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Ribosomal Synthesis of an Amphotericin-B Inspired Macrocycle

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Supporting Information

ABSTRACT: Here we report in vitro ribosomal synthesis of a natural product-like macrocyclic peptide, inspired by the structure of amphotericin B (AmB), an amphiphilic and membrane-interacting antifungal natural product. This AmB-inspired macrocyclic peptide (AmP), one side of which is composed of hydrophobic terpene, and the other side comprises a peptidic chain, was synthesized utilizing flexizyme-assisted in vitro translation via an unusual but successful initiation with a D-cysteine derivative. The established method for the synthesis of AmPs is applicable to the generation of a diverse AmP library coupled with an in vitro display format, with the potential to lead to the discovery of artificial bioactive amphiphilic macrocycles.

Natural product (NP) analogues or mimetic molecules have recently attracted much attention as a source of biologically active compounds.[1] This is because NPs offer more diverse structural scaffolds than traditional small-molecular drugs, which are often aromatic-ring-rich and therefore planar. However, at the same time, the complicated structures of NPs, which are rich in stereocenters and sp^3 carbons, hamper the construction of diverse NP-like libraries. Consequently, screening of commercially available synthetic compounds or intermediates in the total syntheses of NPs has been the major source of libraries for the discovery of drug candidates.

In order to generate an NP-like peptide library, a facile but robust methodology is critical to supply diverse compounds with the functional structure of a mother NP. Among membrane-acting NPs, we have considered amphotericin B (AmB), an amphiphilic polyene−polyol macrolide produced by *Streptomyces nodosus*, as a model for mimicry. AmB has been used clinically over 50 years as one of the most potent antifungals.[2,3] Many lines of evidence indicate that AmB exerts its antifugal activity via a unique mode[4,5] in which the hydrophilic polyol side forms an ion-channel inner face selectively in the fungal biomembrane. The hydrophobic polyene moiety synergistically interacts with alkyl chains of lipids and sterols on the outer face of the channel. Therefore, molecular mimicry of the polyene−polyol structural feature of AmB could add a new layer of available scaffolds for macrocyclic peptide libraries to be used for the discovery of bioactive molecules.

Here we report a unique synthetic approach for the ribosomal preparation of AmB-inspired macrocyclic peptides, referred to as AmPs (**1**), by means of a technology involving a reconstituted custom-made translation system assisted by flexible tRNA acylation ribozymes ("flexizymes"), referred to as a flexible in vitro translation (FIT) system.[6] The flexibility of this system for initiation with various kinds of amino acid derivatives, including naturally occurring L-amino acids, *N*-acyl-L-amino acids, *N*-acyl-D-amino acids,[7] and exotic peptides,[8] enables us to express peptides with various *N*-terminal functionalities. In this study, we have applied the technological advantage of the FIT system to investigate initiator designs having a derivative of unsaturated hydrocarbons and demonstrate the expression of a model AmP.

Inspired by the structure of AmB, we designed an AmP in which the AmB's polyene moiety is replaced with a stable and synthetically accessible farnesyl group (Figure 1) and the hydrophilic polyol region is substituted with peptide sequences whose structural and physical properties are potentially tunable. Retrosynthetically, the cyclic structure of **1** can be constructed via the spontaneous post-translational S_N2 reaction of **2** occurring between the *N*-terminal chloroacetamido (ClAc) moiety positioned at the edge of the farnesyl group and the mercapto group of a downstream cysteine.[9] The acyclic precursor peptide **2** would be expressed in the FIT system under the initiation codon reprogramming when a cysteine derivative of *N*-acetyl-*S*-12-(ClAc)farnesyl group (**L-3a**) is charged onto the initiator $tRNA^{fMet}_{CAU}$ by means of a flexizyme.

Our first task commenced with the synthesis of cyanomethyl ester **L-3b** as a substrate recognized by one of our flexizymes, eFx.[10] We carried out synthesis of the precursor carboxylic acid (**L-3a**), followed by cyanomethyl esterification, to yield **L-3b** as a mixture of *E* and *Z* isomers (*E*:*Z* ≈ 1:2) at the terminal olefin possessing a ClAc group (Supporting Information (SI), Scheme S1). We next examined if **L-3b** could be a substrate for eFx using a microhelix (a short RNA analogue of tRNA) assay method (SI, Figure S1).[6e]

After optimization of conditions, a yield of approximately 20% of **L-3a**−microhelix was observed over the total input of microhelix. Since this yield should be sufficient for testing the initiation reprogramming experiment, we then performed the expression of a model peptide with a sequence of **L-3a**-K-K-K-T-Flag (**pL-3a**) from an mRNA template, **mR1**,[11] using the FIT system in the presence of **L-3a**−$tRNA^{fMet}$ prepared under the optimized eFx conditions (Figure S1A). However, the expression of a peptide initiated with **L-3a** was not detected as a band corresponding to the desired **pL-3a** via denaturing tricine-SDS-PAGE analysis (Figure S1B, lane 3); i.e., the initiation using **L-3a**−$tRNA^{fMet}$ was unsuccessful.

