

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Preliminary Communications

Synthesis of fused tricyclic peptides using a reprogrammed translation system and chemical modification



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ARTICLE INFO

Article history:

Received 31 January 2015

Revised 12 June 2015

Accepted 13 June 2015

Available online 20 June 2015

Keywords:

Peptides

Translation

Cyclic peptides

ABSTRACT

Here we report a unique method of ribosomally synthesizing fused tricyclic peptides. Flexizyme-assisted *in vitro* translation of a linear peptide with the N-terminal chloroacetyl group and four downstream cysteines followed by the addition of 1,3,5-tris(bromomethyl)benzene results in selective production of the fused tricyclic peptide. This technology can be used for the ribosomal synthesis of fused tricyclic peptide libraries for the *in vitro* selection of bioactive peptides with tricyclic topology.

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1. Introduction

Some naturally occurring peptides consist of complex macrocyclic structures [1]. For instance, conotoxins, cyclotides and defensins possess multicyclic frameworks and exhibit remarkable thermostability, serum stability and in some cases oral bioavailability [2–4]. Such peptides are also composed of unique structural elements such as head-to-tail cyclization, cysteine (Cys) knot motifs and multiple intramolecular disulfide bonds. These structural elements are thought to be what give rise to their various biological activities. Hence, these frameworks have gained interest and have been investigated for use as scaffolds for drug discovery [5].

Several methods involving the chemical macrocyclization of peptides to mimic the benefits seen in naturally occurring peptides have been reported [6–10]. One reliable method is to take advantage of the selective reactivity of Cys residues with appropriate chemical reagents, such as benzyl halides [6,8–11]. Heinis et al. have used such macrocyclization methods with phage display to select for bicyclic peptides using libraries of peptides which contain three Cys residues that are bicyclized by the post-translation addition of 1,3,5-tris(bromomethyl)benzene (TBMB) [9]. This method was able to select for a bicyclic peptide with a remarkable IC₅₀ of 1.5 nM against human plasma kallikrein. Macrocyclic peptides inhibiting thrombin via mRNA displayed libraries containing N- and C-terminal Cys residues and monocyclized using α,α -dibromoethylene have been also reported [10].

Cyclization strategies using genetic code reprogramming have also made it possible to generate macrocyclic peptide libraries with diversities up to the trillions [12]. These methods have been coupled with *in vitro* display technologies to rapidly select for cyclic peptides with exceptional bioactivity [13–18]. A very robust method of generating macrocyclic peptides by means of genetic code reprogramming via the FIT (Flexizyme-assisted *in vitro* translation) system [19,20] is to aminoacylate the initiator tRNA^{Met}_{CAU} with an amino acid containing an N-chloroacetyl (ClAc) group. The N-terminal ClAc group is able to form a sulfide bond with the sidechain sulfhydryl group of a downstream Cys resulting in thioether macrocyclic peptides [19]. When a single Cys exists in a downstream position, the selective intramolecular formation of the thioether bond occurs to yield a monocyclic structure. When more than one Cys exists on the peptide, the foremost N-terminal Cys selectively cyclizes with the N-terminal ClAc group [21]. However, cyclization between the ClAc group and an adjacent Cys at the second amino acid position cannot occur due, most-likely, to steric hindrance or prohibited ring-constrain. This specificity has been applied to forming fused bicyclic peptides by forming a thioether macrocycle between the N-terminal ClAc group and a second Cys and a disulfide bond macrocycle between a Cys in the second amino acid position and a third Cys [21].

To generate peptides with a more complex cyclic scaffold, we here report a method that enables us to ribosomally synthesize fused tricyclic peptides via *in vitro* translation systems. Taking advantage of the aforementioned cyclization selectivity, we envisioned that by translating peptides with the N-terminal ClAc group, one Cys residue at the second position and three more arbitrarily spaced downstream Cys residues, followed by the addition

