学位論文（要約）

Development of cyclic peptide scaffolds against large undruggable transmembrane proteins

（アンドラッガブルな膜タンパク質を標的にした特殊環状ペプチド創薬）

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Abstract (abridged)

In this thesis, I will discuss my work toward discovering cyclic peptides against three large transmembrane proteins. The transmembrane proteins targeted in this study were chosen because they are only involved in protein-protein interactions and no drugs have been developed at this time to inhibit them from doing so. This is due to the fact that small molecules do not have a sufficient amount of possible contacts to get in the way of such interactions. Thus, peptides, which are slightly larger in size, are a great candidate drug class to fill this need for protein-protein interaction inhibitors. The RaPID (Random nonstandard Peptide Integrated Discovery) system has previously been shown to be a reliable platform for the discovery of cyclic peptides that bind with high affinity to enzymes and transporters as well as exerting varying degrees of inhibitory activity upon them. I used the RaPID system to generate macrocyclic peptides that bind to the three transmembrane proteins.

In chapter 1, I discuss the current situation in drug discovery and the unmet challenges of generating drugs that block protein-protein interactions and how traditional drug discovery methods such as small molecule screens have not been adequate in addressing these challenges. The relevance and renewed focus on peptides as a drug class to meet these inadequacies of small molecules, and the superior features of in vitro display platforms such as the RaPID system compared to traditional high throughput small molecule screens are also discussed.

In chapter 2, results of my selection for macrocyclic peptides that bind Plexins are described.

In chapter 3, an attempt at discovering peptides with inhibitory activity against integrins is discussed.

In chapter 4, a method to ribosomally synthesize fused tricyclic peptide libraries was developed and this is discussed in this section.

In chapter 5, selection of macrocyclic peptides that bind subunits of secretase complexes are described

Chapter 6 is the conclusion of this thesis. Achievements and novel findings will be discussed. The implications of my findings and future possibilities stemming from the results of my studies will also be discussed

Chapters 1, 2, 3, 5 and 6 are not shown due to reasons involving patent applications. References of chapters 1, 2, 3, 5 and 6 are included in this text.

Table of Contents (abridged)

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Chapter 4

Ribosomal synthesis of fused tricyclic peptides

Introduction

In nature, several examples of polycyclic peptides exist, such as conotoxins, cyclotides and defensins[1–3] and have a suprising variety of biological activities such as protease resistance, protein-protein interaction inhibition and, in some cases, oral bioavailability[4,5]. A method of ribosomally synthesizing such polycyclic peptides in a way that is compatible with mRNA display would be a very powerful tool in possibly discovering such peptides. However, these polycyclic peptides are often head-to-tail cyclized and contain multiple cysteines[6]. To be compatible with mRNA display, it is necessary to have a free C-terminus for puromycin attachment[7]. Additionally, scaffolds with multiple cysteines which have easily reversible disulfide bonds which make structural deconvolution a great challenge[8]. In an aim to develop a methodology to synthesize polycyclic peptides that are mRNA display compatible and have predictable yet complex topologies, the following method was established.

Previous reports have shown that ribosomally synthesized peptides with an N-terminal chloroacetyl group can be cyclized using the sulfhydryl group of downstream cysteines[9]. Additionally, it has been shown that there is some selectivity regarding the positioning of the cysteine in relation to the N-terminal chloroacetyl group[10]. When more than one cysteine is present, the N-terminal chloroacetyl group cyclizes with the foremost N-terminal cysteine. However, due possibly to steric reasons, a cysteine in the second amino acid position is not able to cyclize with the N-terminal chloroacetyl group. Therefore, an N-terminally chloroacetylated peptide containing a cysteine in the second amino acid position and three more arbitrarily spaced downstream cysteines would result in the formation of a cycle between the N-terminal chloroacetyl group and the second cysteine. The remaining unreacted first, third and fourth cysteines can then be reacted with 1,3,5-tris(bromomethyl)benzene (TBMB) to result in a fused tricyclic peptide. Such a methodology can be applied to the synthesis and screening of large fused tricyclic libraries.

Results and discussion

Cyclizing peptides using an N-terminal chloroacetyl group and a downstream thiol is a very robust method of generating libraries of macrocyclic peptides[9]. Previous reports have identified that the N-terminal chloroacetyl group is not able to react with the sulfhydryl group of a cysteine at the second amino acid position[10]. Further, when multiple cysteines appear further downstream, the chloroacetyl group will cyclize with the foremost N-terminal cysteine. Thus if an N-terminally chloroacetylated peptide is designed to have a cysteine in the second amino acid position followed by three more randomly spaced cysteines, the cyclization will occur between the *N*-chloroacetyl group and the second cysteine leaving the first, third and fourth cysteines unreacted. Similar to the *N*-chloroacetyl group, TBMB can react with three sulfhydryl groups. Application of TBMB with the aforementioned peptide can result in a tricyclic peptide with a fused topology (Figure 1). Such a scaffold will make possible larger multicyclic peptides that still retain structural strain as well as the ability to interact with larger surface areas.

