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Concise preparation of N^{α}-Fmoc-N^{ε}-(Boc, methyl)-lysine and its application in the synthesis of site-specifically lysine monomethylated peptide

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Summary. A concise preparation of N^{α}-Fmoc-N^{ε}-(Boc, methyl)-lysine and its application in the synthesis of site-specifically lysine monomethylated peptide is described. N^{α}-Fmoc-N^{ε}-(Boc, methyl)-lysine is obtained, via consecutive reductive benzylation and reductive methylation in a one-pot reaction, followed by debenzylation through catalytic hydrogenolysis and Boc protection in another one-pot reaction. A peptide containing monomethylated lysine is successfully synthesized by incorporating N^{α}-Fmoc-N^{ε}-(Boc, methyl)-lysine as a building block via solid-phase peptide synthesis.

Keywords: Lysine – Methylation – Benzylation – Monomethylated peptide – Synthesis

Abbreviations: Fmoc, 9-fluorenylmethoxycarbonyl; Boc, *tert*-butyloxycarbonyl; TFA, trifluoroacetic acid; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; TEA, triethylamine; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate; HOBt, hydroxybenzotriazole; SPPS, solid-phase peptide synthesis; ESI-MS, electrospray ionization-mass spectrometry; RP-HPLC, reversed-phase high-performance liquid chromatography

Introduction

Lysine methylation, one of the post-translational modifications affecting histones (Murray, 1964; Kouzarides, 2002; Lachner and Jenuwein, 2002; Sims III et al., 2003), can be found in mono-, di-, or trimethylated state in vivo (Fig. 1) (Bannister et al., 2002; Kubicek and Jenuwein, 2004). The precise methylation status of a given histone tail, on a given gene, can change during the process of transcriptional activation (Zhang and Reinberg, 2001; Kouzarides, 2002). Regulation of the transition between mono-, di-, and trimethylation of lysine residues may control dynamic processes such as transcription and DNA repair (Shahbazian et al., 2005). For instance, dimethylation or trimethylation of histone H3 lysine 4 has been correlated with transcriptionally active genes, whereas H3 lysine 4 monomethylation has been implicated in gene silence. This dualistic nature of the H3 lysine 4 methyl mark has thus far remained unresolved (Santos-Rosa et al., 2002; vanDijk et al., 2005).

For years, the lack of site-specifically methylated full-length histones and related peptides negatively impacts the investigation of substrate specificity of histone methyltransferases and demethylases, gene-specific transcription, and chromatin assembly and remodeling (He et al., 2003). Histones isolated from natural sources are highly heterogeneous with respect to post-translational modification state, type, and position. In addition, enzymatic synthesis of full-length histones and related peptides containing complex patterns of sequence specific modifications has been largely precluded because the enzymes that modify histones exhibit high sequence specificity and positional redundancy. Chemical synthesis, combined with native chemical ligation, is an alternative for site-specifically modified peptides and full-length histones.

Synthesizing site-specifically methylated peptides requires a viable and efficient source of different states of N^{ε} methylated lysine. N^{α}-Fmoc-N^{ε}-dimethyl-lysine and trimethylated derivative (Fig. 2, **1** and **2**) and the corresponding peptides derived from histone H3 N-terminal tail have been successfully synthesized in our group (Huang et al., 2006). Dimethylated and trimethylated lysine were directly obtained from N^{α}-Fmoc-lysine as the starting material by reductive methylation with formaldehyde and sodium cyanoborohydride and quarternization with

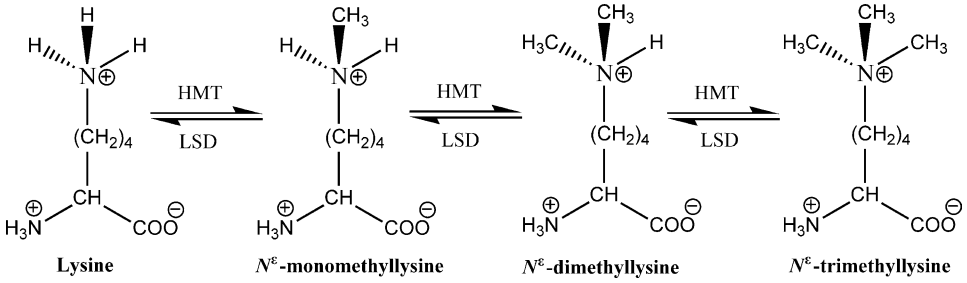


Fig. 1. Chemical structures of lysine and its methylated derivatives. The action of histone methyltransferases (HMTs) and lysine-specific demethylase (LSD) is indicated