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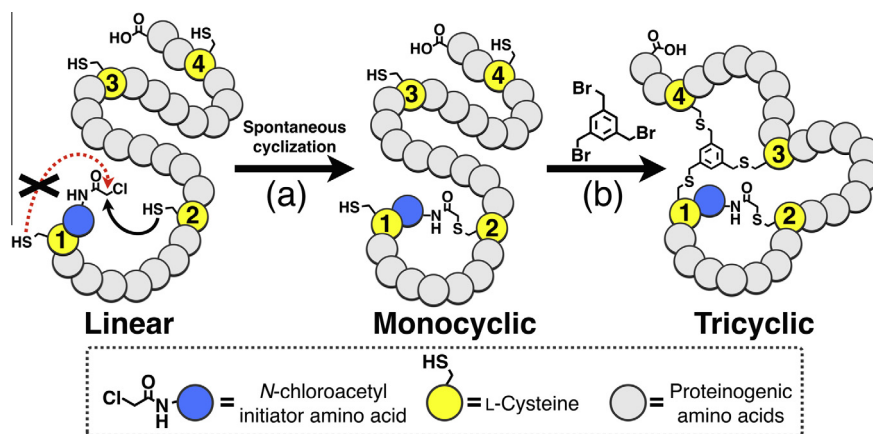


Fig. 1. Schematic representation for the ribosomal production of tricyclic peptides. (a) The Cys in the second amino acid position is not able to react with the N-terminal chloroacetyl group therefore the next closest Cys reacts to form a thioether bond. (b) Addition of TBMB to the monocyclic peptide forms thioethers with the remaining three Cys residues resulting in a peptide with a fused tricyclic topology.

of TBMB, it would be possible to efficiently synthesize fused tricyclic peptides of various sizes and amino acid compositions (Fig. 1).

2. Materials and methods

2.1. Oligonucleotides and DNA templates

DNA oligonucleotides were purchased from Eurofins Genomics and are listed in Table 1. DNA templates used for the translation of peptides in this study were produced via assembly PCR by mixing an FW oligonucleotide with an RV primer with a corresponding name. These products were amplified by using T7gAUG as a forward primer and DGGRK as a reverse primer except in the case of 3C10 where 3C10RV2 was used as a reverse primer.

2.2. Methionine(-) Flexizyme assisted In vitro Translation (FIT) system

All in vitro translations in this study were performed using a methionine-deficient version of the FIT system [20]. The composition of the FIT system, sans pre-aminoacylated tRNAs, are as follows: 1.2 μM ribosome, T7 RNA polymerase, 4 $\mu\text{g}/\text{mL}$ creatine kinase, 3 $\mu\text{g}/\text{mL}$ myokinase, 0.1 μM pyrophosphatase, 0.1 μM nucleotide-diphosphatase kinase, 0.73 μM AlaRS, 0.02 μM CysRS,

0.13 μM AspRS, 0.23 μM GluRS, 0.68 μM PheRS, 0.09 μM GlyRS, 0.02 μM HisRS, 0.4 μM IleRS, 0.11 μM LysRS, 0.04 μM LeuRS, 0.03 μM MetRS, 0.38 μM AsnRS, 0.16 μM ProRS, 0.06 μM GlnRS, 0.03 μM ArgRS, 0.04 μM SerRS, 0.09 μM ThrRS, 0.02 μM ValRS, 0.03 μM TrpRS, 0.02 μM TyrRS, 0.26 μM EF-G, 10 μM EF-Tu, 10 μM EF-Ts, 2.7 μM IF1, 0.4 μM IF2, 1.5 μM IF3, 0.6 μM MTF, 0.25 μM RF2, 0.17 μM RF3, 0.5 μM RRF, 0.1 μM , 1.5 mg/mL *Escherichia coli* total tRNA, 0.5 mM of all 20 proteinogenic amino acids except for methionine, 2 mM ATP, 1 mM UTP, 2 mM GTP, 1 mM CTP, 2 mM spermidine, 20 mM creatine phosphate, 2 mM DTT, 50 mM HEPES-KOH (pH 7.6), 12 mM magnesium acetate, 100 mM potassium acetate.

2.3. Aminoacylation of tRNAs via flexizymes

tRNAs, flexizymes and activated amino acids were synthesized as previously described [20]. tRNA^{Met}_{CAU} was aminoacylated with cyanomethyl ester activated *N*-chloroacetyl *D*-tryptophan (ClAc-^DW) or *N*-chloroacetyl *L*-tyrosine (ClAc-^LF). tRNA^{Met}_{CAU} and eFx were mixed to a final concentration of 25 μM with a MgCl₂ concentration of 600 mM in 50 mM HEPES-KOH pH 7.5. To this, the aforementioned cyanomethyl ester activated amino acid was added to a final concentration of 5 mM. After a 1 h incubation on ice, the reaction contents were precipitated using a 70% ethanol,

Table 1
Sequences of oligonucleotides used in this study.