Conditions of peptide tricyclization using TBMB would ideally not interfere with mRNA display steps as well maintain simplicity as to not reduce library diversity (i.e. buffer exchanges, changing tubes, etc). Several solvents (DMF, DMSO, acetonitrile, ethanol, methanol) were surveyed for their compatibility in reacting TBMB with in vitro translated peptides. In vitro transcription and translation of a DNA template L7WT and the addition of 40 mM TBMB in dimethyl formamide (DMF) following the reduction of disulfide bonds between cysteine residues with 18 mM tris(2carboxyethyl)phosphine (TCEP) showed the cleanest and most reproducible post translational conversion of ribosomally synthesized monocyclic peptides to fused tricyclic peptides, detectable by an increase in 114.01 Da which corresponds to the addition of a mesitylene moiety (Figure 2a, b). To further confirm that this 114.01 Da shift was due to cyclization, MALDI-TOF MS/MS was used to compare the fragmentation pattern of monocyclic and proposed tricyclic peptides. The monocyclic sample should contain a long ‘tail’ which should produce various fragments upon collision induced decomposition. In contrast, if the TBMB treated monocyclic peptide is indeed tricyclized it will have only a short tail and will

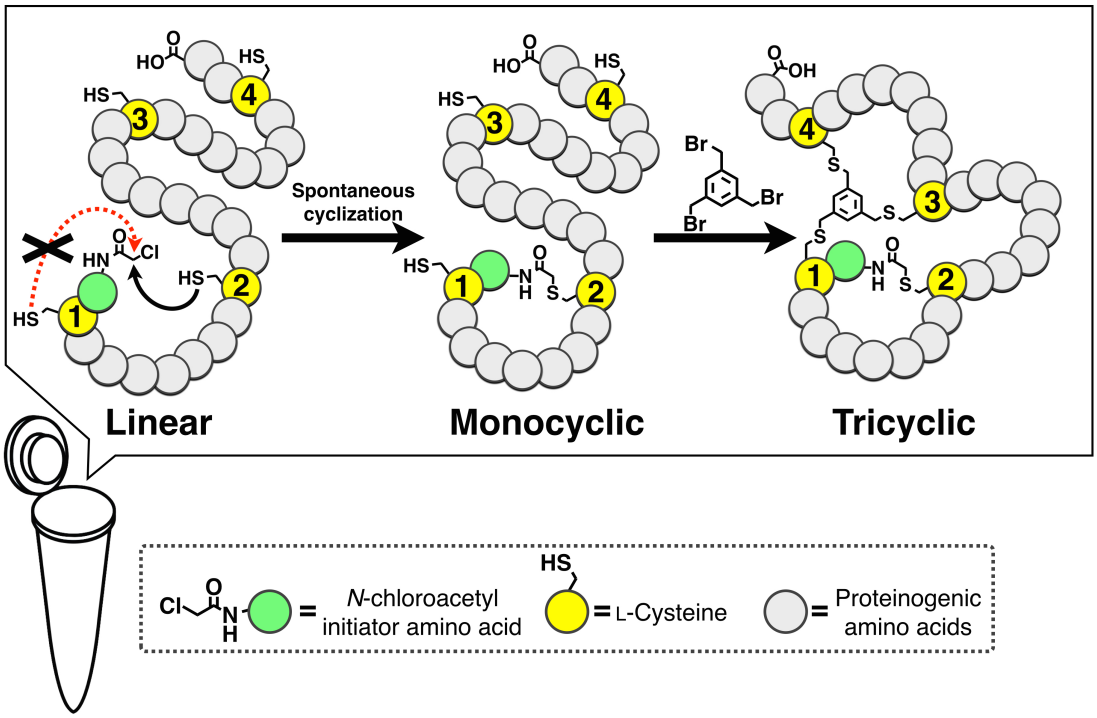


Figure 1. Reaction scheme of forming fused tricyclic peptides via ribosomal synthesis. Peptide translation initiated by an *N*-chloroacetylated amino acid and containing four downstream cysteines (with one of the cysteines in the second amino acid position) creates a monocyclic peptide. Reaction of the remaining three cysteines with tris(bromomethyl)benzene results in a fused tricyclic peptide.

not produce many fragments. MALDI-TOF MS/MS analysis of monocyclic L7WT showed several fragments which corresponded to various 'tail' regions (Figure 2c). However, as predicted, MALDI-TOF MS/MS analysis of TBMB treated L7WT did not produce any visible tail fragments which can be attributed to being tricyclized (Figure 2d).

Although previous reports have determined this cyclization selectivity when multiple cysteines appear in the peptide sequence, the study was not performed with a peptide containing four cysteines. To demonstrate that this selectivity still exists with four cysteines a DNA template encoding a peptide containing a cysteine at the second amino acid position (Cys1) and three more downstream cysteines (from the N-terminus: Cys2, Cys3, Cys4) all spaced seven amino acids apart was designed. Genetic code reprogramming via the FIT system was used to incorporate *N*-chloroacetyl D-tryptophan at the first amino acid position. Further, genetic code reprogramming was used to incorporate L-lactic acid between Cys2 and Cys3. It is

