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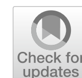
SHORT COMMUNICATION



The tea-bag protocol for comparison of Fmoc removal reagents in solid-phase peptide synthesis

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Abstract

Several factors have influenced the increasing presence of peptides as an important class of Active Pharmaceutical Ingredients. One is the continued development of synthetic methodologies for peptide synthesis. Herein, we investigated the Fmoc removal step, using the tea-bag strategy. In this regard, three different secondary amines: piperidine, 4-methylpiperidine, and piperazine, were evaluated. As a result of this study, 4-methyl piperidine showed to be an excellent alternative to the usually used piperidine in terms of purity and compliance with green chemistry principles as well.

Keywords Piperidine · 4-Methylpiperidine · Piperazine · Parallel synthesis · Green chemistry · Tea-bag protocol · Simultaneous Fmoc synthesis

Introduction

Nowadays, peptides are considered an important class of Active Pharmaceutical Ingredients (APIs) (de la Torre and Albericio 2019). There is not a doubt that this has been in part possible due to the incorporation of the solid-phase peptide synthesis (SPPS) methodology to peptide research and peptide production processes (Merrifield 1963). Although several SPPS strategies are described, the so-called fluorenylmethoxycarbonyl (Fmoc)/*tert*-butyl (tBu) is the strategy of choice based on its performance and simplicity. Basically, the elongation of a peptide by SPPS involves two steps: coupling of the Fmoc-protected amino acid and removal of the Fmoc group after coupling. The widespread acceptance of the Fmoc/tBu/SPPS strategy for all peptide-based drug discovery stakeholders has been possible because of the great performance achieved in peptide synthesis through the continuous methodological work of several groups. In this regard, piperidine related amines were evaluated, selecting the best one for the removal of the Fmoc group. Classically, piperidine in *N,N*-dimethylformamide (DMF) has been used. However, piperidine is a controlled substance to be used as an intermediate for

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the synthesis of narcotic drugs, thus jeopardizing its use in several countries (International Narcotics Control Board for 2014); therefore, its replacement is of practical interest. This study was performed using the "tea-bag" strategy, first described by Houghten et al. (Houghten 1985) for the simultaneous synthesis of peptides. This strategy allows each peptide to be synthesized independently in a "tea bag" compartment where coupling and washing steps are conducted separately while deprotection is carried out using a common solution in a polyethylene bottle. The final global deprotection and cleavage are carried out independently for each peptide. Thus, the "tea-bag" strategy is especially suitable for methodological studies where different reagents, solvents, or protocols are going to be investigated, because they use fully comparable reaction conditions while being also very convenient for running replicates for each condition studied. Additionally, this strategy significantly reduces reagent's usage and waste generation without affecting the quality of the product.

Four small to medium-sized peptide sequences were synthesized, corresponding to the same sequences reported in previous work, namely: NBC112: FISEAIIHVLHSR (Prieto et al. 1995; Santana et al. 2013) NBC155: TLEEFSAKL (Díez et al. 2006; Díez et al. 2007) NBC759: KKWRWWLKALAKK (Murillo et al. 2007) and NBC1951: VAPIAKYLATALAKWALKQGFAKLKS (Segura et al. 2007), using three Fmoc removal reagents: 4-methylpiperidine (4MP), piperidine (PP), and piperazine (PZ).

Methods

Peptide synthesis and characterization

Peptide synthesis was performed according to the Fmoc/tBu standard strategy (Carpino and Han 1970; Wade et al. 2000) using a "tea-bag" protocol (Houghten 1985) adapted and optimized in our laboratory (Carvajal-Rondanelli et al. 2018) (Figure S1). Three Rink Amide resin-filled polypropylene bags per peptide (4) were used with each deprotection reagent (3), rendering a total of 36 parallel syntheses (Table S1).

Removal step of Fmoc group was carried out with either: 20% v/v 4MP in DMF; 20% v/v PP in DMF, or 10% w/v PZ in 9:1 N-methyl-2-pyrrolidone(NMP)/ethanol.

Coupling was performed with 5:5:5:10 equivalents of protected amino acid:activator:Oxyma-Pure®: diisopropylethylamine (DIPEA), and using *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU) and *N*-[(1*H*-6-chlorobenzotriazol-1-yl) (dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HCTU) activators for single and double coupling, respectively. Couplings were monitored using bromophenol blue. Cleavage step was performed with trifluoroacetic acid (TFA) using triisopropylsilane (TIS), and 2,2′-(ethylenedioxy) diethanethiol (DOT) as scavengers, in the proportion TFA/TIS/Water of 95:2.5:2.5, and TFA/TIS/Water/DOT 92.5:2.5:2.5:2.5 to prevent tryptophan oxidation in peptides NBC759 and NBC1951.