Name	Peptide	Oligonucleotide Sequences (5' to 3')
L7FW	P1	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGTGGCACCATCGAAGCAAACACTGCGGCTCCGCCCTTGTG
L7RV		TTTCCGCCCCCGCTTATTTTCGCATCACACGGGATGCGCAGAGCTTCACCACAGTGTGCACAAGGGCGGAGCCGCAG
L7eFW	P1e	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGTGGCACCATCGAAGCAAACACTGCGGCTCCATGCTTGTGCAGCAC
L7eRV		TTTCCGCCCCCGCTTATTTTCGCATCACACGGGATGCGCAGAGCTTCACCACAGTGTGCACAAGCATGGAGCCGCAG
3C1FW	P2	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGTGGCTGCGGTGCGGGTCTG
3C1RV		TTTCCGCCCCCGCTTACGAACCTTTGCGGCTGCGATAATCGCAGCCGCAACCCGACGC
3C5FW	P3	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGTGGCACCATTGCGAGTGTGCGGTTGCTTTAGCCTGTGCGGCCGTGAAGCC
3C5RV		TTTCCGCCCCCGCTTACGAACCTTTGCGGCTGCGATAATCGCAGCCGCTTCACGGCCGCACAGG
3C10FW	P4	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGTGGCACCATTGCGAAATTCGACGGTGGATCTGCGGG
3C10RV		GGCTTGGCAACGCTTCACGGCCGACGTTACGGTGTGTACCAAGGAACCTTTCCCGCAGATCGCACCCGTCG
3C10RV2		TTTCCGCCCCCGCTTACGACCTTTACTCCGAAGGCTTGGCAGCCGCTTCACG
3C5AFW	P5	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGTGGAACGAATGCAAGTTGCGGTAGTTACAGCTTATGTGGG
3C5ARV		TTTCCGCCCCCGCTTACGAGCCTTTGCGGCTACGGAAGTACACAGCCGCTTTGCGCCACATAAGCTGTAAGCTTAACTACCCGAACTTCG
3C5BFW	P6/P6F	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGTGGTACTGATCAAGCTGCGGGAGTTGGTCTTTATGTGG
3C5BRV		TTTCCGCCCCCGCTTACGACCTTTACCCGAGCCGCTTGTGCGAGAGGGCTTCGCGTCCACATAAAGACCAACTCCCGCAGGC
3C5CFW	P7	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGTGGGAACCATCGCCTCTTGGGTAGCCACAGTCTGTGGC
3C5CRV		TTTCCGCCCCCGCTTAAAGAACCTTTACCGAACGTTTATCACAAGAAGCAGGGCCGACAGACTGTGGCTACCGCAAG
T7gAUG	N/A	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATG
DGGRK	N/A	TTTCCGCCCCCGCTTAA

