Amino Acids (2014) 46:367–374
DOI 10.1007/s00726-013-1625-7

ORIGINAL ARTICLE

A mild removal of Fmoc group using sodium azide

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Received: 3 October 2013 / Accepted: 13 November 2013 / Published online: 5 December 2013

Abstract A mild method for effectively removing the fluorenylmethoxycarbonyl (Fmoc) group using sodium azide was developed. Without base, sodium azide completely deprotected N^{α}-Fmoc-amino acids in hours. The solvent-dependent conditions were carefully studied and then optimized by screening different sodium azide amounts and reaction temperatures. A variety of Fmoc-protected amino acids containing residues masked with different protecting groups were efficiently and selectively deprotected by the optimized reaction. Finally, a biologically significant hexapeptide, angiotensin IV, was successfully synthesized by solid phase peptide synthesis using the developed sodium azide method for all Fmoc removals. The base-free condition provides a complement method for Fmoc deprotection in peptide chemistry and modern organic synthesis.

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Electronic supplementary material The online version of this article (doi:10.1007/s00726-013-1625-7) contains supplementary material, which is available to authorized users.

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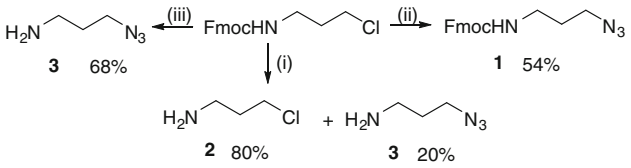
Keywords Fluorenylmethoxycarbonyl (Fmoc) deprotection · Sodium azide · Solid phase peptide synthesis (SPPS)

Introduction

Elegant organic syntheses are based on the clean formation of new bonds without undesired side reactions. However, the presence of multiple functional groups always raises the difficulty in regioselective bond formation (Green et al. 1991; Kocienski 2005). The need for appropriate protective strategies has consequently emerged in synthetic organic chemistry, particularly in carbohydrate (Guo and Ye 2010) and peptide (Isidro-Llobet et al. 2009) chemistry. Protective groups not only allow a particular functional group to persist in various given conditions, but they also regulate selectivity in chemical transformations. Easy protection and deprotection processes are crucial to facilitate the construction of chemical bonds in any order and in the presence of pre-existing bonds. Despite increasing the number of steps and decreasing overall yield in a given synthesis, protective group usage remains an imperative strategy in multistep organic synthesis (Kocienski 2005). For advanced temporary masking of functional groups, mild and orthogonal removal methods are necessary for specificity. Hence, identifying new methods of deprotection is as highly desirable as identifying new protective methods themselves.

The 9-fluorenylmethoxycarbonyl (Fmoc) group is among most widely used protecting groups in the masking of amino groups to prevent undesired reactions. Fmoc is a fitting protective group for amines in many complex natural product syntheses because of its stability under acidic, oxidative and reductive conditions (Carpino and Han 1970,

1972). Because of this impressive stability, the Fmoc protection strategy is also useful in synthesizing many other types of organic molecules, and Fmoc finds particular utility in solid phase peptide synthesis (SPPS) applications. Fmoc functionalities are generally removed by bases that are secondary amines. These secondary amines are chosen because of their adeptness at scavenging the resultant dibenzofulvene (DBF) byproduct, an ability arising from their high nucleophilicity. Liquid NH_3 (Fields 1994), pyridine, ethanolamine (Chang et al. 1980), morpholine (Liebe and Kunz 1997; Katritzky et al. 2005), diethylamine (Sureshbabu et al. 2006; Sudarshan et al. 2007), tetrabutyl ammonium fluoride (TBAF) (Ueki and Amemiya 1987), dimethylacetamide (Butwell et al. 1988) and secondary amines (piperazine or piperidine) immobilized on silica gel or polystyrene (Carpino et al. 1983a, b) have all been successfully applied in wet removals of Fmoc. Recently, dimethylsulfoxonium methylide was used for the simultaneous deprotection of *N*-Fmoc-α-amino acids in which both the amino and carboxyl group of the acids were protected (Spinella et al. 2013). Furthermore, in SPPS applications, several optimized, basic conditions—such as 20 % piperidine in DMF (Atherton et al. 1978; Mergler et al. 2003a, b; Mergler and Dick 2003; Ruczynski et al. 2008), 1–5 % DBU in DMF (Wade et al. 1991; Meldal et al. 1994; Tickler et al. 2001; Zinieris et al. 2005), morpholine-DMF (1:1) (Katritzky et al. 2005), and 25 % *N*-methylpyrrolidine in DMSO-NMP (1:1) (Li et al.)—have exhibited high performance in the removal of Fmoc groups from amino acids attached to resins. The use of base in Fmoc deprotections, however, limits substrate accessibility. Molecules containing base-labile groups are adversely affected by the basic conditions, and the undesired consequences include racemization and byproduct formation. For example, in peptide synthesis, diketopiperazine and aspartimide formation (Mergler et al. 2003a, b; Mergler and Dick 2003; Ruczynski et al. 2008; Bodanszky 1984) along with the racemization of esterified amino acids (Goodman and Stueben 1962) have been reported as side reactions when base was used for Fmoc deprotections. Moreover, base-catalyzed cyclization of resin-bound peptides to diketopiperazines is particularly common in syntheses containing Pro, Gly, D-amino acid, or *N*-methyl amino acid residues (Mazurov et al. 1993). Consequently, methods for Fmoc removal under mild and neutral conditions are highly desirable. Only a few methods have been reported to remove the Fmoc groups under base-free conditions. Maegawa and coworkers used Pd/C for Fmoc deprotection under neutral hydrogenation conditions (Maegawa et al. 2009). Recently, a thermolytic method was reported and used without base or scavengers (Hock et al. 2010). In addition, in an unusual Lewis acid-mediated deprotection, aluminum trichloride exhibited good



