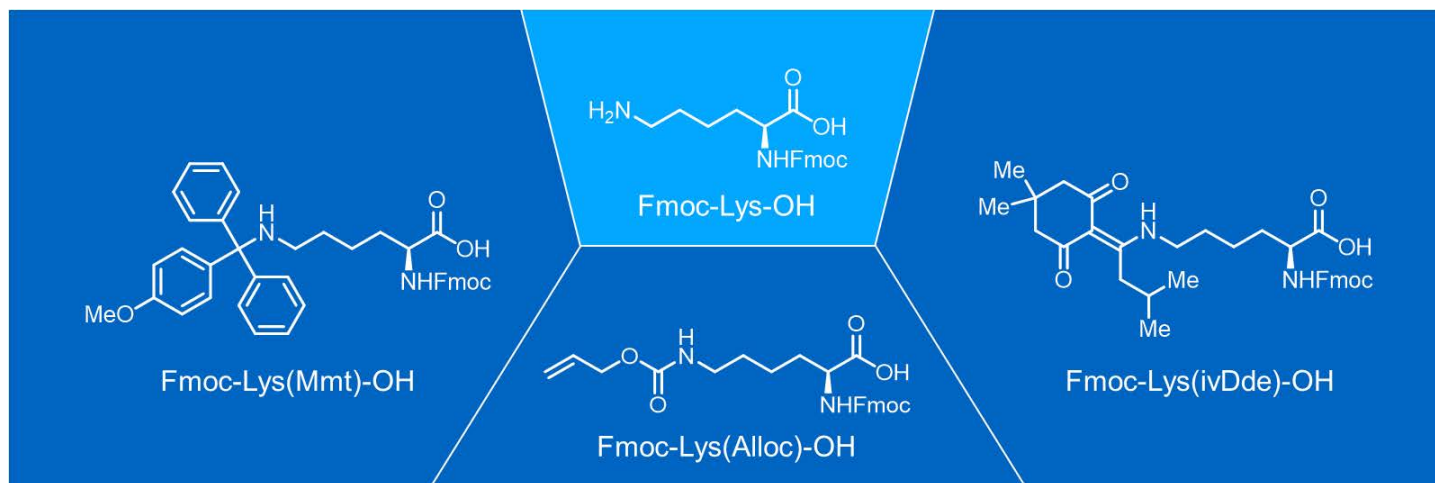


Automated Deprotection of Orthogonal and Non-Standard Lysine Protecting Groups



Summary

The Liberty Blue™ can perform automated deprotection of orthogonal and non-standard side-chain protecting groups, enabling side-chain branching, bioconjugation and cyclization. Microwave-enhanced SPPS on the Liberty Blue produced branched variants of the peptide gp41₆₅₉₋₆₇₁, by Lys(Mmt)-, Lys(Alloc)-, and Lys(ivDde)-deprotection; subsequent coupling produced the variants in 79%, 82%, and 93% purity respectively. The three gp41₆₅₉₋₆₇₁ variants were synthesized in a combined time of 9 h 15 min and required less than 1200 mL main wash (DMF).

Introduction

Peptidyl side-chain functionalizations, such as bioconjugation, branching, and cyclization, are impactful and essential synthetic tools, necessary for pharmaceutical development, medical imaging, materials research, and more.¹⁻⁴ Many amino acid residues can serve as sites for side-chain functionalization; lysine, however, has received particular attention.

To perform side-chain functionalization successfully, the residue in question must be outfitted with an appropriately orthogonal protecting group, which readily undergoes selective deprotection while the rest of the peptide remains unaffected. In the case of lysine (and many other residues), monomethoxytrityl (Mmt), allyl formatyl (Alloc), and 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde), are commonly used orthogonal groups (**Figure 1**), requiring dilute TFA, catalytic Pd(0), and dilute hydrazine for deprotection, respectively.

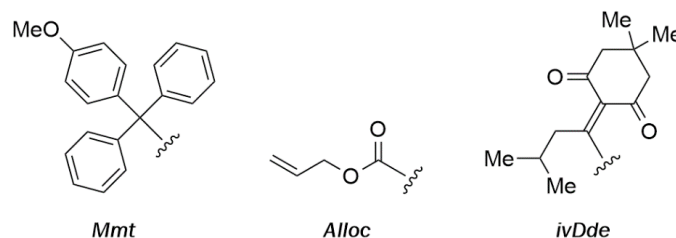


Figure 1: Common, orthogonal lysine protecting groups

To demonstrate the Liberty Blue automated peptide synthesizer's ability to efficiently synthesize and functionalize peptides, branched variants of the HIV-1 antibody epitope gp41₆₅₉₋₆₇₁ (ELLELDKWASLWN) were synthesized.⁵ Lys(Mmt), Lys(Alloc), or Lys(ivDde) were incorporated into the synthesis of gp41₆₅₉₋₆₇₁, and upon selective deprotection, were subsequently coupled with alanine to furnish the branched peptides.