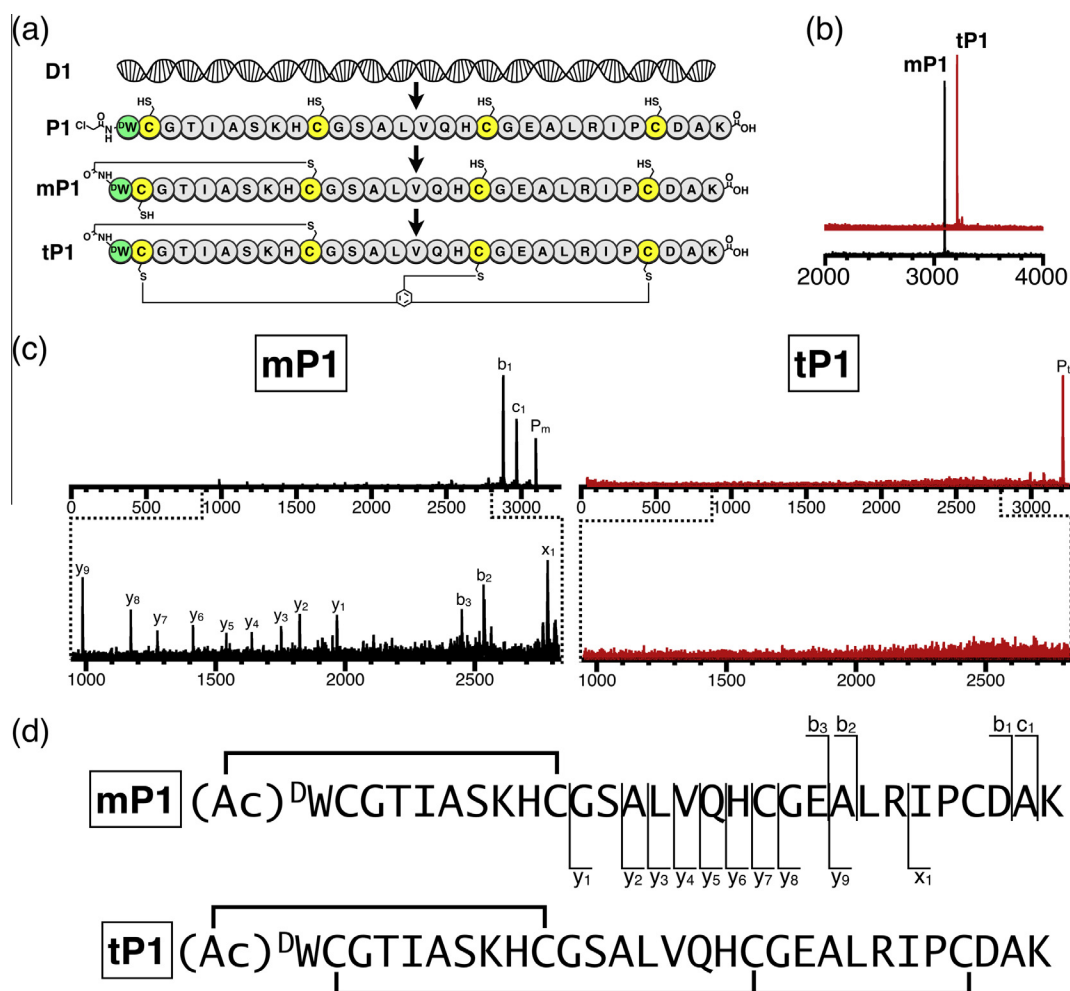


Fig. 2. Peptide tricyclization and MALDI-TOF MS/MS fragmentation analysis. (a) The DNA template D1 was expressed to produce peptide P1. P1 is predicted to spontaneously monocyclize to form mP1 and reaction with 1,3,5-tris-(bromomethyl)benzene (TBMB) results in the tricyclic peptide tP1. (b) MALDI-TOF MS analysis of mP1 (**mP1** $m_{\text{obs}} = 3094.45$ Da $m_{\text{calc}} = 3094.45$) and tP1 (**tP1** $m_{\text{obs}} = 3208.50$ Da $m_{\text{calc}} = 3208.50$). (c) MALDI-TOF MS/MS fragmentation of mP1 (P_m : parent ion) and tP1 (P_t : parent ion). Due to the high intensity of ions b_1 and c_1 , expanded spectra are shown below. Several peaks corresponding to fragments of mP1 were observed whereas only noise was observable in the expanded tP1 spectra. (d) Observed fragmentation patterns of mP1 and tP1. Although the fragmentations of mP1 were observed in the linear peptide region, those of tP1 could not be detected due to the tricyclic topology.

0.1 M sodium acetate at pH 5.2. Pellets were kept dry and at -80°C until use. tRNA^{enAsn}_{CAU} was aminoacylated with L-lactic-acid dinitrobenzyl ester using dF_x by mixing both at a final concentration of 25 μM with a MgCl₂ 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 h followed by ethanol/sodium acetate precipitation.

2.4. Ribosomal synthesis of tricyclic peptides

A methionine deficient FIT system supplemented with a preaminoacylated ClAc-^{DW}-tRNA^{Met}_{CAU} was used to in vitro transcribe and translate a DNA template encoding a peptide with ClAc-^{DW} at the first amino acid position a Cys in the second amino acid positions and three more arbitrarily spaced downstream Cys residues. Following a 5 μl in vitro translation reaction, 1 μl of H₂O, 1 μl 150 mM TCEP in 0.6 M HEPES KOH pH 7.5 and 1 μl 40 mM TBMB in DMF is added and incubated at 25 $^\circ\text{C}$ for 1 h. For MALDI-TOF 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 -cyano-4-hydroxycinnamic acid (Bruker Daltonics) solution of 80% acetonitrile 0.5% sodium acetate.

2.5. Translation, MALDI-TOF MS/MS fragmentation and alkaline hydrolysis of mP1e peptides

D1e DNA template was used to synthesize mP1e for alkaline hydrolysis experiments using the Met-deficient FIT system as described in the previous report [21].

