was demonstrated that the amounts of the undesired protected dipeptide produced by the present

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***Table 1. N*-Protected amino acids prepared by the azide procedure**

no.	compound	reaction conditions (1:1, v/v)	time (day)	yield (%)	purity (%)	Fmoc-dipeptide (%)	commercial Fmoc-dipeptide (%)
	Fmoc-Gln						0.39
1		10% Na_2CO_3−dioxane	5	59	97.6	0.19	
2		10% Na_2CO_3−DMF	5	68	95.7	0.35	
3		10% Na_2CO_3−acetone	5	65	98.2	0.86	
4		10% Na_2CO_3−ACN	5	47	97.8	0.15	
5		5% Na_2CO_3−dioxane	5	71	96.6	not detectable	
6		1% Na_2CO_3−dioxane	5	75	98.8	not detectable	
7		1% Na_2CO_3−acetone	5	56	94.2	0.38	
8		acetone−H_2O, DIEA (1 equiv)	3	50	94.4	2.41	
9		acetone−H_2O, DIEA (2 equiv)	3	82	89.7	2.31	
10		acetone−H_2O, DIEA (3 equiv)	3	90	92.2	2.59	
	Fmoc-Asn						0.65
11		1% Na_2CO_3−dioxane	5	79	98.1	0.11	
	Fmoc-Ala						0.59
12		1% Na_2CO_3−dioxane	3	90	96.2	0.45	
	Fmoc-Gly						0.31
13		1% Na_2CO_3−dioxane	2	93	98.6	0.25	

method were insignificant. In addition, a series of detailed improvements over the previous synthesis of Fmoc/Alloc amino acid were developed, which resulted in an optimized protocol for the preparation of these derivatives. Furthermore, the azide method is less expensive than other acyl derivatives and therefore shows a better suitability for the preparation of *N*-carbamate protected amino acids in large scale.

Results and Discussion

It has been reported that the Fmoc group can be introduced into amino acids using allyloxycarbonyl chloride (Fmoc-Cl) as acylating agent under Schotten−Baumann conditions in the presence of $NaHCO_3$ or Na_2CO_3. However, the formation of protected dipeptide contaminants in high amount represents a real problem in peptide chemistry. This side product is difficult to detect by TLC but appears clearly on C_{18} chromatography.[9] It was demonstrated that a simple crystallization is not effective, making the removal of the contaminant a tedious and expensive process. Most of the reported methods are based on modifications of the acylating agent used for introducing the urethane-type protecting groups. However, the new methods developed were more expensive than the chloroformate method.

To develop an efficient, competitive, and inexpensive procedure, the azide method was studied and optimized. In earlier reports,[9,10] the intermediate 9-fluorenylmethylazidoformate (Fmoc-N_3) was isolated and the formation of the Fmoc-amino acid was accomplished in a separate step. The main drawback of this method is that Fmoc-N_3 needs to be isolated and stored, with the corresponding handling problems that this can cause especially at large scale. Furthermore, the elimination of a reaction step reduces the cost and the time of the total process.

Herein, the protocol is based on the synthesis of these compounds in one pot. Thus, Fmoc-Cl reacts with NaN_3 without isolating the Fmoc-N_3, the reaction with the free amino acids leading to the target compounds. In detail, the Fmoc-Cl is dissolved in dioxane, and an aqueous NaN_3 solution is added. The mixture is left stirring at room temperature, the reaction progress being followed by reversed-phase HPLC. Reaction completion is typically achieved after 1 h. Contrary to previous reports,[9,11] in which acetone was used instead of dioxane, the Fmoc-N_3 does not precipitate, favouring the reaction with the free amino acids. This second reaction was investigated in depth by varying temperatures, solvents, reaction times, and pH.

Using glutamine as a model, an amino acid that can present solubility problems, initial experiments were performed in solvents such as acetonitrile (ACN), *N*,*N*-dimethylformamide (DMF), dioxane, and acetone. Also, different amounts of inorganic base (Na_2CO_3) were added (entries 1−7) (Table 1). According to these results, the use of dioxane and variable amounts of aqueous Na_2CO_3 produced the most favourable results (entries 1, 5, and 6).

