DOI: 10.1002/cbic.200900021

Ribosomal Synthesis of Cyclic Peptides with a Fluorogenic Oxidative Coupling Reaction

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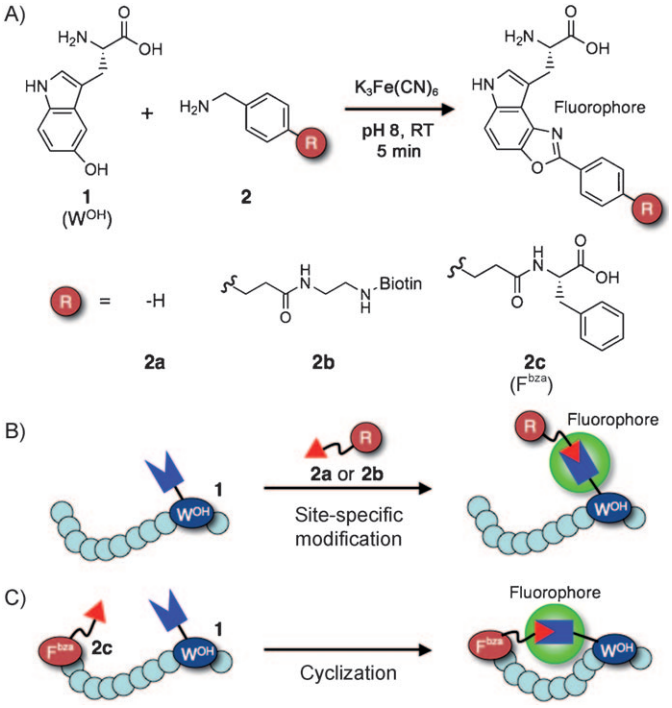
Cyclic peptides often have better physiological properties than linear peptides due to their increased structural rigidity and peptidase resistance.[1–3] Despite the fact that translation provides an attractive platform for the synthesis of cyclic peptides, those expressed by the translation apparatus have cysteine–cysteine (Cys–Cys) disulfide bridges, and this limits their use. However, because substitution of the disulfide bond with a nonreducible bond is expected to give better physiological stability, it has been of interest to develop novel methodologies for generating such nonstandard cyclic peptides expressed by translation. For instance, a non-natural amide linkage was introduced between the N-terminal α-amino and the lysine ε-amino groups by the treatment of peptides with a crosslinking reagent that had been activated by *N*-hydroxysuccinimide.[4,5] Likewise, the sulfhydryl groups of two Cys residues existing in peptides were crosslinked with α,α′-dibromo-*m*-xylene to generate a unique cyclic peptide.[6] In contrast to these approaches, we have devised unique methods that use nonproteinogenic amino acids with sidechains that selectively form linkages to the cognate pair.[7–9] Here, we extend such methodology to incorporate a pair of nonproteinogenic amino acids containing benzylamine and 5-hydroxyindole functionalities into designated sites assigned by reprogrammed codons. Under oxidative conditions, these groups instantly react with each other and form a fluorescent heterocyclic moiety. Thus, this method enables the conversion of a nonfluorescent linear peptide to a fluorescent cyclic peptide.

5-Hydroxytryptamine (serotonin) is known to react rapidly and under mild conditions with benzylamine (**2a**) in the presence of potassium ferricyanide, $K_3Fe(CN)_6$, to yield a benzoxazole derivative (**14**, Figures S1 and S5 in the Supporting Information).[10–12] It is noteworthy that the resulting adduct is fluorescent with an excitation maximum at 345 nm and an emission maximum at 460 nm. We have envisioned that this oxidative reaction would allow us to develop a new class of orthogonal pairs for the intermolecular and intramolecular reactions of polypeptides involving 5-hydroxytryptophan (W^{OH}, **1**) and three benzylamine derivatives (**2a–c**, Scheme 1A). As a preliminary study, W^{OH} was incorporated into a model peptide at a specific site, and the resulting peptide could be site-selectively modified with a benzylamine (**2a**) or its biotin-bearing derivative (**2b**) (Scheme 1B). Upon successful demonstration of the above intermolecular coupling reaction, we attempted cyclization of peptides between the benzylamine attached to the α-amino group of phenylalanine (F^{bza}, **2c**) and W^{OH} in the nascent chain to give fluorescent cyclic peptides (Scheme 1C). In both cases, incorporation of the above nonproteinogenic amino acids into peptides was achieved by genetic code reprogramming by using the flexizyme system,[13,14] and a modified peptide-translation by using the recombinant elements (PURE) system.[15–17] The former system consists of *flexi*ble tRNA acylation ribo*zymes*, and the latter system is a reconstituted



Scheme 1. A) Fluorogenic oxidative coupling reactions between 5-hydroxytryptophan **1** (W^{OH}) and benzylamine derivatives **2**. Reaction between **1** and **2** is achieved by treatment with K_3FeCN_6 in aqueous media to form the fluorophore structure. B) Schematic presentation of the site-specific modification of peptides bearing W^{OH} by intermolecular reaction with benzylamine derivatives (**2a** or **2b**). Blue symbol and red triangle represent 5-hydroxyindole and benzylamine groups, respectively. C) Schematic presentation of the cyclization of peptides by the intramolecular reaction between **1** and **2c** (F^{bza}).