To the best of our knowledge and experience in manipulating the translation apparatus, we hypothesized as the reason for this failure that *S*-alkylation of the sterically demanding farnesyl derivative prevented the initiator L-cysteine from the accommodating the ribosome peptidyl-$tRNA^{fMet}$ (P) site in order to form the initiation complex (Figure 2A).[12] If this hypothesis were

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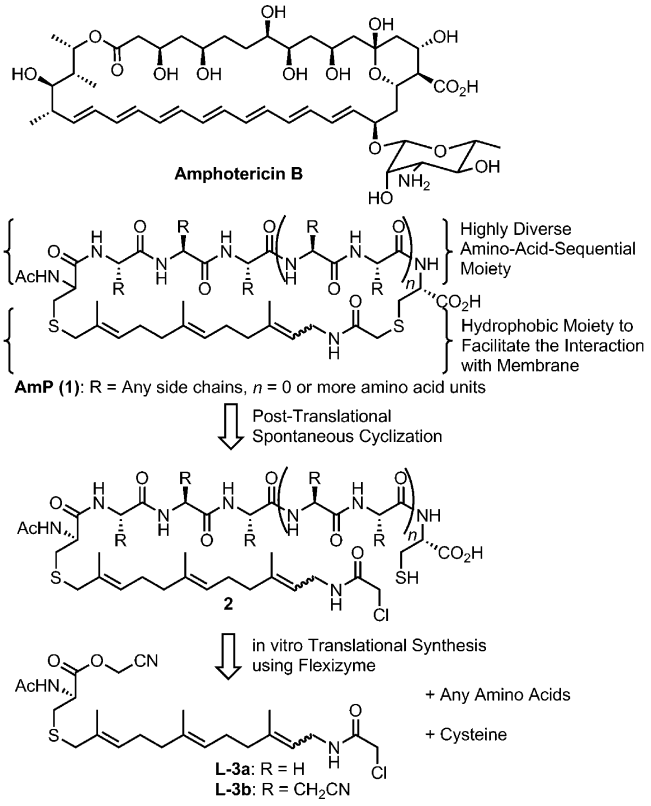

Figure 1. Structure of amphotericin B (top), design concept, and retrosynthetic analysis of AmP.

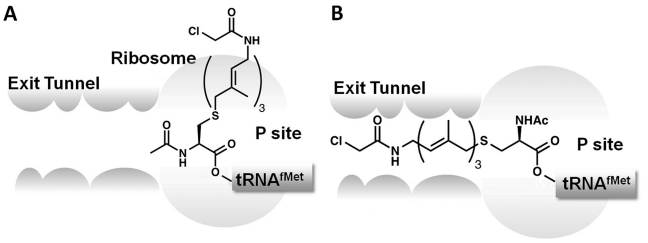

Figure 2. Schematic view of the orientation of peptides in the ribosome P site and exit tunnel. Hypothetical orientations of (A) a peptide possessing **L-3a** and (B) a peptide possessing **D-3a**. Gray wall represents ribosomal residues in the P site and exit tunnel.

correct, switching the initiator's stereochemistry from L- to D-cysteine would accommodate the *S*-farnesyl group at the peptide exit tunnel of ribosome and the *N*-acetyl group at the space for the side chain (Figure 2B), a situation similar to that of the ordinary elongation complex.

We thus redesigned the initiator based on D-cysteine, synthesized **D-3b** (SI, Scheme S2), and then prepared **D-3a**–tRNA^{fMet} by eFx. As expected, tricine-PAGE analysis of translated products showed a peptide band of **pD-3a-1** (Figure 3B, lane 1). The band intensity of **pD-3a-1** was 55% the efficiency of those of the ordinary expression for fMet-initiated peptide (its expression was estimated to be 7.5 pmol/μL based on the method previously reported[8,9]) and the fPhe-initiated peptide upon the reassignment from L-Met to L-Phe at the initiation codon (lanes 4 and 3, respectively), suggesting that the translation efficiency was satisfactory. The observed molecular weight determined by MALDI-TOF mass spectrometry of the isolated peptide was consistent with the calculated value, indicating that the desired

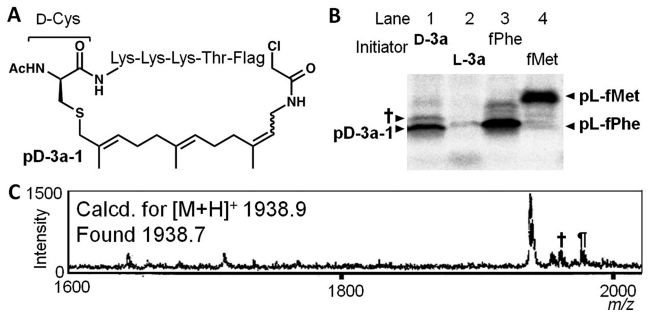

Figure 3. In vitro translational synthesis of **D-3a**-K-K-K-T-FLAG peptide (**pD-3a-1**). (A) Structure of target peptide **pD-3a-1**. (B) Tricine-PAGE of translated peptides: lane 1, **pD-3a-1**; lane 2, **pL-3a** (**L-3a**-K-K-K-T-FLAG); lane 3, formyl-F-K-K-K-T-FLAG; lane 4, formyl-M-K-K-K-T-FLAG. The arrowhead on the left is for lane 1, and those on the right are for lanes 3 and 4. The band marked with † was not elucidated in detail but may correspond to the minor *E* isomer at the terminal olefin possessing ClAc. (C) MALDI-TOF mass spectrum of **pD-3a-1**. Peaks with † (m/z = 1960.7) and ¶ (m/z = 1976.8) correspond to $[M+Na]^+$ (calcd 1960.9) and $[M+K]^+$ (calcd 1976.8), respectively.