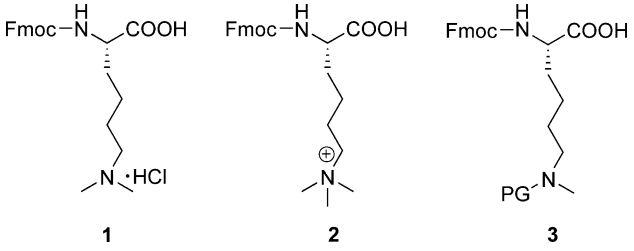
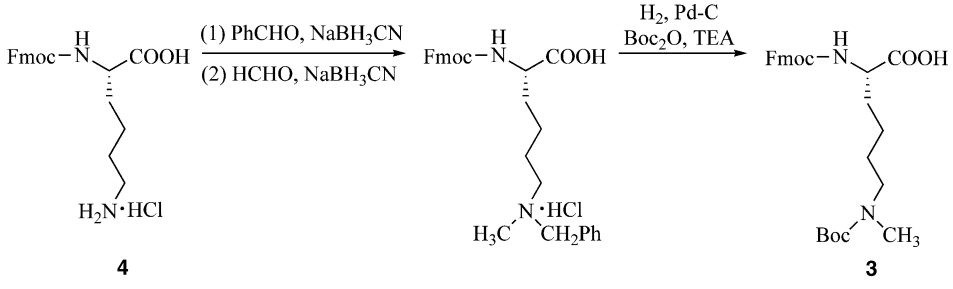


Fig. 2. N^{α}-Fmoc-N^{ε}-mono-, di- and trimethyl-lysine for the synthesis of corresponding peptides. PG represents protecting group

excessive methyl iodide, respectively. Little attention was focused on the preparation of N^{ε} monomethylated lysine derivatives and its corresponding peptides, except that Benoiton described the synthesis of N^{ε}-methyl-lysine by the reaction of N^{α}-benzoyl-lysine with benzaldehyde and sodium borohydride in 2 M sodium hydroxide followed by Clarke-Eschweiler reaction and hydrogenolysis with Pd-C (Benoiton, 1964). N^{ε} monomethylated lysine can not be obtained by direct methylation of N^{α}-Fmoc-lysine, since direct methylation of primary amino group with stoichiometric amount of methylated agents often affords a mixture of mono-, di- and trimethylated products. Also, reductive methylation of N^{α}-Fmoc-lysine with formaldehyde and formic acid (the Clarke-Eschweiler method), which has been proved to be a useful method for the preparation of methylated amines (Clarke et al., 1933), led to complex mixtures resulted from the multiplicity of side reactions. In recent years, several methods for the synthesis of N^{α} methylated amino acids and their derivatives have been developed (Song et al., 2000; Laplante and Hall, 2001; Aurelio et al., 2002; Prashad et al., 2003; White and Konopelski, 2005), however, they were not effective when extended to preparation of N^{ε} monomethylated lysine, because they often gave poor selectivities for monomethylation and some of them resulted in racemization (Aurelio et al., 2004). The methods also had the problem of region-selectivity to N^{α} and N^{ε} in the case of the lysine derivatives preparation. Therefore, a suitable method of selective monomethylation for the synthesis of N^{ε} monomethylated lysine is very desirable.

