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Fmoc-Amox, a Suitable Reagent for the Introduction of Fmoc

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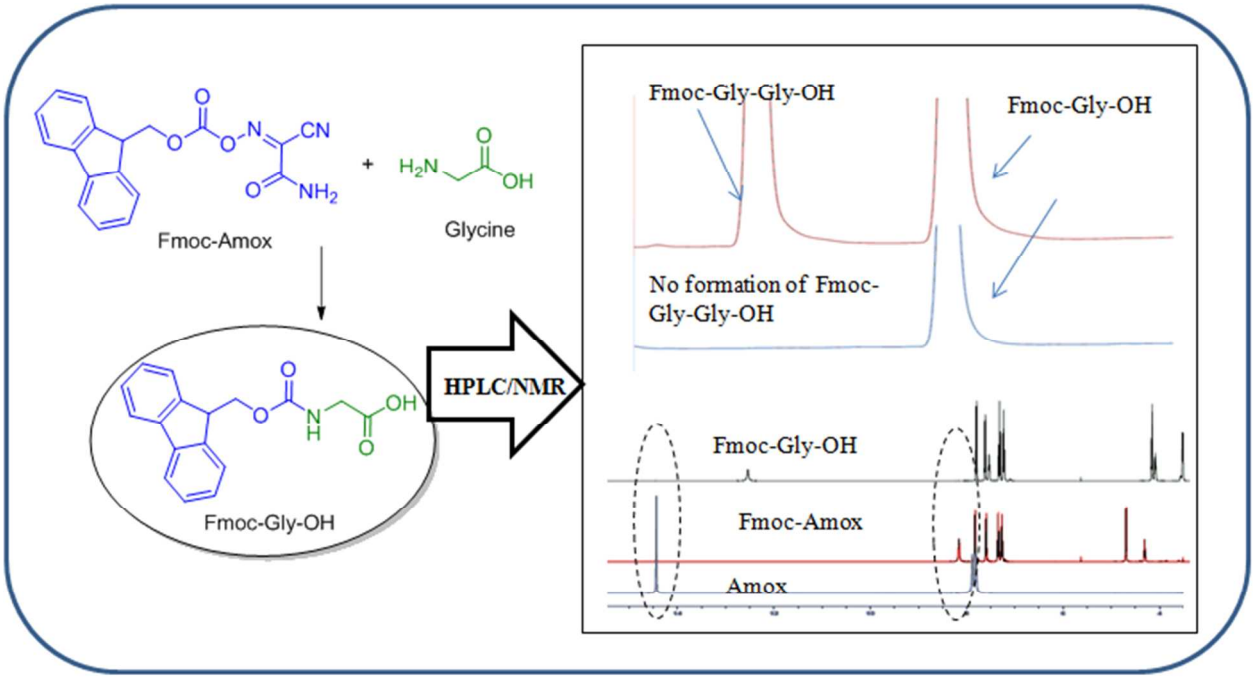
Graphical Abstract



KEYWORDS

Solid-phase peptide synthesis, Fmoc, protecting groups, side reactions, dipeptide formation, β-alanine.

ABSTRACT

Synthesis of most peptides is achieved using SPPS employing Fmoc/tBu strategy. However, introduction of Fmoc in N-unprotected amino acids seems to be challenging due to the formation of dipeptides and sometimes tripeptides as impurities, and β-alanyl impurities when Fmoc-OSu is used as well. Herein, we report an efficient and successful method using Fmoc-Amox, which is an oxime based derivative, towards the synthesize of Fmoc-glycine with no traces of side reactions. Fmoc-Amox is inexpensive and Amox can be easily removed after the reaction, thus affording pure Fmoc-Gly-OH devoid of any detrimental impurities or contamination, mainly dipeptide or Amox itself, as shown by HPLC and NMR, respectively.

In 1963, Merrifield described a whole new concept of chemical synthesis,[1] with first of all great impact in research, but that at the end has changed the *pharmacopoea*.[2-3] Nowadays, there are drugs in market whose active pharmaceutical ingredients (APIs) are TIDES (Oligonucleotides and Peptides Therapeutics), containing up to 30-40 monomers,[4] which have been produced using the solid-phase methodology described first by Merrifield.[1] Although at the beginning, the solid-phase peptide synthesis (SPPS) strategy was in some manner criticized by his European colleagues,[5] currently it has been totally adapted in research field as well as in the production.[6-8]

The idea is simple, as the name indicates, all amino acids have at least two functions, a carboxylic acid and an amine, and therefore if the reactivity of carboxylic acid of the amino acid *C*-terminal is masked with an insoluble polymeric permanent group and the amine with a temporary protecting groups, the stepwise synthesis is performed by sequential cycles which involves removal of the protecting group from amine followed by coupling of the next *N*-protected amino acid.[1, 8-9] However, in case of trifunctional amino acids, side chain is protected with a permanent (or semi permanent) protecting group has been employed.[10-11] During the early years, Merrifield used benzyl (Bzl) type groups for permanent protection and *tert*-butyloxycarbonyl (Boc) as temporary one.[1, 11] However, Boc and Bzl are not orthogonal as they both can be cleaved under acidolytic condition: trifluoroacetic acid (TFA) for Boc and stronger acids such as HF or trifluoromethanesulfonic acid (TFMSA) for Bzl.[12] In the earliest 70's, Carpino, who had also described the Boc group, revolutionized the area of peptide chemistry by proposing the fluorenylmethoxycarbonyl (Fmoc) group as temporary *N*-amino protection which is removed in presence of bases and hence is orthogonal to *tert*-butyl (*t*Bu) group.[13-14] In addition, Sheppard and Atherton in Europe[15] parallel to Chang and Meienhofer in USA[16], developed the so-called Fmoc/*t*Bu strategy, which at the end liberates the peptide after treatment

with TFA solution.[17] The implementation of this methodology signified a *democratization* of the peptide synthesis, because the use of Boc/Bzl strategy implied the concourse of well-trained chemists and special equipment for the cleavage reaction, while with the Fmoc/*t*Bu strategy, other laboratories with more bio focus are also able to synthesize peptides.[12] Furthermore, it also allows the production of peptides in multi-kilogram scale.[6-7]

At the beginning, commercial Fmoc-amino acids were prepared by the reaction of amino acids with Fmoc-Cl in basic pH following Schotten-Baumann conditions (**Figure 1A)**.[13-14] However, in the earlier 80's, our group[18] and Verlander at BioResearch, Inc., (later Bachem Americas) in collaboration with Goodman[19] described independently that most of the commercial Fmoc-amino acids contains dipeptide and even tripeptides. These impurities come from the high reactivity of Fmoc-Cl, which could also react with the carboxylic group of the amino acid to be protected rendering an anhydride, which in turn could react with the amine terminus of another molecule of amino acid leading to the formation of dipeptide according to the mechanism as shown in **Figure 1B**.

