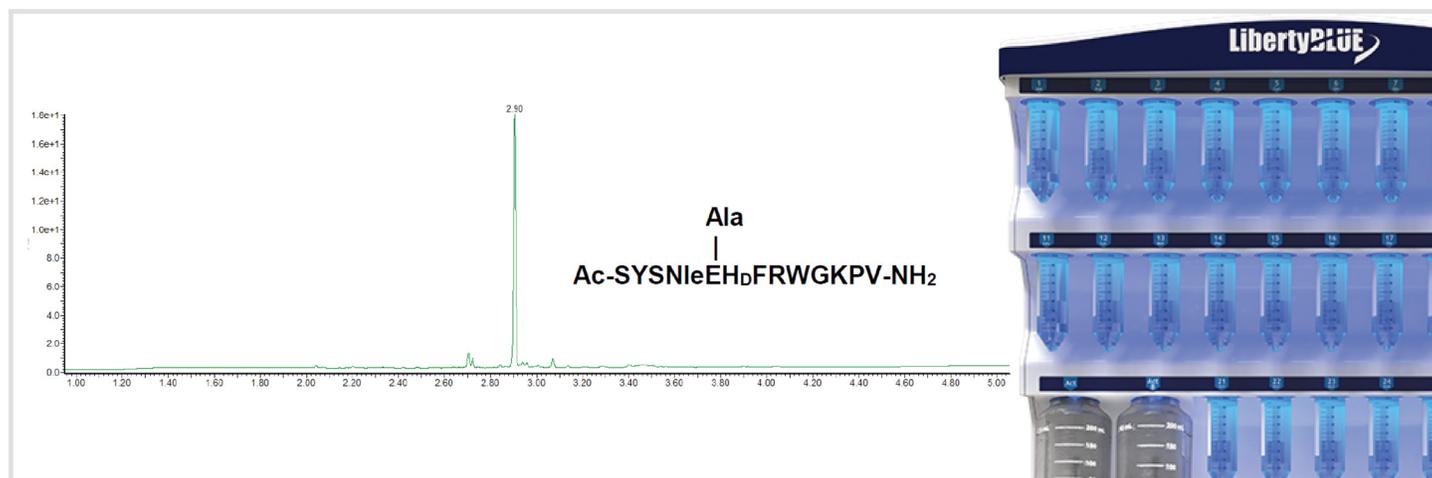


Automated Orthogonal Deprotection of Glu(OAllyl) and Peptide Stapling via Lactamization



Summary

Orthogonal deprotection of Glu(OAllyl) and peptide stapling via lactamization can be readily performed on the Liberty Blue™ automated microwave peptide synthesizer. Branched and lactam-stapled variants of both the HIV-1 antibody epitope gp41₆₅₉₋₆₇₁ and Afamelanotide were synthesized, with each of the four peptides synthesized between 80 – 87% purity and requiring 3 – 3.5 h of synthesis time, generating only 700 – 750 mL of total waste per synthesis.

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, including glutamic acid.

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 glutamic acid, the allyl ester (OAllyl) is commonly used (**Figure 1**), requiring catalytic Pd(0) and phenylsilane for deprotection.

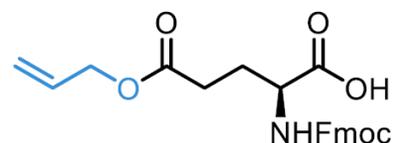


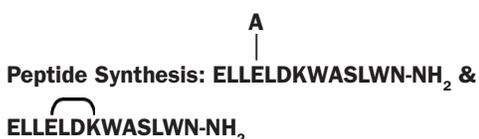
Figure 1: Fmoc-Glu(OAllyl)-OH

To demonstrate the Liberty Blue automated peptide synthesizer's ability to efficiently synthesize and functionalize peptides, branched and lactam-stapled variants of the HIV-1 antibody epitope gp41₆₅₉₋₆₇₁ (ELLELDKWASLWN) and Afamelanotide (Ac-SYSNleEHdFRWGKPV-NH₂) were synthesized. For branched variants, H-Ala-OtBu was coupled to the orthogonally deprotected Glu side-chain. For cyclized variants, Lys(Alloc) was incorporated into the linear synthesis and simultaneously deprotected with Glu(OAllyl) prior to lactam-stapling.