Materials and Methods

Reagents

Fmoc-Lys(ivDde)-OH was obtained from CEM Corporation (Matthews, NC). Fmoc-Lys(Mmt)-OH and Fmoc-Lys(Alloc)-OH were obtained from Novabiochem–MilliporeSigma (Burlington, MA). All other amino acids were obtained from CEM Corporation (Matthews, NC) and contained the following side chain protecting groups: Asn(Trt), Asp(OMpe), Glu(OtBu), Ser(OtBu), and Trp(Boc). Oxyma Pure and Rink Amide ProTide™ LL resin were obtained from CEM Corporation (Matthews, NC). N,N-Diisopropylcarbodiimide (DIC) was obtained from CreoSalus (Louisville, KY). Piperidine was obtained from Alfa Aesar (Ward Hill, MA). Trifluoroacetic acid (TFA), 3,6-dioxo-1,8-octanedithiol (DODT), hydrazine, phenylsilane, tetrakis(triphenylphosphine)-palladium(0) (Pd(PPh₃)₄), triisopropylsilane (TIS), and acetic

acid were obtained from Sigma-Aldrich (St. Louis, MO). Dichloromethane (DCM), N,N-dimethylformamide (DMF), and anhydrous diethyl ether (Et₂O) were obtained from VWR (West Chester, PA). HPLC-grade water (H₂O), and HPLC-grade acetonitrile (MeCN) were obtained from Fisher Scientific (Waltham, MA).

Peptide Synthesis: ELLELDK(-A)WASLWN-NH₂ via Mmt Deprotection

The peptide was prepared at 0.1 mmol scale using the CEM Liberty Blue automated microwave peptide synthesizer on 0.526 g Rink Amide ProTide LL resin (0.19 meq/g substitution). Fmoc-Deprotection was performed with 20% piperidine and 0.1 M Oxyma Pure in DMF. Coupling reactions were performed with 0.2 M Fmoc-AA-OH, 1.0 M DIC in DMF and 1.0 M Oxyma Pure in DMF.⁶ Mmt-Deprotection was performed with 2% TFA in DCM. Cleavage was performed using the CEM Razor™ high-throughput peptide cleavage system with 92.5:2.5:2.5:2.5 TFA/H₂O/TIS/DODT. Following cleavage, the peptide was precipitated in Et₂O and lyophilized overnight.

Note: To prevent undesired coupling, the N-terminus (E) remained Fmoc-protected until completion of Mmt-deprotection and lysine side-chain functionalization.

Peptide Synthesis: ELLELDK(-A)WASLWN-NH₂ via Alloc Deprotection

The peptide was prepared at 0.1 mmol scale using the CEM Liberty Blue automated microwave peptide synthesizer 0.526 g Rink Amide ProTide LL resin (0.19 meq/g substitution). Fmoc-Deprotection was performed with 20% piperidine and 0.1 M Oxyma Pure in DMF. Coupling reactions were performed with 0.2 M Fmoc-AA-OH, 1.0 M DIC in DMF and 1.0 M Oxyma Pure in DMF.⁶ Alloc-Deprotection was performed with 0.0312 M Pd(PPh₃)₄ in DCM and 0.750 M phenylsilane in DCM. Cleavage was performed using the CEM Razor high-throughput peptide cleavage system with 92.5:2.5:2.5:2.5 TFA/H₂O/TIS/DODT. Following cleavage, the peptide was precipitated in Et₂O and lyophilized overnight.

Note: To prevent undesired coupling, the N-terminus (E) remained Fmoc-protected until completion of Alloc-deprotection and lysine side-chain functionalization.

Peptide Synthesis: Ac-ELLELDK(-A)WASLWN-NH₂ via ivDde Deprotection

The peptide was prepared at 0.1 mmol scale using the CEM Liberty Blue automated microwave peptide synthesizer on 0.526 g Rink Amide ProTide LL resin (0.19 meq/g substitution). Fmoc-Deprotection was performed with 20% piperidine and 0.1 M Oxyma Pure in DMF. Coupling reactions were performed with 0.2 M Fmoc-AA-OH, 1.0 M DIC in DMF and 1.0 M Oxyma Pure in DMF.⁶ N-Terminal acetylation was performed with 10% acetic anhydride in DMF. ivDde-Deprotection was performed with 5% hydrazine in DMF. Cleavage was performed using the CEM Razor high-throughput peptide cleavage system with 92.5:2.5:2.5:2.5 TFA/H₂O/TIS/DODT. Following cleavage, the peptide was precipitated in Et₂O and lyophilized overnight.

