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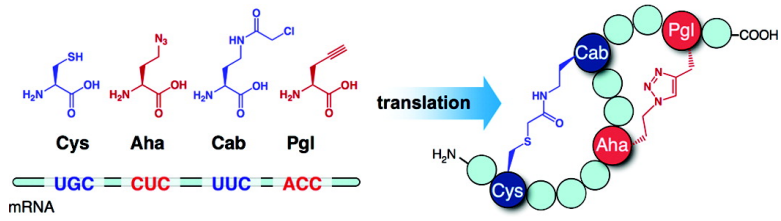
J. Am. Chem. Soc., **2008**, 130 (23), 7232-7234 • DOI: 10.1021/ja800953c • Publication Date (Web): 14 May 2008



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Published on Web 05/14/2008

Ribosomal Synthesis of Bicyclic Peptides via Two Orthogonal Inter-Side-Chain Reactions

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Many naturally occurring peptides possess two pairs of disulfide bonds, representing a bicyclic structural feature.[1] Such a structure rigidifies the peptide conformation into a specific active form and consequently plays a crucial role in the biological function of the peptide. To form the two disulfide bonds, two particular pairs of cysteines (Cys) out of four Cys residues must selectively react with each other, avoiding two other possible combinations[2] (Figure 1A, a−d = Cys). Specific formation of such disulfide bonds in naturally occurring peptides is generally dictated by the proximity of the Cys residues in the tertiary structure[3] or in cells it is occasionally driven by a disulfide isomerase that rearranges kinetically trapped incorrect disulfide pairs to the correct pairs.[4] Although it is of great interest to generate libraries of such bicyclic peptides using the translation apparatus and subsequently screen their biological functions, upon randomization of the sequence, the disulfide bond formation would very likely be scrambled (Figure 1A); thus the 4-Cys-containing peptide scaffold cannot be utilized as a reliable bicyclic platform. We here report a new peptide scaffold containing a single Cys and three nonproteinogenic amino acids (Figure 1B), selectively forming the desirable cross-links. Significantly, this peptide scaffold can be expressed by using a reconstituted *Escherichia coli* cell-free translation system (called PURE[5]) under the reprogrammed genetic code. Therefore, it is readily applicable to the preparation of libraries of bicyclic peptides with a uniform skeleton.

We designed and synthesized two pairs of amino acids containing orthogonal functional groups (Figure 1B). 4-(2-Chloroacetyl)aminobutyric acid (Cab) bears the thiol-reactive functional group in its side chain. We have previously reported that, upon the site-specific incorporation of Cab paired with a Cys into the peptide chain, these two side chains spontaneously react with each other to give a thioether linkage.[6] Because this chemistry requires no additional reagent, the Cab−Cys pair is a convenient and reliable amino acid pair to generate the desired monocyclic peptides closed by a nonreducible bond. The functional groups of the other pair, azidohomoalanine (Aha) and propargylglycine (Pgl), are known to react with each other via Cu(I)-catalyzing azide−alkyne cycloaddition, and this type of chemistry has been extensively used in bioconjugate applications.[7] Despite the fact that the individual molecules were shown to be compatible with translation,[8] their site-specific double incorporations have not yet been demonstrated. Therefore, we first attempted to establish the assignment of Aha and Pgl to certain codons by genetic code reprogramming and demonstrate their double incorporations into the nascent chain of a model peptide, and then perform the intramolecular cyclization.

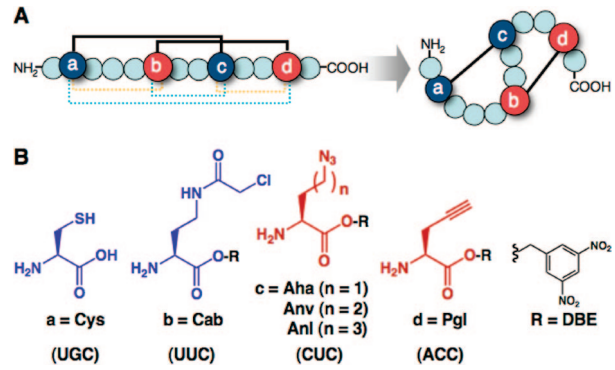

Figure 1. Ribosomal synthesis of bicyclic peptides. (A) Schematic representation of a bicyclic peptide closed by two orthogonal inter-side-chain reactions. (B) Structure and assignment of amino acids to the reprogrammed genetic code. Cab, 4-(2-Chloroacetyl)aminobutyric acid; Aha, azidohomoalanine; Anv, azidonorvaline; Anl, azidonorleucine: Pgl, propr-gylglycine; DBE, 3,5-dinitrobenzyl ester. Codon assignment of each amino acid is shown in parentheses.