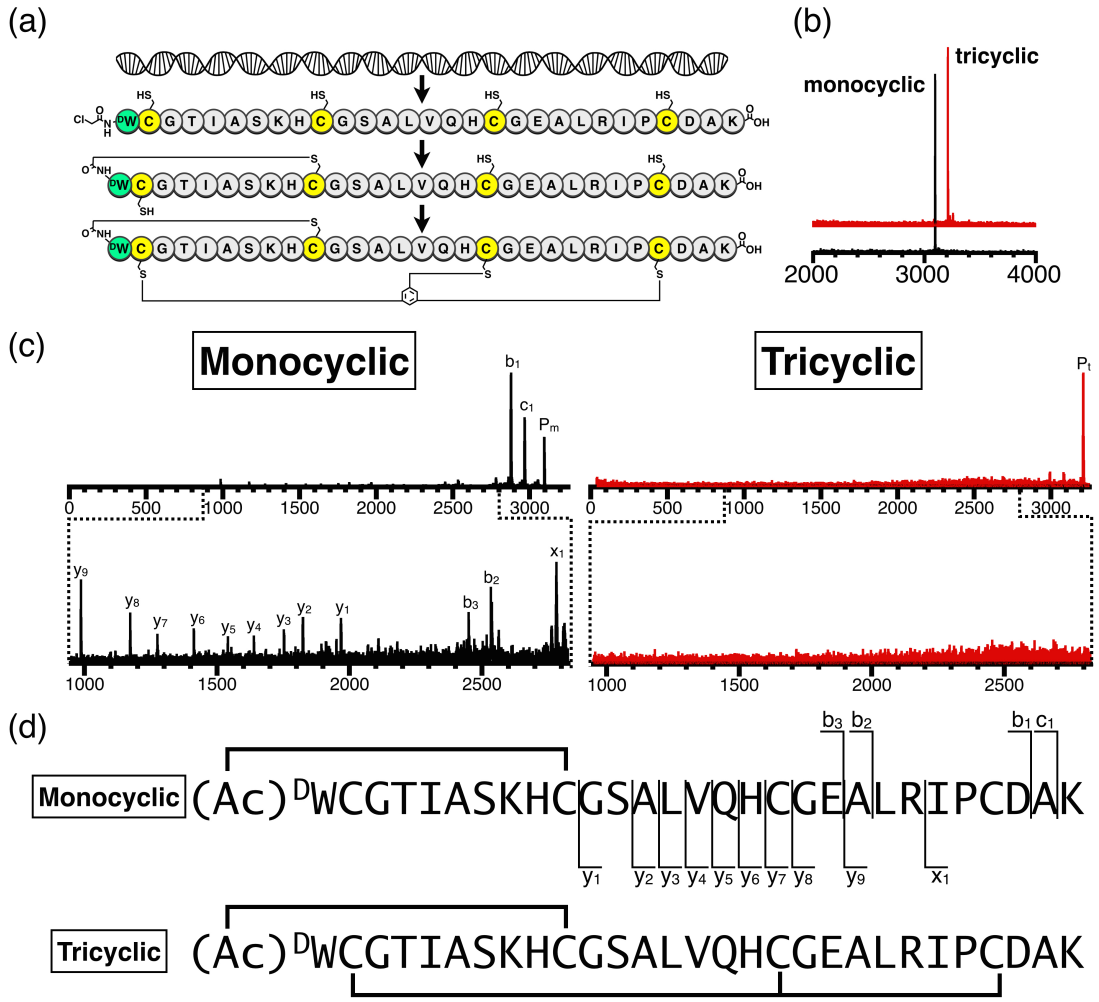


Figure 2. Synthesis and MALDI-TOF MS/MS analysis of ribosomally synthesized monocyclic and tricyclic peptide L7WT. (a) Cyclization method of DNA encoded peptide L7WT. (b) MALDI-TOF MS analysis of monocyclic (**monocyclic** m_{obs} = 3094.45 Da m_{calc} = 3094.45) and tricyclic (**tricyclic** m_{obs} = 3208.50 Da m_{calc} = 3208.50) L7WT. (c) MALDI-TOF MS/MS fragmentation of monocyclic ($\mathbf{P_m}$: parent ion) and tricyclic ($\mathbf{P_t}$: parent ion) L7WT. Due to the high intensity of ions b_1 and c_1, expanded spectra are shown below. Several peaks corresponding to fragments of monocyclic L7WT were observed whereas only noise was observable in the expanded tricyclic L7WT spectra. (d) Observed fragmentation patterns of monocyclic and tricyclic L7WT.

established that the ribosome is able to catalyze ester bond formation[11,12]. Ester bonds hydrolyze much more readily relative to peptide bonds and therefore this peptide can be fragmented via alkaline hydrolysis and analyzed using MALDI-TOF MS to determine whether the initial cyclization event occurs between Cys2, Cys3 or Cys4

(Cys1 is not able to react due, most likely, to steric reasons)[10] (Figure 3a). In vitro translation of this peptide followed by MALDI-TOF MS analysis showed a single peak corresponding to the full length monocyclic peptide (Figure 3b). Upon alkaline hydrolysis with sodium carbonate, two peaks, one with a mass corresponding to an N-terminal fragment of a cyclization event with Cys2 and another with a mass corresponding to a peptide fragment downstream of the ester bond was observed. A small peak corresponding to the full length unhydrolyzed product was observed as well. Importantly, no peak corresponding to a full length hydrolyzed peak was observed confirming that even with a peptide containing four cysteines, the same cyclization selectivity is retained. DNA templates to encode the same peptide with ester bonds in differing positions all showed no peaks to contradict this cyclization phenomenon.

Using the established tricyclization conditions, four model peptides were tested with 1, 2, 5, and 10 amino acids between each cysteine residue to examine what size scaffolds are possible (Figure 4). In vitro translation followed by the addition TBMB to each model peptide showed complete conversion of monocyclic peaks to tricyclic peaks. Additionally, three model peptides which together comprise all proteinogenic amino acids (with the exception of methionine which must be excluded to incorporate *N*-chloroacetyl D-tryptophan) to confirm that TBMB will not result in any unwanted side reactions with other residues. MALDI-TOF MS analysis of these peptides showed clean conversion of all three model-peptides from monocylic to tricylic peptides (Figure 4).

To determine whether this tricyclization chemistry is compatible with the conditions of the RaPID system, a puromycin ligated mRNA template encoding a peptide containing three cysteines with 4 amino acids between each, an N-terminal *N*-biotinyl-L-phenylalanine and a C-terminal GS linker was used to make two samples of peptide-mRNA fusions two be tested for recovery from streptavidin-conjugated magnetic beads. One sample was reacted with TBMB while DMF was added to another sample. These reacted and unreacted samples were first mixed with streptavidin-conjugated magnetic beads pre-blocked with biotin. Next, the peptide-mRNA fusions were transferred to unblocked streptavidin-conjugated magnetic beads. The peptide-mRNA fusions were then removed from the unblocked

