Synthesis of therapeutic stapled peptides: Fully automated peptide cyclization via olefin metathesis

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Abstract

Cyclic peptides are an important tool for development of peptide therapeutics. Their increased rigidity allows for increased membrane permeability and increased stability to proteases, two major drawbacks of peptide drug development. Peptide cyclization can be achieved several ways, including head-to-tail cyclizations or side chain-to-side chain cyclizations, which can be done by lactam bridge formation, disulfide bonds or hydrocarbon stapling via olefin metathesis, to name a few. Hydrocarbon stapling stabilizes α-helical structures and thus increases cell permeability and stability. Other advantages of stapled peptides include the targeting of protein-protein interactions, important for targeting many disease states such as cancer.

Zhang et al. have described the synthesis and properties of an i, i+4 stapled peptide, NYAD-1 (Figure 1), which targets the capsid and inhibits HIV-1 in cell culture [1].

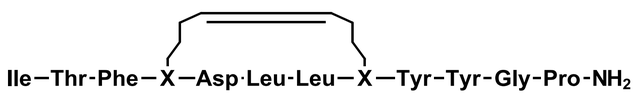

Figure 1. NYAD-1 stapled peptide structure.
X = (S)-2-(4′-pentenyl)alanine

A small GTPase RAB25, has been found to have both pro-oncogenic and anti-oncogenic phenotypes in specific cellular contexts. Mitra et al. have shown several cell permeable peptides, including RFP14 peptide (Figure 2), that disrupt RAB25:FIP complex formation in vitro and in situ and compete with the context-specific phenotypes associated with RAB25 function in ovarian and breast cancer cell lines [2].

Figure 2. RFP14 peptide structure

X= (S)-2-(4′-pentenyl)alanine

Here we show the fully automated synthesis, from linear, on-resin cyclization, and resin cleavage, of NYAD-1 and RFP14.

Method & Analysis

Protocol A: NYAD-1 was synthesized on Rink MBHA resin (0.33 mmol/g) at 10 µmol scale. Deprotection was done with 20% piperidine in DMF for 2 x 5 min. The coupling reaction was done with 100 mM amino acid and HATU, 400 mM NMM for 2 x 20 min with ten-fold excess for natural amino acids, 1 x 30 min with five-fold excess for unnatural amino acid.

Protocol B: RFP14 was synthesized using Rink Amide ChemMatrix Resin (0.54 mmol/g substitution) at a 25 umol scale. Deprotection time was 2 x 1:30 min at 25°C, 60°C, or 90°C using 20% Piperidine in DMF. Amino acids were coupled using a six-fold excess and final concentration of 100 mM for amino acid and HCTU and 200 mM for DIEA. Coupling time was 2:30 min at 25°C, 60°C, or 90°C. A capping step was done after every coupling reaction for 5 min. DMF washes followed deprotect, coupling, and capping steps with 3 repetitions at 30 seconds each.

Metathesis Reactions: The resin bound Fmoc-protected peptide was treated with a 10 mM solution of Grubbs catalyst (Bis(tricyclohexylphosphine) benzylidene ruthenium(IV) dichloride) in 1,2-Dichloroethane (2 mL) for 2 X 2 hours.

Cleavage: Final cleave used TFA:TIS:EDT:Water (95:1:2.5:2.5) for 2 hours at 25°C on the instrument followed by precipitation in ether.

Analysis

NYAD-1 was analyzed using a C18, 300 Å, 5 m, 250 x 4.6 mm column (Varian Microsorb-MV), with a gradient of 5-95%B in 60 min using Water (0.1%TFA):ACN(0.1%TFA) at 1ml/min. A 1:10 dilution of a standard sample of 3 mg/ml was run on a Phenomenex Kinetex 2.6 um C18 100A 50x2.1 mm column for LCMS with a gradient of 5-50%B in 7 min using Water(0.1%FA):ACN(0.1%FA) at 1 ml/min.

RFP14 was analyzed using a C18, 180 Å, 4.6 um, 250 X 4.6 mm Polaris column (Agilent), over 30 min with a flow rate of 1 mL/min and a gradient of 5-95% B where A is 0.1% TFA in water and B is 0.1% TFA in acetonitrile. Detection was done at 214 nm. Mass analysis was done on a Shimadzu LCMS-2020 Single-Quad mass spectrometer, equipped with a C18, 100 Å, 2.6 um, 250 x 2.1 mm Polaris column (Agilent), over 30 min with a flow rate of 1 mL/min and a gradient of 5-95% B where A is 0.1% formic acid in water and B is 0.1% formic acid in acetonitrile.

Results

NYAD-1

NYAD-1 was successfully synthesized on an automated peptide synthesizer with a 31.4% crude purity using HATU and a conservative protocol.