Scheme 1 **a** Reaction conditions *(i)* NaN_3 (1.2 equiv.) DMF, r.t. *(ii)* NaN_3 (1.2 equiv.), PrOH/H_2O, 90 °C *(iii)* NaN_3 (5 equiv.) DMF, 90 °C

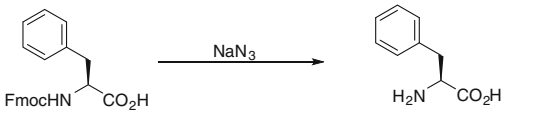
performance in deprotecting N^{α}-Fmoc-isoleucine and N^{α}-Fmoc-alanine (Leggio et al. 2000). However, the harsh conditions or highly reactive reagents of these base-free strategies may limit their applications. A mild and efficient Fmoc deprotection method with broad substrate applicability would remarkably increase the usefulness of the Fmoc protecting group in modern organic synthesis.

In exploring preparative methods for the Fmoc-protected 3-azidopropylcarbamate **1**, the desired substitution compound was obtained in 54 % yield by the treatment of Fmoc-protected 3-chloropropan-1-amine with sodium azide (NaN_3) in a propanol/water (1:1) co-solvent system (Scheme 1). Surprisingly, when DMF was used, NaN_3 drove the cleavage of the Fmoc group, forming the amino compound **2** (80 %) bearing the original chloro-group and the minor azide compound **3** (20 %). Using NaN_3 from different sources [including Acros (99 %, extra pure) and Aldrich (ReagentPlus®, ≧99.5 %)] afforded similar results. Preliminary observation suggests depending upon the solvent choice, NaN_3 can act as a nucleophile to yield the corresponding azide product by substitution, or act as a deprotection agent to produce the corresponding amine. Polar protic solvents, such as propanol and water, solvate the sodium and azide ions well. The energy provided by heating disrupts the tightly bound azide solvent cage, allowing the nucleophilic substitution to occur. On the other hand, the polar aprotic solvent DMF solvates the sodium ion well but leaves the azide ion free for reaction. The primary chloride of the starting material is susceptible to S_N2 reactions. This reaction, however, needs a strong nucleophile to occur, and the azide ion possesses only weak nucleophilicity. Because of the release of CO_2 during the elimination, the azide ion preferentially abstracts the proton (9-H) of the fluorenyl group and thereby removes the Fmoc group. Nevertheless, in the heating condition at 90 °C, the pure amino compound **3** carrying the azide group was obtained in 68 % yield by treatment with NaN_3 (5 equiv.) in DMF. Encouraged by this result, a new NaN_3- mediated Fmoc deprotection method was carefully developed in this study of amino acid chemistry and SPPS. The mild and near-neutral conditions employed will be highly attractive to organic chemists for the construction of many different organic molecules.