Herein a novel and concise method for the synthesis of monomethylated lysine, N^{α}-Fmoc-N^{ε}-(Boc, methyl)-lysine (Fig. 2, **3**, PG = Boc), was described. Compound **3** was obtained, without resorting to carboxyl group protection, via a sequence of reductive benzylation, reductive methylation, debenzylation and Boc protection using N^{α}-Fmoc-lysine (**4**) as the starting material in two one-pot reactions as depicted in Scheme 1. Monomethylation of lysine side chain was specifically achieved by selective removal of the benzyl group after sequential reductive benzylation and reductive methylation of **4**. The synthesized monomethylated lysine **3** was efficiently incorporated as a building block into peptide chain with the standard Fmoc-based strategy.



Scheme 1. Synthetic route for N^{α}-Fmoc-N^{ε}-(Boc, methyl)-lysine

Materials and methods

Materials

N^{α}-Fmoc-lysine hydrochloride (99.4%) and Fmoc protected amino acids were purchased from GL Biochem (Shanghai) Ltd. Corporation (China). Benzaldehyde, formaldehyde (37 wt.% aqueous solution), Boc anhydride, sodium cyanoborohydride and Pd-C were from ACROS (U.S.A.). 3 Å molecular sieve was dried under vacuum for 8 h before being used. All reagents and solvents were analytically pure.

General methods

Melting points of the compounds were measured with WRS-1A digital microscopic melting-point apparatus without correction. Optical rotation was measured at room temperature with a Perkin Elmer model 343 polarimeter by using the Sodium line. Mass spectrometry was performed on a Brucker ESQUIRE∼LC ESI ion trap spectrometry equipped with a gas nebulizer probe. Nitrogen was used as drying gas. All NMR spectra were acquired on a JEOL 300 in methanol-d_3.

Synthesis of building blocks

One-pot synthesis of N^{α}-Fmoc-N^{ε}-(benzyl, methyl)-lysine: benzylation and methylation

To a stirred solution of N^{α}-Fmoc-lysine hydrochloride (203 mg, 0.5 mmol) in 6 ml of ethanol in the presence of 3 Å molecular sieves at room temperature was added freshly distilled benzaldehyde (64 mg, 0.6 mmol). After stirring for 30 min, sodium cyanoborohydride (38 mg, 0.6 mmol) was added. The reaction mixture was allowed to stir overnight. TLC detection (methanol/dichloromethane 1:4) indicated that N^{α}-Fmoc-lysine ($R_f \approx 0.3$) had disappeared and a new product N^{α}-Fmoc-N^{ε}-benzyl-lysine ($R_f \approx 0.65$) had formed. 37% aqueous formaldehyde (80 µl, 1.0 mmol) was then added followed by sodium cyanoborohydride (44 mg, 0.7 mmol) treatment. After stirring for additional 30 min, N^{α}-Fmoc-N^{ε}-benzyl-lysine was completely transformed to another new product N^{α}-Fmoc-N^{ε}-(benzyl, methyl)-lysine ($R_f \approx 0.55$) by TLC detection. 0.2 M hydrochloric acid was then added dropwise to acidify the reaction solution and some by-product white solid precipitated. The reaction mixture was filtrated to remove the solid. The filtrate was concentrated to afford crude product under reduced pressure. 216 mg pure N^{α}-Fmoc-N^{ε}-(benzyl, methyl)-lysine hydrochloride as white powder was isolated by purification with silica gel column chromatography using methanol/dichloromethane 1:2 as eluent. Mp 90–92 °C, $[\alpha]_D^{20} + 5.0 \pm 0.2°$ ($c = 0.005$ in methanol). The total yield was 85%. In a separate reaction, pure N^{α}-Fmoc-N^{ε}-benzyl-lysine was isolated by purification with silica gel column chromatography using methanol/dichloromethane 1:1 as eluent. Mp 185–187 °C, $[\alpha]_D^{20} - 2.0 \pm 0.1°$ ($c = 0.011$ in methanol). Both N^{α}-Fmoc-N^{ε}-(benzyl, methyl)-lysine and N^{α}-Fmoc-N^{ε}-benzyl-lysine were confirmed with ESI-MS, ^{1}H-NMR and ^{13}C-NMR.

N^{α}-Fmoc-N^{ε}-(benzyl, methyl)-lysine

ESI-MS (m/z): 473.6 $[M+H]^+$, 495.5 $[M+Na]^+$, 511.5 $[M+K]^+$.