2.6. Translation and LC-ESI-MS of peptide P6F

The DNA template for P6 was in vitro translated as in 2.4 at 22 μl scale using a preaminoacylated ClAc-^L-F-tRNA^{Met}_{CAU}. The translation was split into two 10 μl aliquots and tricyclized as in 2.4 or left in monocyclic form by adding only DMF (negative control). 45 μl of 50% acetonitrile, 0.1% formic acid aq. was added to each reaction for LC-ESI-MS analysis on a Thermo Accela liquid chromatograph interfaced to a Thermo Exactive Orbitrap mass spectrometer. LC was performed on a Phenomenex Aeris Peptide column (XB-C18, 150 mm \times 2.1 mm, dp = 3.6 μm) using a 25 min gradient from 100% solvent A (5% acetonitrile, 10 mM ammonium formate, 10 mM formic acid aq.) to 100% solvent B (95% acetonitrile, 10 mM ammonium formate, 10 mM formic acid aq.) at a 700 $\mu\text{l}/\text{min}$ flow rate.

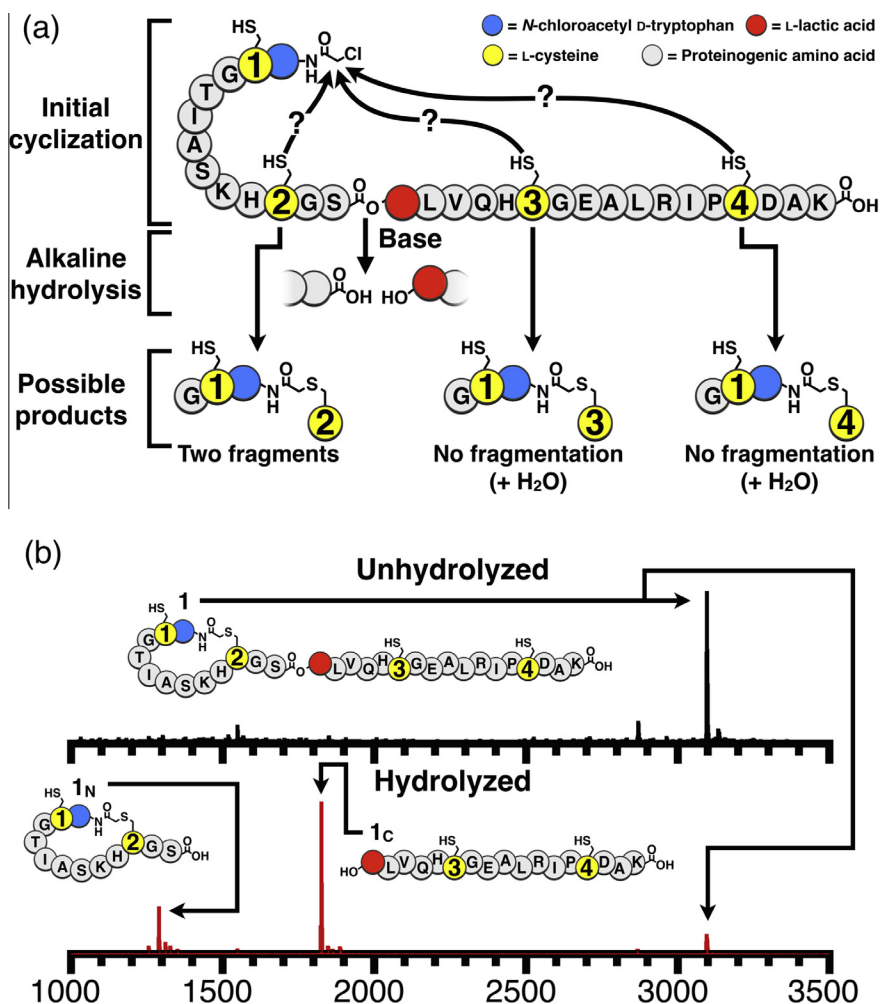


Fig. 3. Determination of monocyclization selectivity in a peptide containing four Cys residues. (a) The mode of initial cyclization of a peptide containing an ester bond between Cys2 and Cys3 will effect the fragmentation pattern upon alkaline hydrolysis. Cyclization with Cys2 will result in two fragments upon alkaline hydrolysis while cyclization with Cys3 or Cys4 will result in the opening of the cycle and the addition of H₂O. (b) MALDI-TOF analysis of peptide mP1e. The black spectrum shows the peak corresponding to the full length unhydrolyzed peptide **1** ($m_{\text{obs}} = 3095.46$ Da $m_{\text{calc}} = 3095.43$). The red spectrum shows peaks corresponding to the N-terminal (**1_N** $m_{\text{obs}} = 1289.60$ Da $m_{\text{calc}} = 1289.54$) and C-terminal (**1_C** $m_{\text{obs}} = 1825.01$ Da $m_{\text{calc}} = 1824.91$) fragments of mP1e when the ClAc groups reacts with Cys2. A peak corresponding to remaining unhydrolyzed mP1e **1** ($m_{\text{obs}} = 3095.62$ Da $m_{\text{calc}} = 3095.43$) was observed but no peak corresponding to a hydrolyzed mP1e resulting from cyclization between Cys3 or Cys4 ($m_{\text{calc}} = 3113.45$ Da) was observed.