While rationalizing the removal mechanism of Fmoc group, apparently the treatment of N^{α}-Fmoc-amino acid with NaN_3 in DMF produces the free amino acid carrying negative charge on the nitrogen, accompanying with the release of CO_2, hydrazoic acid (HN_3), and DBF. The resultant HN_3 could act as a proton source to generate the desired free amino acid. Therefore, the toxic HN_3 byproduct would soon be consumed in the reaction and regenerated as NaN_3. For achieving successful deprotection in the NaN_3 method without the need of DBF scavenger, the acidic quenching conditions were important to prevent the formation of DBF adduct. Little or no distinguishable DBF adduct was observed during the reaction monitoring; however, considerable amounts of DBF adducts were present after the subsequent water quench and concentration process in reduced pressure. To prevent the formation of undesired DBF adduct, 10 % HCl solution was used to prevent the side reaction by completely protonating the amino acids which was added to the reaction mixture after the completion of the reaction. Subsequently, the undesired DBF was removed by ethyl acetate extraction and left the protonated amino acids in the aqueous solution. The collected aqueous solution was then basified to neutral to give the native amino acids and quench the hazardous HN_3 formed during the work-up process. A screening of reaction conditions such as reaction temperature, time, the amount of NaN_3 and solvents was carefully conducted using N^{α}-Fmoc-phenylalanine (Fmoc-Phe-OH) (Table 1).

Table 1 summarizes the optimization of the Fmoc deprotection of Fmoc-Phe-OH using NaN_3. Initially, the attempts were made at ambient temperature (entries 1–7) using different additives: 18-crown-6 and 15-crown-5 as cation scavengers; DMSO and 10 % H_2O in DMF to increase the desired NaN_3 solvation properties; and TBAI as a phase transfer catalyst. None of these additives gave the expected results even after prolonged reaction time at room temperature—only trace amount of product formation was observed by TLC. However, when the reaction temperature was increased to 50 °C and DMF was used as the solvent, the Fmoc group was completely removed in 1 h. The use of 1.2 equiv. of NaN_3 was found to be sufficient to drive the reaction into completion. According to the proposed mechanism, the use of NaN_3 in this reaction should be in the catalytic manner. The use of 0.6 equiv. of NaN_3 in DMF at 50 °C did not drive the reaction into completion in 1 h (entry 9); nevertheless, extending the reaction time to 24 h allowed a complete conversion. Unfortunately, a considerable increase of DBF adduct was observed. Hence, the use of slightly more than one equivalent of NaN_3 was found to effectively deprotect the Fmoc group of Fmoc-Phe-OH in short time while avoiding DBF adduct formation. Because the DMF is known to potentially degrade to dimethylamine, a control reaction was performed by heating of the Fmoc-protected amino acid in DMF at 50 °C. No new product was observed after reacting for 24 h and therefore excluded the participation of dimethylamine in the reaction (entry 10). Less polar solvents (THF, ACN, and 1,4-dioxane), nonpolar solvents (toluene and hexane) and polar protic solvents (H_2O and MeOH) also afforded no desired deprotection product (entries 11–17). In conclusion, treatment with NaN_3 (1.2 equiv.) in DMF at 50 °C can effectively transform Fmoc-Phe-OH to the desired free phenylalanine in 1 h. Due to the base-free reaction condition, the consistency of chirality of deprotected amino acids was reasonably expected. To examine this stereochemical integrity, the compound **5a** and the methyl ester of DL phenylalanine (DL-Phe-OMe; purchased from AK Science, Inc.) were quantitatively analyzed by chiral HPLC. Considering the solubility in organic mobile phase, the DL phenylalanine methyl ester was treated with acetic anhydride in the presence of

Table 1 Optimization of Fmoc deprotection of Fmoc-Phe-OH using NaN_3



Fmoc-Phe-OH L-phenylalanine **(4)**

Entry	NaN_3 (equiv.)	Additives (equiv.)	Solvent	Temp (°C)	Time (h)	Yield (%)
1	1.2	18-crown-6 (0.1)	DMF	rt	24	Trace
2	1.2	15-crown-5 (0.1)	DMF	rt	24	Trace
3	1.2	DMSO (cat.)	DMF	rt	24	Trace
4	1.2	Iodine (1.0)	DMF	rt	24	Trace
5	1.2	–	10 % H_2O in DMF	rt	24	Trace
6	1.2	TBAI (1.0)	DMF	rt	24	Trace
7	1.2	–	DMF	rt	24	Trace
8	1.2	–	DMF	50	1	95
9	0.6	–	DMF	50	1	45
10	0	–	DMF	50	24	NR
11	1.2	–	THF	50	24	NR
12	1.2	–	MeOH	50	24	NR
13	1.2	–	Toluene	50	24	NR
14	1.2	–	Hexane	50	24	NR
15	1.2	–	1,4-dioxane	50	24	NR
16	1.2	–	ACN	50	24	NR
17	1.2	–	H_20	50	24	NR