^{1}H NMR (methanol-d_3, 300 M): δ (ppm) 7.26–7.78 (m, 13H, Ar-H/Fmoc), 4.32 (d, 2H, $J = 6.8$ Hz, CH_2/Fmoc), 4.18 (t, 1H, $J = 6.7$ Hz, CH/Fmoc), 4.03 (m, 1H, αCH/Lys), 3.95 (s, 2H, PhCH_2N), 2.79 (t, 2H, $J = 7.9$ Hz, εCH_2/Lys), 2.49 (s, 3H, NCH_3), 1.84 (m, 1H, βCH/Lys), 1.69 (m, 3H, βCH′/Lys, δCH_2/Lys), 1.38 (m, 2H, γCH_2/Lys).

^{13}C NMR (methanol-d_3, 300 M): δ (ppm) 179.3, 158.1, 145.4, 145.2, 142.5, 134.1, 131.6, 129.9, 129.8, 128.7, 128.1, 126.2, 120.9, 67.6, 61.4, 57.3, 48.4, 40.7, 33.7, 25.8, 24.2.

N^{α}-Fmoc-N^{ε}-benzyl-lysine

ESI-MS (m/z): 459.3 $[M+H]^+$, 481.2 $[M+Na]^+$.

^{1}H NMR (methanol-d_3, 300 M): δ (ppm) 7.27–7.79 (m, 13H, Ar-H/Fmoc), 4.35 (d, 2H, $J = 6.8$ Hz, CH_2/Fmoc), 4.19 (t, 1H, $J = 6.8$ Hz, CH/Fmoc), 4.18 (s, 2H, PhCH_2), 4.11 (m, 1H, α CH/Lys), 3.02 (t, 2H, $J = 7.5$ Hz, εCH_2/Lys), 1.88 (m, 1H, βCH/Lys), 1.74 (m, 3H, βCH′/Lys, δCH_2/Lys), 1.47 (m, 2H, γCH_2/Lys).

^{13}C NMR (methanol-d_3, 300 M): δ (ppm) 176.9, 158.7, 145.3, 145.1, 142.6, 132.6, 131.0, 130.6, 130.2, 128.8, 128.2, 126.2, 120.9, 67.8, 55.5, 52.2, 48.4, 48.3, 32.3, 26.6, 24.0.

One-pot preparation of N^{α}-Fmoc-N^{ε}-(Boc, methyl)-lysine: debenzylation and Boc protection

To a stirred mixture of N^{α}-Fmoc-N^{ε}-(benzyl, methyl)-lysine hydrochloride (203 mg, 0.4 mmol), TEA (125 µl, 0.9 mmol) and Boc anhydride (109 mg, 0.5 mmol) in 20 ml of anhydrous dichloromethane, Pd-C and hydrogen was added. The reaction was monitored by TLC. After completion of the reaction, Pd-C was removed by filtration through a celite pad, which was then washed with 20 ml dichloromethane. The filtrate was washed with 0.2 M hydrochloric acid 10 ml × 3, saturate sodium bicarbonate 10 ml × 3, and brine 10 ml × 2, dried over anhydrous magnesium sulfate and concentrated to give crude product. The crude product was purified by silica gel column chromatography using a mixture of methanol and dichloromethane (1:100, v/v) as eluent to afford 179 mg pure N^{α}-Fmoc-N^{ε}-(Boc, methyl)-lysine as white powder. Mp 85–87 °C, $[\alpha]_D^{20} - 1.7 \pm 0.2°$ ($c = 0.005$ in methanol). The yield was 93%. N^{α}-Fmoc-N^{ε}-(Boc, methyl)-lysine was confirmed with ESI-MS, ^{1}H-NMR and ^{13}C-NMR. Reaction of N^{α}-Fmoc-N^{ε}-(benzyl, methyl)-lysine hydrochloride with Pd-C and hydrogen without adding TEA and Boc anhydride gave Fmoc-N^{ε}-methyl-lysine as a product, which was isolated and confirmed with ESI-MS and ^{1}H-NMR.

