Stapled Peptides: Optimizing Ring-Closing Metathesis for Automation

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Introduction

Peptides are well suited for use as tool compounds to interrogate biological systems and processes due to their chameleonic ability to mimic a potential protein partner within the biological system of interest. Their use in this application continues to grow as the rules governing permeability across the plasma membrane are further elucidated. Unfortunately, a well-defined secondary structure is often required for productive interaction with the other protein partners within the system of interest.

Many covalent stapling methods have been introduced that stabilize and rigidify secondary structures within short peptide sequences since the pioneering working was published by Miller, Blackwell, and Grubbs[1]. The most popular of strategy involves a cross coupling reaction between two unnatural amino acids bearing an alkenyl side chain, mediated by a 1st Generation Grubbs ruthenium catalyst. Herein, we present work further optimizing the ring closing metathesis reaction for expedient use, toward an end goal of fully automating both the synthesis and ring-closing metathesis reactions.

Experimental protocol

Peptide Synthesis and Analysis

Peptides were synthesized with a Biotage® Initiator+ Alstra™ microwave assisted automated peptide synthesizer on Rink amide Chemmatrix® resin using DIC, Oxyma, and Fmoc-protected amino acids. The unnatural, α,α-disubstituted olefin bearing amino acids were coupled using three equivalents relative to resin load while all other amino acids were coupled with five equivalents. All amino acids were coupled using standard methods.

A solution containing 20 mol percent Grubbs catalyst dissolved in 1 mL dichloroethane was added to a 2 mL reactor vial containing approximately 20 mg peptidyl resin. Individual reaction conditions are outlined further.

Peptide cleavage occurred in a cocktail of 95% TFA, 2.5% TIS and 2.5% H_2O for 2 hours at room temperature. The cleavage cocktail was rapidly evaporated using the Biotage® V-10 evaporation system and the resulting residue was washed with cold diethyl ether and dissolved for HPLC analysis. Crude peptides were analyzed with an Agilent 1260 Infinity series HPLC and AB Sciex MS equipped with Restek ARCC18 column (2.7 µm particles, 2.1 x 50 mm).

Results and discussion

Linear synthesis optimization

For these experiments, I decided to use the BIM peptide sequence published previously by LaBelle et al[2]. One step that is often overlooked when optimizing secondary chemistry is the optimization of the linear sequence itself. This becomes even more important for a peptide of this nature, which contains two, relatively expensive, unnatural amino acids. The first synthesis, while containing the desired linear sequence, also contained a significant amount of a deletion sequence. Simply increasing the reaction time for the final two amino acids gave the desired linear sequence as the predominant compound in the crude sample.

Optimizing Ring Closing Metathesis

The stapled peptide contains two (S)-pentenylalanine residues at positions nine and thirteen (*i*, *i*+4 spacing) to stabilize the helical secondary structure. Substituting these residues, even with fewer equivalents did not compromise the linear synthesis efficiency, Figure 1.

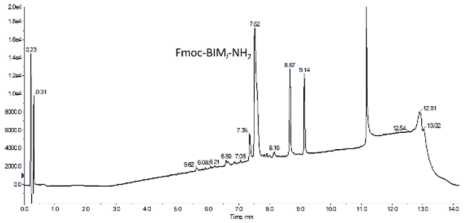

Figure 1. *Crude analytical HPLC chromatogram for Fmoc-protected linear BIM peptide containing two olefin-bearing unnatural amino acids.*

For optimization of the ring-closing metathesis reaction, the Fmoc-protected peptidyl-resin was divided into approximately equivalent fractions and subjected to various reaction conditions and evaluation for extent of conversion, Table 1.

Trial	Reaction Time (min)	Reaction Temperature	Reaction Iterations	Inert Gas	Percent completion
1	120	r.t.	2	Y	100
2	60	40C	1	Y	99
3	60	40C	1	N	96.8
4	30	40C	2	Y	100
5	30	40C	2	N	100
6	30	40C	1	Y	95.0
7	30	40C	1	N	78.5

Table 1. *Ring closing metathesis reaction conditions evaluated with the goal of minimizing overall reaction time, while still achieving a high degree of reaction completion.*

As this peptide has been published previously, it is not surprising that the established, manual protocol achieved complete conversion of the linear peptide to a fully stapled peptide.

