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Facile Synthesis and Altered Ionization Efficiency of Diverse N^ε-Alkyllysine-containing Peptides†

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Abstract

Protein lysine methylation is a biologically-important posttranslational modification. Given the increased use of alkyllysine-containing peptides to study protein methylation, we described an efficient solid-phase synthesis to access various alkyllysine-containing peptides. As an exemplary application, we evaluated how ionization efficiency of the alkyllysine peptides affects mass spectrometric analysis.

Lysine-N^ε amino groups of proteins undergo diverse posttranslational modifications such as ubiquitination, sumoylation, neddylation, acetylation and methylation.¹ Among the most known lysine posttranslational modifications is methylation, which is featured by enzymatic installation of a methyl group from the biological methyl donor *S*-adenosyl-*L*-methionine (SAM) (Fig. 1).² Despite its stable chemical nature, this modification can be removed by demethylases (Fig. 1).² The dynamic methylation and demethylation of targeted proteins play essential roles in epigenetic regulation via modulating their localization, stability or interacting partners.³ Their deregulation has been linked to many diseases including cancer.³ For instance, histone H3 lysine 9 (H3K9) can be methylated by 7 human protein lysine methyltransferases (PKMTs).⁴ This modification is associated with gene silencing at euchromatin, facultative/constitutive heterochromatin, or virus-integrating sites to maintain normal cellular functions.^{5, 6} In contrast, the same events can be hijacked by cancer cells to silence tumor suppressor genes.⁷

Given the importance of protein methylation, multiple approaches were described for site-specific incorporation of methylated lysines into peptides or proteins.^{8–10} Complementary biochemical assays were also developed to trace these events.¹⁰ Recently, the peptides that contain lysine-N^ε-β unsaturated substituents have emerged as valuable chemical entities to study protein methylation and demethylation.^{11–15} For example, several successes have been reported to utilize SAM derivatives, in combination with native or engineered PKMTs, to transfer allyl derivatives to PKMT substrates (Fig. 1).^{13–15} The resultant lysine-N^ε derivatization enabled the PKMT substrates to be readily characterized.^{13, 14} The peptides carrying lysine-N^ε-propargyl/allyl derivatives are potent inhibitors of the protein lysine demethylase LSD1 (Fig. 1).^{11, 12} These applications thus triggered us to explore efficient chemical methods to assemble N^ε-β-unsaturated moieties onto lysine side chain.^{11, 12}

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Among known synthetic strategies to access N^ε-alkyllysines are base-promoted lysine-N^ε alkylation, lysine-N^ε reductive alkylation, and leaving-group-facilitated S_N2 C-amination of 6-hydroxynorleucine.^{11, 12, 16, 17} Base-promoted lysine-N^ε-alkylation is less selective for the degree of alkylation and often leads to mono/di/tri-alkylated product mixtures.^{8, 9} Lysine-N^ε reductive alkylation is facile to prepare N^ε-monomethyllysine. The monoalkylation selectivity was achieved by reductive benzylation and then methylation through imine intermediates followed by debenylation.^{8, 9} However, this approach is not suitable to install lysine-N^ε-β-unsaturated substituents because of the undesirable loss of the unsaturated moieties via 1,4-hydride reduction of the imine intermediates.^c So far, the reported approach to chemically install lysine-N^ε-β-sp/sp² substituents is to mesylate 6-hydroxynorleucine of resin-immobilized peptides, followed by solution-phase S_N2 displacement of the mesylate with N^β-unsaturated amines (S_N2-type C-amination), in which multiple HPLC purifications were involved.^{11, 12}

Here we described a concise, efficient chemical approach to install lysine-N^ε-β-unsaturated substituents via solid-phase Dess-Martin oxidation/reductive amination of hydroxynorleucine. Characterizing these lysine-N^ε-derivatized peptides also allowed us to compare ionization efficiency of diverse N^ε-alkyllysine-containing N-terminal H3 peptides on matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Although most H3K9-N^ε alkyl substitutions (*e.g.* Me-, CF₃CH₂-, propargyl, allyl, 2-butenyl and 2-butenyl) have no significant effect on the ionization efficiency, ethylation and benzylation alter the ionization efficiency by 2.5–5-fold. These results therefore claimed the need to validate ionization efficiency of native and alkyllysine peptides for direct quantification through their MALDI-MS peak intensities.