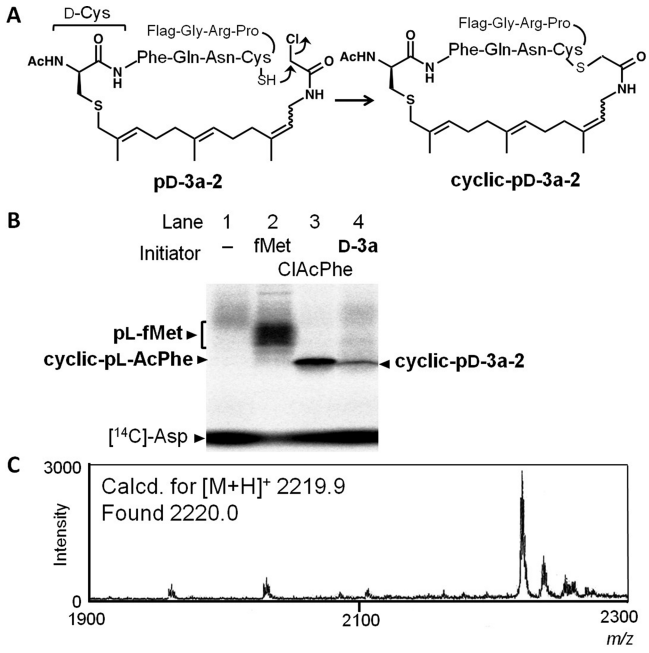

Figure 4. In vitro translational synthesis of AmP. **D-3a**-F-Q-N-C-P-R-G-FLAG peptide was translated. (A) Structures of translated peptide and its spontaneous cyclization furnishing **cyclic-pD-3a-2**. (B) Tricine-PAGE of translated peptides: lane 1, translation product with mR2 in the absence of L-Met; lane 2, L-M-Q-N-C-P-R-G-FLAG (a broad band corresponds to many *S*,*S*-bridged species between free Cys in the peptides and mercaptoethanol, Cys, etc. in the FIT system); lane 3, *N*-(2-chloroacetyl)-F-Q-N-C-P-R-G-FLAG (this peptide also cyclized to form **cyclic-pAcF**); lane 4, **pD-3a-2** was translated and spontaneously converted to **cyclic-pD-3a-2**. (C) MALDI-TOF mass spectrum of **cyclic-pD-3a-2**.

peptide **pD-3a-1** was successfully expressed without concomitant formation of truncated peptides, having Lys instead of **D-3a** at their *N*-termini. (This phenomenon, as a result of translation starting with skipping the first exotic amino acid, is occasionally observed when the efficiency of initiation codon reprogramming is low.) In contrast, the initiation with **L-3a** yielded only a very faint band, probably corresponding to **pL-3a-1** (lane 2), but its

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formation could not be confirmed by mass spectrometry (SI, Figure S2).

Having the above preliminary data, we designed another mRNA template (**mR2**, see SI) expressing **D-3a**-F-Q-N-C-P-R-G-FLAG peptide, where macrocyclization between the ClAc group at the *N*-terminal **D-3a** and the mercapto group of a downstream cysteine residue could take place (Figure 4A, **pD-3a-2** to **cyclic-pD-3a-2**). We thus evaluated the peptide expression level compared that of with the ClAc-L-Phe-initiated peptides (**cyclic-pAcF**). Although the expression level of **cyclic-pD-3a-2** (lane 4) was ca. 19% of fMet initiation (lane 2) (that of **cyclic-pAcF**, lane 3, was ca. 32%), mass analysis revealed that cyclization took place to form the desired AmP structure (Figure 4C).[13]

Here, we have demonstrated the ribosomal synthesis of a model AmP that possesses both peptidic macrocycle[14] and hydrophobic terpene structures to gain the property of amphiphilic. A virtue of this methodology is the facile alteration of peptidic sequences by the simple design of the corresponding mRNAs. This in turn means that the preparation of randomized mRNA sequences encoding the peptidic region allows us to express vast libraries of Amps. Most importantly, this method can be rapidly coupled with an mRNA-display selection format (RaPID system), enabling us to select active species against variously chosen protein targets.[1g,15] Such advancement may yield a unique platform for the discovery of drug leads with amphiphilic property.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic schemes for **L-3b** and **D-3b**; aminoacylation and translation using L-derivative; MALDI-TOF MS spectra of controlling peptides; experimental details; and NMR spectra of **L-3b** and **D-3b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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