3. Results and discussion

Reassignment of the initiation codon (AUG) with ClAc-^DW was facilitated by the use of a Met-deficient FIT system in the presence of ClAc-^DW-tRNA^{Met}_{CAU} prepared by the flexizyme, eFx [19]. In vitro transcription/translation of a DNA template (D1) encoding a linear 29-mer peptide (P1) successfully expressed the monocyclic peptide (mP1) via thioether macrocyclization between the N-terminal ClAc group and a downstream Cys most likely at position 10 (Fig. 2a and b). mP1 was then treated with tris(2-carboxyethyl)phosphine (TCEP) followed by addition of TBMB in DMF. The MALDI-TOF MS analysis of the resulting product revealed a new peak with a mass shift of 114 Da from that of P1. This mass difference corresponded to the addition of a mesitylene moiety to the remaining three Cys residues resulting in a tricyclic peptide tP1 (Fig. 2a and b).

We have previously demonstrated that the MALDI-TOF MS/MS fragmentations of macrocyclic peptides allows us to identify the macrocyclic region over the linear region due to less susceptibility of the macrocyclic region to fragmentation [22]. Therefore we performed MALDI-TOF MS/MS analysis of tP1 in comparison to mP1.

As expected, the linear tail region of mP1 was fragmented to yield peaks corresponding to the expected residual fragments (Fig. 2c). On the other hand, tP1 remained intact as one molecule under the same fragmentation conditions (Fig. 2d). The results suggested that mP1 was most likely converted to the desired tricyclic structure of tP1.

Based on our previous experimental data for the ribosomal synthesis of bicyclic peptides [23], we were fairly confident that the first thioether-macrocylation took place between the N-terminal ClAc and the Cys2 residue at position 10, but we felt that it would be necessary to obtain further evidence to confirm the fused tricyclic topology. The incorporation of an L-lactic acid (^HO) at a designated position can be achieved by genetic code reprogramming. Since we utilized the Met-deficient FIT system for the reassignment of ClAc-^DW, the elongator AUG codon is left vacant; therefore, this vacant codon can be utilized for the assignment for ^HO, similar to the strategy reported elsewhere [18,24]. We thus designed a DNA template (D1e) based on the sequence of D1 to include an elongator AUG codon between Cys2 and Cys3, which would be suppressed with ^HO by the use of ^HO-tRNA^{enAsn}_{CAU}. When ^HO was incorporated into this position