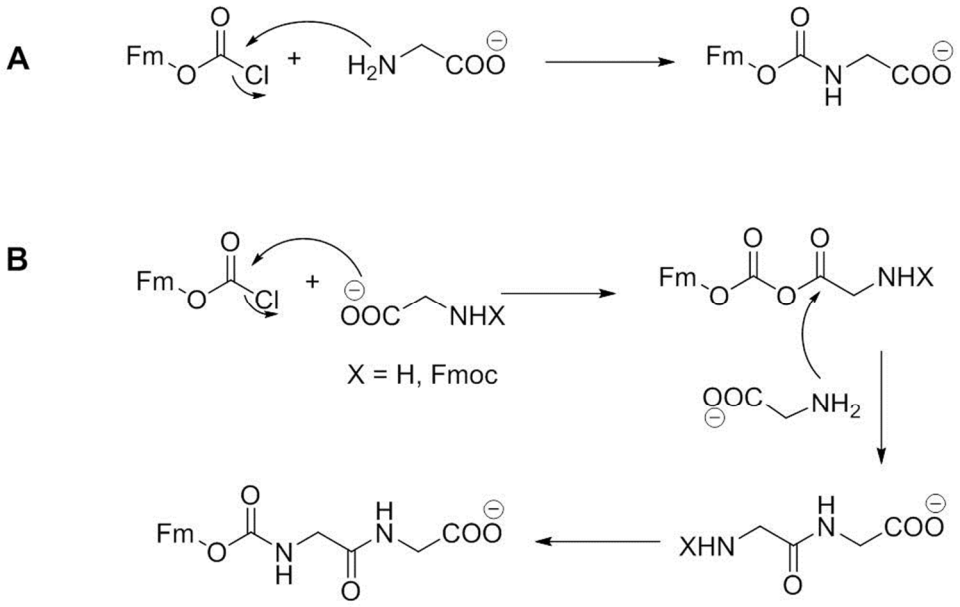


Figure 1: Fmoc protection of Amino acids: **A**. Mechanism of Fmoc protection. **B**. Mechanism for the formation of protected dipeptides during the protection of amino acids.

Taking into consideration that even a small percentage of impurities can lead to decrease of the final yield and purity, both groups came to a common conclusion of avoiding the use of Fmoc-Cl. Since these side-reactions are associated with the quality of the leaving group, we proposed the use Fmoc-N_3,[18] which had been already proposed by Carpino in his seminal paper.[13] and Verlander, Goodman, *et al* suggested the use of Fmoc-succinimidyl carbonate (Fmoc-OSu), after screening different leaving groups.[19] To avoid the preparation and storage of Fmoc-N_3, our group proposed *in situ* preparation of Fmoc-N_3 from Fmoc-Cl and NaN_3. This strategy render Fmoc-amino acids with high range of purity.[20] Parallel to this, Bolin *et al.* proposed the use of Fmoc-Cl with an *in situ* protection of carboxyl group with silylating reagent.[21] Years later, Barlos *et al*. also proposed this method for the preparation of trityl (Trt)-amino acid from Trt-Cl to avoid the presence of Trt esters as impurities.[22] Although, this strategy renders good results, it involves an extra step in addition to the use of organic solvents such as dichloromethane (DCM).[21-22] Years later, Suresh *et al.* proposed the preparation of Fmoc-amino acids with Fmoc-Cl in the presence of activated Zn-dust, which allows to carry out the reaction under neutral conditions.[23-24]

However, most commonly used method for the synthesis of Fmoc-amino acids out of the mentioned above protocols, is Fmoc-OSu (or NHS) which was questioned when Hlebowicz *et al.* at Bachem Europe[25] showed that Fmoc-AA-OH prepared from Fmoc-OSu contained Fmoc-β-Ala-OH and Fmoc-β-Ala-AA-OH formed through a Lossen rearrangement after the successive attack of OSu on one of the carbonyls of the HOSu moiety present in Fmoc-OSu (**Figure 2**).

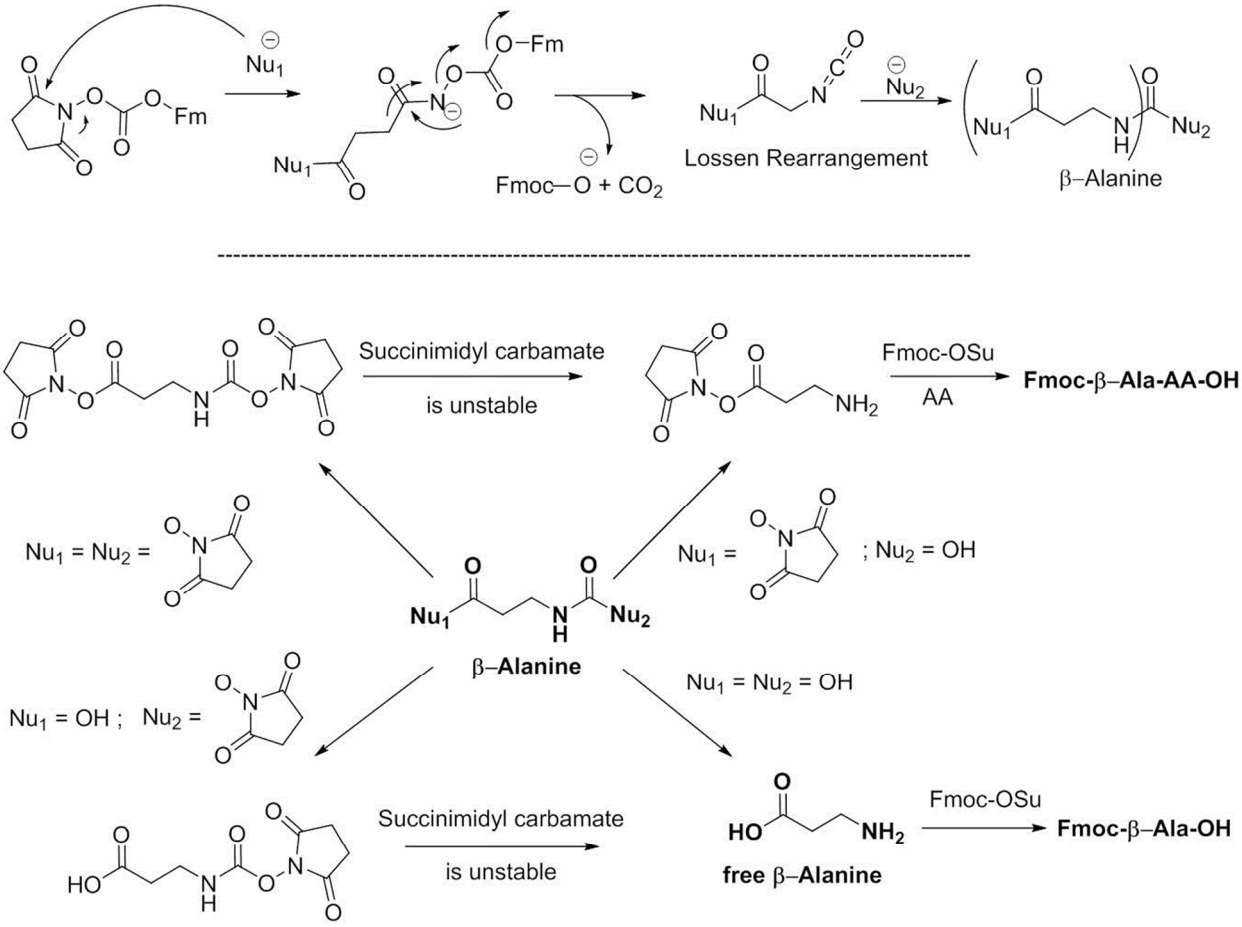
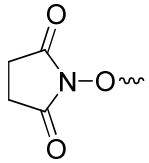
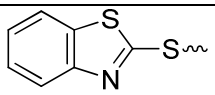
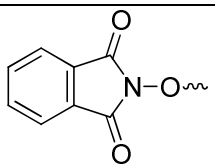