To develop strategies to access lysine derivatives containing N^ε-β-unsaturated substituents, we first focused on solution-phase synthesis of N^ε-allyllysine as a proof-of-concept target (Scheme 1). The reductive alkylation reaction of Fmoc-protected lysine and acrolein underwent undesirable 1,4- and then 1,2-imine reduction to give N^ε-propyl rather than N^ε-allyllysine.^c To avoid this 1,4-hydride reduction, we envisioned a strategy through switching the positions of the amine-aldehyde pair (reductive alkylation versus reductive amination). Here N^α-Fmoc lysine was converted to N^α-Fmoc hydroxynorleucine **1** via spontaneous hydrolysis of a diazonium intermediate (Scheme 1).^{11, 12, 18} A subsequent direct oxidation of hydroxynorleucine's primary alcohol to aldehyde proved to be challenging. All the attempts for such oxidation, concomitant with the loss of **1**, gave a mixture of products, which either showed complex profiles (*e.g.* with *tetra-n*-propyl ammonium perruthenate, pyridinium chlorochromate, or chromium(VI) oxide/pyridine as oxidants) or decomposed in the course of purification (*e.g.* oxalyl chloride/DMSO for Swern oxidation or periodinane for Dess-Martin oxidation).

Given that the product profile under the Dess-Martin condition is simpler than others, we reasoned that the failure of Dess-Martin periodinane to yield the desirable aldehyde could be due to the challenge of product purification. This problem was gratifyingly circumvented using a step-wise one-pot reaction (Scheme 1). Here N^α-Fmoc hydroxynorleucine was first oxidized to the corresponding aldehyde **2** with Dess-Martin periodinane. After simple concentration, the aldehyde product was reacted with allyl amine (2 equiv. to suppress multi-alkylation), followed by cyanoborohydride-mediated reduction, to yield N^ε-allyllysine **3**. Without further purification, N^ε-allyllysine was masked by Boc-carbamate with a final yield of 15% from N^α-Fmoc hydroxynorleucine **1** to **4a**. The generality of this synthesis was also confirmed by its application to prepare N^α-Fmoc-N^ε-(Boc, propargyl)-lysine **4b** and

^cUpon reacting N^α-Fmoc-lysine with acrolein, NaCNBH₃ and then Boc₂O, N^α-Fmoc-N^ε-(Boc, propyl)lysine was isolated as the only major product, which arises from 1,4-imine reduction (Section 2a of ESI).

N^α-Fmoc-N^ε-(Boc, 2-butyryl)-lysine **4c** in comparable 15–20% yields (Scheme 1, Section 2b in ^{ESI}). Despite less promising yields, the one-pot strategy served as a key starting point to develop more efficient solid-phase synthesis (results below).

Encouraged by the success of solution-phase synthesis of N^ε-allyl/propargyl/2-butyryl lysine analogues **4a–c**, we then explored such chemical transformation of a peptide substrate in solid phase (Scheme 2). The N-terminal H3 peptide (3–13 aa, **5**) with its H3K9 replaced by hydroxynorleucine was assembled on Wang resin. In comparison with the prior solid-phase peptide synthesis (SPPS) for hydroxynorleucine-containing peptides,^{11, 12} the current approach is featured by incorporating hydroxynorleucine and other amino acids without protecting hydroxynorleucine ε-hydroxyl group. Similar to our solution-phase strategy, the hydroxynorleucine moiety of resin-immobilized peptide was efficiently converted to aldehyde by Dess-Martin periodinane (from **5** to **6**). Subsequent solid-phase reductive amination of the aldehyde with 2 equiv. allyl amine and then TFA-mediated global deprotection yielded the desirable allyl peptide **8a** with a remarkable yield of 30% for 23-step solid-phase reactions from Wang resin. The same approach also allowed the synthesis of 7 other H3K9-alkylated peptides (Me-, ethyl, CF₃CH₂-, propargyl, allyl, 2-butyryl, 2-butenyl and benzyl) from respective primary amines (30–50% yields from **1** to **8a–h** based on Wang Resin, Scheme 2, Fig. S2 and Section 2c in ^{ESI}). The current strategy is convenient because of its ability to access multiple products from the commonly-shared hydroxynorleucine peptide via simple solid-phase washing and a single HPLC purification. In addition, Thr(tBut), Lys(Boc), Gln(Trt), Arg(Pbf), Ser(tBut), Gly and Cys(Trt) are tolerant to Dess-Martin oxidation/reductive amination conditions.^d The robust and general applicability of the solid-phase Dess-Martin oxidation/reductive amination thus presents a short and efficient route to accessing various alkyllysine-containing peptides.