Additionally, the substitution of an inorganic base (Na_2CO_3) by an organic base such as *N*,*N*-diisopropylethylamine (DIEA) was studied. The DIEA base was added in increasing amounts, and when 2 or 3 equiv were used, an improvement in the yield and reaction rate were detected (entries 9, 10 vs 3; Table 1). However, as a concomitant result, the Fmoc amino acid became contaminated with a substantial amount of protected dipeptide (2−3%). Besides, the use of organic base leads to the premature cleavage of the Fmoc group, and its use was discontinued.

Several Fmoc amino acids were synthesized using the reaction conditions described above (free amino acids were dissolved in 1% aqueous Na_2CO_3), and good yields were obtained (entries 11−13, Table 1). Moreover, in all cases studied, the amounts of Fmoc dipeptide contaminant found in these compounds was less than those in commercial samples.[21]

(21) To quantify the formation of protected dipeptide as contaminants, the corresponding protected dipeptides (Fmoc-Gln-Gln-OH, Fmoc-Asn-Asn-OH, Fmoc-Ala-Ala-OH, Fmoc-Gly-Gly-OH, Alloc-Gly-Gly-OH, and Alloc-Val-Val-OH) were synthesized on solid phase using Fmoc/Alloc chemistry and ClTrt-Cl-resin. The protected dipeptides were cleaved from the resin and characterized by analytical HPLC.

Table 2. **Synthesis of Fmoc/Alloc amino acids via the azide method[a]**

no.	compound	reaction condition (1:1, v/v)	yield (%)	purity (%)	Fmoc-dipeptide (%)
14	Fmoc-Gln	1% Na_2CO_3−dioxane	71	99.8	0.09
15	Fmoc-Asn	1% Na_2CO_3−dioxane	60	99.37	not detectable
16	Fmoc-Ala	1% Na_2CO_3−dioxane	81	98.9	0.25
17	Fmoc-Gly	1% Na_2CO_3−dioxane	85	99.4	0.10
18	Fmoc-Gly large scale	1% Na_2CO_3−dioxane	92	98.3	0.36
19	Alloc-Gly	1% Na_2CO_3−dioxane	96	98.6	0.03
20	Alloc-Val	1% Na_2CO_3−dioxane	95	98.7	0.05

[a] Reaction time: 48 h and pH controlled between 8 and 10.

To develop an efficient and economic methodology, the reaction time and the basic conditions were also studied and optimized. Long reaction times promote the formation of side products, which are difficult to remove by workup. Another important factor is the basic pH, which enhances the reaction rate. Furthermore, a more basic pH improves the solubility of the free amino acid. However, it has been observed that highly basic conditions (pH > 11) might conceivably lead to premature degradation of the azidoformate to dibenzofulvene and other byproducts.[11] Therefore, to avoid this phenomenon, the pH reaction was kept between 8 and 10 by controlled addition of 10% aqueous Na_2CO_3.

With these considerations taken into account, Fmoc amino acids were obtained in good yields (entries 14−18, Table 2). Besides, the formation of protected dipeptide contaminant was either marginal or not detectable.

As a scaled up example of this method, the preparation of Fmoc-Gly-OH was carried out. Due to its reduced sterical hindrance, glycine appears to be one of the most problematic amino acids in terms of dipeptide and tripeptide formation. However, by the present method Fmoc-Gly-OH was obtained in very high yield and with acceptably low formation of Fmoc-Gly-Gly-OH (entry 18, Table 2).

All the Fmoc amino acids prepared by this procedure were found to be exceptionally pure (Table 3). Analysis by TLC, using conditions that separate monomer from dimer derivatives, indicated the presence of only one spot. Figure 1 shows the HPLC profiles of some of these compounds.

To extend this method to other *N*-protecting amino groups, Alloc amino acids were prepared. Alloc-Gly-OH and Alloc-Val-OH were obtained using the azide method in one-pot. Thus, starting from allyloxycarbonyl chloride (Alloc-Cl), after 1 h of reaction with NaN_3, allyloxycarbonyl azide (Alloc-N_3) was obtained. Then, the free amino acid, dissolved in 1% aqueous Na_2CO_3-dioxane, was added and stirred for 48 h. The Alloc-Gly-OH and Alloc-Val-OH were obtained with good yields and high purities (entries 19 and 20, Table 2). These were characterized satisfactorily by reversed-phase HPLC, TLC, and other physical methods (Table 3). The Alloc-Gly-OH and Alloc-Val-OH prepared by the azide method did not contain significant amounts of protected dipeptide as determined by HPLC.