In the present study, we chose the Leu codon (CUC) for the assignment of Aha and the Thr codon (ACC) for Pgl. Each amino acid activated with a 3,5-dinitrobenzyl ester (DBE) (Figure 1B) was charged onto orthogonal $tRNA^{Asn\text{-}E1}$ using flexizyme,[9] a tRNA acylation ribozyme (Supporting Information Figure S1). Then, these aminoacyl-tRNAs, Aha-$tRNA^{Asn\text{-}E1}_{GAG}$ and Pgl-$tRNA^{Asn\text{-}E1}_{GGU}$, were added in a PURE system in which both Thr and Leu were withdrawn (referred to as *w*PURE system). We observed double incorporations of ^{5}Aha and ^{11}Pgl into the nascent chain of a model peptide **1** (Figure 2) in 36% yield relative to the yield of wild-type expressed under the normal genetic code (Supporting Information Figure S2A,B); this value translated to the quantity of **1** with an approximately 0.64 pmol/μL compared with 1.75 pmol/μL of the wild-type.[10] MALDI-TOF analysis of the peptide showed a single major peak corresponding to **1** accompanied by a minor peak corresponding to an amine molecule **3** (see more details in the legend of Figure 2).[11] We also demonstrated that the replacement of Aha with its derivatives bearing a longer arm (Figure 1B, Anv, n = 2, and Anl, n = 3) yielded the desired peptides with nearly equivalent quantities and qualities to **1** (Supporting Information Figure S2B,C). Thus, the genetic code reprogramming approach enables us to prepare Aha/Pgl-containing peptides.

To test if the inter-side-chain closure of ^{5}Aha/^{11}Pgl-containing peptide **1** could be achieved by the cycloaddition, **1** was treated with $CuSO_4$ and ascorbate (Figure 2, **1**→**2**). Because we expected that both the unreacted linear form and the cyclic form generated by the cycloaddition would show no change in molecular weight, the occurrence of cycloaddition was confirmed by reducing the azide

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10.1021/ja800953c CCC: $40.75

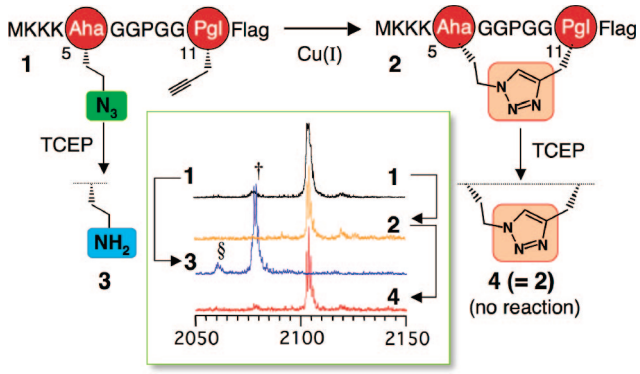

Figure 2. Cyclization via the Cu(I)-catalyzing azide−alkyne cycloaddition. The observed molecular mass (m_{obs}) of **1** is 2103.52 Da, while the calculated molecular mass (m_{calc}) is 2102.96 Da. Likewise, **2** is m_{obs} = 2103.47 and m_{calc} = 2102.96, while **3** is m_{obs} = 2076.54 Da and m_{calc} = 2076.97 Da. Upon treatment of **2** with TCEP, no change in m_{obs} was detected (m_{obs} = 2102.56). The minor peak shown by † in the spectrum of **1** is consistent with m_{obs} of **3**, possibly generated by the reduction of the azide group by DTT and/or mercaptoethanol contained in the translation buffer;[11] § corresponds to a peak of an imido ester likely produced by a similar mechanism reported by Back et al.[12]