To examine the activities of protein methyltransferases, a common strategy is to quantify enzymatic modification with MALDI-MS. Some prior experiments relied on direct comparison of ion intensities of modified peptides (products) versus unmodified peptides (starting materials).¹⁹ However, given uncertain ionization efficiency of the alkylated peptides, the caution should be made for such direct comparison.^{13, 15, 20} For example, prior to MALDI-based MS quantification, comparable ionization efficiency on MALDI-MS was confirmed for N-terminal H3 peptide and its (*E*)-hex-2-en-5-ynyl derivative.¹³

Given our newly-gained ability to access various alkyllysine peptides, their ionization efficiency on MALDI-MS were evaluated in a systematic manner. Upon co-injecting equal-molar N-terminal H3 3–13 aa peptide and its H3K9-alkylated derivatives (Me-, Et-, CF₃CH₂-, propargyl, allyl, 2-butyryl, 2-butenyl and benzyl) on MALDI-MS, their ion intensities were compared (Figs. 2, S2 in ^{ESI}). Although the H3K9 peptides containing Me-, CF₃CH₂-, propargyl, allyl, 2-butyryl and 2-butenyl modifications showed MALDI-MS ionization efficiency comparable to that of the native peptide, the ethylation reduces and the benzylation enhances the ionization efficiency by 2.5- and 5-fold, respectively (Fig. 2). This finding thus argues that, although H3K9 methylation, allylation and propargylation have negligible effect on the peptide's MALDI-MS ionization efficiency, other H3K9 alkylations (*e.g.* ethylation or benzylation) may alter such parameter dramatically.

[†]Electronic Supplementary Information available: General materials & methods, synthetic protocols and product characterization. See DOI: 10.1039/b000000x/

^dThr(tBut), Lys(Boc), Gln(Trt), Arg(Pbf), Ser(tBut) and Gly were installed in the course to synthesizing the 3–13 aa H3 peptides. The success of the peptide synthesis therefore indicated that these amino-acid building blocks are tolerant to Dess-Martin oxidation/reductive amination. The tolerance of Cys(Trt) was confirmed independently by synthesizing C-terminal-Cys(Trt)-appended 3–13 H3 peptide (data not shown). Other amino acids (Met, Tyr and Trp) remained to be examined.

In conclusion, a convenient synthesis has been developed through consecutive solid-phase Dess-Martin oxidation and reductive amination. The unprecedented approach enables the ready access to alkyllysine-containing peptides via SPPS, in particular those containing lysine-N^ε-β-allyl/propargyl analogues. Given that similar peptide derivatives have been explored as intermediates for labelling PKMT substrates and as inhibitors of histone-modifying enzymes, these applications can be benefited by diversifying the peptide entities with the delineated synthesis. Analyzing ionization efficiency of alkyllysine-containing peptides also underscores the importance of validating the MS parameter upon applying MALDI-MS to quantify alkylated peptides. Here N^ε-alkyllysine-containing peptides can be readily prepared as the MS standards for such validation through the described synthesis.