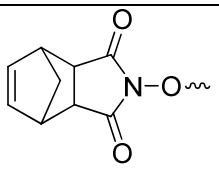
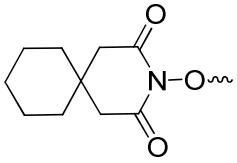
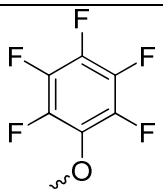
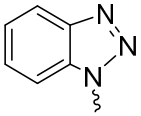
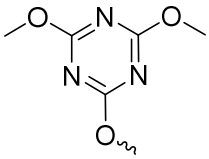
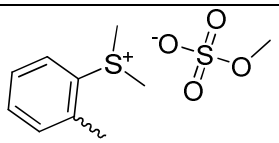
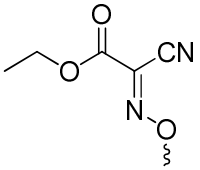
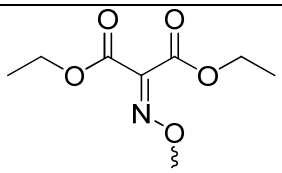
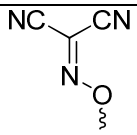
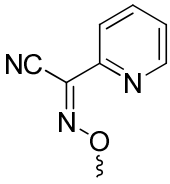
Figure 2: Mechanism of formation of β-Alanine *via* Lossen's rearrangement

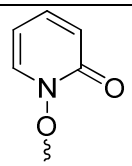
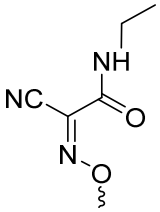
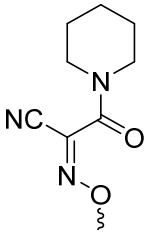
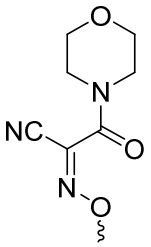
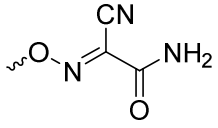
These findings revitalized again the research for developing new reagents and/or strategies for a safe introduction of the Fmoc group. **Table 1** enlists different Fmoc derivatives used for the protection of amino acids and results of their application for the preparation of Fmoc-Gly-OH. Although, this reaction could take place with all amino acids,[18-19] it is more pronounced with Gly, since its low sterical hindrance favors a high percentage of oligomerization. These Fmoc-introducing derivatives should present two key characteristics: (i) no special high reactivity to avoid the formation of oligopeptides and (ii) the leaving group, normally a hydroxyl compound, which is displaced by the amino acid, should be easily removable during the work-up. Thus, our

group, first proposed Fmoc-2-mercaptobenzothiazole (Fmoc-MBT), which give rise to the Fmoc derivatives with minimal contamination of oligomers[26] (Table 1, #4). However, the poor solubility of the released 2-MBT byproduct required washings with organic solvents for its total removal, which also dissolve the prepared Fmoc-amino acid, and therefore was detrimental for the final yield. Similar problem was encountered by Verlander, Goodman, *et al* where the alcohol was contaminating the final product with low overall yields (4-30%) when (poly)chlorophenyl derivatives were used[19].

Table 1. Fmoc-protecting reagents (Fmoc-OX)

#	Fmoc-X	Fmoc-Gly-Gly-OH (%)	Fmoc-Gly-OH		[Ref.]
			Scale (Mmol)	Yield (%)	
1		10-20	7.6	89	14
		2-6	25	NA[a]	18
		10-20	NA[a]	NA[a]	19
2		ND[b]	25	78	18
		0.25	11	98.6	20
3		20	0.55	68	19
		Traces	550	86	27
4		ND[b]	4.24	55[c]	26
		ND[b]	10	88	28
5		NA[a]	NA[a]	74	19
		NA[a]	NA[a]	88	29

6		NA[a]	10	81	30
7		ND[b]	11.2	93	31
8		NA[a]	NA	68	19
		NA[a]	6	84	32
9		ND[b]	1	94	33
10		ND[b]	0.13	96	34
11		NA[a]	10	94	35
12		0.57	20	92.1	36
13		0.17	20	82.9	36
14		0.22	20	48.5	36
15		0.01	20	91.6	36

16		0.41	20	92.8	36
17		0.17	20	91.8	37
18		0.06	20	92.1	37
19		0.08	20	93.5	37
20		0.06	20	90.8	37

[a]NA: not available; [b]ND: no detected; [c]to remove the 2-MBT, which was not soluble in the aqueous phase, the final product should be washed with solvent, which also dissolved the Fmoc-amino acid.

Other succinimidyl based derivatives such as the Phtalimide,[19, 29] the norbornenyl (which is the radical derived from norbornene),[30] and the corresponding spiro analogue, a six member derivative[31] were also assayed but with no remarkable advantage (Table 1, #5-7). In parallel, our group detected an important formation of a substituted β-alanine residue when the norbornenyl was used in combination with EDC for the solid-phase peptide synthesis (SPPS) in H_2O.[38] In order to overcome the challenging problem of β–alanine residues insertion when succinimidyl derivatives are used, Najera *et al.* has described a polymeric version of the reagent in which the

β-alanine contamination remained anchored to the polymeric support.[39-40] This bright strategy limits its usage in the preparation of small amounts of protected amino acids.