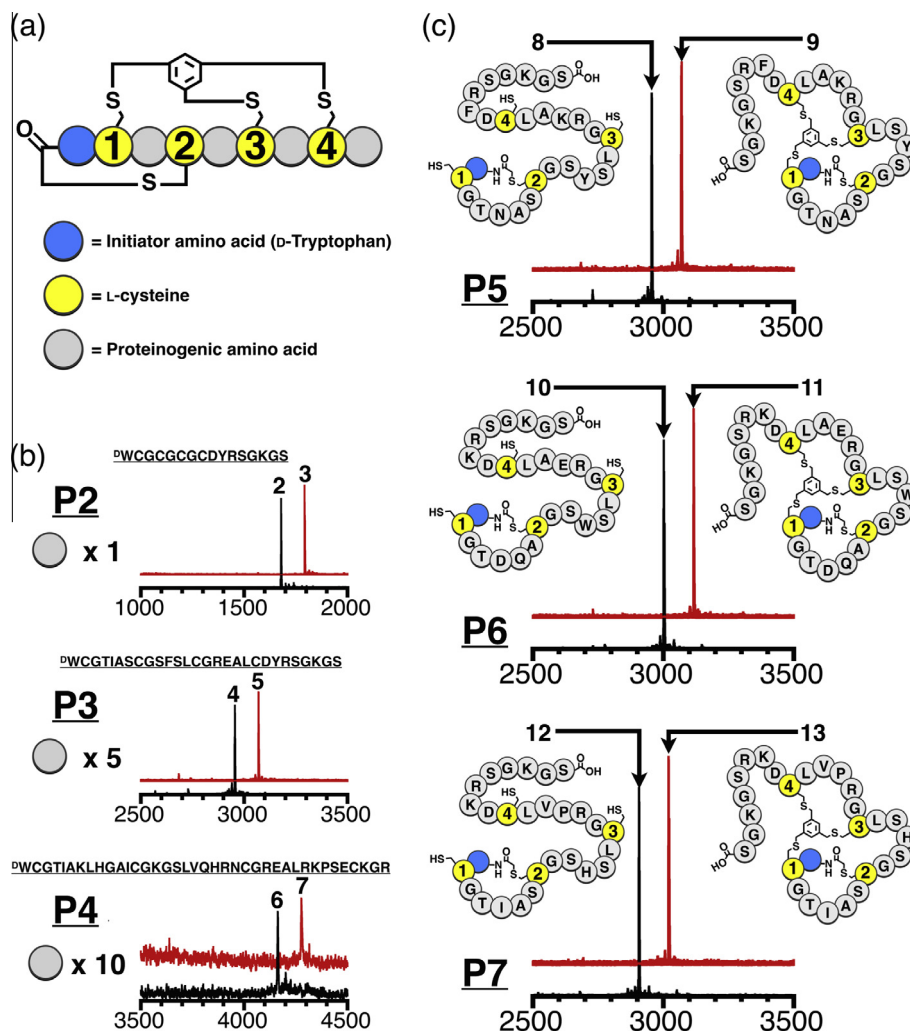


Fig. 4. Ribosomal synthesis of tricyclic peptides with various cycle sizes and amino acid composition. (a) A schematic representation of the topology and the constituents of ribosomally synthesized tricyclic peptides. (b) MALDI-TOF analysis of peptide P2, P3 and P4 which contain 1, 5 and 10 amino acids between each Cys, respectively (amino acid sequences are shown above each spectra). Peaks 2, 4 and 6 in the black spectra correspond to monocyclized peptides. Peaks 3, 5 and 7 in the red spectra correspond to tricyclic peptides resulting from the reaction of TBMB with 2, 4 and 6, respectively. The molecular weights of the observed peaks are as follows: 2, $m_{\text{obs}} = 1678.55$ Da ($m_{\text{calc}} = 1678.59$); 3, $m_{\text{obs}} = 1792.61$ Da ($m_{\text{calc}} = 1792.64$); 4, $m_{\text{obs}} = 2954.32$ Da ($m_{\text{calc}} = 2954.27$); 5, $m_{\text{obs}} = 3068.41$ Da ($m_{\text{calc}} = 3068.32$); 6, $m_{\text{obs}} = 4162.74$ Da ($m_{\text{calc}} = 4160.11$); 7, $m_{\text{obs}} = 4277.26$ Da ($m_{\text{calc}} = 4274.16$). Tricyclization of P2, P3 and P4 gave a MW increase corresponding to the addition of a mesitylene moiety ($m_{\text{calc}} = 114.05$ Da; 2 \rightarrow 3 $m_{\text{obs}} = 114.06$; 4 \rightarrow 5 $m_{\text{obs}} = 114.09$; 6 \rightarrow 7 $m_{\text{obs}} = 114.52$). (c) MALDI-TOF analysis of P5, P6 and P7 which all together comprise all proteinogenic amino acids expect for methionine which must be removed to incorporated CIAC-DW. The peaks in the black spectra (8, 10 and 12) correspond to monocyclic peptides and peaks in the red spectra (9, 11 and 13) represent corresponding tricyclic peptides. The molecular weights of the observed peaks are as follows: 8, $m_{\text{obs}} = 2954.28$ Da ($m_{\text{calc}} = 2954.28$); 9, $m_{\text{obs}} = 3068.44$ Da ($m_{\text{calc}} = 3068.33$); 10, $m_{\text{obs}} = 3001.32$ Da ($m_{\text{calc}} = 3001.28$); 11, $m_{\text{obs}} = 3115.40$ Da ($m_{\text{calc}} = 3115.33$); 12, $m_{\text{obs}} = 2905.40$ Da ($m_{\text{calc}} = 2905.34$); 13, $m_{\text{obs}} = 3019.48$ Da ($m_{\text{calc}} = 3019.38$). Tricyclization of each peptide gave a MW increase corresponding to the addition of a mesitylene moiety ($m_{\text{calc}} = 114.05$ Da; 8 \rightarrow 9 $m_{\text{obs}} = 114.16$; 10 \rightarrow 11 $m_{\text{obs}} = 114.08$; 12 \rightarrow 13 $m_{\text{obs}} = 114.08$).