Conclusions

Our studies have shown that the Fmoc/Alloc amino acids can be obtained in one pot using Fmoc/Alloc-azide as an acylating agent. This procedure does not require the temporary protection of the carboxyl function of the amino acid. The formation of Fmoc/Alloc-dipeptide contaminants using the azide method in one pot is significantly reduced compared to the higher degree of contaminant obtained employing Fmoc/Alloc-Cl as an acylating agent.[9,12,20] The contaminant level is comparable to that obtained by the Fmoc-Cl method in the presence of Zinc dust[22] or to that of the succinimidyl procedure.[23] Handling and storage of the organic azide reagent is circumvented, since the latter is not isolated in this method. In addition, in the synthesis using the azide method, the byproducts (azide) can be easily removed in the workup. In contrast to the succinimidyl method, it is often necessary to resort to additional crystallization steps for the removal of byproducts (*N*-hydroxysuccinimide).

From these results, we conclude that the azide method is a simple and convenient procedure to obtain Fmoc/Alloc amino acids, with good yields, minimum side products, and easy scalability. Other urethane-type protecting groups

Table 3. ***N*-Protected amino acids characterization**

compound	retention time (min)[a]	MW[b] (*m*/*z*)	melting point[c] (°C)	^{1}H NMR (δ)
Fmoc-Gln	9.03	369.46 (368.14)	219−220 (221−223)	1.74 (1H, m), 1.93 (1H, m), 2.13 (2H, t), 3.92 (1H, m), 4.19 (1H, t) 4.23 (2H, d), 6.74 (2H, broad s), 7.30−7.87 (8H, m)
Fmoc-Asn	9.11	355.41 (354.12)	187−188 (186−186)	2.46 (2H, m), 4.19 (1H, m), 4.24 (1H, t), 4.32 (2H, d), 6.89 (2H, broad s), 7.30−7.87 (8H, m)
Fmoc-Ala	10.85	312.42 (311.12)	143−145 (143−144)	1.24 (3H, d), 3.99 (1H, m), 4.19 (1H, t), 4.24 (2H, d), 7.30−7.87 (8H, m)
Fmoc-Gly	10.33	298.39 (297.10)	174−176 (173−174)	3.65 (2H, d), 4.19 (1H, t), 4.27 (2H, d), 7.30−7.87 (8H, m)
Alloc-Gly	4.98	160.27 (159.05)	(oil)	4.03 (2H, d), 4.60 (2H, m), 5.23 (1H, d), 5.30 (1H, m), 5.32 (1H, d), 5.92 (1H, m).
Alloc-Val	7.57	202.0 (201.10)	(oil)	0.94 (3H, d), 1.01 (3H, d), 2.23 (1H, m), 4.33 (1H, m), 4.588 (2H, d), 5.23 (1H, dd), 5.32 (1H, d), 5.92 (1H, m).

[a] Retention time was determined by RP-HPLC. [b] The molecular weight was measured by MS−HPLC; the values in parentheses are calculated by the ChemDraw program. [c] Values in parentheses are reported by Chang. et al.[24]