Other active species commonly used with carbodiimide based couplings such as pentafluorophenyl (Pfp)[19, 32] or benzotriazole (Bt)[33] were also addressed, with good yields, though the reagents are either expensive (Pfp) or explosive (Bt), respectively (Table 1, #8,9). Even Fmoc-triazine derivative was investigated[34] (Table 1, #10) as a method.

For Fmoc introduction using neat aqueous solution, Fmoc-phenyldimethylsulfonium methyl sulfate (Fmoc-ODsp)[35] has been used but no formation of dipeptides was studied (Table 1, # 11).

Recently, our group described OxymaPure® as superior additive for the carbodiimide based coupling strategy.[41] This reagent competes favorably in front of the traditionally used HOBt,[42] which is being discontinued due to its explosive properties.[43] Inspired by the impressive reactivity of OxymaPure® and it's fast assimilation in the peptide industry, we investigated other less reactive oxime derivatives for the introduction of Fmoc group.[36-37] From the first screening (Table 1, #12-16),[36] which also included 2-hydroxypyridine N-oxide (HOPO), the OxymaPure® derivative (Table 1, #12), as expected due to its high reactivity, gave the largest amount of dipeptide, followed by HOPO (Table 1, #16). The best candidate was the cyanopyridyl oxime (Table 1, #15), but this is an expensive derivative and it can be difficult to remove from the reaction medium. The second screening gave overall less amount of dipeptide formation (Table 1, #17-20)[37] and from this the cyano amide derivative (Amox) (Table 1, #20) was selected as the candidate to substitute HOSu for the introduction of Fmoc. Amox synthesis is inexpensive and can be easily removed after the reaction as the solubility of Amox in water was found to be 0.9 M which assures that it will not contaminate the final Fmoc-amino acid, unlike the case of MBT (Table 1, #4).

Initially Fmoc-Amox was prepared following the similar methodology used for other derivatives with slight modifications. The reaction of Fmoc-Cl and Amox in presence of sodium carbonate (aq solution) using DCM as solvent was carried out for 20 h at room temperature. After completion of reaction (monitored by HPLC for complete consumption of Fmoc-Cl), the reaction mixture was diluted with DCM. The reaction mixture in separating funnel prolonged the separation of DCM from aqueous phase. However, after separation the organic phase was collected and solvent was removed under vacuum. The HPLC chromatogram clearly showed high purity of Fmoc-Amox synthesis. Fmoc-Amox has been shown to be stable at room temperature for several months.

Fmoc-Gly-OH was prepared at two different scales using 1 and 40 g with similar results. In terms of Fmoc-dipeptide formation, H-Gly-OH proves to be an excellent candidate for evaluating the performance of Fmoc-Amox, since its low sterical hindrance favors a high percentage of oligomerization.[39] The reaction procedure is described as follows: The solution of Fmoc-Amox in acetone was added slowly to a stirring solution of H-Gly-OH and Na_2CO_3 in water. The pH of the reaction mixture was maintained at 9-10 by successive addition of Na_2CO_3. The reaction was monitored by TLC as well as by pH stability (decrease of pH was measured as a sign which indicated that the reaction was taking place). After completion of the reaction (4 h), solvent was removed and the remaining aqueous layer was washed with DCM, followed by addition of 1N HCl (till pH <2) which afforded off-white precipitate. The precipitate was then filtered and recrystallized using ethylacetate and *n*-Hexane (93 %) (see Experimental Section for more detailed procedure).

