Fast Solid Phase Peptide Synthesis Method Development for the Synthesis of Complex Peptides

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Abstract

With the advancement of peptides into the clinic the complexity of peptide sequences has continued to increase. With more complex sequences, synthesis difficulties arise, for example, sterically hindered coupling reactions, increased hydrophobicity, among others. Parallel synthesis optimization with induction heating was performed on the Solid Phase Peptide Synthesis (SPPS) of peptides with sterically hindered sequences looking for improvements in crude peptide purity. Several conditions were tested in parallel, including different temperatures, reaction times, and different coupling reagents. For example, the crude purity for Aib-ACP was improved from 7.1% using HCTU to 92.5% by changing the coupling reagent from HCTU to COMU and increasing the reaction temperature to 75°C using short reaction times (2 x 3 min).

Aib-ACP Sequence:

H-Val-Gln-Aib-Aib-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2

Other examples including cyclic peptides like APY-d4. Abnormal EphA4 receptor tyrosine kinase activity can inhibit neutral neural repair after injury and intensify neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and Alzheimer's. Peptide stability is important when developing potential drug candidates and Olson et al. found a cyclic peptide that had good binding affinity with reduced plasma stability[1]. Introducing a b-Ala modification resulted in improved plasma stability and increased receptor affinity.

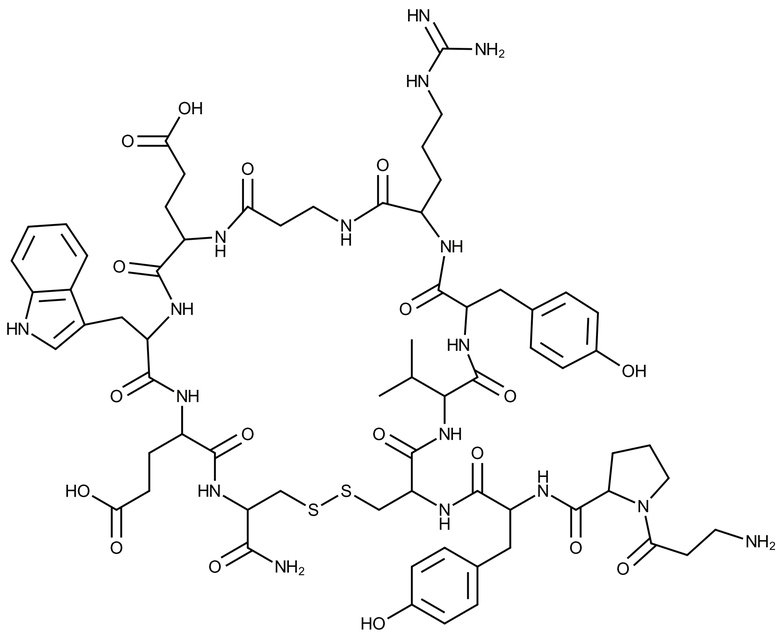

Figure 1. *APY-d4 cyclic structure.*

Method & Analysis

Protocol A: Aib-ACP was synthesized using Rink Amide MBHA low loaded Resin (0.27mmol/g loading) at a 25 µmol scale. Deprotection time was 2 x 1 minute at 25°C, 75°C, or 90°C using 20% Piperidine in DMF. Amino acids were coupled using a six-fold excess and final concentration of 100mM for Amino Acids and Activators and 200mM for DIEA. Coupling time was 1 x 10 min or 2 x 10 min at 25°C, 75°C, or 90°C for the second Aib and 1 x 3 min or 2 x 3 min at 25°C, 75°C, or 90°C for all other amino acids in the sequence. DMF washes followed both deprotect and coupling steps with 3 repetitions at 30 seconds each.

Protocol B: Using Rink Amide ChemMatrix resin (0.47 mmol/g) APY-d4 was synthesized in a 50 µmol scale using 300 mM amino acids and HCTU, and 600 mM DIPEA. Deprotections were done as 2 x 1 min using the UVXtend on the Prelude® X at 25°C, 60°C, 90°C. Coupling reactions were done for 3 min at 25°C, 60°C, 90°C. DMF washes followed both deprotect and coupling steps with 3 repetitions at 30 seconds each.

Cyclization: The resin bound Fmoc-protected peptide was treated with 2 equivalents of fresh $Tl(CF_3CO_2)_3$ for 2 x 20 min on the Prelude X.

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

Analysis: APY-d4 and Aib-ACP were analyzed using a C18 Kinetex Evo, 2.1 um, 50 x 4.6 mm column with a gradient of 5-95%B in 5 min using Water (0.1%TFA):ACN(0.1%TFA) at 0.8 ml/min on a ThermoScientific U3000RS. A 1:10 dilution of a standard sample of 3 mg/ml was run on a Shimadzu LCMS-2020 Single-Quad mass spectrometer using a C18, 300 Å, 5 µm, 50 x 4.6 mm column (Varian Microsorb-MV), with a gradient of 5-95%B in 9 min using Water(0.1%FA):ACN(0.1%FA) at 1 ml/min.

Results

Difficult couplings – Aib-ACP synthesis optimization

Synthesis of sterically hindered Aib-ACP at room temperature was significantly higher when using COMU as the coupling reagent (Table 1) regardless of the coupling time.

In the search for optimized synthesis time of difficult peptides, induction heating was assessed. Single coupling reactions at 75°C resulted in similar crude purity to double couplings of 30 min at 25°C. With double coupling the crude purity of Aib-ACP was significantly improved up to 91% (Table 2).

The highest crude purity was obtained at 75°C, while increasing the temperature further to 90°C did improve crude purities compared to 25°C, it was not the best overall for the synthesis of Aib-ACP.