NR no reaction

triethylamine to afford Ac-DL-Phe-OMe which allowed the analysis in normal-phase HPLC. Similarly, compound **5a** (in Table 2) obtained by NaN_3-mediated Fmoc deprotection was also acetylated to get Ac-L-Phe-OMe. Two peaks were observed in Fig. 1b which clearly define the retention time of two enantiomers, Ac-D-Phe-OMe and Ac-L-Phe-OMe. Remarkably, the single peak of acetylated **5a** (Ac-L-Phe-OMe) was obtained to strongly support the chiral integrity without undesired racemization (Fig. 1c). Therefore, the treatment of NaN_3 induced no racemization in the Fmoc deprotection process of amino acids.

Table 2 Screening of Fmoc deprotection on standard amino acids and esters

Entry	Substrate	Product	Time (h)	Yield (%)
1	Fmoc-Phe-OMe	5a	3	97
2	Fmoc-lle-OMe	5b	3	87
3	Fmoc-Val-OMe	5c	4	94
4	Fmoc-Leu-OMe	5d	3	99
5	Fmoc-Ala-OBu	5e	3	91
6	Fmoc-Gly-OBu	5f	3	93
7	Fmoc-Thr-OBu	5g	4	90
8	Fmoc-Ser-OBu	5h	3.5	94
9	Fmoc-Asp-OH	5i	24	90
10	Fmoc-Asn-OH	5j	20	90
11	Fmoc-Gln-OH	5k	24	97
12	Fmoc-Arg-OH	5l	3	95
13	Fmoc-Lys-OH	5m	4	90
14	Fmoc-Met-OH	5n	16	95
15	Fmoc-Pro-OH	5o	3	92

Because the Fmoc protecting group is popularly used in solid-phase and solution-phase peptide synthesis, the developed method was used in a series of common Fmoc-protected amino acids (products **5a**–**5o**, Table 2). Amino acids in entries 1–8 are protected as the ester forms at their C terminus. Notably, the treatment of NaN_3 showed no side reaction from the possible azide substitution. After the completion of the reaction monitoring by TLC, different substrate-dependent work-up methods were used to purify the products. For example, a conventional acid–base work-up was employed for the ester analogs to remove the side product (i.e., DBF) formed during the process. In general, DBF is highly electrophilic and induces addition reactions with nucleophiles, particularly with free amines. In these cases, however, such side reactions were not observed; therefore, the need for scavengers to trap DBF was obviated. Addition of dilute hydrochloric acid solution (10 % HCl solution) protonated the amines and brought the product into the aqueous phase. Residual DBF was then separated from the aqueous layer in ethyl acetate. Finally, the product-containing aqueous phase was basified with $NaHCO_{3\ (aq)}$, and the product was then extracted into ethyl acetate, affording the deprotected amines. To the free C-terminal amino acids (**5i**–**5o**), reverse phase silica gel column chromatography was necessary to rid the free amino acids of the inorganic salts (e.g., sodium chloride and excess NaN_3). During the work-up process, the free amino acid was extracted into the aqueous phase and thereby separated from the side product DBF in organic solvent. The amino acids of interest were then obtained by the neutralization and concentration of the aqueous phase followed by reverse phase silica gel column