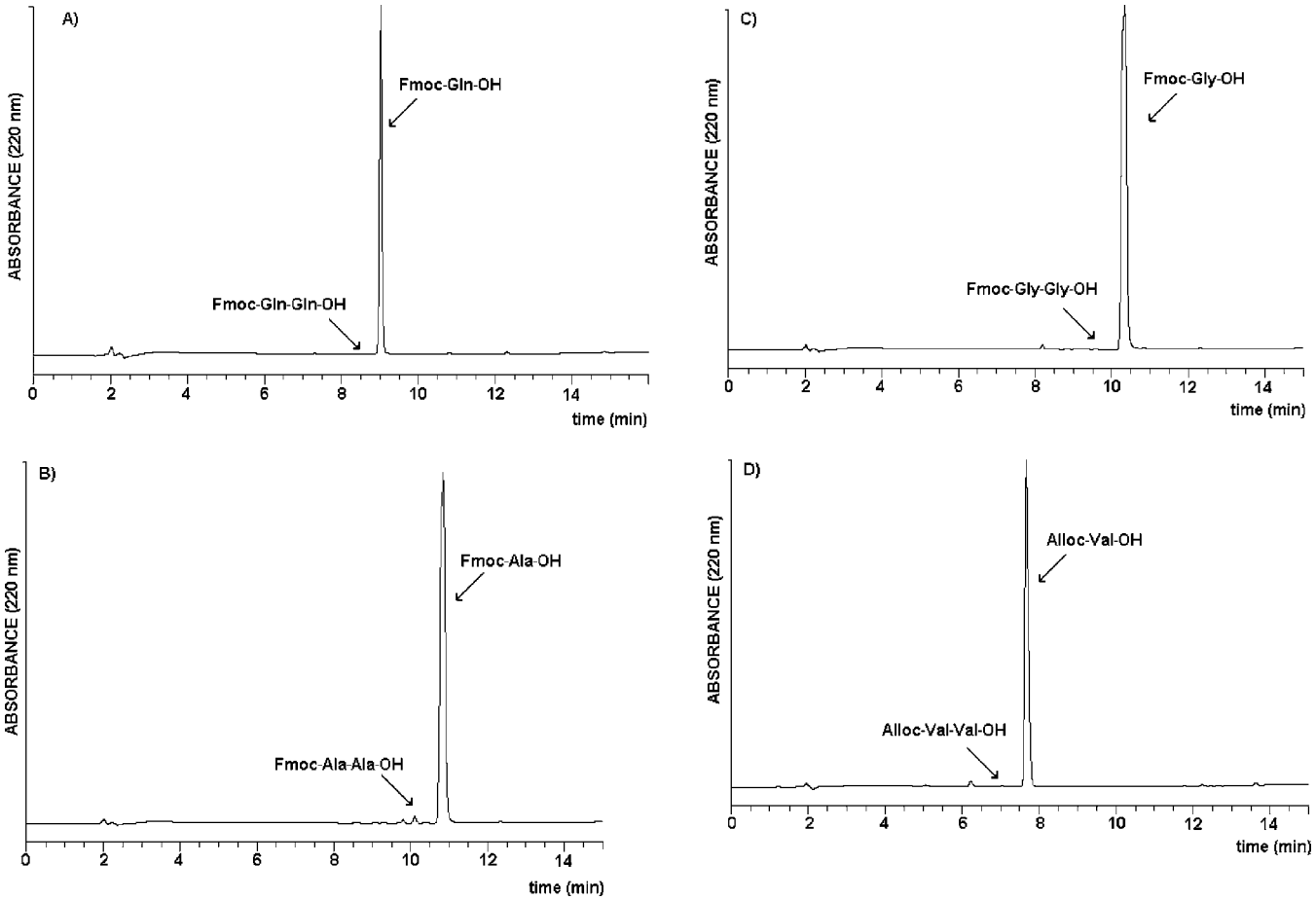

Figure 1. **HPLC chromatograms of (a) Fmoc-Gln-OH, (b) Fmoc-Ala-OH, (c) Fmoc-Gly-OH, (d) Alloc-Val-OH. All the Fmoc/Alloc amino acids were prepared by the azide method in one pot. Samples were applied onto a Waters system C_{18} Symmetry column (4.6 × 150 mm, 5 μm). Conditions: λ = 220 nm, flow = 1 mL/min, linear gradient = 5−100% B in 15 min (B = ACN).**

introduced through the procedure described herein are currently being studied.

Experimental Section

General. Melting points were determined on a Buchi capillary tube melting point B-540 apparatus and are uncorrected. Thin-layer chromatography (TLC) was performed on precoated TLC plates (Merck, silica gel 60, F_{254}), using as eluent CH_2Cl_2−MeOH−AcOH (94:5:1). The plates were visualized by using a 254 nm UV lamp. HPLC reversed-phase Symmetry columns C_{18} 4.6 × 150 mm, 5 μm were from Waters (Ireland). Analytical HPLC was carried out on a Waters 996 instrument provided with a photodiode array detector and equipped with a Waters 2695 separation module controlled by the Millennium software. NMR spectra were taken on a (400 MHz) Varian spectrometer and expressed in ppm from Me_4Si as internal standard. HPLC−MS analysis were carried out on an Alliance system (Waters 2795 Separation Module, MA, U.S.A.) coupled to a double wavelength UV detector (Waters 2487 dual λ Absorbance Detector, MA) and a ZQ4000 mass spectrometer (Waters, Micromass ZQ, MA).

