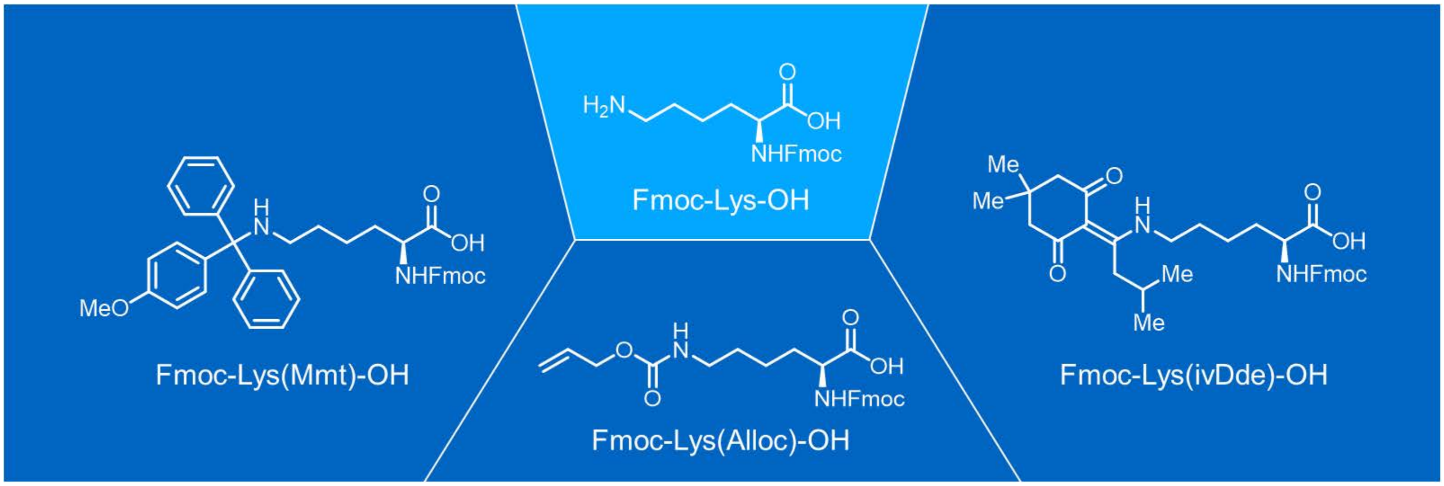
Automated Deprotection of Orthogonal and Non-Standard Lysine Protecting Groups



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 gp41$_{659–671}$, by Lys(Mmt)-, Lys(Alloc)-, and Lys(ivDde)-deprotection; subsequent coupling produced the variants in 79%, 82%, and 93% purity respectively. The three gp41$_{659–671}$ 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.

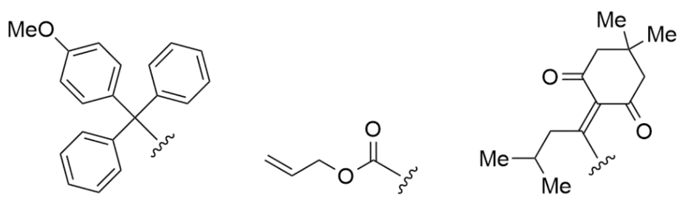


Figure 1: Common, orthogonal lysine protecting groups

To demonstrate the Liberty Blue automated peptide synthesizer's ability to efficiently synthesize and functionalize peptides, branched variants of the HIV-1 antibody epitope gp41$_{659–671}$ (ELLELDKWASLWN) were synthesized.[5] Lys(Mmt), Lys(Alloc), or Lys(ivDde) were incorporated into the synthesis of gp41$_{659–671}$, 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-dioxa-1,8-octanedithiol (DODT), hydrazine, phenylsilane, tetrakis(triphenylphosphine)-palladium(0) ($Pd(PPh_3)_4$), triisopropylsilane (TIS), and acetic acid were obtained from Sigma-Aldrich (St. Louis, MO). Dichloromethane (DCM), N,N-dimethylformamide (DMF), and anhydrous diethyl ether (Et_2O) were obtained from VWR (West Chester, PA). HPLC-grade water (H_2O), and HPLC-grade acetonitrile (MeCN) were obtained from Fisher Scientific (Waltham, MA).