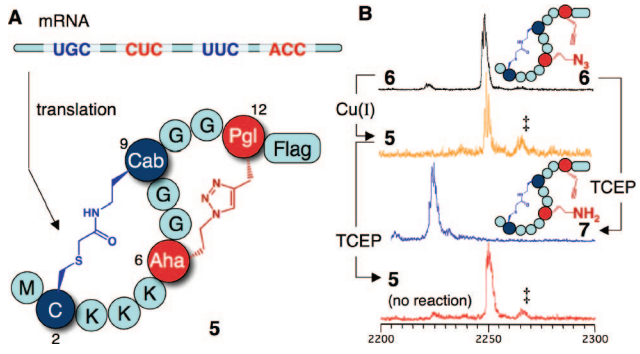

Figure 3. Ribosomal synthesis of bicyclic peptides. (A) Schematic structure of the bicyclic peptide **5** containing ^{9}Cab, ^{6}Aha, and ^{12}Pgl. (B) MALDI-TOF analysis of the nascent peptide **6** (m_{obs} = 2248.29 Da, m_{calc} = 2248.97 Da), which was spontaneously cyclized by the thioether bond between Cab and Cys side chains. TCEP reduction of peptide **6** resulted in the formation of the reduced peptide **7** (m_{obs} = 2222.94 Da, m_{calc} = 2222.98 Da), while bicyclic peptide **5** produced by the copper-catalyzed cycloaddition of **6** was not reduced (m_{obs} = 2248.36); ‡ denotes a peak of peptide **5** in which Met was oxidized during the MALDI-TOF analysis.

group by incubating with tris(carboxyethyl)phosphine (TCEP). Prior to the cycloaddition, the azide group of **1** was readily reduced by TCEP to yield the corresponding amine **3**, consistent with its MALDI-TOF analysis (Figure 2, **1**→**3**). In contrast, the TCEP treatment of the product generated by incubation of **1** with Cu(I) showed no change in the molecular weight (Figure 2, **2**→**4**). This result confirmed that **1** was converted to **2** by the cycloaddition. Likewise, ^{5}Anv/^{11}Pgl- or ^{5}Anl/^{11}Pgl-containing peptide, in which the ring structure was assisted by a longer arm, also afforded the corresponding cyclic peptide upon the Cu(I)-catalyzing reaction (Supporting Information Figure S4).

With the above cyclization methodology in our hands, we next applied it to the synthesis of bicyclic peptides in combination with the intramolecular thioether formation between Cab and Cys. We prepared an mRNA template for the expression of peptide **5** containing ^{2}Cys, ^{6}Aha, ^{9}Cab, and ^{12}Pgl in the peptide sequence (Figure 3A). ^{9}Cab was assigned to a Phe codon (UUC) by charging onto tRNA$^{Asn\text{-}E1}_{GAA}$ using flexizyme. We then performed the triple sense suppressions of ^{6}Aha, ^{12}Pgl, and ^{9}Cab using a *w*PURE system lacking Leu, Thr, and Phe. MALDI-TOF analysis of the peptide showed a major peak corresponding to the monocyclic **6** closed by a thioether bond between the side chains of ^{2}Cys and ^{9}Cab (Figure 3B, **6**). These results indicated that the "first" eight-membered ring cyclization occurred in the translation mixture in situ cleanly. **6** was then subjected to the "second" seven-membered ring cyclization step in the presence of Cu(I) to yield **5**, which was further treated with TCEP. As expected, the molecular weight of the product (**5**) did not show any changes before or after TCEP treatment (Figure 3B, **6**→**5**→**5**), as opposed to the observation that the Cu(I)-untreated **6** was reduced to the corresponding amine **7** by TCEP (**6**→**7**). This result indicates that peptide **5** has the expected bicyclic structure with an eight/seven-membered ring fusion.

Using the same DNA template that expressed **5**, Aha was substituted with Anv or Anl by simply altering the use of the corresponding Anv- or Anl-tRNA$^{Asn\text{-}E1}_{GAG}$ in the translation. The respective precursor peptide containing ^{2}Cys, ^{9}Cab, and ^{12}Pgl along with ^{6}Anv or ^{6}Anl was expressed under the reprogrammed genetic code and treated with Cu(I). Again, to ensure the cycloaddition, these peptides as well as the precursor monocyclic peptides were treated with TCEP, and the resulting peptides were analyzed by MALDI-TOF (Supporting Information Figure S5). The data were indeed consistent with the idea that the desired bicylic peptides were produced upon the Cu(I) treatment.