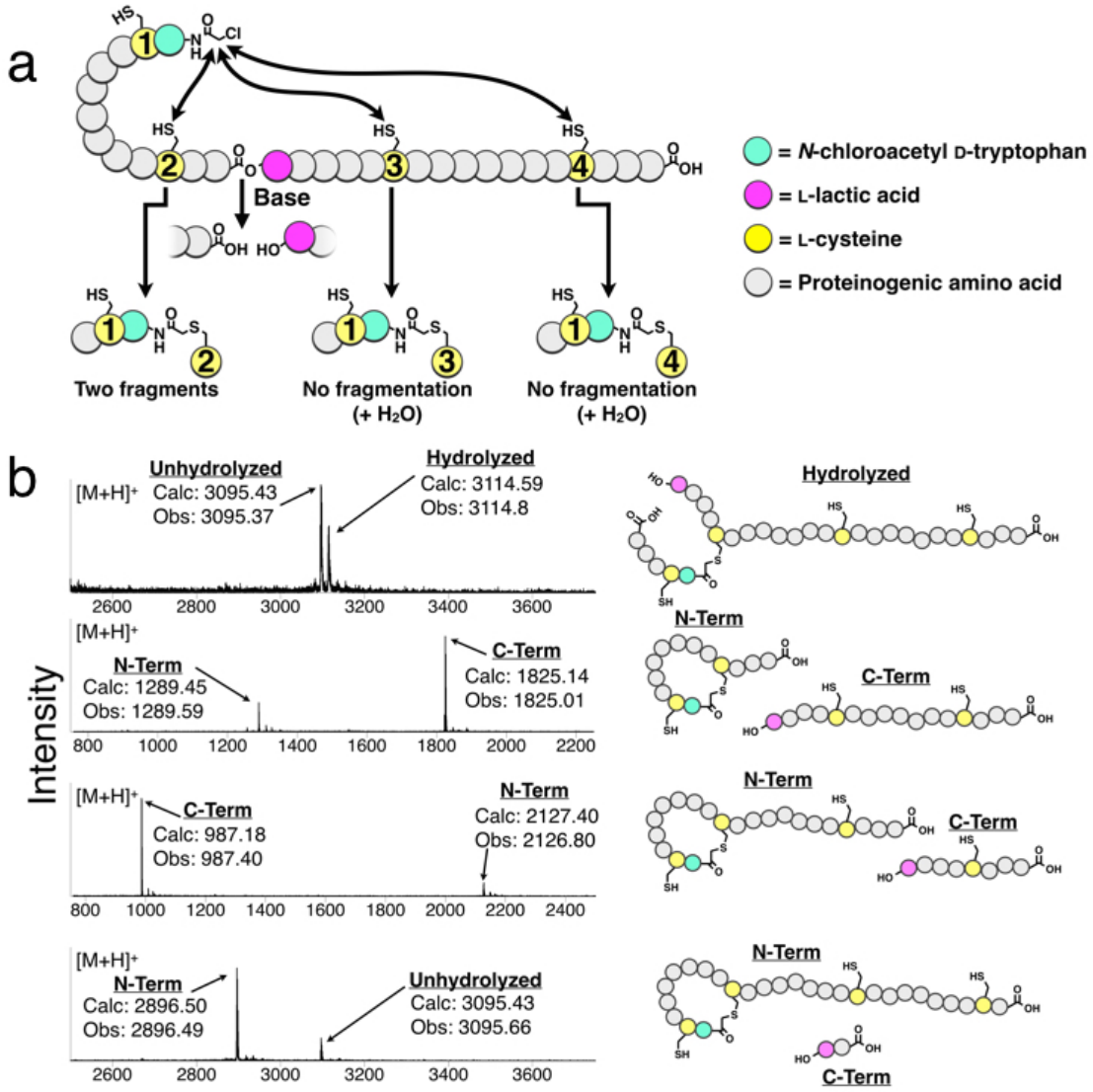
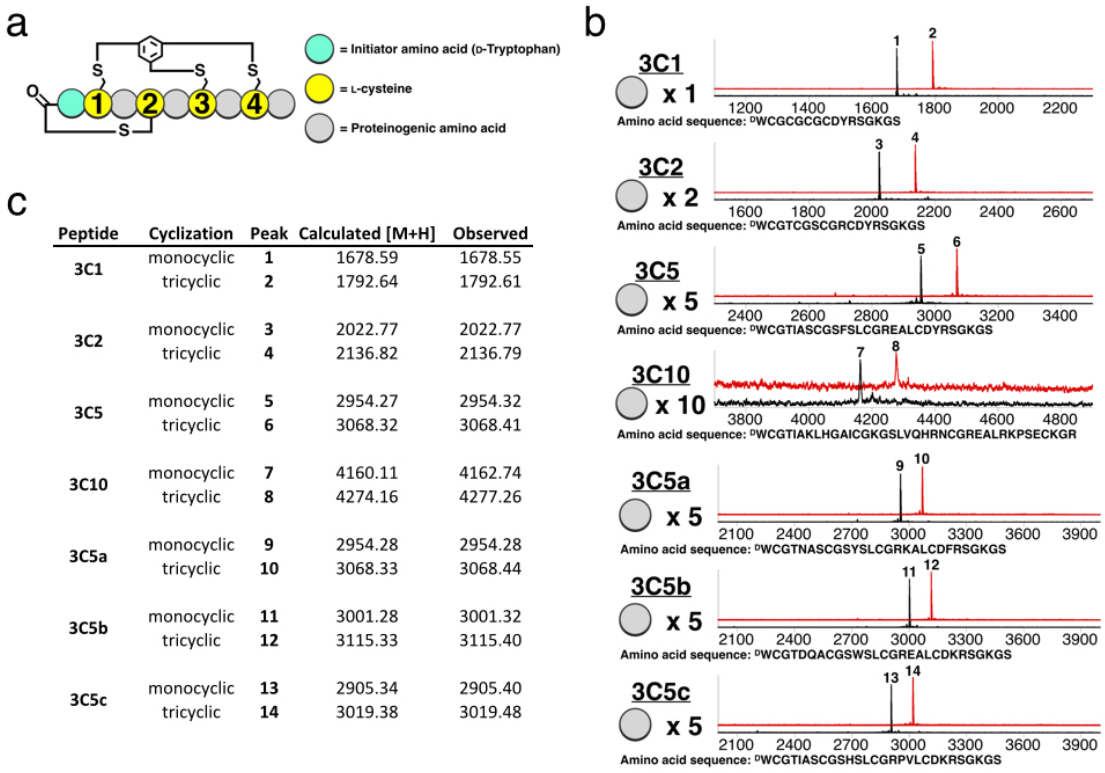