Materials and Methods

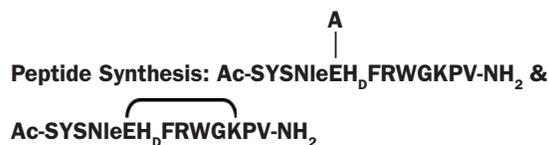
Reagents

Fmoc-Glu(OAllyl)-OH, Fmoc-Lys(Alloc)-OH, Fmoc-Nle-OH, Fmoc-D-Phe-OH, and H-Ala-OtBu · HCl (free-based prior to use) 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), Arg(Pbf), Glu(OtBu), His(Boc), Lys(Boc), Ser(tBu), and Tyr(tBu). Oxyma Pure and Rink Amide ProTide™ LL resin were obtained from CEM Corporation (Matthews, NC). N,N-Diisopropylcarbodiimide (DIC) and hydroxybenzotriazole (HOBt) were obtained from CreoSalus (Louisville, KY). Piperidine was obtained from Alfa Aesar (Ward Hill, MA). Phenylsilane, tetrakis(triphenylphosphine)-palladium(0), acetic anhydride, trifluoroacetic acid (TFA), 3,6-dioxa-1,8-octanedithiol (DODT), 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).



The peptides were prepared at 0.1 mmol scale using the CEM Liberty Blue automated microwave peptide synthesizer on 0.556 g Rink Amide ProTide LL resin (0.18 meq/g substitution). Deprotection was performed with 20% piperidine and 0.1 M Oxyma Pure in DMF. Coupling reactions were performed with a 5-fold excess of Fmoc-AA-OH, 1.0 M DIC in DMF and 1.0 M Oxyma Pure in DMF.⁵ Alloc-Deprotection was performed with 0.025 M Pd(PPh₃)₄ in DCM and 0.50 M phenylsilane in DCM. Peptide stapling via lactamization was performed with a solution of 0.34 M DIC and 0.17 M HOBt 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: The conditions employed in these Alloc-deprotection methods require less Pd than those in the following section, but longer heating periods.



The peptides were prepared at 0.1 mmol scale using the CEM Liberty Blue automated microwave peptide synthesizer on 0.556 g Rink Amide ProTide LL resin (0.18 meq/g substitution). Deprotection was performed with 20% piperidine and 0.1 M Oxyma Pure in DMF. Coupling reactions were performed with a 5-fold excess of Fmoc-AA-OH, 1.0 M DIC in DMF and 1.0 M Oxyma Pure in DMF.⁵ Alloc-Deprotection was performed with 0.0312 M Pd(PPh₃)₄ in DCM and 0.750 M phenylsilane in DCM. Peptide stapling via lactamization was performed with a solution of 0.34 M DIC and 0.17 M HOBt in DMF. N-terminal acetylation was performed with 10% acetic anhydride 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: The conditions employed in these Alloc-deprotection methods require more Pd equivalents than those in the previous section, but shorter heating periods.



Peptide to AA Coupling:



Deprotection (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 coupling reaction.

Peptide to Glu(OAllyl) Coupling:



Deprotection (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 Glu(OAllyl) Deprotection.

Note: It is necessary to deprotect and functionalize immediately upon Glu(OAllyl) incorporation to prevent undesired capping due to pyroglutamic acid formation.

Glu(OAllyl) Deprotection:⁶



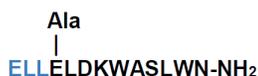
The peptide was washed with DMF (4 x 4 mL), and then DCM (4 x 4 mL). Then, 0.500 M phenylsilane in DCM (3 mL) was added to the reaction vessel. After a 90 s wait period, 0.025 M Pd(PPh₃)₄ in DCM (1 mL) was added to the reaction vessel and the solution was microwaved for 12 min at 30°C. Upon completion, the reaction vessel was drained and the peptide washed with DCM (4 x 4 mL). This procedure was performed two additional times. Upon completion, two wash thru manifolds were performed (4 mL) and the peptide was washed with DMF (4 x 4 mL), preparing the peptide for side-chain coupling.

Side-Chain Coupling:



Amino acid (H-Ala-OtBu, 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 (H-Ala-OtBu, 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, preparing the peptide for the next coupling reaction.

Peptide to AA Coupling:



Deprotection (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 coupling reaction.

Method Programming: ELLELDKWASLWN-NH₂

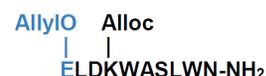
Peptide to AA Coupling:



Deprotection (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 coupling reaction.

Peptide to Glu(OAllyl) Coupling:



Deprotection (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 Glu(OAllyl) and Lys(Alloc) Deprotection.

Note: It is necessary to deprotect and functionalize immediately upon Glu(OAllyl) incorporation to prevent undesired capping due to pyroglutamic acid formation.