Peptide Synthesis: ELLELDK(-A)WASLWN-NH_2 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/H_2O/TIS/DODT. Following cleavage, the peptide was precipitated in Et_2O 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-NH_2 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 $Pd(PPh_3)_4$ 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/H_2O/TIS/DODT. Following cleavage, the peptide was precipitated in Et_2O 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-NH_2 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/H_2O/TIS/DODT. Following cleavage, the peptide was precipitated in Et_2O and lyophilized overnight.

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

Method Programming

Peptide to AA Single Coupling

Mmt, Alloc, or ivDde
KWASLWN-NH_2

Fmoc-Deprotection solution (4 mL) was added to the peptide-containing reaction vessel and the solution was microwaved for 1 min at 90 °C. Following deprotection, the peptide was washed with DMF (4 x 4 mL). Then, amino acid (2.5 mL), DIC (1 mL) and Oxyma Pure (0.5 mL) were added to the reaction vessel and the solution was microwaved for 2 min at 90 °C. Upon completion, the reaction vessel was drained, preparing the peptide for the next synthetic transformation.

Peptide to AA Double Coupling

Mmt, Alloc, or ivDde
ELLELDKWASLWN-NH_2

Fmoc-Deprotection solution (4 mL) was added to the peptide-containing reaction vessel and the solution was microwaved for 1 min at 90 °C. Following deprotection, the peptide was washed with DMF (4 x 4 mL). Then, amino acid (2.5 mL), DIC (1 mL) and Oxyma Pure (0.5 mL) were added to the reaction vessel and the solution was microwaved for 2 min at 90 °C, whereupon the reaction vessel was drained and additional amino acid (2.5 mL), DIC (1 mL) and Oxyma Pure (0.5 mL) were added. The solution was microwaved for another 2 min at 90 °C. Upon completion, the reaction vessel was drained, preparing the peptide for the next synthetic transformation.

Mmt Deprotection

Mmt → NH_2
ELLELDKWASLWN-NH_2 → ELLELDKWASLWN-NH_2

2% TFA in DCM (4 mL) was added to the peptide-containing reaction vessel and the solution was bubbled for 2 min at 23 °C, whereupon the reaction vessel was drained and a wash thru manifold was performed. The peptide was washed with DCM (5 x 3 mL), and then with DMF (5 x 4 mL). The entire procedure was performed four additional times, preparing the peptide for side-chain coupling.

Alloc-Deprotection[7]

Alloc → NH_2
ELLELDKWASLWN-NH_2 → ELLELDKWASLWN-NH_2

The peptide was washed with DMF (4 x 4 mL), and then DCM (4 x 4 mL). Then, 0.750 M phenylsilane in DCM (2 mL) was added to the reaction vessel. After a 90 s wait period, 0.0312 M $Pd(PPh_3)_4$ in DCM (8 mL) was added to the reaction vessel and the solution was microwaved for 5 min at 35 °C. Upon completion, the reaction vessel was drained, the peptide was washed with DCM (4 x 4 mL), and additional 0.750 M phenylsilane in DCM (2 mL) was added to the reaction vessel. After another 90 s wait period, additional 0.0312 M $Pd(PPh_3)_4$ in DCM (8 mL) was added to the reaction vessel and the solution was microwaved for 5 min at 35 °C. The reaction vessel was drained, the peptide was washed with DCM (4 x 4 mL), two wash thru manifolds were performed, and the peptide was washed with DMF (4 x 4 mL), preparing the peptide for side-chain coupling.

N-Terminal Acetylation

ivDde → ivDde
ELLELDKWASLWN-NH_2 → Ac-ELLELDKWASLWN-NH_2

Note: This procedure was performed prior to ivDde-deprotection. Hydrazine will deprotect Fmoc; if not considered, undesired coupling will occur at the peptide's N-terminus.

Fmoc-Deprotection solution (4 mL) was added to the peptide-containing reaction vessel and the solution was microwaved for 1 min at 90 °C. Following deprotection, the peptide was washed with DMF (4 x 4 mL). Then, 10% v/v acetic anhydride in DMF was added to the reaction vessel and the solution was microwaved for 2 min at 65 °C. Upon completion, the reaction vessel was drained and a Wash Thru Manifold was performed. The peptide was washed with DMF (4 x 4 mL), preparing the peptide for ivDde-deprotection.