Table 1*. Effect of coupling reagents and reaction time on crude purity of* ***Aib-ACP*** *at 25°C.*

Reagent	2 x 3 min	2 x 30 min
HCTU	7.8%	45.2%
COMU	35.5%	74.3%

Table 2*. Effect of temperature and couplings on crude purity of* ***Aib-ACP****, using 10 min couplings for Aib-Aib with COMU.*

Coupling	25°C	75°C	90°C
Single couplings	34.1%	74.0%	65.9%
Double couplings	74.5%	91.1%	78.3%

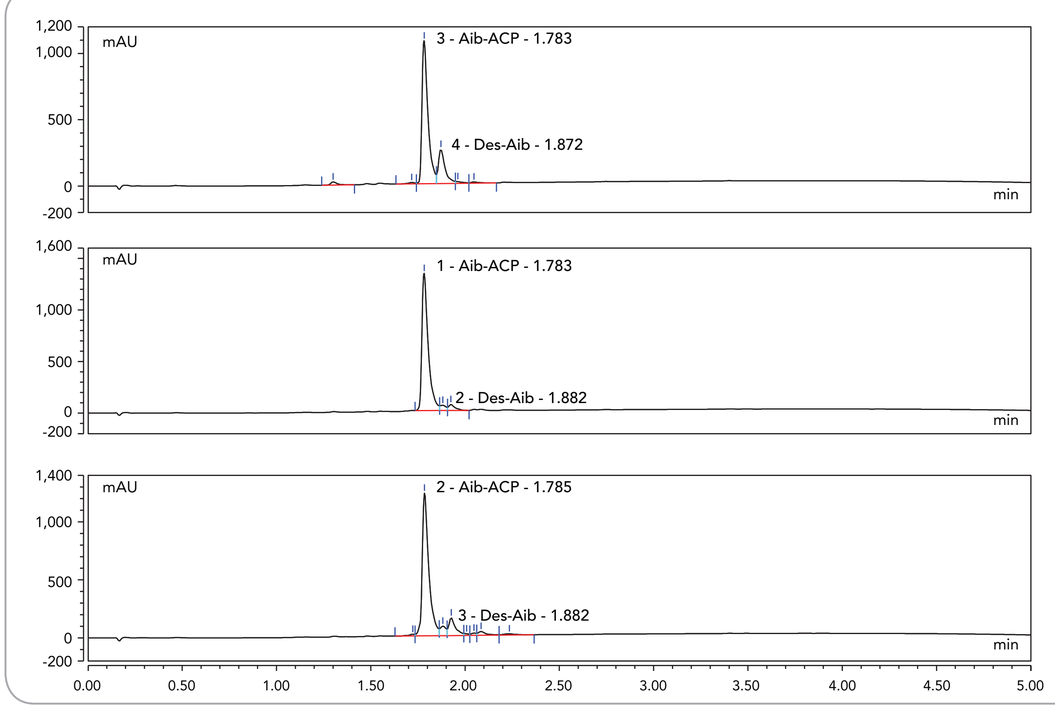

Figure 2. *Crude purity profiles of Aib-ACP synthesized using 2 x 3 min with COMU, and 2 x 10 min for Aib-Aib coupling at A) 25°C, B) 75°C and C) 90°C.*

APY-d4 synthesis optimization

APY-d4 was successfully synthesized on an automated peptide synthesizer with an overall **58.2%** crude purity using HCTU using a fast protocol. The linear crude purity was slightly improved by increasing the temperature to 90°C.

Table 3*. Crude purity of linear APY-d4*

Peptide	25°C	60°C	90°C
Linear APY-d4	75.3%	78.3%	80.2%

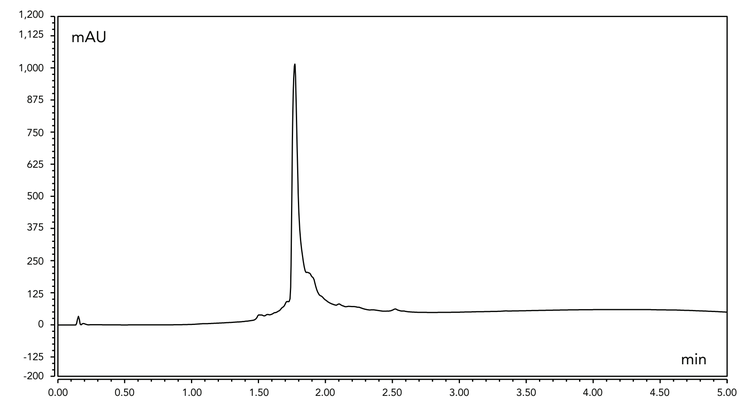

Figure 3. *Crude purity profile of cyclized APY-d4.*

Conclusion

- Independent induction heating on the Prelude X allowed multiple temperatures to be screened simultaneously on the synthesis of the linear peptides for increased crude purity in a reduced synthesis time
- Sterically hindered Aib-ACP was successfully synthesized in high purity using short coupling times using COMU at 75°C on Rink Amide MBHA low-loaded resin
- Cyclized APY-d4 was fully synthesized on the Prelude X with 58.4% crude purity using a fast protocol with a highly reactive coupling reagent, HCTU
- APY-d4 linear crude purity improved with an increase in temperature up to 90°C using Rink Amide ChemMatrix resin
- Future studies include optimization of the cyclization reaction on the automated synthesizer
- Increased crude purity with increased temperature is peptide sequence dependent, Aib-ACP had highest crude purity at 75 °C, while APY-d4 had highest crude purity at 90 °C

Reference

1. Olson EJ, et al. ACS Med. Chem. Lett. 2016; 7: 841–846.

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