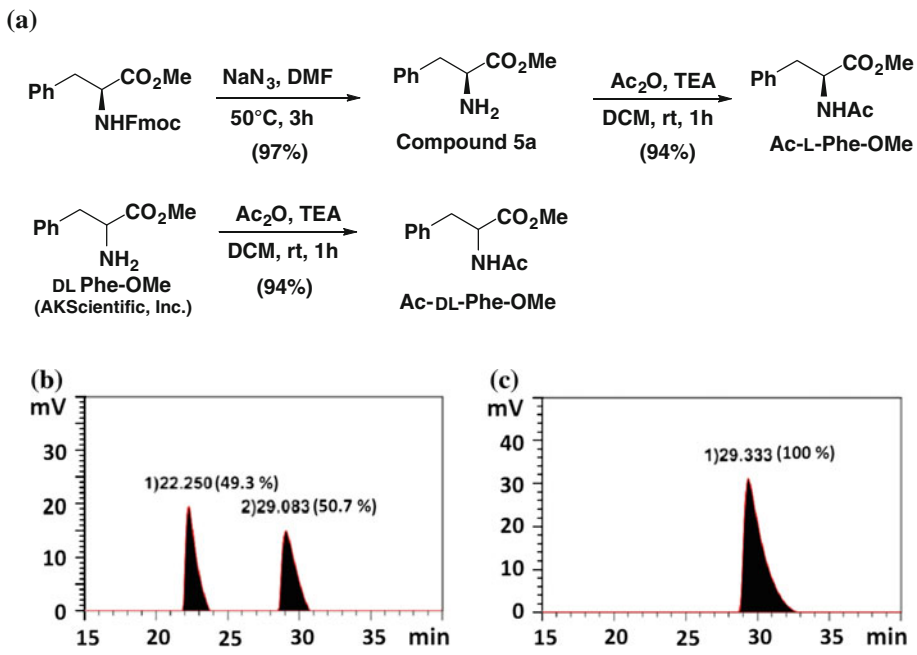


Fig. 1 **a** Preparation ofAc-L-Phe-OMe and Ac-DL-Phe-OMe, Chiral HPLC data for **b** Ac-DL-Phe-OMe and **c** Ac-L-Phe-OMe (CHIRALPAKAD-H, 22 × 0.5 cm)

chromatography using water as the mobile phase afforded the desired products. The common N^{α}-Fmoc-amino acids in Table 2 were efficiently deprotected by NaN_3 in 3–24 h for complete conversion and with yields of 87–99 %.

After reasonable results were obtained in the Fmoc deprotection of simple amino acids and their ester analogs, we evaluated the extendibility of this methodology to systems containing different functionalities. Table 3 lists cases in which the Fmoc-protected amino acids contain functional groups such as trityl (**6a–6e**), acetyl group (**6f**) and *tert*-butyl ethers (**6g and 6h**). The trityl and *tert*-butyl protecting groups are generally used in amino acid chemistry to mask non-amino nucleophilic centers, such as -OH and -SH, and can be easily removed under mild acidic conditions. As expected, these functional groups were unaffected by the reaction conditions. Because of the presence of these bulky functional groups, these substrates are relatively less polar than those described in Table 2. After Fmoc removal using the optimized conditions, the resultant amino acids were extracted into the organic phase using a conventional work-up, and then the DBF was removed using silica gel column chromatography to afford the desired products in high yields. Similarly, the base-labile acetyl group in the Fmoc-protected threonine (**6f**) showed high compatibility with the developed NaN_3 deprotection method. The *Pbf* group in the Fmoc-protected arginine survived the reaction conditions (**6i**). *Pbf* (Carpino et al. 1993), which can be removed by TFA scavengers, is the best Arg protecting group for the Fmoc/tBu solid-phase strategy. Furthermore, the *tert*-butyl and allyl groups (**6j**, **6k and 6l**) in Fmoc-protected aspartic acid and glutamic acid successfully resisted the ester hydrolysis and obtained good yields of 97, 92 and 94 %. Remarkably, the *tert*-butyl ester groups of Fmoc-Asp(O^{t}Bu)-OH (entry 10) and Fmoc-

Table 3 Fmoc deprotection of various amino acids in the presence of other labile groups

Entry	Substrate	Product	Time (h)	Yield (%)
1	Fmoc-Gln(Trt)-OH	6a	9	90
2	Fmoc-Asn(Trt)-OH	6b	12	95
3	Fmoc-Lys(Mtt)-OH	6c	24	85
4	Fmoc-His(Trt)-OH	6d	10	91
5	Fmoc-Cys(Trt)-OH	6e	9	82
6	Fmoc-Thr(Ac)-OH	6f	3	94
7	Fmoc-Thr(^{t}Bu)-OH	6g	12	97
8	Fmoo-Tyr(^{t}Bu)-OH	6h	5	98
9	Fmoc-Arg(Pbf)-OH	6i	6	92
10	Fmoc-Asp(O^{t}Bu)-OH	6j	4	97
11	Fmoc-Gluf(O^{t}Bu)-OH	6k	4	92
12	Fmoc-Asp(OAII)-OH	6l	3	94
13	Fmoc-Ser(TBS)-OEt	6m	4	91
14	Fmoc-Lys(TCA)-OEt	6n	3	90