Figure 3. Confirmation of initial cyclization topology. (a) Fragmentation of ester bonds placed between various cysteines via L-lactic acid will result in different fragment sizes dependent on cyclization topology. (b) MALDI-TOF MS analysis of the fragments formed depending on ester bond positioning. All peaks support the formation of a cycle with cysteine 2.

streptavidin-conjugated magnetic beads and the amount of cDNA recovered from both blocked and unblocked beads were determined via RT-PCR (Figure 5a). Approximately 0.05 and 0.6% of the unreacted peptide-mRNA fusion sample was recovered from the blocked and unblocked streptavidin-conjugated magnetic beads, respectively. For the reacted peptide-mRNA fusion samples, approximately 0.025%



c

Peptide	Cyclization	Peak	Calculated [M+H]	Observed
3C1	monocyclic	1	1678.59	1678.55
	tricyclic	2	1792.64	1792.61
3C2	monocyclic	3	2022.77	2022.77
	tricyclic	4	2136.82	2136.79
3C5	monocyclic	5	2954.27	2954.32
	tricyclic	6	3068.32	3068.41
3C10	monocyclic	7	4160.11	4162.74
	tricyclic	8	4274.16	4277.26
3C5a	monocyclic	9	2954.28	2954.28
	tricyclic	10	3068.33	3068.44
3C5b	monocyclic	11	3001.28	3001.32
	tricyclic	12	3115.33	3115.40
3C5c	monocyclic	13	2905.34	2905.40
	tricyclic	14	3019.38	3019.48

Figure 4. Tricyclization of peptides with various size lengths and amino acid composition. (a) Schematic of tricyclized peptides in this study. (b) MALDI-TOF MS analysis of monocyclic (black peaks) and corresponding tricyclic peptides (red peaks). Amino acid sequences are shown below each spectrum. (c) Calculated and observed masses of peaks in (b).

and 0.35% was recovered from the blocked and unblocked streptavidin-conjugated magnetic beads, respectively. Although the amount of peptide-mRNA fusions recovered from the reacted samples were slightly lower than the unreacted, the difference was low and the fold difference between the unblocked and blocked beads were 10.2 for the unreacted peptide-mRNA fusions and 14.0 for the reacted peptide-mRNA fusions. These results show that the chemistry involved in the tricyclization of peptides by TBMB does not interfere with the steps required for the RaPID system. Conversely, to see if the tricyclization reaction is compatible with the conditions of the RaPID system the tricyclization reaction was carried out on a peptide after reagents for reverse transcription were added. MALDI-TOF MS analysis showed a peak with a mass corresponding to a tricyclized peptide which was shifted 114 MW from an unreacted sample confirming that this reaction can still proceed under conditions of the RaPID system (Figure 5b).