in the peptide backbone, the hydrolytically labile ester bond could be selectively cleaved by alkaline hydrolysis, allowing us to verify the cyclic topology (Fig. 3a). The D1e template was expressed in the presence of both CIAC-DW-tRNA^{Met}_{CAU} and ^HO^A-tRNA^{Met}_{CAU}. To facilitate the hydrolysis of the ester bond, we chose position 13 for ^HO^A incorporation neighboring a serine residue at position 12. If macrocyclization occurs as predicted between the N-terminal CIAC group and the sulfhydryl group on Cys2, we predicted that alkaline hydrolysis of the ester bond at position 13 would give two peptide fragments detectable by MALDI-TOF MS. On the other hand, macrocyclization between the CIAC group and the sulfhydryl group on Cys3 or Cys4 will not result in the formation of two fragments by the ester hydrolysis.

Ribosomal synthesis of mP1e showed the expected full-length peak upon MALDI-TOF MS analysis (Fig 3b). Upon alkaline hydrolysis of mP1e, we detected two peaks (one with a mass shift of

18 Da corresponding to the addition of H₂O) corresponding to the fragments upstream and downstream of the ester bond, in addition to a minor peak representing unhydrolyzed product. Notably, no peak representing a hydrolyzed full-length peptide with a 18 Da mass shift from the addition of H₂O was observed, indicating that selective cyclization occurred between the N-terminal CIAC group and Cys2, and not between the N-terminus and Cys3 or Cys4. Thus, we concluded that the first thioether-macrocylation occurred between the N-terminus and Cys2, and the following TBMB-mediated cyclization occurred between the sulfhydryl groups on Cys1, Cys3, and Cys4, to yield the tricyclic peptide with the topology as predicted.

We finally examined to see whether the same method could be applied to peptides composed of various lengths between Cys2, Cys3, and Cys4. We designed three DNA templates encoding peptides P2, P3 and P4 composed of 1, 5 and 10 amino acids between

each Cys, respectively (Fig. 4). As expected, each tricyclized sample showed a 114 Da increase compared to their monocyclized counterparts on MALDI-TOF MS analysis. Moreover, we designed another set of three DNA templates varying in amino acid composition based on the framework of P3 (P5, P6 and P7). Indeed, we were able to detect a peak with a 114 Da increase from the monocyclic peptide, indicating the formation of tricyclic peptides. These results confirmed that the tricyclization method was applicable to various peptides regardless of length and amino acid composition. Further, to test for any possible side reactions, LC-ESI-MS (Liquid Chromatography-Electrospray Ionization-Mass Spectrometry) analysis was performed using the template for P6 with ClAc-^LF as the initiator amino acid (P6F) and only negligible amounts of side reaction products were observed (Fig. S1, see more details in the [supplementary discussion](#)). This analysis indicates that the tricyclization method reported here allows us to cleanly convert the linear peptide expressed in the FIT system to the topologically controlled tricyclic peptide via spontaneous thioether-macrocylation between the N-terminal ClAc and Cys2 followed by TBMB-mediated Cys1-Cys3-Cys4 thioether-crosslinking.

4. Conclusion

To summarize, we have demonstrated a unique method of converting ribosomally synthesized thioether monocyclic peptides into fused thioether tricyclic peptides by the addition of TBMB. This method is compatible with peptides of various lengths and amino acid compositions, and therefore it can be readily adapted to the ribosomal synthesis of DNA-encoded tricyclic peptide libraries. Moreover, because of the methodological simplicity and clean conversion from linearly expressed peptides to complex fused tricyclic peptides, the method can be coupled with in vitro display platforms, such as the RaPID (Random non-standard Peptides Integrated Discovery) system. Upon constructing such a platform, we will be able to perform selection of bioactive species that tightly bind to drug targets and readily deconvolute their novel peptide sequences.

Acknowledgments

We thank Dr. Yuki Goto, Dr. Nikita D. Loik and other members of the Suga group for invaluable discussions. This work was supported by a Grant-in-Aid for Scientific Research S (23220204),

Japan Society for the Promotion of Science (JSPS), and Platform for Drug Discovery, Informatics, and Structural Life Science from MEXT, Japan, and JSPS Grants-in-Aid for Fellows to M.N. (253534).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bioorg.2015.06.002>.

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