Simultaneous Glu(OAllyl) and Lys(Alloc) Deprotection:⁶

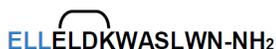


The peptide was washed with DMF (4 x 4 mL), and then DCM (4 x 4 mL). Then, 0.500 M phenylsilane in DCM (3 mL) was added to the reaction vessel. After a 90 s wait period, 0.025 M Pd(PPh₃)₄ in DCM (2 mL) was added to the reaction vessel and the solution was microwaved for 12 min at 30°C. Upon completion, the reaction vessel was drained and the peptide washed with DCM (4 x 4 mL). This procedure was performed two additional times. Upon completion, two wash thru manifolds were performed (4 mL) and the peptide was washed with DMF (4 x 4 mL), preparing the peptide for side-chain stapling via lactamization.

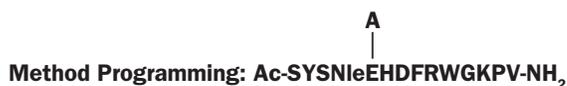
Side-Chain Stapling via Lactamization:



A solution of 0.34 M DIC and 0.17 M HOBT in DMF (8 mL) was added to the reaction vessel and the solution was microwaved for 10 min at 90 °C, whereupon the reaction vessel was drained. This procedure was performed two additional times. Upon completion, the peptide was washed with DMF (4 x 4 mL), preparing the peptide for the next coupling reaction.

Peptide to AA Coupling:

Deprotection (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 coupling reaction.

Peptide to AA Coupling:

Deprotection (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 coupling reaction.

Peptide to Glu(OAllyl) Coupling:

Deprotection (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 Glu(OAllyl) Deprotection.

Note: It is necessary to deprotect and functionalize immediately upon Glu(OAllyl) incorporation to prevent undesired capping due to pyroglutamic acid formation.

Glu(OAllyl) Deprotection:⁶

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(PPh₃)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 and the peptide

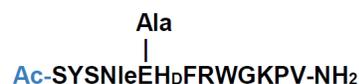
washed with DCM (4 x 4 mL). This procedure was performed one additional time. Upon completion, two wash thru manifolds were performed (4 mL) and the peptide was washed with DMF (4 x 4 mL), preparing the peptide for side-chain coupling.

Side-Chain Coupling:

Amino acid (H-Ala-OtBu, 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 (H-Ala-OtBu, 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, preparing the peptide for the next coupling reaction.

Peptide to AA Coupling:

Deprotection (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 coupling reaction.

N-Terminal Acetylation:⁷

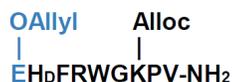
Deprotection (4 mL) was added to the peptide-containing reaction vessel and the solution was microwaved for 1 min at 90 °C. Following deprotection, the peptide was washed with DMF (4 x 4 mL). Then, 10% v/v acetic anhydride in DMF (4 mL) was added to the reaction vessel and the solution was microwaved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

Method Programming: Ac-SYSNleEHDFRWGKPV-NH₂Peptide to AA Coupling:

Deprotection (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 coupling reaction.

Peptide to Glu(OAllyl) Coupling:



Deprotection (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 Glu(OAllyl) and Lys(Alloc) deprotection.

Note: It is necessary to deprotect and functionalize immediately upon Glu(OAllyl) incorporation to prevent undesired capping due to pyroglutamic acid formation.

Simultaneous Glu(OAllyl) and Lys(Alloc) Deprotection:⁶



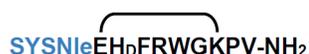
The peptide was washed with DMF (4 x 4 mL), and then DCM (4 x 4 mL). Then, 0.750 M phenylsilane in DCM (2 mL) was added to the reaction vessel. After a 90 s wait period, 0.0312 M Pd(PPh₃)₄ in DCM (8 mL) was added to the reaction vessel and the solution was microwaved for 5 min at 35°C. Upon completion, the reaction vessel was drained and the peptide washed with DCM (4 x 4 mL). This procedure was performed one additional time. Upon completion, two wash thru manifolds were performed (4 mL) and the peptide was washed with DMF (4 x 4 mL), preparing the peptide for side-chain stapling via lactamization.

Side-Chain Stapling via Lactamization:



A solution of 0.34 M DIC and 0.17 M HOBT in DMF (8 mL) was added to the reaction vessel and the solution was microwaved for 10 min at 90 °C, whereupon the reaction vessel was drained. This procedure was performed two additional times. Upon completion, the peptide was washed with DMF (4 x 4 mL), preparing the peptide for the next coupling reaction.