Peptides were characterized by high-performance liquid chromatography (HPLC) in a JASCO system (JASCO Corp., Tokyo, Japan) and molecular mass of the peptides was determined by electrospray-mass spectrometry (ESI–MS) in a LCMS-2020 ESI–MS equipment (Shimadzu Corp., Kyoto, Japan).

Purification and quantification

Peptides were purified using preparative Clean-Up® CEC18153 C-18 columns (UCT. Bristol. PA. USA) and eluted with an acetonitrile/water gradient from 10 to 60% (v/v). Fractions were analyzed by HPLC and ESI–MS, to determine the main fraction containing the expected peptide.

A calibration curve was constructed by preparing 50 µL of 0.125, 0.25, 0.5, 0.75, 1 and 1.5 µg/µL peptide solutions in milliQ water. Data analysis was performed using the ChromNAV Chromatography Data System v 2.02.05 Build 4. Crude peptide yield is calculated according to Eqs. 1 and 2.

$$\text{mg Theoretical Yield} = \text{g resin} \times \text{resin substitution} \times \text{peptide molecular weigth} \quad (1)$$

$$\%\text{Crude Yield} = \frac{\text{mg peptide obtained}}{\text{theoretical yield}} \times 100 \quad (2)$$

Statistical analysis

Graphics and statistical calculations were performed by the Graphpad Prism v6.1 for Windows. (GraphPad software. San Diego. CA. USA). Yield and purity results for each deprotecting reagent were expressed as mean plus standard deviation and analyzed by two-way ANOVA. Results showing significant differences were analyzed by Tukey´s multiple comparison test. Significant differences were determined at $p<0.0001$, $p<0.001$, $p<0.01$, and $p<0.05$.

Results

Yield and purity of synthesized peptides

After peptide synthesis, the product in each individual bag was weighed for the determination of peptide crude yield in

relation to the theoretical value (Eqs. 1 and 2 in Methods). Purity was obtained from the calibration curve performed with purified peptides as described in Methods, and a new parameter, peptide-specific yield, was calculated by considering purity in the crude product mass (Eq. 3).

$$\%\text{Peptide - specific Yield} = \frac{\text{purity} \times \text{mg peptide obtained}}{\text{theorteical yield}} \times 100 \quad (3)$$

As can be seen in Table 1 and Fig. 1, the use of the different deprotection reagents has a slight influence in purity. Two of the peptides, NBC759 and NBC1951, were not affected by the deprotection strategy; however, purity was markedly different for peptide NBC155 and peptide NBC112, peptide-specific yield being significantly increased using 4MP.

HPLC and mass spectrometry

Results of HPLC and mass spectrometry for crude synthetic peptides are summarized in Figure S2 and Table S2 of Supplementary Material. As observed, the chromatograms and mass spectra for the three deprotection strategies are superimposable. The HPLC data of the crude product showed a main peak that corresponds to the peptide, and some minor peaks that correspond to impurities. According to mass spectra, some of them correspond to atomic or molecular ions such as sodium or formic salt, others correspond to amino acid deletions. The results are summarized in Table S3.

Discussion

Using the tea-bag strategy, the simultaneous synthesis of 36 peptides (4 different sequences) was performed to determine the best piperidine derivative for the Fmoc removal stage in SPPS.

Table 1 Crude peptide yield, purity and peptide-specific yield obtained using 4-methylpiperidine (4MP), piperidine (PP) and piperazine (PZ) as deprotection reagents

Peptide	Total crude yield (mg), [%][a]			Purity %			Peptide-specific yield %		
	4MP	PP	PZ	4MP	PP	PZ	4MP	PP	PZ
NBC112	24.5 [67]	11.9**** [32.8]	14.5**** [39.6]	24.4 ± 0.8	23.9 ± 9.6	25.2 ± 5.4	16.4 ± 0.4	8.0* ± 4.0	10.0 ± 2.3
NBC155	23.9 [95.9]	15.3**** [61.4]	15.5**** [62.1]	65.1 ± 4.5	51.3* ± 3.7	51.4* ± 9.4	62.4 ± 4.6	31.4**** ± 2.0	31.8**** ± 5.2
NBC759	22.2 [53.1]	24.1 [57.7]	22.6 [54]	64.4 ± 5.4	62.1 ± 3.1	62.8 ± 2.8	34.2 ± 3.0	36.0 ± 6.6	33.9 ± 2.6
NBC1951	30.1 [45]	30.3 [45.3]	29.71 [44.4]	56.1 ± 2.2	62.2 ± 5.5	63.9 ± 7.4	25.2 ± 1.2	28.1 ± 2.3	28.3 ± 2.6

Each value is the average of the three tea bags used in synthesis. Statistical analysis was made by the Tukey multiple comparison test, significant differences indicated in bold as follows: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$