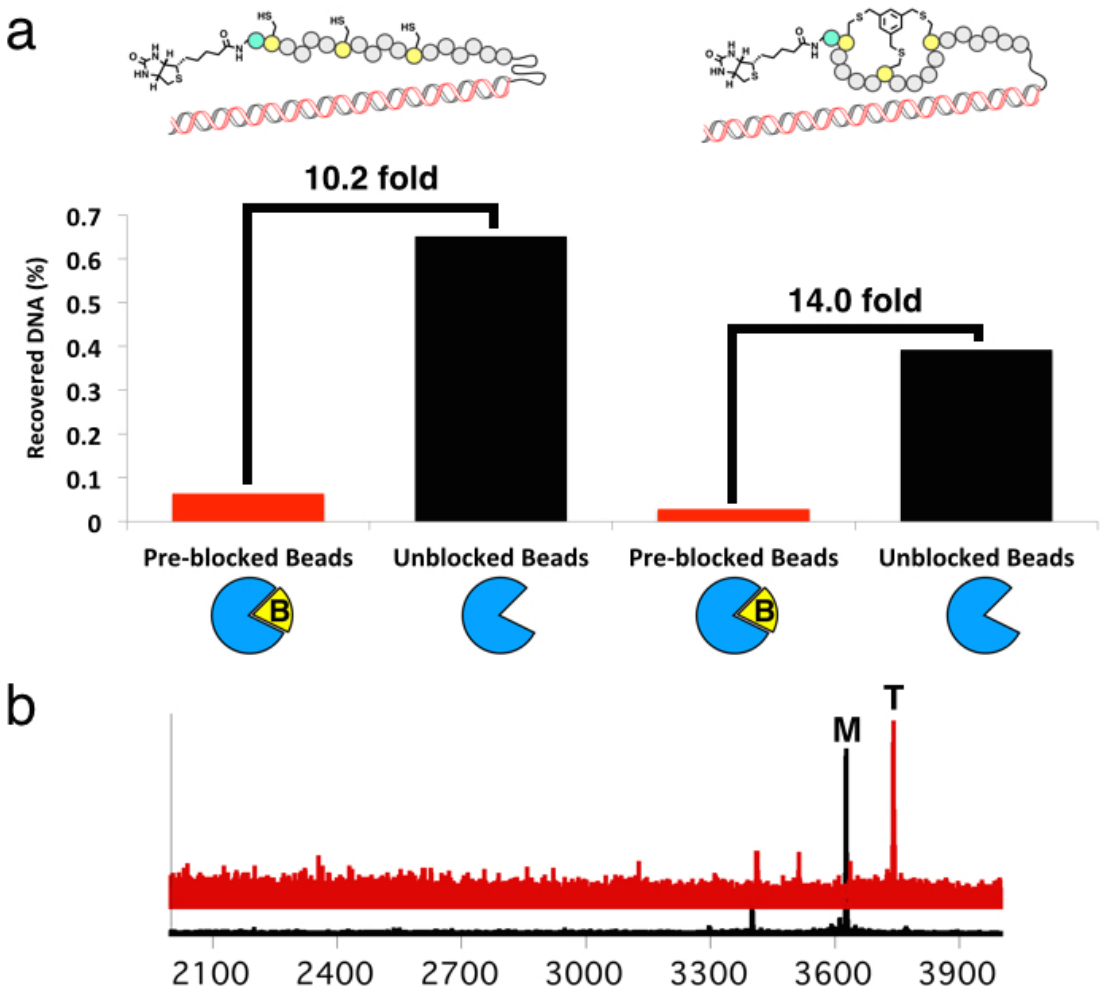


Figure 5. Compatibility of tricyclization chemistry with the RaPID system. (a) N-terminally biotinylated peptide-mRNA fusions were with and without the addition of tris(bromomethyl)benzene were subjected to streptavidin conjugated magnetic beads to determine if cDNA can still be recovered. (b) MALDI-TOF MS analysis of peptide tricyclization under mock RaPID system conditions. Peaks representing the conversion of the monocyclic peptide (**M**, black peak) to a tricyclic peptide (**T**, red peak) was observed. **M** m_{obs} = 3625.56 Da (m_{calc} = 3625.46); **T** m_{obs} = 3739.61 Da (m_{calc} = 3739.51)

Conclusion

In this chapter I described my work in developing a general and robust method of producing fused tricyclic peptides of ribosomal origin. This method can be applied to ribosomally synthesize vast libraries of fused tricyclic peptides in an mRNA display compatible manner. The fused tricyclic topology allows one to design larger more complex peptides while still maintaining the structural strain seen in smaller monocyclic peptides which is established as a necessity for high binding affinity. This method is compatible with all proteinogenic amino acids with the exception of methionine which was excluded to allow genetic code reprogramming. This tricyclization method can be used for another attempt at selecting for peptides that bind α6β1-integrin and inhibit its interaction with laminin-511 (Chapter 3). Additionally, the larger size may make it applicable to select for tricyclic peptides that bind unstructured peptides. The human genome encodes for approximately 300 SH3 domains which are composed of ~60 amino acids and no disulfide bonds[13]. These SH3 domains are capable of binding a short 5mer linear motif and thus tricyclic peptides of similar size may be used to bind a variety of linear peptides. This can open up doors for non-invasive binding of GPCR N-termini to add a fluorescence tag, highly selective histone tail binders for ChIP-seq[14] or structural investigations of intrinsically disordered proteins[15].

Materials and methods

EnAsn-3'.R20-OMe: 5’-TGGCGGCTCTGACTGGACTC-3’

EnAsn-3'.R38: 5’-TGGCGGCTCTGACTGGACTCGAACCAGTGACATACGGA-3’

EnAsn-5'.F49:

5’-

GTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGA-3’

EnAsn CAU.R43:

5’-GAACCAGTGACATACGGATTNNNAGTCCGCCGTTCTACCGACT-3’

dFx.R46:

5’-ACCTAACGCCATGTACCCTTTCGGGGATGCGGAAATCTTTCGATCC-3’

dFx.R19: 5’-ACCTAACGCCATGTACCCT-3’

3C-1-ext1:

5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACAT

ATGTGCGGCTGCGGTTGCGGCTGC-3’

3C-1-ext2:

5’-

TTTCCGCCCCCCGTCTTACGAACCTTTGCCGCTGCGATAATCGCAGCCGCA

ACCGCAGCC-3’

3C-2-ext1:

5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACAT

ATGTGCGGCACCTGCGGTTCGTGCGGCCGTTGC-3’

3C-2-ext2:

5’-

TTTCCGCCCCCCGTCTTACGAACCTTTGCCGCTGCGATAATCGCAACGGCC

GCACGAACC-3’

3C-5-ext1:

5’-TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACAT

ATGTGCGGCACCATTGCGAGCTGCGGTTCGTTTAGCCTGTGCGGCCGTGA

AGCG-3’

3C-5-ext2:

5’-

TTTCCGCCCCCCGTCTTACGAACCTTTGCCGCTGCGATAATCGCACAGCGC

TTCACGGCCGCACAGG-3’

3C-10 new FW

5’-

TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTG

CGGCACCATTGCGAAATTGCACGGTGCGATCTGCGGG-3’

3C-10 new RV1

5’-

GGCTTGCGCAACGCTTCACGGCCGCAGTTACGGTGCTGTACCAAGGAACC

TTTCCCGCAGATCGCACCGTGC-3’

3C-10 new RV2

5’-

TTTCCGCCCCCCGTCTTAGCGACCTTTACACTCCGAAGGCTTGCGCAACGC

TTCACG-3’

3C-5A FW

5’-

TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTG

TGGAACGAATGCAAGTTGCGGTAGTTACAGCTTATGTGGG-3’

3C-5A RV

5’-

TTTCCGCCCCCCGTCTTAGGAGCCTTTGCCGCTACGGAAGTCACACAGCGC

TTTGCGCCCACATAAGCTGTAACTACCGCAACTTGC-3’

3C-5B FW

5’-

TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTG

TGGTACTGATCAAGCCTGCGGGAGTTGGTCTTTATGTGG-3’