N^{α}-Fmoc-N^{ε}-(Boc, methyl)-lysine

ESI-MS (m/z): 483.2 $[M+H]^+$, 505.2 $[M+Na]^+$, 521.1 $[M+K]^+$, 383.2 $[M+H\text{-Boc}]^+$, 405.2 $[M+Na\text{-Boc}]^+$, 421.1 $[M+K\text{-Boc}]^+$.

^{1}H NMR (methanol-d_3, 300 M): δ (ppm) 7.27–7.81 (m, 8H, Ar-H/Fmoc), 4.34 (m, 2H, CH_2/Fmoc), 4.20 (t, 1H, $J = 6.8$ Hz, CH/Fmoc), 4.07 (m, αCH/Lys), 3.21 (t, 2H, $J = 7.2$ Hz, εCH_2/Lys), 2.82 (s, 3H, NCH_3), 1.85 (m, 1H, βCH/Lys), 1.68 (m, 1H, βCH′/Lys), 1.54 (m, 2H, δCH_2/Lys), 1.37 (m, 2H, γCH_2/Lys), 1.43 (s, 9H, C(CH_3)$_3$.

^{13}C NMR (methanol-d_3, 300 M): δ (ppm) 178.9, 158.6, 157.6, 145.3, 145.2, 142.6, 128.8, 128.6, 128.1, 126.2, 126.0, 120.9, 80.8, 67.9, 56.4, 48.4, 34.6, 33.0, 28.7, 24.2.

Fmoc-N^{ε}-methyl-lysine

ESI-MS (m/z): 383.3 $[M+H]^+$, 405.3 $[M+Na]^+$.

^{1}H NMR (methanol-d_3): δ (ppm) 7.27–7.81 (m, 8H, Ar-H/Fmoc), 4.33 (m, 2H, CH_2/Fmoc), 4.20 (t, 1H, $J = 6.8$ Hz, CH/Fmoc), 4.01 (m, αCH/Lys), 2.93 (t, 2H, $J = 7.5$ Hz, εCH_2/Lys), 2.65 (s, 3H, NCH_3), 1.82 (m, 1H, βCH/Lys), 1.68 (m, 3H, βCH′/Lys, δCH_2/Lys), 1.42 (m, 2H, γCH_2/Lys).

Peptide synthesis

Peptide was assembled on Rink Amide resin (substitution 0.8 mmol g^{-1}) by manual solid-phase peptide synthesis (SPPS) using Fmoc/piperidine strategies. Entering amino acids were preactivated with HOBt, HBTU, and DIEA in DMF for 5 min, and couplings were run for 1.5–2 h. Coupling efficiencies were monitored by Kaiser analysis. When the peptide chain assembly was completed, the peptide was cleaved from the resin with TFA containing phenol (5%), thioanisole (5%), ethanedithiol (2.5%) and water (5%), with simultaneous removal of side chain functional protecting groups. The peptide was precipitated by dry ethyl ether.

Crude peptide was purified by RP-HPLC (Vydac C_{18}) using linear gradients from 65% buffer A (water/TFA 99.9:0.1) and 35% buffer B

(acetonitrile/water/TFA 90:10:0.1) to 40% buffer A and 60% buffer B. The peptide identities were confirmed by ESI-MS.

Results and discussion

N^{α}-Fmoc-N^{ε}-(Boc, methyl)-lysine was obtained by two one-pot reactions. In the first one-pot reaction, N^{α}-Fmoc-lysine was benzylated by reductive benzylation with benzaldehyde and sodium cyanoborohydride in the presence of 3 Å molecular sieves to form N^{α}-Fmoc-N^{ε}-benzyl-lysine, which was then transformed to N^{α}-Fmoc-N^{ε}-(benzyl, methyl)-lysine by another reductive methylation with formaldehyde and sodium cyanoborohydride. Both reductive alkylations were performed in the same flask without isolation. In addition, reductive alkylation using sodium cyanoborohydride was a mild reaction (Borch et al., 1971). Fmoc group was just tolerable to such condition and no trace of racemization was observed. Benzyl group served as a great protecting group, because it can be conveniently incorporated and removed (Greene and Wuts, 1999). Moreover, an important advantage of benzyl over other protecting groups for lysine side chain such as Boc, Z and Trt was that another methyl group can be further introduced by reductive methylation. Reductive methylation was possible for the benzylated lysine because it was a secondary amine, which had the potential of being transformed further to a tertiary amine.