[a]Values are in mg and percentage in square brackets

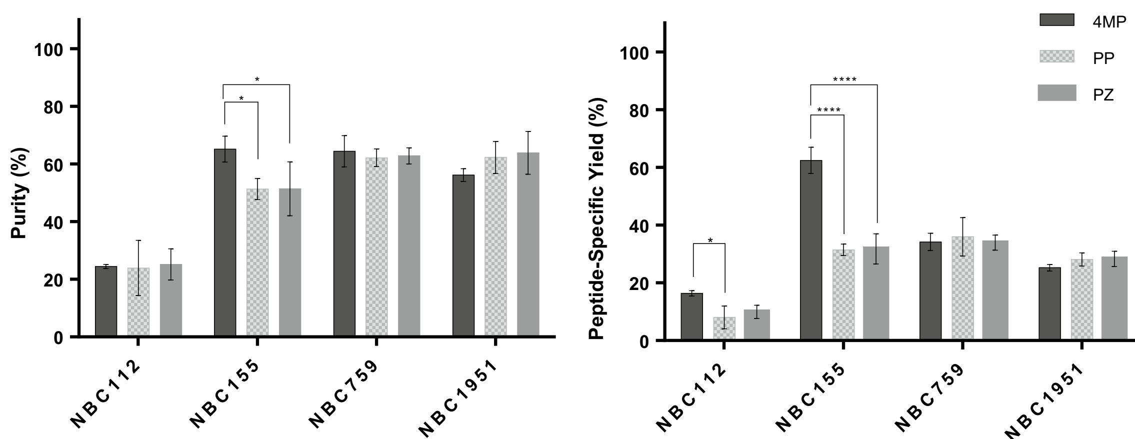

Fig. 1 Purity and peptide-specific yield (%) for the synthesized peptides obtained with the three different deprotection reagents. The values were obtained by HPLC quantification with a calibration curve. Percentages values were calculated from theoretical yield and the obtained peptide-specific yield. Each bar represents the average of the three bags for each peptide and their respective standard deviation. Significant differences are shown as ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$

For two peptides (NBC759 and NBC1951), which in our experience are relatively straightforward or easy sequences, no differences in yield were obtained among the three deprotection reagents. For NBC759, 22 mg of crude product was obtained with a crude yield of almost 53%, a purity above 75%, and a peptide-specific yield above 40%. In the case of NBC1951, the longest peptide (26 residues), 30 mg were obtained with a yield of 44–45%, a purity above 70% and a peptide-specific yield between 32 and 36%. The other two sequences (NBC 155 and NBC 112) presented different and more complex features. Peptide NBC155 is also considered an easy sequence to synthesize, but it is a short 9-residues peptide, which influences its yield. In this case, the use of 4MP significantly improved the process, allowing to obtain 23 mg of the crude peptide with a yield and purity above 60%. Peptide NBC112 is a short 13-residue sequence, but it is a difficult sequence to be synthesize. In this case, 4MP presented significant differences with piperazine only in the peptide-yield.

As can be seen, in Figure S2, when superimposing HPLC data for each peptide and analyzing such data, the Fmoc removal reagent used does not seem to affect the purity of the crude peptide product, so it is unnecessary to consider this factor in the choice of the reagent. A mass spectrometry analysis revealed a deletion of the last coupled amino acid, threonine, in the peptide NBC155, which is present in the crude product regardless of the deprotection strategy employed. This deletion could be related to the coupling step, which is highly dependent on the sequence and the amino acid.

In our previous work (Luna et al. 2016), the results obtained by microwave-assisted synthesis showed that deprotection reagents can be used interchangeably; in the present case, the synthesis was performed individually for each sequence and, in addition, the use of high temperatures can mask the differences in the effect of the different deprotection reagents. The use of the tea-bag protocol showed only slight differences in purity for NBC155 and NBC1951 when using 4MP. In this report differences between deprotection reagents are more evident than in our previous work, because all the peptides were synthesized simultaneously under the same coupling and deprotection conditions, with a greater influence of the hydrophobic character of the peptide, because the homogenizing effects of high temperatures are absent in this study.

Surprisingly, the yield of two of the peptides showed significant differences in the synthesis in which 4MP was used. Although not fully understood, this supports the use of 4-MP as a deprotection reagent.

Conclusions

The tea-bag protocol proved to be the strategy of choice for this kind of methodological work because it allowed to perform several studies simultaneously using different reagents and peptides, as well as allowing the repetitive synthesis of the same sequence. As a result of this study, 4-methyl piperidine showed to be an excellent alternative to the usually used piperidine in terms of purity and compliance with green chemistry principles.

Furthermore, 4MP does not have the status of restricted substance as is the case of PP (International Narcotics Control Board for 2014).

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical standards This study did not involve research with humans or animals, nor biological samples. Informed consent was not required.

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