General Procedure for the Preparation of Fmoc Amino Acids by the Azide Method. Fmoc-Cl (2.58 g, 10 mmol) was dissolved in dioxane (5 mL), and NaN_3 (0.78 g, 12 mmol) in water (4 mL) was added. The mixture was left to stir at room temperature, and the reaction was followed by HPLC, which showed that it was complete after 1 h (no precipitate appeared). Next, the amino acid [H-Gln-OH, H-Asn-OH, H-Ala-OH, and H-Gly-OH (11 mmol)] was added to the previous mixture dissolved in 1% aqueous Na_2CO_3−dioxane (1:1, v/v) (the amount of the latter depending on the solubility of the respective amino acid: 30 mL for H-Ala-OH and H-Gly-OH; 60 mL for H-Gln-OH and H-Asn-OH). The reaction was stirred at room temperature for 48 h, during which the pH (controlled by pH paper) was kept between 8 and 10 by the addition of 10% aqueous Na_2CO_3. The progress of the reaction was monitored by RP-HPLC.

Once the reaction was completed, the mixture was poured into 100 mL of water (keeping a basic pH) and extracted 4 times with *tert*-butyl methyl ether to remove side products. The aqueous solution was cooled to 4 °C and acidified to pH 2.0 with 2 N aqueous HCl. The white precipitate was filtered off and washed with ice water made slightly acidic (pH 4−5) with dilute HCl. The solid is dried under vacuum overnight, and the purity was measured by HPLC. Yields and purities are shown in Tables 1 and 2. Additional analytical data are shown in Table 3.

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Scale-up Preparation of Fmoc-Gly-OH by the Azide Method. Fmoc-Cl (258.70 g, 1 mol) was dissolved in dioxane (250 mL), and NaN_3 (78.0 g, 1.2 mol) in water (250 mL) was added. The mixture was left to stir at room temperature, and the reaction was complete after 1 h. Next, the H-Gly-OH (82.6 g, 1.1 mol) dissolved in 1% aqueous Na_2CO_3−dioxane (1:1, v/v) (500 mL) was added to the previous mixture, while the solution was stirred at 25 °C. The reaction mixture was kept at pH 8−10 by the addition of solid Na_2CO_3. The progress of the reaction was monitored by TLC and RP-HPLC.

Once the reaction was completed, the mixture was poured into 400 mL of water (keeping a basic pH) and extracted four times with *tert*-butyl methyl ether to remove side products. The aqueous solution was cooled to 4 °C and acidified to pH 2.0 with 6 N aqueous HCl. The white precipitate was filtered off and washed with ice water made slightly acidic (pH 4−5) with dilute HCl. [The cold aqueous solution was immediately neutralized with NaOH, to avoid subjecting the released azide anions to acidic conditions at room temperature.] The solid was dried under vacuum overnight, and the purity was measured by HPLC. Yields and purities are shown in Table 2 (entries 18).

General Procedure for the Preparation of Alloc Amino Acids by the Azide Method. Alloc-Cl (3.81 mL, 36 mmol) was disolved in dioxane (10 mL), and NaN_3 (3.5 g, 53 mmol) in water (8 mL) was added. The mixture was left to stir at room temperature for 1 h. Next, the amino acid H-Val-OH and H-Gly-OH (43 mmol) dissolved in 1% aqueous Na_2CO_3−dioxane (1:1, v/v) (125 mL) was added to the previous mixture. The reaction was stirred at room temperature during 48 h, and the pH of the reaction was kept between 8 and 10 (controlled by pH paper) by addition of 10% aqueous Na_2CO_3. The progress of the reaction was monitored by RP-HPLC.

Once the reaction was completed, the mixture was poured into water (100 mL) (keeping the pH between 9 and 10) and extracted several times with *tert*-butyl methyl ether to remove the byproducts. The aqueous solution was acidified to pH 2.0 with 2 N aqueous HCl and was extracted several times with ethyl acetate. The organic phase was dried with $MgSO_4$, filtered, and concentrated under vacuum. The purity of the oil was measured by HPLC. Yields and purities are shown in Table 2. Additional analytical data are shown in Table 3.

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