What is most interesting is the role that an inert environment plays for the success of this reaction. Using nitrogen bubbling not only provides a mixing mechanism during manual RCM reactions, but it also drives off generated ethylene by product, furthering reaction completion. In the absence of nitrogen bubbling, one could assume that the reaction efficiency would decline. As expected, the conversion efficiency declined, but most significantly for the short reaction times, Figure 2.

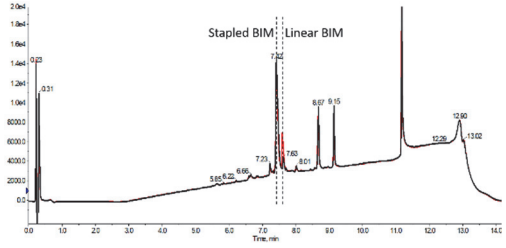

Figure 2. *Impact of providing an inert environment, particularly for short reaction times. When conducted with nitrogen continuously purging the microwave cavity, the RCM reaction can be driven to 95% completion with a single iteration for only 30 minute (black). In the absence of nitrogen purging, the conversion of the linear peptide to the stapled peptide drops to approximately 78% (red).*

While plausible, it is unlikely that this affect is attributed to the environmental sensitivity of the Grubbs ruthenium catalyst is at play, given that simply extending the reaction time improves the percent conversion to near completion even in the absence of an inert atmosphere, Figure 3.

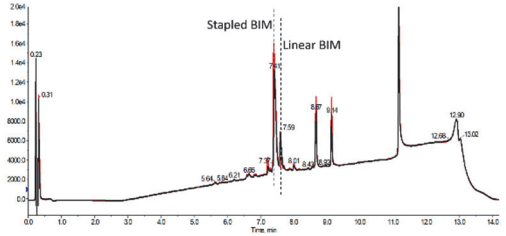

Figure 3. *Comparison of conversion efficiency with respect to reaction time. Simply extending reaction time from 30 min (black) to 60 minutes (red) drives the RCM reaction to near completion even in the absence of an inert gas purging the microwave cavity.*

Given these observations, the environmental stability of the Grubbs catalyst appears sufficient to continue with efforts toward automating not only the peptide synthesis, but also the RCM reaction.

Fully Automating Synthesis and Ring Closing Metathesis

Using the most optimal reaction conditions (Trial 5), the Grubbs catalyst solution was prepared as previously described and placed in a designated position on the amino acid rack, allowing the Initiator+ Alstra™ to add the solution automatically. Surprisingly, conversion efficiency dropped significantly, to approximately 50% Figure 4.

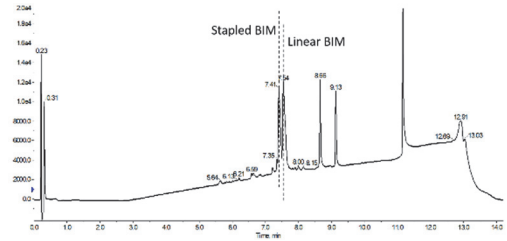

Figure 4. *Conversion of the linear BIM peptide to the desired stapled peptide using full automation. The reaction efficiency decreased significantly from 100 % to approximately 50% simply due to the reagent addition mechanism.*

Despite the resin being equilibrated with dichloroethane, the small amount of residual DMF remaining within the needle assembly appears to poison the Grubbs catalyst. All conditions investigated here were also attempted using full automation and a 50% conversion was the highest level achieved. Future experiments will investigate a method which overcomes the observed DMF catalyst poisoning towards a fully automated method.

Conclusions

Microwave reaction conditions were evaluated with the goal of reducing reaction time while maintaining reaction efficiency towards a fully automated strategy. Microwave heating shortens reaction time while driving efficiency such that only a single reaction is necessary. However, once those optimized conditions were applied to a fully automated process, the reaction efficiency dropped significantly. DMF poisoning of the Grubbs catalyst is the probable cause and further development is necessary to establish an efficient, yet fully automated process.

[1] Miller, S.J., Blackwell, H.E., and Grubbs, R.H. *JACS* 1996, 118, 9606-9614.

[2] LaBelle, J.L. et al, *J Clin Invest* 2012, 122, 2018-2031.

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