Evaluation of On-Resin Head-to-Tail Cyclization Protocols of Peptides Using Automated Peptide Synthesizers

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Introduction

Intracellular protein-protein interactions (PPI) have been considered "undruggable" targets for traditional drug modalities such as small molecules and monoclonal antibodies. The reason being that PPIs do not contain well-defined binding pockets common to small molecule targets nor is it easy for monoclonal antibodies to reside intracellularly due to their large size.

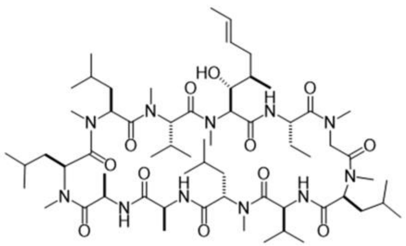

Figure 1: Naturally occurring cyclic peptide capable of passive diffusion across a plasma membrane; Cyclosporine A.

Cyclic peptides have emerged as new nature-inspired derivatives capable of crossing the plasma membrane of cells through proposed mechanisms such as passive diffusion and endosomal uptake. Cyclic peptides also show the ability to release into the cytoplasm rather than residing in vesicle carriers which can lead to premature degradation through lysosomal digestion. Cyclic peptides also improve on some of the limitations of linear peptides such as reduced proteolytic degradation and lower immunogenicity.

To synthesize cyclic peptides, automated peptide synthesizers using solid phase supports have been the method of choice utilizing cyclization techniques such as lactam and disulfide bridges to impart cyclic structure. Head-to-tail cycles have proved difficult to perform on solid supports due to the C-terminus being bound to the solid support. More often than not, the cyclization reaction is performed in solution at high dilution levels. Some techniques have been developed that cleave and cyclize in a single reaction step however these reactions typically run overnight and result in a lower crude purity. Here we evaluated conditions to automate head-to-tail cyclization reactions while leaving the peptide on-resin to improve recovery and decrease synthesis times.

Experimental protocol

Peptide Synthesis and Analysis

Linear peptide sequences were synthesized using a Biotage® Initiator+ Alstra™ peptide synthesizer on 0.3-1 mmol scale in 30 mL reactor vials. Couplings proceeded using 5 equivalents of amino acid (0.5 M concentration), with DIC/Oxyma according to manufacturer's protocols. Each peptide was synthesized with the same lot of Rink amide polystyrene resin (0.49 mmol/g) to minimize synthesis variations. Peptides were bound to resin using the side chain carboxylic acid of either Asp or Glu residues which contained an llyl protected carboxy terminus. This was then selectively deprotected two times using 0.25 equivalents Pd(PPh3) and 15 equivalents of phenylsilane heated at 40°C for 10 min. Final cyclization occurred using DIC/Oxyma couplings at varied temperatures and times (Table 1).

Microcleavages occurred in a 2mL cocktail of 95% TFA, 2.5% TIS and 2.5% H_2O for 1 hour at room temperature. The cleaved peptide solutions were collected in 20 mL scintillation vials and evaporated with a Biotage® V10-Touch. Crude peptides were analyzed via analytical HPLC using an Agilent 1260 Infinity series HPLC equipped with Restek® ARC18 column (2.7 µm particles, 2.1 x 50 mm) connected in-line with an Advion® expression® CMS mass detector.

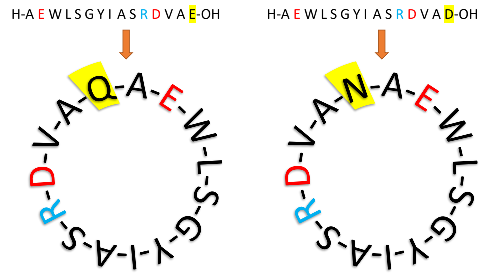

Figure 2: Linear peptide is synthesized on resin followed by on-resin cyclization to yield the final product. After cleavage from Rink Amide resin, Asp and Glu are converted to Asn and Gln respectively.

Results and Discussion

Peptide Design

Cyclic peptides generally come in lengths of 6-15 aa residues in length. We targeted a sequence that contained reasonable charge dispersion, bulky side chains and a length of 15 aa to ensure robustness and generality of our synthesis conditions.

The C-terminus residues here contained Asp and Glu that after cleavage from the Rink Amide resin would be converted to Asn and Gln respectively; this offered a simple cleavage and coupling scheme. It would also let us evaluate steric hinderance due to the resin-linked side chain, theorizing Glu would offer better results than Asp. We hypothesized the extra methylene unit in the side chain would allow further spacing from the resin to improve cyclization conversion.