Encouraged by the above observations, we next attempted to apply this methodology to the expression of peptides composed of different sequences with other combinations of ring sizes. We designed three DNA templates (see Tables 1 and 2 in Supporting Information) that would produce precursor peptides **8**, **11**, and **14** (Figure 4A−C) under the assignments of Aha, Cab, and Pgl to the Leu, Phe, and Thr codons. It was expected that these precursor peptides were spontaneously closed by the first sulfhydryl−chloroacetyl cyclization upon the translation and then converted to bicyclic peptides **10**, **13**, and **16**, respectively, via the second azide−alkyne cycloaddition catalyzed by Cu(I) (Figure 4A−C, left panels). Consequently, peptide **10** had the same bicyclic structure as **5** with an eight/seven-membered ring fusion, but its sequence composition of amino acids in each ring differed from that of **5**. Moreover, peptides **13** and **16** were composed of not only different amino acid sequences but also different bicyclic structures with eight/eight- and seven/seven-membered ring fusions, respectively. To confirm the occurrence of azide−alkyne cycloaddition, the precursor as well as cyclized peptides were treated with TCEP, and these peptides were analyzed by MALDI-TOF (Figure 4A−C, right panels). As expected, the precursor peptides **8**, **11**, and **14** were reduced by TCEP to yield the corresponding monocyclic amino peptides, **9**, **12**, and **15**. In contrast, no change in molecular weight was observed for the Cu(I)-treated peptides, indicating that the cyclization of **8**, **11**, and **14** took place to yield bicyclic peptides **10**, **13**, and **16**, respectively. These results have clearly shown the versatility of this methodology to the synthesis of bicyclic peptides with various sequences and ring sizes in the 7/7, 8/7, and 8/8 fusions.

In conclusion, we have developed a new methodology to construct bicyclic scaffolds by the combination of peptide translation under a reprogrammed genetic code with post-translational cyclizations involving two pairs of amino acids, Cys−Cab and Aha−Pgl. Because the sulfhydryl−chloroacetyl reaction and the azide−alkyne cycloaddition are orthogonal, they act as the specific pairs to yield only the desirable bicyclic peptide with fairly clean conversions of each step. Most importantly, we have shown the versatility of this methodology to the peptide synthesis of various bicyclic peptides bearing fused ring scaffolds, which are controlled by the side chain length of the azide-containing nonproteinogenic amino acids and even the amino acid sequence compositions between the cyclizing