3C-5B RV

5’-

TTTCCGCCCCCCGTCTTACGACCCTTTACCCGAGCGCTTGTCGCAGAGGGC

TTCGCGTCCACATAAAGACCAACTCCCGCAGGC-3’

3C-5C FW

5’-

TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTG

CGGAACCATCGCCTCTTGCGGTAGCCACAGTCTCTGTGGC-3’

3C-5C RV

5’-

TTTCCGCCCCCCGTCTTAAGAACCTTTACCAGAACGTTTATCACAAAGAAC

AGGGCGGCCACAGAGACTGTGGCTACCGCAAG-3’

L7 WT FW

5’-

TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTG

TGGCACCATCGCAAGCAAACACTGCGGCTCCGCCCTTGTG-3’

L7 WT RV

5’-

TTTCCGCCCCCCGTCTTATTTCGCATCACACGGGATGCGCAGAGCTTCACC

ACAGTGCTGCACAAGGGCGGAGCCGCAG-3’

L7 E1 FW

5’-

TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTG

TGGCACCATCATGAGCAAACACTGCGGCTCCGCCCTTGTG-3’

L7 E2 FW

5’-

TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTG

TGGCACCATCGCAAGCAAACACTGCGGCTCCATGCTTGTGCAGCAC-3’

L7 E3 RV

5’-

TTTCCGCCCCCCGTCTTATTTCGCATCACACGGGATGCGCAGCATTTCACC

ACAGTGCTGCACAAGGGCGGAGCCGCAG-3’

L7 E4 RV

5’-

TTTCCGCCCCCCGTCTTATTTCATATCACACGGGATGCGCAGAGCTTCACC

ACAGTGCTGCACAAGGGCGGAGCCGCAG-3’

3C-4FW:

5’-

TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTG

TTGGACGGGCGGTCCGCAGTGCGGCGGTTCCCATTATACG-3’

3C-4RV:

5'-
TTTCCGCCCCCCGTCTTAAGACCCGCGACCACTACGATAATCACACGTATA
ATGGGAACCGCCGCACTG-3'

Synthesis of L-lactic acid dinitrobenzyl ester

1 equivalent of L-lactic acid (29 mg) was reacted with 1.5 equivalents of dinitrobenzyl chloride (111 mg) with 2 equivalents of diethylamine (117 µl) in DMF (Total volume: 2 ml) and stirred for 12 hrs. The reaction was confirmed on TLC using 1:1 hexane:ethyl acetate. The reaction was quenched with 350 µl 1 M HCl followed by the addition of 1 ml diethyl ether. The reaction was transferred to a 100 ml separating flask containing ~80 ml 50% diethyl ether aq. Contents were then mixed and the aqueous phase was drained. The remaining organic phase was brought to 80 ml with 1 M HCl aq. and the aqueous phase was drained. This was repeated with H_2O, saturated sodium bicarbonate and saturated NaCl. The remaining aqueous phase was further dehydrated with anhydrous $MgSO_4$. The contents were filtered to remove $MgSO_4$ and the evaporated on the rotovap followed by an oil pump vacuum. The product weighed ~120 mg at this point and was resuspended in 1 ml DCM. The product was run on two preparative TLC plates using 1:1 hexane:ethyl acetate as mobile phase. The single band was cut out, filtered and eluted with diethyl ether (~200 ml). The product remained a viscous liquid following evaporation and 53 mg remained at this point. The product was resuspended in 2 ml chloroform-D and subjected to NMR analysis (Figure 6). The NMR spectra showed impurities and following the testing of several mobile phase options, 1:1 hexane:acetone was used to re-purify the product via preparative TLC. This purification resulted in a clean NMR spectra of L-lactic acid dinitrobenzyl ester.

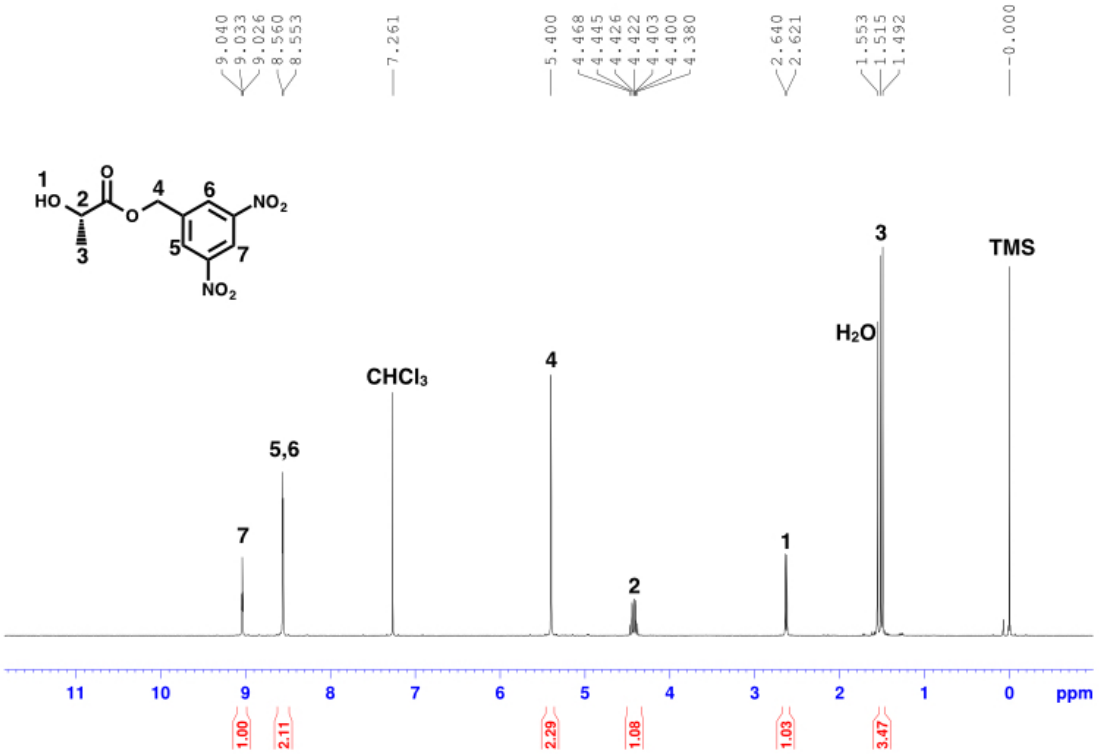


Figure 6. ^{1}H-NMR spectrum of L-Lactic acid dinitrobenzyl ester in D-chloroform