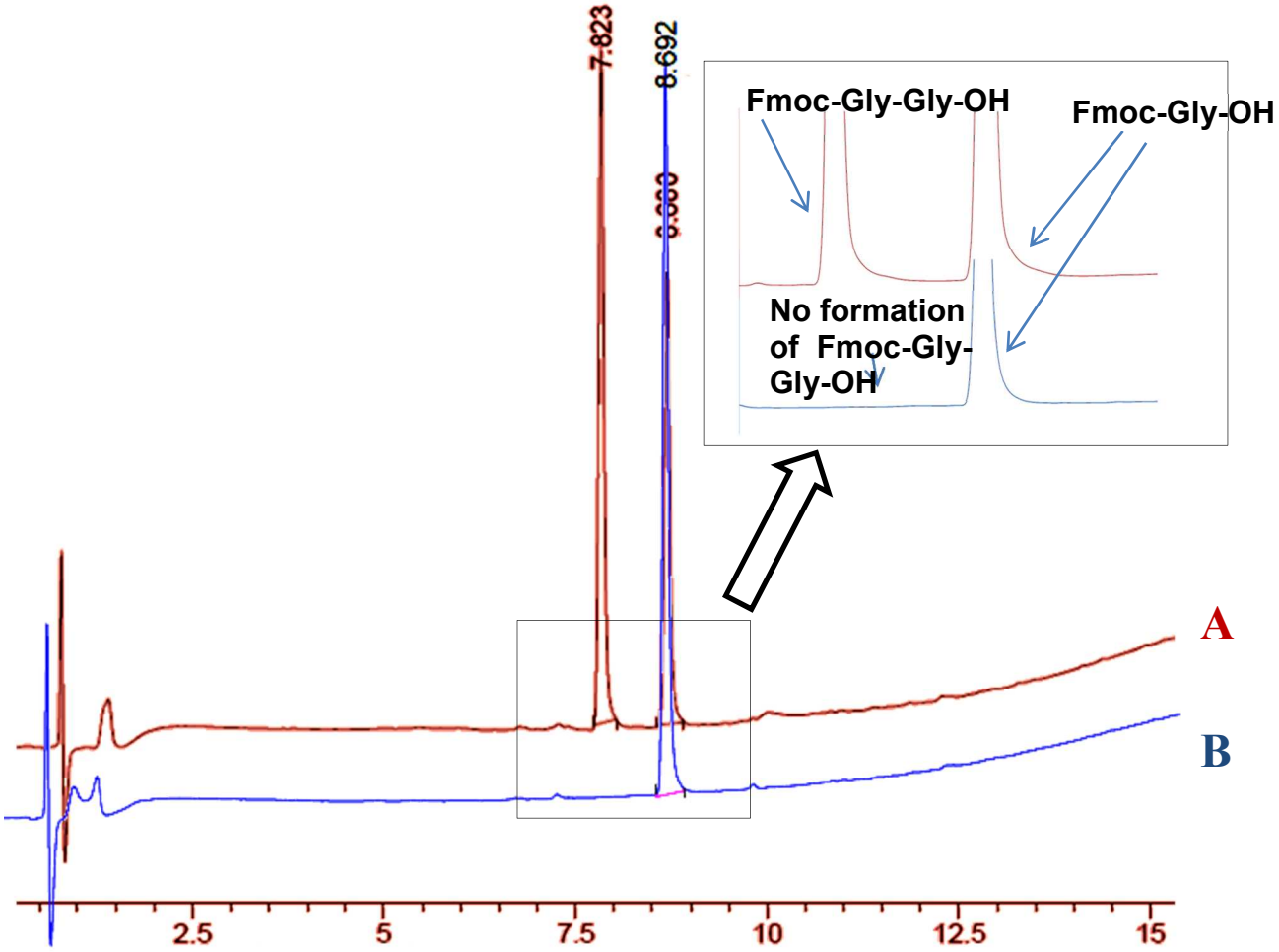


Figure 3: **A**. HPLC Chromatogram of Fmoc-Gly-OH and co-eluted Fmoc-Gly-Gly-OH. **B**. HPLC Chromatogram of synthetic Fmoc-Gly-OH.

The purity of Fmoc-Gly-OH was monitored by HPLC. Fmoc-Gly-Gly-OH, which was prepared by solid-phase technique, was also eluted along with Fmoc-Gly-OH to indicate the formation of any detrimental impurity of dipeptide. As shown in **Figure 3**, there was no trace of dipeptide observed in the chromatogram (**Figure 3**). Futhermore, ^{1}H-NMR did not showed the presence of any contamination of Amox (**Figure 4**), which clearly indicates the advantage of Amox for the use in the synthesis of Fmoc-amino acids.

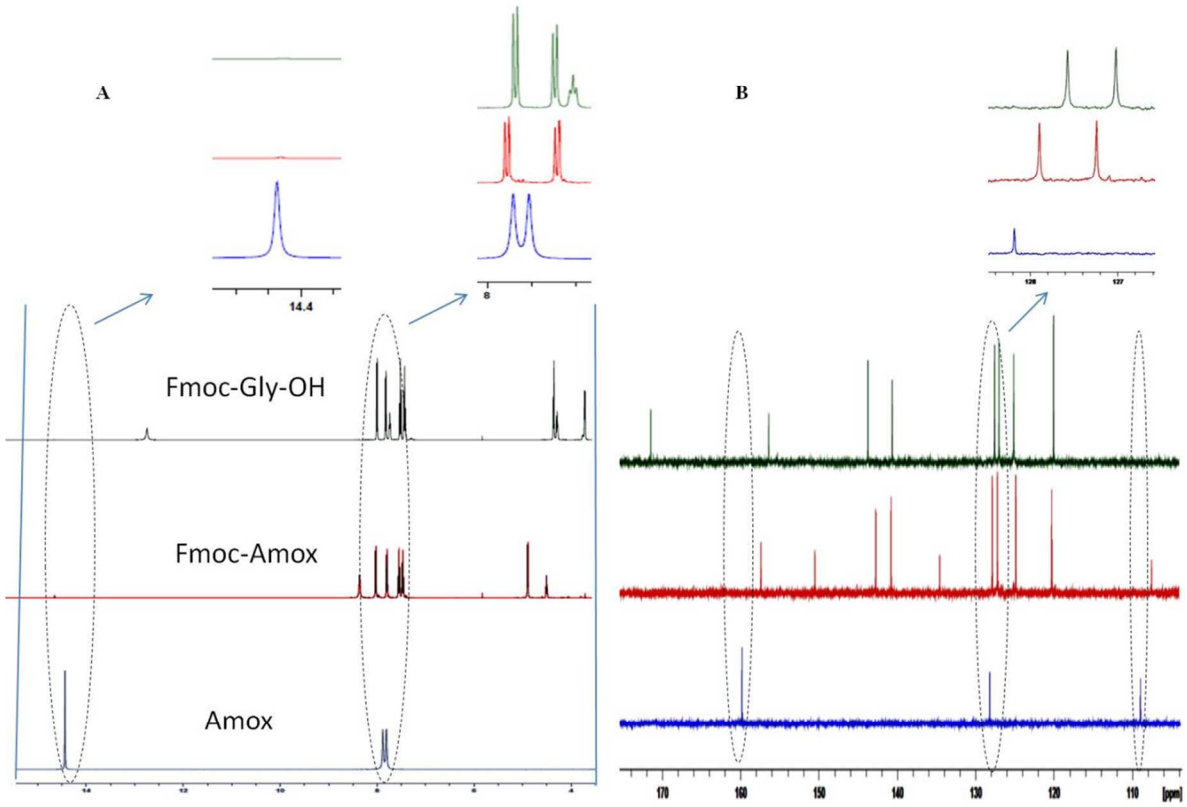


Figure 4: Comparison of ^{1}H and ^{13}C NMR of Fmoc-Gly-OH, Fmoc-Amox and Amox.

Prompted by these results, Fmoc-Phe-OH and Fmoc-Val-OH (10 g scale each) were also prepared using the procedure described above (**Figure 5** and **6**). The purity of the final product was confirmed by HPLC and NMR which shows no trace formation of dipeptide. Importance of solvent during workup was also studied as DCM is considered to be toxic and not suitable for bulk scale up reactions. Ethyl acetate was found to be a suitable substitute for DCM. Ethyl acetate layer when used during workup was collected, dried over $MgSO_4$, filtered and concentrated under vaccum. However, it was found to contain trace amount of Amox (as observed by NMR), which is not detrimental in the peptide synthesis. Therefore, the obtained product was dissolved in DCM and extracted using distilled water. The DCM workup helps in

the removal of trace amount of Amox yielding pure compound as confirmed by NMR (**Figure 7**).

