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Preliminary Communications

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

Nasir Kato Bashiruddin, Masanobu Nagano, Hiroaki Suga *

Department of Chemistry, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

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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.

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 IC_{50} 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-dibromoxylene 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 $tRNA^{fMet}_{CAU}$ 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

* Corresponding author.
E-mail address: hsuga@chem.s.u-tokyo.ac.jp (H. Suga).

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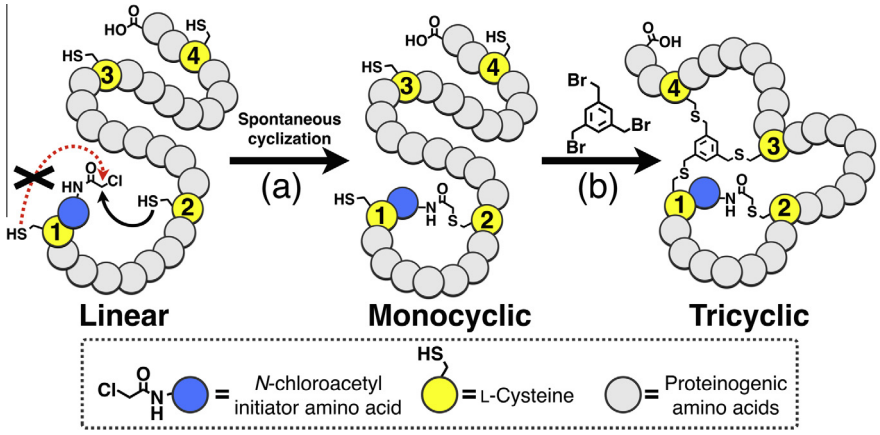

Fig. 1. Schematic representation for the ribosomal production of tricyclic peptides. (a) The Cys in the second amino acid position is not able to react with the N-terminal chloroacetyl group therefore the next closest Cys reacts to form a thioether bond. (b) Addition of TBMB to the monocyclic peptide 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 μg/mL creatine kinase, 3 μg/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]. $tRNA^{fMet}_{CAU}$ was aminoacylated with cyanomethyl ester activated *N*-chloroacetyl D-tryptophan (ClAc-^{D}W) or *N*-chloroacetyl L-tyrosine (ClAc-^{L}F). $tRNA^{fMet}_{CAU}$ 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 acid was added to a final concentration of 5 mM. After a 1 h incubation on ice, the reaction contents were precipitated using an 70% ethanol,

Table 1
Sequences of oligonucleotides used in this study.

Name	Peptide	Oligonucleotide Sequences (5′ to 3′)
L7FW	P1	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGTGGCACCATCGCAAGCAAACACTGCGGCTCCGCCCTTGTG
L7RV		TTTCCGCCCCCCGTCTTATTTCGCATCACACGGGATGCGCAGAGCTTCACCACAGTGCTGCACAAGGGCGGAGCCGCAG
L7eFW	P1e	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGTGGCACCATCGCAAGCAAACACTGCGGCTCCATGCTTGTGCAGCAC
L7eRV		TTTCCGCCCCCCGTCTTATTTCGCATCACACGGGATGCGCAGAGCTTCACCACAGTGCTGCACAAGCATGGAGCCGCAG
3C1FW	P2	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGCGGCTGCGGTTGCGGCTGC
3C1RV		TTTCCGCCCCCCGTCTTACGAACCTTTGCCGCTGCGATAATCGCAGCCGCAACCGCAGCC
3C5FW	P3	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGCGGCACCATTGCGAGCTGCGGTTCGTTTAGCCTGTGCGGCCGTGAAGCG
3C5RV		TTTCCGCCCCCCGTCTTACGAACCTTTGCCGCTGCGATAATCGCACAGCGCTTCACGGCCGCACAGG
3C10FW	P4	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGCGGCACCATTGCGAAATTGCACGGTGCGATCTGCGGG
3C10RV		GGCTTGCGCAACGCTTCACGGCCGCAGTTACGGTGCTGTACCAAGGAACCTTTCCCGCAGATCGCACCGTGC
3C10RV2		TTTCCGCCCCCCGTCTTAGCGACCTTTACACTCCGAAGGCTTGCGCAACGCTTCACG
3C5AFW	P5	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGTGGAACGAATGCAAGTTGCGGTAGTTACAGCTTATGTGGG
3C5ARV		TTTCCGCCCCCCGTCTTAGGAGCCTTTGCCGCTACGGAAGTCACACAGCGCTTTGCGCCCACATAAGCTGTAACTACCGCAACTTGC
3C5BFW	P6/P6F	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGTGGTACTGATCAAGCCTGCGGGAGTTGGTCTTTATGTGG
3C5BRV		TTTCCGCCCCCCGTCTTACGACCCTTTACCCGAGCGCTTGTCGCAGAGGGCTTCGCGTCCACATAAAGACCAACTCCCGCAGGC
3C5CFW	P7	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATGTGCGGAACCATCGCCTCTTGCGGTAGCCACAGTCTCTGTGGC
3C5CRV		TTTCCGCCCCCCGTCTTAAGAACCTTTACCAGAACGTTTATCACAAAGAACAGGGCGGCCACAGAGACTGTGGCTACCGCAAG
T7gAUG	N/A	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATG
DGGRK	N/A	TTTCCGCCCCCCGTCTTA