Table 1. *Crude purity of stapled **NYAD-1.***

Peptide	Crude Purity
Stapled NYAD-1	31.4%

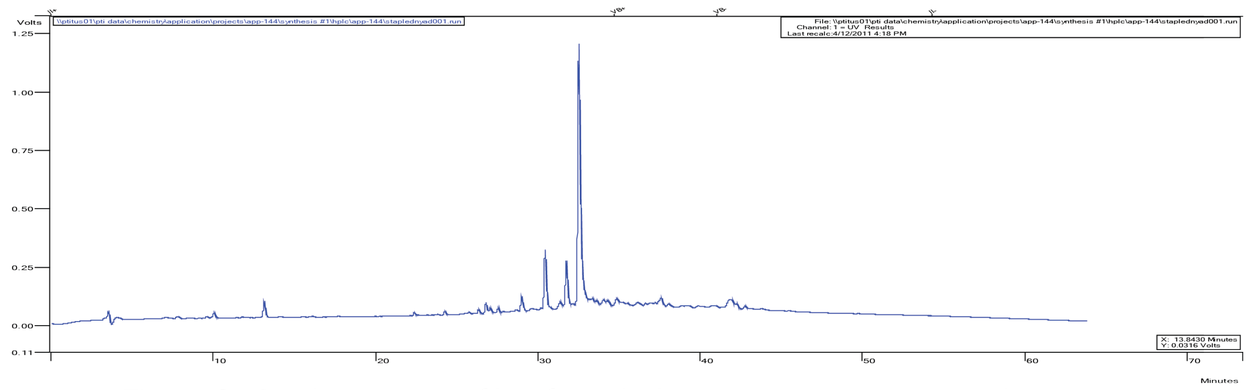

Figure 3. *Crude purity profile of stapled NYAD-1 using HATU and NMM.*

RFP14

RFP14 was synthesized in parallel at three different temperatures, 25°C, 60°C, and 90°C using a fast protocol of 2:30 min coupling time using HCTU. The best crude purity was seen at the higher 90°C temperature (Table 2, Figure 4A).

Peptide	25°C	60°C	90°C
Linear RFP14	28.7%	27.7%	59.6%

Table 2. *Effect of temperature on crude purity of linear **RFP14.***

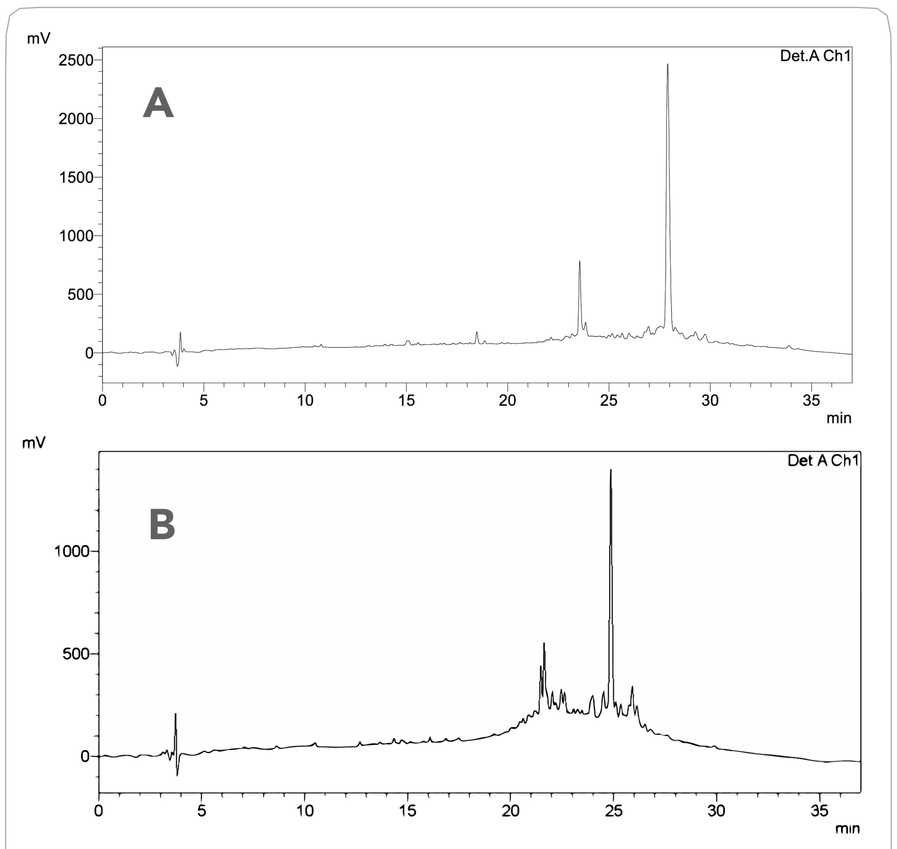

Figure 4. *Crude purity profiles of:*
A) linear RFP14 (at 90°C) and B) stapled RFP14

Conclusion

- Therapeutic stapled peptides were successfully synthesized under variable conditions on automated peptide synthesizers
- NYAD-1 was synthesized with 31.4% crude purity using a conservative protocol with a highly reactive coupling reagent, HATU
- Independent induction heating on the Prelude X allowed multiple temperatures to be screened simultaneously on the synthesis of the linear RFP14 peptide
- RFP14 crude purity improved with an increase in temperature up to 90°C

References

1 Zhang, H. et al. Dual-acting stapled peptides target both HIV-1 entry and assembly. Retrovirology 10, 1–20 (2013).

2 Mitra, S. et al. Nat. Commun. 2017, 8 (1).

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