Resin bound AA	Coupling Scheme	Temperature(°C)	Coupling 1 (min)	Coupling 2 (min)
Glu	Double	50	30	60
Glu	Double	50	30	10
Glu	Single	75	10	-
Asp	Double	50	30	60
Asp	Single	75	10	-

Table 1: Reaction conditions evaluated for cyclization of Asp and Glu bound peptides.

Since cyclic peptides are in fact cyclic, this strategy could work for a multitude of sequences as the location of the N-C cyclic coupling is irrelevant compared to the sequence order (e.g. wherever Q or N are in the sequence, the neighboring residue would simply need to be on the N terminus of the linear form). Reactions conditions performed can be found in Table 1. Here we started with relatively long reaction times for SPPS before moving to more conventional synthesis conditions for microwave SPPS.

On Resin Cyclization with Glutamic Acid Linker

Analyzing the chromatograms for the Glu linked peptide (Figure 3) we can see significant conversion from linear peptide to cyclic peptide and some side product formation. Crude purity of final cyclic samples was ~22%-28% compared to ~64% in our linear sample. Our best crude purity was a result of our 50°C double coupling condition.

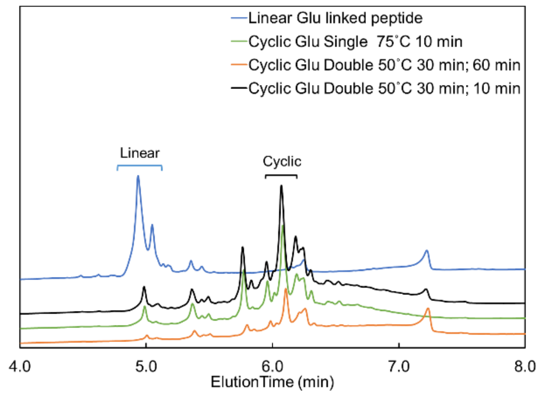

Figure 3: Chromatogram comparing elution times of linear vs cyclic peptides containing a C-terminal Glu under different reaction conditions.

On Resin Cyclization with Aspartic Acid Linker

For the Asp linked peptide (Figure 4) we see poorer crude purity compared to the Glu peptide after coupling with poorer conversion from linear to cyclic form. Considering that the Asp linear peptide began with higher crude purity (Table 2) this indicates that there may be an effect of amino acid linker on cyclization efficiency. We do maintain a similar trend between the reaction conditions from slow to fast coupling where the longer reaction time produced a higher conversion to cyclic product.

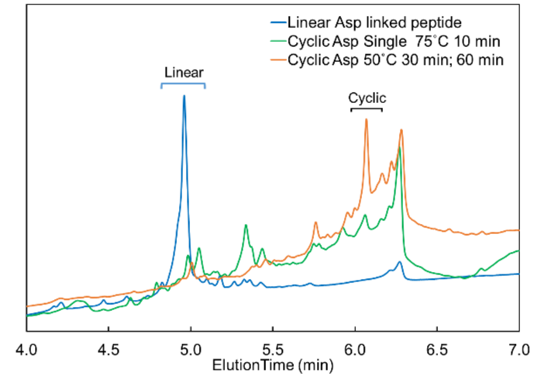

Figure 4: Chromatogram comparing elution times of linear vs cyclic peptides containing a C-terminal Asp under different reaction conditions.

Conclusion

Resin bound AA	Coupling Scheme	% Cyclic in Crude	% Linear in crude
Glu	Double Long	28.0	4.0
Glu	Double Short	22.5	4.5
Glu	Single	22.8	4.3
Asp	Double Long	16.6	3.3
Asp	Single	13.3	3.0

Table 2: % of cyclic product in crude samples; Linear crude purity for Glu and Asp peptides were 64% and 78% respectively.

Head to tail cycles utilizing side chain carboxylic acid bound Asp and Glu has been shown here to be a potential method for on-resin cyclization. While both Asp and Glu linked peptides showed the ability to cyclize, the longer side chain of Glu is proposed to provide better cyclization potential compared to that of Asp. Further study of these efficiency will focus on resin loading level and evaluate if lower loading will provide better conversion to cyclic product due to the relative increase in spacing of reactive sites.

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