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Reference

1. Smith BC, Denu JM. *Biochim. Biophys. Acta.* 2009; 1789:45–57. [PubMed: 18603028]
2. Cole PA. *Nat. Chem. Biol.* 2008; 4:590–597. [PubMed: 18800048]
3. Bhaumik SR, Smith E, Shilatifard A. *Nat. Struct. Mol. Biol.* 2007; 14:1008–1016. [PubMed: 17984963]
4. Kouzarides T. *Cell.* 2007; 128:802–803. [PubMed: 17320515]
5. Leung DC, Dong KB, Maksakova IA, Goyal P, Appanah R, Lee S, Tachibana M, Shinkai Y, Lehnertz B, Mager DL, Rossi F, Lorincz MC. *Proc. Natl. Acad. Sci. USA.* 2011; 108:5718–5723. [PubMed: 21427230]
6. Cheng X, Blumenthal RM. *Biochemistry.* 2010; 49:2999–3008. [PubMed: 20210320]
7. Spannhoff A, Sippl W, Jung M. *Int. J. Biochem. Cell Biol.* 2009; 41:4–11. [PubMed: 18773966]
8. Huang ZP, Du JT, Su XY, Chen YX, Zhao YF, Li YM. *Amino Acids.* 2007; 33:85–89. [PubMed: 17031472]
9. Huang ZP, Su XY, Du JT, Zhao YF, Li YM. *Tetrahedron Lett.* 2006; 47:5997–5999.
10. Wang Y-S, Wu B, Wang Z, Huang Y, Wan W, Russell WK, Pai P-J, Moe YN, Russell DH, Liu WR. *Mol. Biosyst.* 2010; 6:1575–1578.
11. Culhane JC, Szewczuk LM, Liu X, Da GP, Marmorstein R, Cole PA. *J. Am. Chem. Soc.* 2006; 128:4536–4537. [PubMed: 16594666]
12. Culhane JC, Wang DQ, Yen PM, Cole PA. *J. Am. Chem. Soc.* 2010; 132:3164–3176. [PubMed: 20148560]
13. Islam K, Zheng W, Yu H, Deng H, Luo M. *ACS Chem. Biol.* 2011; 6:679–684. [PubMed: 21495674]
14. Wang R, Ibáñez G, Islam K, Zheng W, Blum G, Sengelaub C, Luo M. Submitted. 2011
15. Wang R, Zheng W, Yu H, Deng H, Luo M. *J. Am. Chem. Soc.* 2011; 133:7648–7651. [PubMed: 21539310]
16. AbdelMagid AF, Carson KG, Harris BD, Maryanoff CA, Shah RD. *J. Org. Chem.* 1996; 61:3849–3862. [PubMed: 11667239]
17. Sajiki H, Ikawa T, Hirota K. *Org. Lett.* 2004; 6:4977–4980. [PubMed: 15606114]
18. Thompson PR, Kurooka H, Nakatani Y, Cole PA. *J. Biol. Chem.* 2001; 276:33721–33729. [PubMed: 11445580]
19. Collins RE, Tachibana M, Tamaru H, Smith KM, Jia D, Zhang X, Selker EU, Shinkai Y, Cheng XD. *J. Biol. Chem.* 2005; 280:5563–5570. [PubMed: 15590646]

20. Patel A, Dharmarajan V, Vought VE, Cosgrove MS. J. Biol. Chem. 2009; 284:24242–24256. [PubMed: 19556245]
21. Barlesi F, Giaccone G, Gallegos-Ruiz MI, Span SW, Lefesvre P, Krut FAE, Rodriguez JA. Int. J. Cancer. 2008; 122:1441–1442. [PubMed: 18027872]

Figure 1.

Methylation, alkylation and demethylation on protein lysine. PKMTs or their variants account for site-specific lysine modifications. Demethylation is carried out by demethylases and can be inhibited by N^ϵ - propargyl/allyllysine-containing peptides.

Figure 2.

Comparison of MALDI-MS ionization efficiency of H3K9 with representative H3K9-alkyllysine derivatives. Equal molar native and alkyllysine peptides were co-injected into MS. Peptides **8a** (allyl) and **8d** (methyl) displayed comparable ionization efficiency to that of the native peptide (upper left and right). In contrast, ionization efficiency of **8e** (ethyl) and **8h** (benzyl) altered by 2.5–5 fold (lower left and right).

Scheme 1.

Synthesis of lysine analogues containing N^ε-β-unsaturated substituents via consecutive Dess-Martin oxidation/reductive amination of hydroxynorleucine.

Scheme 2.

Solid-phase synthesis of alkyllysine-containing H3 N-terminal peptides via Dess-Martin oxidation/reductive amination. NL=norleucine.