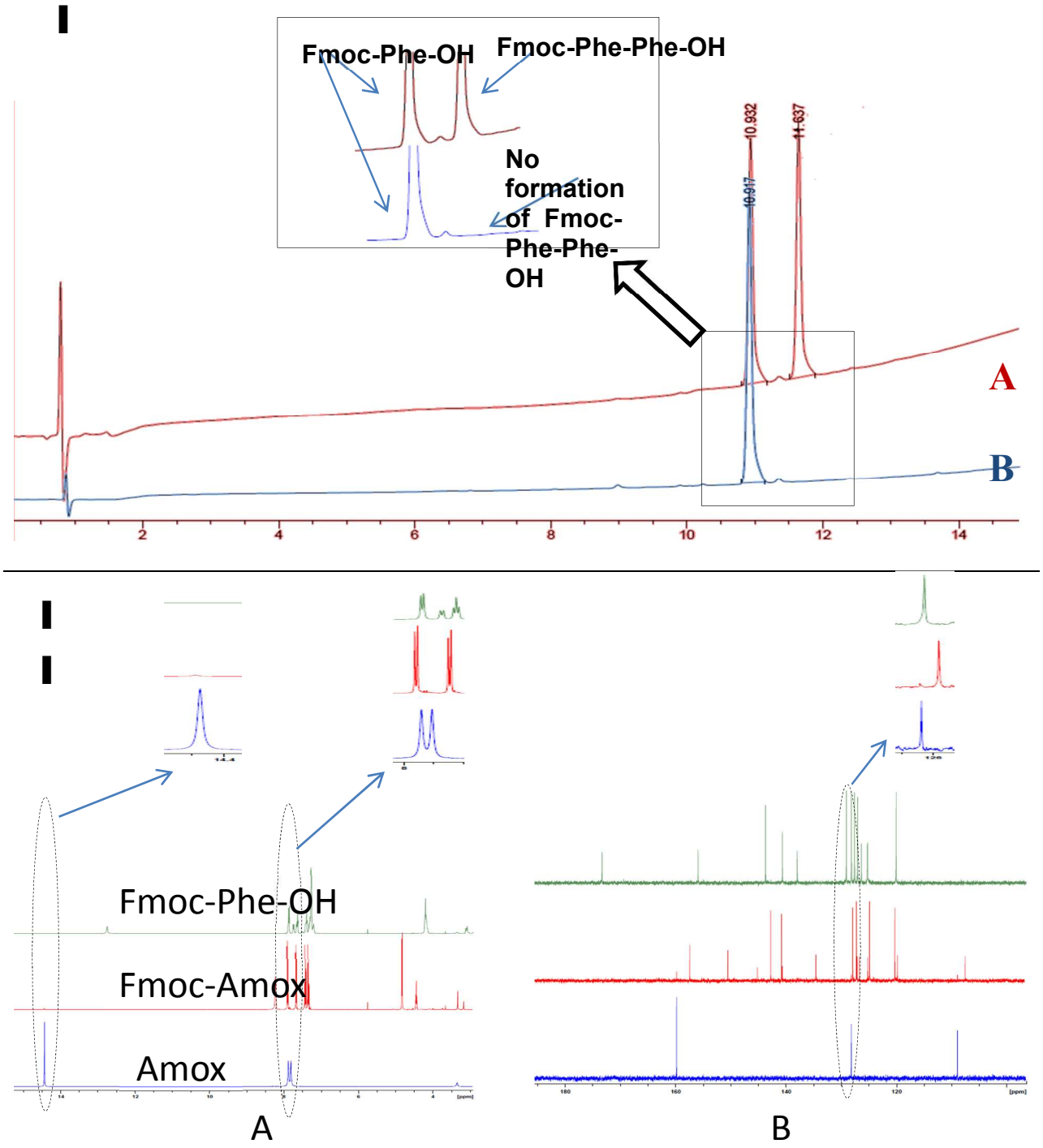


Figure 5I: A. HPLC Chromatogram of Fmoc-Phe-OH co-eluted with Fmoc-Phe-Phe-OH. B. HPLC Chromatogram of Fmoc-Phe-OH

Figure 5II. Comparison of ^{1}H and ^{13}C NMR of Fmoc-Phe-OH, Fmoc-Amox and Amox