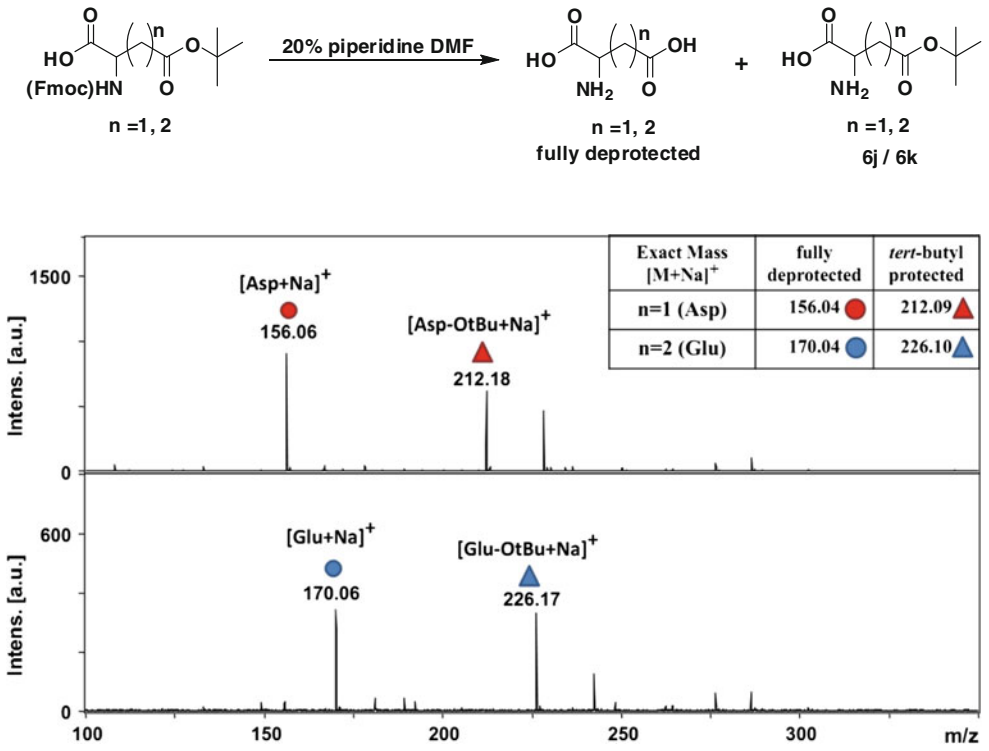
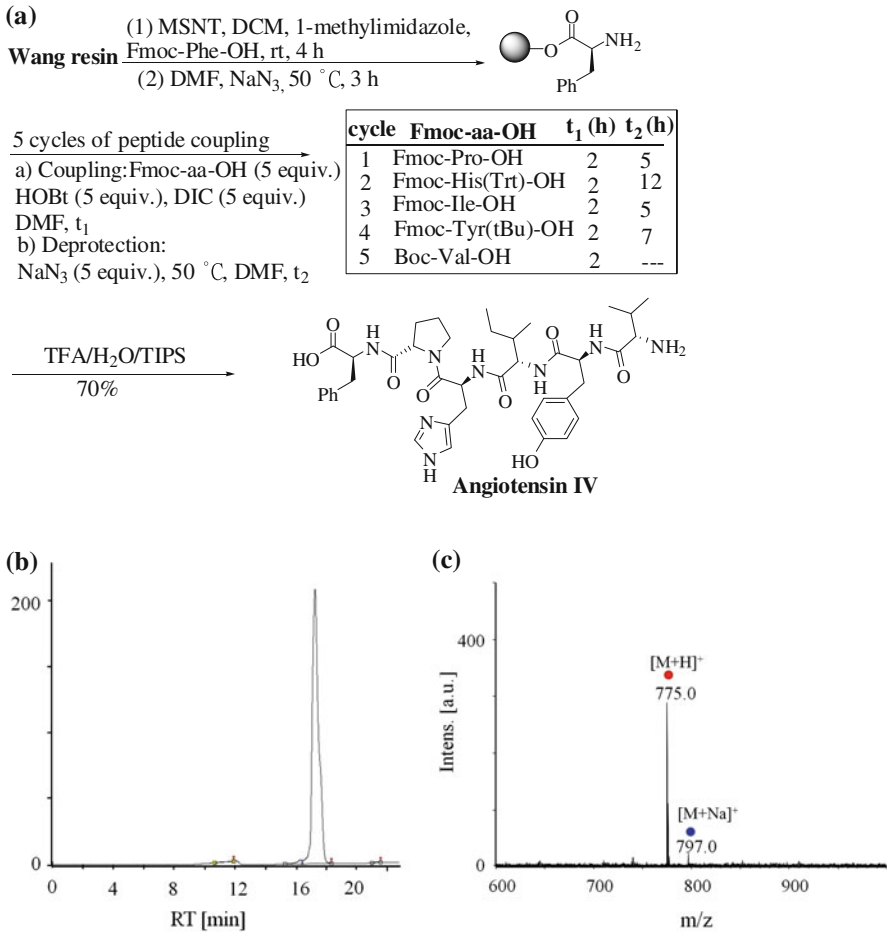