Synthesis of dFx, tRNA$^{enAsn}_{CAU}$ and DNA templates.

dFx was synthesized in the same manner as in Chapter 2 with the following exceptions: The extension step was performed with Fx-5'.F36 and dFx.R46. The following PCR amplification was performed with T7ex5.F22 and dFx.R19.

tRNA$^{enAsn}_{CAU}$ was synthesized in the same manner as in Chapter 2 with the following exceptions: The exception step was performed with EnAsn-5'.F49 and EnAsn CAU.R43. The first PCR amplification was performed with T7ex5.F22 and EnAsn-3'.R38. The second PCR amplification was performed with T7ex5.F22 and EnAsn-3'.R20-OMe.

DNA templates for tricyclic peptide experiments (3C-1, 3C-2, 3C-5, 3C-10, 3C-5A, 3C-5B, 3C-5C, L7WT, L7E1, L7E2, L7E3, L7E4 and 3C-4) were all synthesized as the above templates with primers named accordingly.

Aminoacylation of tRNAs via flexizymes.

tRNA$^{fMet}_{CAU}$ was aminoacylated with cyanomethyl ester activated *N*-chloroacetyl D-tryptophan or *N*-Biotinyl-L-phenylalanine which were synthesized in house as previously described. tRNA$^{fMet}_{CAU}$ and eFx were mixed to a final

concentration of 25 μM with a $MgCl_2$ concentration of 600 mM in 50 mM HEPES-KOH pH 7.5. To this, the aforementioned cyanomethyl ester activated amino acids were added to a final concentration of 5 mM. After a 1 hr incubation on ice (or 2 hrs for *N*-Biotinyl-L-phenylalanine), the reaction contents were precipitated using an ethanol/sodium acetate mixture at pH 5.2. Pellets were kept dry and at -80ºC until use. $tRNA^{enAsn}_{CAU}$ was aminoacylated with L-lactic-acid dinitrobenzyl ester using dFx by mixing both at a final concentration of 25 μM with a $MgCl_2$ concentration of 600 mM in HEPES-KOH pH 7.5. To this, L-lactic acid dinitrobenzyl ester was added to a concentration of 5 mM and incubated on ice for 3 hrs followed by ethanol/sodium acetate precipitation.

Ribosomal synthesis of tricyclic peptides.

Using the FIT system, a monocyclic peptide is translated using a DNA or mRNA template encoding a peptide with a *N*-chloroacetylated amino acid in the first amino acid position, a cysteine in the second amino acid positions and three more arbitrarily spaced downstream cysteines. Following a 5 μl in vitro translation reaction, 1 μl of H_2O, 1 μl 150 mM tris(2-carboxyethyl)phosphine in 0.6 M HEPES KOH pH 7.5 and 1 μl 40 mM tris(bromomethyl)benzene in DMF is added and incubated at 25ºC for 1 hr. For MALDI-TOF MS or MS/MS analysis, 12 μl of HBS was added to each sample and desalted on a C-Tip column (AMR inc.) and eluted with a 50% saturated α-hydroxycinammic acid (Bruker Daltonics) solution of 80% acetonitrile 0.5% sodium acetate.

Translation and alkaline hydrolysis of L7 peptides.

DNA templates encoding L7 peptides were synthesized using the methionine(-) FIT system used in Chapter 2 with the following exceptions: L-lactic acid-$tRNA^{enAsn}_{CAU}$ was added to the translation mixture at a final concentration of 50 μM and translation was performed at 37ºC for 1 hr due to compensate for the slow formation of ester bonds by the ribosome. Following translation, any intramolecular disulfide bonds were reduced by treatment with tris(2-carboxyethyl)phosphine. 1 μl of 1 M sodium carbonate was then added and the mixture was incubated at 37ºC for 1 hr to hydrolyze the ester bonds. Following hydrolysis, the sample was neutralized with 1 μl 2% trifluoroacetic acid and desalted on a C-Tip column for MALDI-TOF MS analysis.

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List of accomplishments

Journal Publications

1. Hipolito, C. J., Bashiruddin, N. K. & Suga, H. Protein cocrystallization molecules originating from in vitro selected macrocyclic peptides. *Curr. Opin. Struct. Biol.* **26,** 24–31 (2014).

2. Bashiruddin, N. K. & Suga, H. Construction and screening of vast libraries of natural product-like macrocyclic peptides using in vitro display technologies. *Curr. Opin. Chem. Biol.* **24C,** 131–138 (2014).

3. Bashiruddin, N. K., Nagano, M. & Suga, H. DNA-encoded fused tricyclic peptides via ribosomal synthesis and chemical modification. Bioorganic Chemistry. Accepted with minor revisions (2/2015).

Books

1. Suga, H., John Hipolito, C., Goto, Y., Katoh, T. & Kato Bashiruddin, N. in *Synthetic Biology: Volume 1* **1,** 126–163 (The Royal Society of Chemistry, 2014).

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Nasir Kato Bashiruddin

Figure 1. My father Aasim Zaki Bashiruddin (5/26/1929 – 6/22/2014)