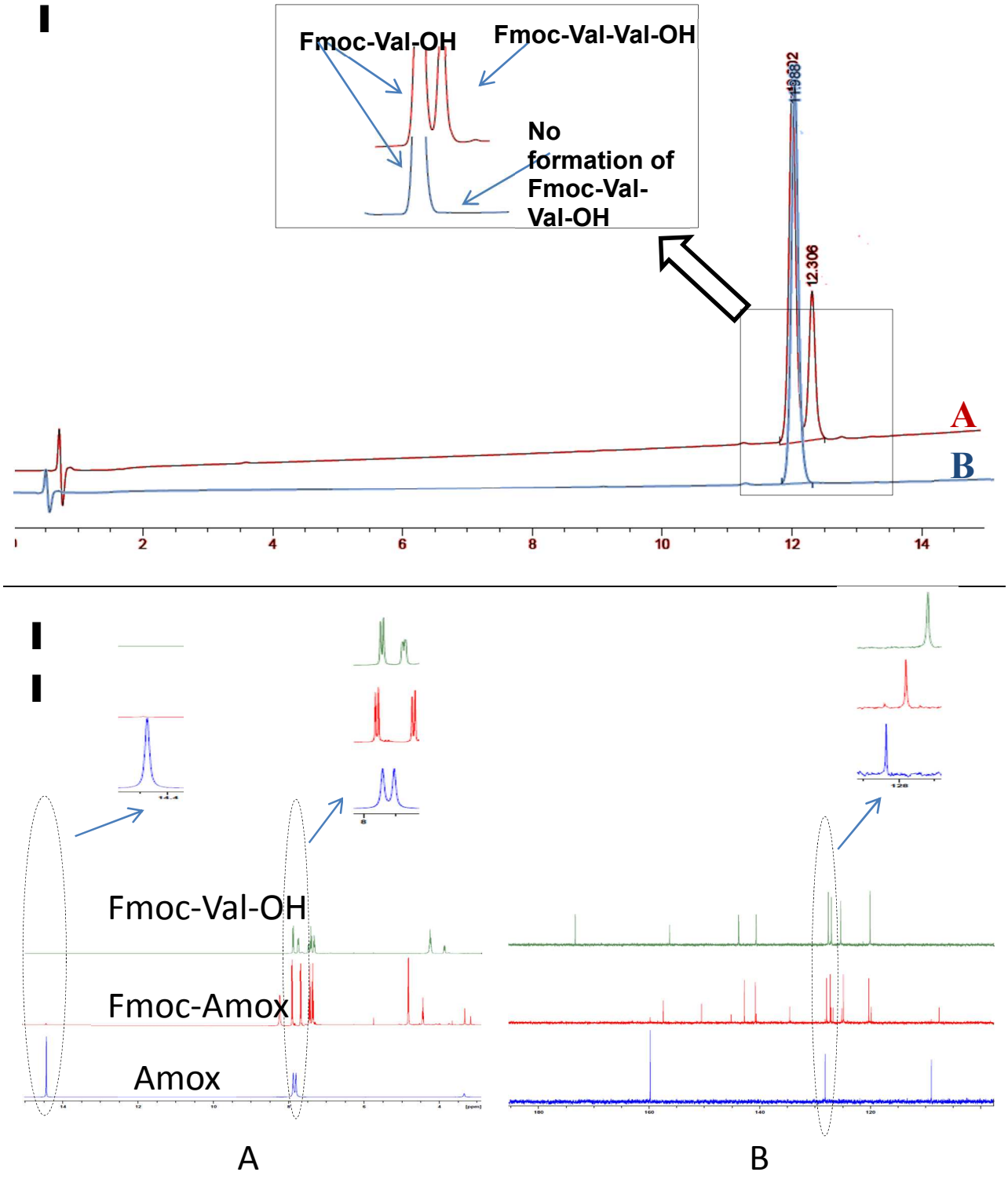


Figure 6I: A. HPLC Chromatogram of Fmoc-Val-OH co-eluted with Fmoc-Val-Val-OH and B. HPLC Chromatogram of Fmoc-Val-OH
Figure 6II. Comparison of ^{1}H and ^{13}C NMR of Fmoc-Val-OH, Fmoc-Amox and Amox

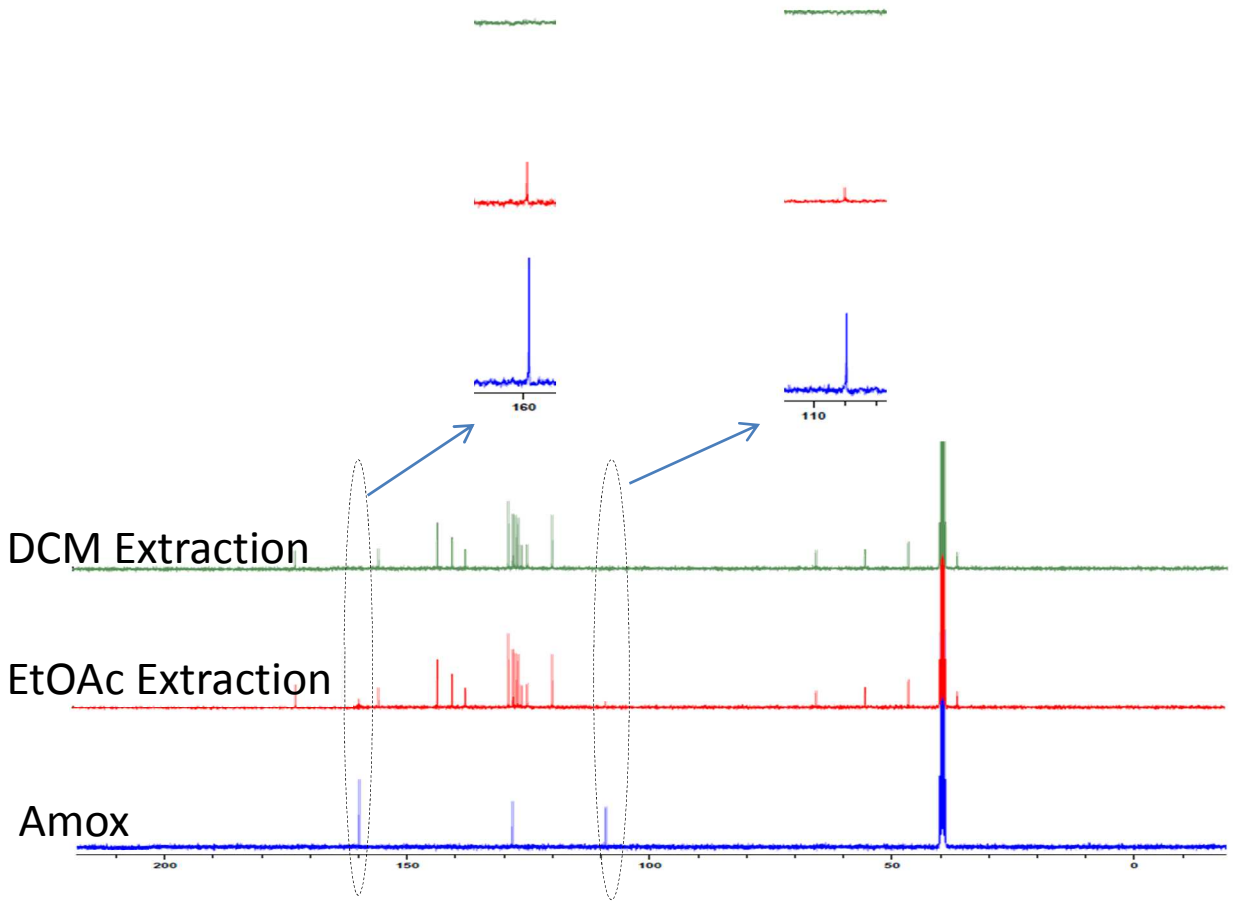


Figure 7: Comparison of workup using ethyl acetate and DCM for extraction of Fmoc-Phe-OH

These results clearly indicate the proficiency and superiority of Fmoc-Amox in synthesizing Fmoc-amino acids, which are most prone to give the side-reaction.

Conclusion

In order to overcome one of the biggest challenges in the synthesis of peptides building blocks, which is the undesired formation of di- and tripeptides during the protection of amino acids,

various methods incorporating large array of Fmoc-Leaving Groups have been intensively studied. Although few of the addressed reagents showed promising result, none of them has turned on to be a potential candidate for the industrial application. In this work, we are proposing Fmoc-Leaving Group derivative, Fmoc-Amox, based on the structure of well-known carbodiimide additive family – oxime. Fmoc-Amox has been used for Fmoc protection in case of H-Gly-OH, a challenging model which favors the formation of side reactions, showed outstanding performance with excellent yield (93 %) and total absence of detrimental impurities in the form of Fmoc-dipeptide, as supported by the HPLC and NMR analysis. Furthermore, it is envisaged that Amox derivatives will be useful for the introduction of other protecting groups such as pNZ, Alloc, Boc.

Experimental Procedures

Materials

The solvents used were of HPLC reagent grade and purchased from commercial suppliers. Magnetic resonance spectra (^{1}H, ^{13}C, DEPT90, DEPT135) were recorded with Bruker 400 MHz, chemical shift values reported in δ units (ppm) using TMS as internal standard. Follow-up of the reactions and checks of the purity of the compound was done by TLC on silica-gel-protected aluminum sheets 60 F254 (Merck), and the spots were detected by exposure to UV light at λ = 254 nm. Analytical HPLC was performed on Agilent 1100 system using Phenomenax C_{18} column (3µm, 4.6 × 50 mm) and for data processing Chemstation software was used. Buffer A: 0.1% TFA in H_2O, buffer B: 0.1% TFA in CH_3CN were used in HPLC. LCMS was performed on Shimadzu 2020 UFLC using YMC- Triart C18 (5µm, 4.6 × 150 mm) column and data processing was carried out by Lab Solution software. Buffer A: 0.1% formic acid in H_2O, buffer B: 0.1% formic acid in CH_3CN were used.