Fig. 2 Observed side products (fully deprotected and *tert*-butyl protected) in the cases of Fmoc-Glu(O^{t}Bu)-OH and Fmoc-Asp(O^{t}Bu)-OH during Fmoc cleavage using 20 % piperidine in DMF



cycle	Fmoc-aa-OH	t_1 (h)	t_2 (h)
1	Fmoc-Pro-OH	2	5
2	Fmoc-His(Trt)-OH	2	12
3	Fmoc-Ile-OH	2	5
4	Fmoc-Tyr(tBu)-OH	2	7
5	Boc-Val-OH	2	---

Scheme 2 **a** solid phase synthesis of angiotensin IV; Characterization of obtained Angiotensin IV by **b** HPLC chromatogram and **c** MALDI-MS of synthesized angiotensin IV. The *red symbol* indicates the proton adduct of Angiotensin IV and the *blue symbol* points its sodium adduct (color figure online)

Glu(O^{t}Bu)-OH (entry 11) were partially removed by treating with 20 % piperidine solution, and then identified by mass analysis (Chen et al. 2013). In Fig. 2, the circle marked peaks indicated the fully deprotected aspartic acid and glutamic acid which lost the *tert*-butyl groups in the presence of 20 % piperidine. The popular silyl protecting group in the case of Fmoc-Ser(TBS)-OEt (**6m**) and the very sensitive base-labile trichloro acetyl (TCA) group in the case of Fmoc-Lys(TCA)-OEt (**6n**) were also tolerated in the treatment of NaN_3.

The effective removal of Fmoc groups in the presence of other labile groups assured the utility of our method in complex, multistep syntheses involving many functional groups. The deprotection protocol can be used not only for amino acids but also for simple aromatic and aliphatic amines. The developed deprotection conditions were tested on substituted anilines bearing halogen and other functional groups in different positions on the benzene ring. As expected, the results showed that the inductive and resonance effects of the substituent groups have no obvious impacts on the Fmoc deprotection. The deprotection reaction proceeded faster than that with the amino acids, and the yields were high.

To demonstrate the applicability of this developed NaN_3-mediated Fmoc deprotection in SPPS, a hexapeptide, angiotensin IV, which is known to be closely associated with acquisition, consolidation and recall of learning and memory in animal models (Albiston et al. 2011; Gard 2008), was synthesized. The sequence of angiotensin IV contains six different amino acids carrying aliphatic, aromatic and cyclic side chains which is in the sequence of VYIHPF. As illustrated in Scheme 2, the synthesis of angiotensin IV was started by treating Wang resin with Fmoc-Phe-OH in the presence of 1-(2-Mesitylenesulfonyl)-3-nitro-1H-1,2,4-triazole (MSNT) and 1-methylimidazole. A Bromophenol blue (BPB) test was used to check reaction progress until the coupling was completed in 4 h. The resultant resin-attached N^{α}-Fmoc-Phe was then treated with NaN_3 in DMF at 50 °C for 3 h to expose the amino terminus for subsequent coupling. To complete the SPPS, the coupling and deprotection steps were repeated an additional five times with Fmoc-Pro-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Tyr(^{t}Bu)-OH, and Boc-Val-OH. Reaction times are listed in the inserted table of Scheme 2a. Subsequently, the crude angiotensin IV mixture was collected by treating with the cleavage solution