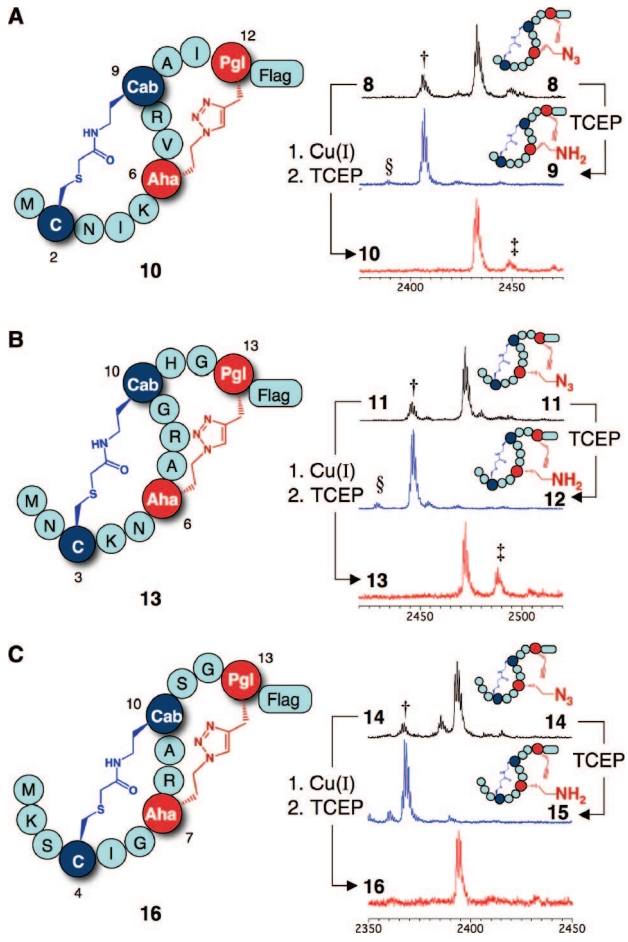

Figure 4. Ribosomal synthesis of bicyclic peptides from three different template DNAs. Left panel shows the schematic structures of bicyclic peptides **10**, **13**, and **16**. Right panel shows MALDI-TOF analysis of the precursor monocyclic peptides **8**, **11**, and **14**, the TCEP-reduced peptides **9**, **12**, and **15**, and Cu(I) followed by TCEP-treated peptides **10**, **13**, and **16**. (A) Formation of bicyclic peptide **10**. Molecular weights of the observed peaks are as follows: **8**, m_{obs} = 2430.58 Da (m_{calc} = 2431.12); **9**, m_{obs} = 2405.82 Da (m_{calc} = 2405.13); bicyclic peptide **10**, m_{obs} =2431.46 Da (m_{calc} = 2431.12). The minor peak indicated by † (m_{obs} = 2404.57 Da) was possibly generated due to the reduction of the azide group by DTT and/or mercaptoethanol contained in the translation buffer.[11] The minor peak indicated by ‡ (m_{obs} = 2448.52 Da) was likely generated by the oxidation of Met of **10** during the MALDI-TOF analysis; § denotes an imido ester produced by a similar mechanism reported by Back et al.[12] (B) Formation of bicyclic peptide **13**. Molecular weights of the observed peaks are as follows: **11**, m_{obs} = 2470.67 Da (m_{calc} = 2471.02); **12**, m_{obs} = 2444.85 Da (m_{calc} = 2445.03); bicyclic peptide **13**, m_{obs} =2471.23 Da (m_{calc} = 2471.02). The minor peaks shown by † and ‡ are m_{obs} = 2444.58 and 2487.75 Da, respectively. (C) Formation of bicyclic peptide **16**. Molecular weights of the observed peaks are as follows: **14**, m_{obs} = 2392.76 Da (m_{calc} = 2393.03); **15**, m_{obs} = 2366.95 Da (m_{calc} = 2367.04); bicyclic peptide **16**, m_{obs} =2393.16 Da (m_{calc} = 2393.03). The minor peak indicated by † corresponds to m_{obs} = 2366.79 Da, whereas the other minor peak observed near 2390 Da was unknown.

residues. Notably, the libraries of bicyclic peptides can be easily constructed by synthesis of the corresponding mRNA libraries. Screening of such peptide libraries with uniform bicyclic scaffolds, perhaps in combination with an appropriate in vitro peptide-display technique, will quickly lead us to discover unique peptidyl drugs against various therapeutic targets.

Acknowledgment. We thank Dr. P. C. Reid for critical proofread. This work was supported by grants of Japan Society for the promotion of Science Grants-in-Aid for Scientific Research (S) (16101007) to H.S., Grants-in-Aid for JSPS Fellows to Y.S. (17-11918), and a research and development projects of the Industrial Science and Technology Program in the New Energy and Industrial Technology Development Organization (NEDO).

Supporting Information Available: Experimental details and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) Our general method for the peptide quantification was as follows: Radioisotope (RI) counts of [^{14}C]-Asp were plotted against its known concentrations to determine the calibration line. Then, RI counts of the expressed wild-type peptide and cyclic peptides were fitted to the calibration line to estimate the individual concentration of peptides. We found that this quantification method was reliable to assess the quantity of peptide previously proven by performing activity assays using a known bioactive peptide.[6].

(11) During the translation, the azide group of Aha, Anv, and Anl was very likely reduced by DTT and/or mercaptoethanol included in the translation buffer. See the following reference as an example of chemistry: Meinjohanns, E.; Meldal, M.; Jensen, T.; Werdelin, O.; GalliStampino, L.; Mouritsen, S.; Bock, K. *J. Chem. Soc., Perkin Trans. 1* **1997**, 871–884.

(12) Back, J. W.; David, O.; Kramer, G.; Masson, G.; Kasper, P. T.; de Koning, L. J.; de Jong, L.; van Maarseveen, J. H.; de Koster, C. G. *Angew. Chem., Int. Ed.* **2005**, *44*, 7946–7950. We found that the reported mechanism herein also proceeds in the translated peptides containing appropriate nonproteinogenic amino acids (Nakajima, E.; et al. unpublished results). More detailed studies of this work will be reported elsewhere.

JA800953C