Peptide to AA Coupling:



Deprotection (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 coupling reaction.

N-Terminal Acetylation:⁷



Deprotection (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 (4 mL) 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 performed (4 mL). The peptide was washed with DMF (4 x 4 mL), preparing the peptide for cleavage.

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 the Glu-branched variant of the HIV-1 antibody epitope gp41₆₅₉₋₆₇₁ on the Liberty Blue automated microwave peptide synthesizer produced the target peptide in 82% purity (**Figure 2**). Microwave-enhanced SPPS of the lactam-stapled variant of the HIV-1 antibody epitope gp41₆₅₉₋₆₇₁ on the Liberty Blue automated microwave peptide synthesizer produced the target peptide in 87% purity (**Figure 3**). Microwave-enhanced SPPS of the Glu-branched variant of Afamelanotide on the Liberty Blue automated microwave peptide synthesizer produced the target peptide in 85% purity (**Figure 4**). Microwave-enhanced SPPS of the lactam-stapled variant of Afamelanotide on the Liberty Blue automated microwave peptide synthesizer produced the target peptide in 80% purity (**Figure 5**).

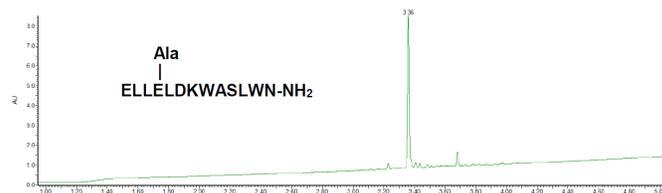


Figure 2: UPLC Chromatogram of the Glu-branched variant of HIV-1 antibody epitope gp41₆₅₉₋₆₇₁.

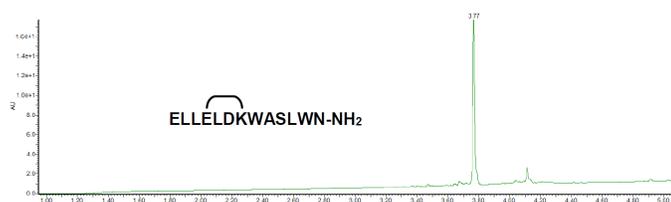


Figure 3: UPLC Chromatogram of the lactam-stapled variant of HIV-1 antibody epitope gp41₆₅₉₋₆₇₁.

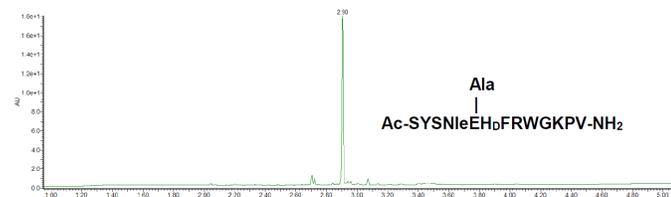


Figure 4: UPLC Chromatogram of the Glu-branched variant of Afamelanotide.

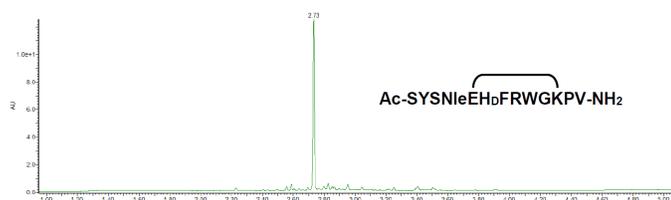


Figure 5: UPLC Chromatogram of the lactam-stapled variant of Afamelanotide.

Conclusions

The Liberty Blue automated peptide synthesizer enables the simple and efficient synthesis of variously functionalized peptides. With the capability of performing automated orthogonal deprotections (and contingent synthetic manipulations), branched and lactam-stapled variants of both the HIV-1 antibody epitope gp41₆₅₉₋₆₇₁ and Afamelanotide were synthesized. Each of the 4 peptides were synthesized between 80–87% purity and required 3–3.5 h of synthesis time, generating only 700 – 750 mL of total waste per synthesis.

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- (5) CEM Application Note (APO124) - "CarboMAX - Enhanced Peptide Coupling at Elevated Temperature."
- (6) Automated Alloc-deprotection adapted from: Wilson, K. R.; Sedberry, S.; Pescatore, R.; Vinton, D.; Love, B.; Ballard, S.; Wham, B. C.; Hutchison, S. K.; Williamson, E. J. *J. Pep. Sci.* **2016**, *22*, 622–627.
- (7) CEM Application Note (APO114) - "Automated N-Terminal Acetylation."

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