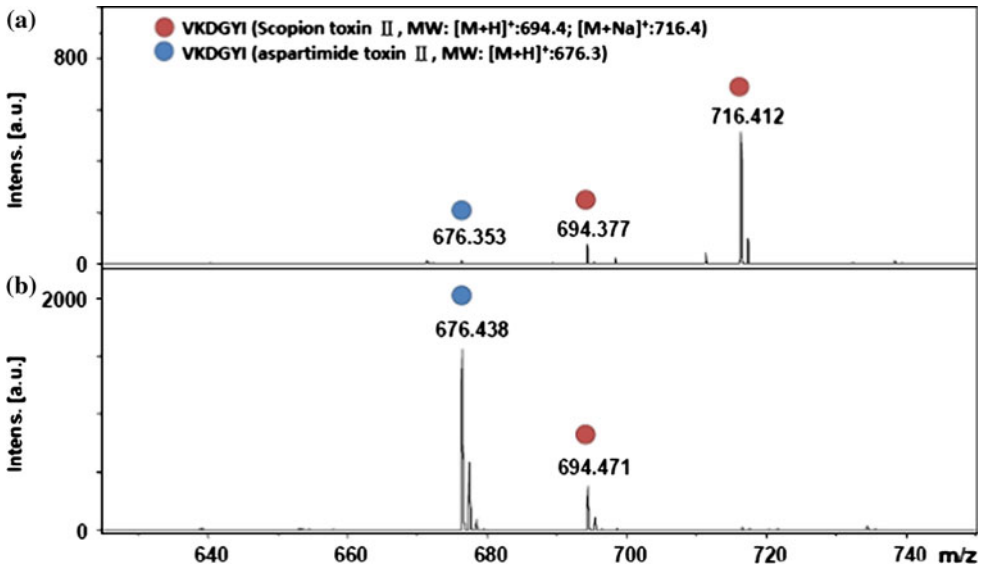


Fig. 3 MALDI-MS of obtained peptides scorpion toxin II **(a)** using the developed NaN_3 method and **(b)** deprotected by conventionally used 1 % DBU condition

[trifluoroacetic acid (TFA)/water/triisopropylsilane(TIPS)]. The product was further purified by reverse phase high performance liquid chromatography (RP-HPLC), affording angiotensin IV in 70 % yield. The purity of the obtained hexapeptide was evaluated by RP-HPLC, which showed a single peak (Scheme 2b). The identification was further assessed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) (Scheme 2c), which indicated molecular weights corresponding to the proton and sodium adducts.

To further demonstrate the importance of this mild deprotection method in preventing the formation of aspartimide formation during SPPS, a hexapeptide scorpion toxin II (VKDGYI) which is known to be a subject in the formation of aspartimide in the basic condition (Mergler et al. 2003a, b; Mergler and Dick 2003) has been prepared. To a similar procedure in the preparation of angiotensin IV, two different Fmoc deprotection methods, NaN_3 and DBU/piperidine, were processed individually. After precipitating with cold ether, the obtained peptides were identified by MALDI MS. In Fig. 3, the peaks marked in red indicated the desired scorpion toxin II and the blue marked peaks were the undesired aspartimide byproducts. Only a very weak peak of aspartimide byproduct can be observed using the developed NaN_3-mediated Fmoc deprotection. On the contrary, the formation of undesired aspartimide byproduct was dramatically increased under the basic deprotection condition (DBU/piperidine/DMF = 1/20/79) which is consistent with the reported result (Mergler and Dick 2003).

In conclusion, NaN_3 in DMF was found to effectively remove the Fmoc group of various N^{α}-Fmoc-amino acids without adding base or auxiliaries. Compared to the conventional basic condition, the neutral condition prevents the undesired racemization, aspartimide formation and accommodates a wide variety of protecting and functional groups. Depending on the chosen solvent, azide ions can function as bases or nucleophiles and thereby selectively participate in elimination or substitution reactions. The cheap and easily handled NaN_3 salt makes the developed deprotection a feasible method for a variety of organic syntheses. However, the potentially explosive property of NaN_3 should be carefully operated, especially for the large-scale synthesis. According to the optimized condition, Fmoc group was successfully removed from amino acids containing various protected side chains without affecting their acid or base-labile protecting groups. Remarkably, we provide an orthogonal method to allow the presence of pH-sensitive groups in the removal of Fmoc groups. Finally, two biologically significant hexapeptides, angiotensin IV and scorpion toxin II, were synthesized by SPPS with the use of NaN_3 without the formation of aspartimides. This work constitutes a mild strategy in protecting group chemistry and provides a new Fmoc removal method in SPPS.

Acknowledgments This work is supported by Grants from National Science Council (NSC 101-2113-M-110-007- MY2) and National Sun Yat-sen University (01C030703 and 01A06802). Authors thank Prof. Shiue-Shien Weng's help in chiral HPLC experiments. Authors thank Prof. Chi-Wi Ong's help in the preparation of manuscript.

Conflict of interest The authors declare no conflict of interest. The authors alone are responsible for the content and writing of this manuscript.

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