Additionally, the sequence of reductive benzylation and reductive methylation was crucial to the success of this method. When reductive methylation of N^{α}-Fmoc-lysine with formaldehyde and sodium cyanoborohydride was performed first, two methyl groups were incorporated into N^{α}-Fmoc-lysine to form N^{α}-Fmoc-dimethyl-lysine (ESI-MS 397.5 $[M+H]^+$) (Huang et al., 2006). Consequently, the benzyl group can not be further introduced to provide N^{α}-Fmoc-N^{ε}-(benzyl, methyl)-lysine. However, when reductive benzylation with appropriate amount of benzaldehyde was performed first, only one benzyl group was attached to amino group as expected, and no dibenzylated derivative was observed. The steric hindrance of benzaldehyde precluded dibenzylation, and methylation to the benzylated secondary amine was still available. Therefore, reductive benzylation must proceed first, followed by reductive methylation.

In another one-pot reaction, two reactions including catalytic debenzylation of N^{α}-Fmoc-N^{ε}-(benzyl, methyl)-lysine and Boc protection of debenzylated product were also performed in the same flask without isolation. N^{α}-Fmoc-N^{ε}-(benzyl, methyl)-lysine was firstly converted to N^{α}-Fmoc-N^{ε}-methyl-lysine through debenzylation by hydrogenolysis with Pd-C, and then transformed to N^{α}-Fmoc-N^{ε}-(Boc, methyl)-lysine in the presence of Boc anhydride and TEA. As a result, Boc was introduced in situ.

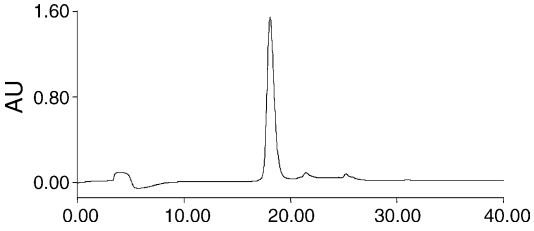


Fig. 3. RP-HPLC chromatograms of crude peptide

In order to synthesize a fragment derived from histone H3 N-terminal tail 1–6 residues (Ala-Arg-Thr-Lys(CH_3)-Gln-Thr) containing monomethylated lysine, N^{α}-Fmoc-N^{ε}-(Boc, methyl)-lysine was used as a building block in the peptide chain assembly on Rink-Amide resin with the standard Fmoc-based chemistry. When the peptide chain assembly was completed, a peptide H_2N-Ala-Arg-Thr-Lys(CH_3)-Gln-Thr-NH_2, in which the lysine residue was methylated, was obtained upon TFA-based cleavage from the resin. Simultanously, Boc group at the methylated lysine side chain as well as the protecting groups for the other amino acid residues was cleaved by TFA-based cleaving solution. The synthetic building block was efficiently coupled into peptide chain according to Kaiser Test result. RP-HPLC traces (Fig. 3) and ESI-MS data (717.5 $[M+H]^+$, 739.3 $[M+Na]^+$) of the synthetic peptide indicated high purity (95%) and homogeneity of the crude peptide and thus confirmed the reliability and efficiency of the method.

Conclusion

In conclusion, a concise, rapid and efficient method for the preparation of N^{ε} monometylated lysine, N^{α}-Fmoc-N^{ε}-(Boc, methyl)-lysine, was described. N^{α}-Fmoc-N^{ε}-(Boc, methyl)-lysine was obtained via a consecutive process of benzylation, methylation, debenzylation and Boc protection of N^{α}-Fmoc-lysine in two one-pot reactions. Monomethylation of lysine side chain was specifically acquired by using benzyl group as an excellent protecting group. The synthetic monomethylated lysine derivative was successfully incorporated into peptide and TFA cleavage gave corresponding site-specifically monomethylated peptide.

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