Note: To prevent undesired coupling, the N-terminus (E) was acetylated prior to ivDde-deprotection and lysine side-chain functionalization

Peptide Analysis

The peptides were analyzed on a Waters Acquity UPLC system with PDA detector equipped with an Acquity UPLC BEH C8 column (1.7 mm and 2.1 x 100 mm). The UPLC system was connected to a Waters 3100 Single Quad MS for structural determination. Peak analysis was achieved on Waters MassLynx software. Separations were performed with a gradient elution of 0.1% TFA in (i) H₂O and (ii) MeCN.

Results

Microwave-enhanced SPPS of ELLELELDK(-A)WASLWN-NH₂ via Mmt-Deprotection on the Liberty Blue automated microwave peptide synthesizer produced the target peptide in 79% purity (**Figure 2**). Microwave-enhanced SPPS of ELLELELDK(-A)WASLWN-NH₂ via Alloc-Deprotection on the Liberty Blue automated microwave peptide synthesizer produced the target peptide in 82% purity (**Figure 3**). Microwave-enhanced SPPS of Ac-ELLELELDK(-A)WASLWN-NH₂ via ivDde-Deprotection on the Liberty Blue automated microwave peptide synthesizer produced the target peptide in 93% purity (**Figure 4**).

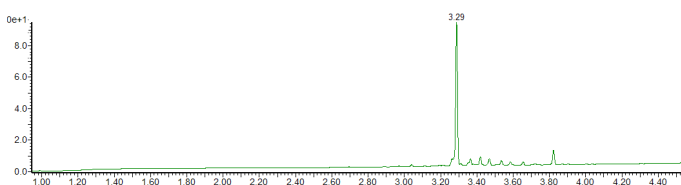


Figure 2: UPLC Chromatogram of ELLELDK(-A)WASLWN-NH₂, synthesized via Mmt-deprotection.

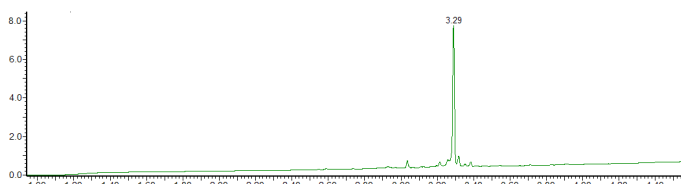


Figure 3: UPLC Chromatogram of ELLELDK(-A)WASLWN-NH₂, synthesized via Alloc-deprotection.



Figure 4: UPLC Chromatogram of Ac-ELLELDK(-A)WASLWN-NH₂, synthesized via ivDde-deprotection.

Conclusion

The Liberty Blue automated peptide synthesizer enables the efficient and successful synthesis of functionalized peptides, compatible with the employment and deprotection of orthogonal and non-standard side-chain protecting groups. Microwave-enhanced SPPS produced branched variants of the HIV-1 antibody epitope gp41₆₅₉₋₆₇₁ by selective deprotection of Lys(Mmt), Lys(Alloc), and Lys(ivDde) and subsequent coupling;

the resulting peptides were furnished in 79%, 82%, and 93% purity respectively. In total, the three peptide variants required less than 9.5 h of synthesis time and less than 1200 mL of wash (DMF) solvent, with each synthesis requiring around 3 h of synthesis time and between 340–450 mL of wash solvent. Importantly, the carbodiimide-activation coupling methodology employed, which increases coupling efficiency and suppresses epimerization, is compatible with these non-standard protecting groups and most-notably, the hyper acid-sensitive Mmt protecting-group.

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United States (Headquarters)

800-726-3331
 704-821-7015
 Fax: 704-821-7894
 info@cem.com

France

33 (01) 69 35 57 80
 Fax: 33 (01) 60 19 64 91
 info.fr@cem.com

Germany, Austria, Switzerland

(49) 2842-9644-0
 Fax: (49) 2842-9644-11
 info@cem.de

Ireland

+353 (0) 1 885 1752
 Fax: +353 (0) 1 885 1601
 info.ireland@cem.com

Italy

(39) 35-896224
 Fax: (39) 35-891661
 info.srl@cem.com

Japan

+81-3-5793-8542
 Fax: +81-3-5793-8543
 info@cemjapan.co.jp

United Kingdom

(44) 1280-822873
 Fax: (44) 1280-822873
 info.uk@cem.com

www.cem.com

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