Synthesis

Synthesis of Fmoc-Amox: A solution of Fmoc-Cl (5 mmol) in 15 mL DCM was added slowly to a solution of 5.5 mmol of Amox and sodium carbonate (10 mmol) in 10 mL water with stirring at 0 °C for 30 min and then at room temperature for 20 h. After dilution with DCM, the organic phase was collected and washed with water and saturated brine solution. DCM layer was dried over $MgSO_4$, filtered and the solvent was removed to obtain pure Fmoc-Amox (80% yield).

HPLC [5 - 95 % of CH_3CN (0.1% TFA)/ H_2O (0.1 % TFA)] retention time (t_R) = 10.22 min. **^{1}H NMR (400 MHz, DMSO-d_6)** 4.45 (1H, t, J = 6.0 Hz), 4.83 (2H, d, J = 6.0 Hz), 7.36 (2H, t, J = 7.2 Hz), 7.44 (2H, t, J = 7.2 Hz), 7.70 (2H, d, J = 7.4 Hz), 7.92 (2H, t, J = 7.4 Hz), 8.24 (2H, brs NH). **^{13}C NMR (100 MHz, DMSO-d_6)** 157.4 (C), 150.5 (C), 142.7 (C), 140.7 (C), 134.5 (C), 127.9 (CH), 127.2 (CH), 124.8 (CH), 120.2 (CH), 107.5 (C), 70.8 (CH_2), 46.0 (CH)

Synthesis of Fmoc-Amino acid: A solution of Fmoc-Amox (119.4 mmol in case of Gly and 30 mmol in case of Phe and Val) in acetone was slowly added to the stirred solution of amino acid (131.34 mmol in case of Gly and 33 mmol for Phe and Val) and sodium carbonate (106 mmol in case of Glycine and 75 mmol in case of Phe and Val) in distilled water. Constantly, pH (9-10) was monitored and reaction mixture was maintained basic. Reaction was monitored by TLC till the complete consumption of Fmoc-Amox. After 4 h stirring at room temperature, the reaction mixture was concentrated under reduced pressure to remove acetone and then extracted with DCM to remove unreacted Fmoc-Amox. The reaction mixture was then acidified with 1N HCl to give off-white solid, which was filtered and washed with water several times. The filtrate was dried and recrystallized (ethyl acetate/ hexane) to afford pure Fmoc protected amino acid. The purity of Fmoc-amino acids were determined by HPLC analysis using a Phenomenax C_{18} column (3 µm, 4.6 × 50 mm) with a linear gradient over 15 min, flow rate: 1.0 mL/min, detection at 220 nm and were characterized by ^{1}H, ^{13}C, DEPT90 & DEPT135 NMR (Spectra attached in Supporting information).

Fmoc-Gly-OH: **HPLC** [5 - 95 % of CH_3CN (0.1% TFA)/ H_2O (0.1 % TFA)] retention time (t_R) = 8.69 min. **^{1}H NMR (400 MHz, DMSO-d_6)** 3.67 (2H, d, *J* = 6 Hz), 4.24 (1H, t, *J* = 6.4 Hz), 4.31 (2H, d, *J* = 7.2 Hz), 7.34 (2H, t, *J* = 7.2 Hz), 7.43 (2H, t, *J* = 7.2 Hz), 7.64 (NH, t, *J* = 6 Hz), 7.72 (2H, d, *J* = 7.2 Hz), 7.90 (2H, d, *J* = 7.2 Hz), 12.56 (COOH). **^{13}C NMR (100 MHz, DMSO-d_6)** 171.5 (C), 156.4 (C), 143.8 (C), 140.6 (C), 127.5 (CH), 127.9 (CH), 125.1 (CH), 120.0 (CH), 65.6 (CH_2), 46.5 (CH), 42.0 (CH_2).

Fmoc-Phe-OH: **HPLC** [5 - 95 % of CH_3CN (0.1% TFA)/ H_2O (0.1 % TFA)] retention time (t_R) = 10.92 min. **^{1}H NMR (400 MHz, DMSO-d_6)** 2.88 (1H, t, *J* = 11.4 Hz), 3.09 (1H, d, *J* = 13.8 Hz), 4.19 (4H, m), 7.28 (7H, m) 7.40 (2H, t, *J* = 7.2 Hz), 7.65 (2H, t, *J* = 7.4 Hz), 7.74 (NH, d, *J* = 8.3 Hz), 7.88 (2H, d, *J* = 7.5 Hz), 12.75 (COOH). **^{13}C NMR (100 MHz, DMSO-d_6)** 173.3 (C), 155.9 (C), 143.7 (C), 140.6 (C), 137.9 (C), 129.0 (CH), 128.1 (CH), 127.5 (CH), 127.0 (CH), 126.3 (CH), 125.2 (CH), 120.0 (CH), 65.5 (CH_2), 55.4 (CH), 46.5 (CH), 36.4 (CH_2).

Fmoc-Val-OH: **HPLC** [20 - 65 % of CH_3CN (0.1% TFA)/ H_2O (0.1 % TFA)] retention time (t_R) = 11.99 min. **^{1}H NMR (400 MHz, DMSO-d_6)** 0.90 (6H, d, *J* = 6.7 Hz), 2.08 (1H, m), 3.87 (1H, m), 4.25 (3H, m), 7.32 (2H, m) 7.41 (2H, t, *J* = 7.4 Hz), 7.48 (NH, d, *J* = 8.6 Hz), 7.74 (2H, m), 7.89 (2H, d, *J* = 7.5 Hz). **^{13}C NMR (100 MHz, DMSO-d_6)** 173.3 (C), 156.3 (C), 143.8 (C), 140.6 (C), 127.5 (CH), 127.0 (CH), 125.3 (CH), 120.0 (CH), 65.6 (CH_2), 59.7 (CH), 46.6 (CH), 29.7 (CH), 19.2 (CH_3), 18.0 (CH_3).

ASSOCIATED CONTENT

Electronic Supplementary Information (ESI) available: [Experimental details and HPLC chromatograms] and these materials are available free of charge via the Internet at http://pubs.acs.org."

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Author Contributions

Preparation of Fmoc-Amox was done by EH, the rest of the experimental was carried out by AK and AS. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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