Note: Due to the highly polar nature of acetic anhydride, a modified microwave method (**Table 1**) was used to minimize temperature overshoot

Table 1. N-Terminal Acetylation Microwave Method

Stage	Temperature (°C)	Power (W)	Hold Time (sec)	ΔT (°C)
1	65	40	30	5
2	65	0	30	5
3	65	40	30	5
4	65	0	30	5

ivDde-Deprotection

ivDde → NH_2
Ac-ELLELDKWASLWN-NH_2 → Ac-ELLELDKWASLWN-NH_2

5% Hydrazine in DMF (4 mL) was added to the peptide-containing reaction vessel and the solution was microwaved for 3 min at 90 °C, whereupon the reaction vessel was drained and a wash thru manifold was performed. The peptide was then washed with DMF (5 x 4 mL). This procedure was performed two additional times, preparing the peptide for side-chain coupling.

Side-Chain Coupling

NH_2 → A
ELLELDKWASLWN-NH_2 → ELLELDKWASLWN-NH_2
and
NH_2 → A
Ac-ELLELDKWASLWN-NH_2 → Ac-ELLELDKWASLWN-NH_2

Amino acid (2.5 mL), DIC (1 mL) and Oxyma Pure (0.5 mL) were added to the reaction vessel and the solution was microwaved for 4 min at 90 °C, whereupon the reaction vessel was drained and additional amino acid (2.5 mL), DIC (1 mL) and Oxyma Pure (0.5 mL) were added. The solution was microwaved for another 4 min at 90 °C. Upon completion, the reaction vessel was drained and a final Fmoc-deprotection performed, preparing the peptide for cleavage and analysis.

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) H_2O and (ii) MeCN.

Results

Microwave-enhanced SPPS of ELLELEDK(–A)WASLWN-NH_2 via Mmt-Deprotection on the Liberty Blue automated microwave peptide synthesizer produced the target peptide in 79% purity (**Figure 2**). Microwave-enhanced SPPS of ELLELEDK(–A)WASLWN-NH_2 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-ELLELEDK(–A)WASLWN-NH_2 via ivDde-Deprotection on the Liberty Blue automated microwave peptide synthesizer produced the target peptide in 93% purity (**Figure 4**).

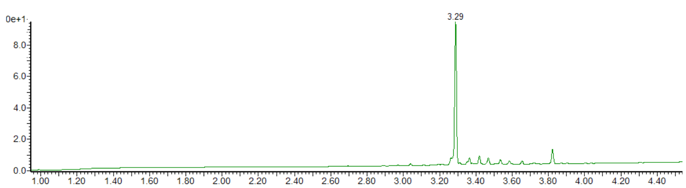


Figure 2: UPLC Chromatogram of ELLELDK(–A)WASLWN-NH_2, synthesized via Mmt-deprotection.

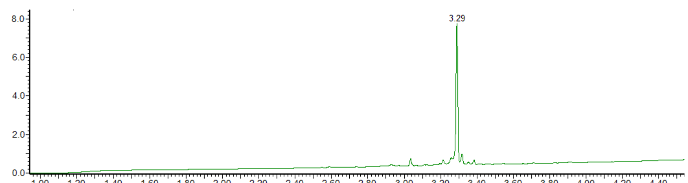


Figure 3: UPLC Chromatogram of ELLELDK(–A)WASLWN-NH_2, synthesized via Alloc-deprotection.

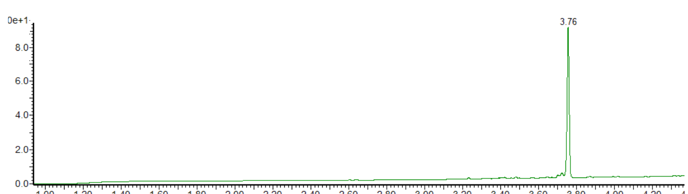


Figure 4: UPLC Chromatogram of Ac-ELLELDK(–A)WASLWN-NH_2, synthesized via ivDde-deprotection.

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 $gp41_{659–671}$ 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.

References

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