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.200900021: further details of all experiments.

cell-free translation system in which isoleucine (Ile) and/or methionine (Met) were *w*ithdrawn, referred to as *w*PURE system.

To reassign W^{OH} to AUC (one of the vacant Ile codons), W^{OH}-$tRNA^{Asn\text{-}E1}_{GAU}$ prepared by the flexizyme was added to a *w*PURE system lacking Ile. An mRNA template expressing a dodecamer model peptide containing the Ile codon at position 11 (**3**, Figure S5) was subjected to translation. The expressed peptide was desalted by using a conventional C_{18}-resin filter, and the isolated peptide was analyzed by using a MALDI-TOF mass spectrometer. The observed molecular mass (ms) was consistent with the expected ms of peptide **3** containing W^{OH}11 (Figure S5B). The expression level of **3** was approximately 51% relative to that of the wild-type peptide containing Ile11 expressed in the ordinary PURE system (Figures S3 and S4); this value translated to approximately 0.46 pmol μL^{-1} of **3** compared with 0.91 pmol μL^{-1} of the wild-type as determined by the quantification method reported elsewhere.[18]

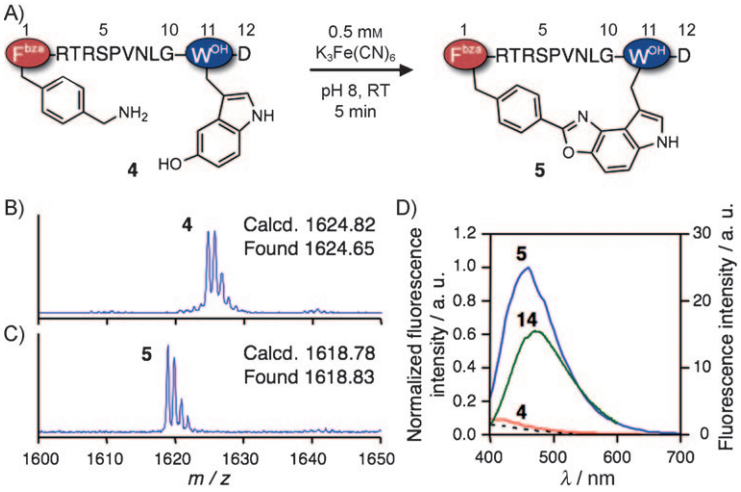

Figure 1. A) Intramolecular cyclization of peptide **4** through an oxidative coupling reaction. B) MALDI-TOF spectra of linear peptide **4** and C) cyclic peptide **5**. The calculated molecular mass (Calcd.) and found molecular mass (Found) for singly charged species, $[M+H]^+$ are shown in the spectra. D) Emission spectra of **4**, **5**, and **14**. The spectra were recorded in 250 mM borate buffer and DMSO 10% (*v*/*v*) adjusted pH to 8.0. The green line indicates the fluorescent profile of 300 nM **14** at an excitation wavelength of 326 nm (right axis), and other lines show the emission profiles at an excitation wavelength of 345 nm (left axis). The dashed line shows a blank profile obtained in the above buffer, while red and blue lines indicate the profiles of **4** and **5**, respectively.

For the conjugation, the translation solution containing crude peptide **3** was treated with the benzylamine derivatives **2a** or **2b** in the presence of $K_3Fe(CN)_6$ at room temperature for 5 min (Figure S5A) and then desalted. MALDI-TOF analysis revealed that **3** was modified by the respective reagents to yield **3a** and **3b** without undesirable side-reactions (Figure S5C and S5D). To further confirm the formation of the conjugation through a heterocyclic structure, we next examined the fluorescent properties of peptides **3**, **3a**, and **3b** (Figure S5E). The unmodified peptide **3** showed nearly the same emission profile as a blank sample; this indicates that it does not exhibit any fluorescent properties under such conditions (Figure S5E, **3**). In contrast, both modified peptides **3a** and **3b** exhibited similar fluorescent profiles with an excitation maximum at 345 nm and an emission maximum at 460 nm (Figure S5E, **3a** and **3b**). The above fluorescent profiles were nearly identical to that of fluorophore **14** (Figures S1and S5E), which is derived from 5-hydroxyindole and **2a**; this implies that the same fluorophore was generated. Because the molecular absorption coefficient and fluorescent quantum yield were not available in the literature, we determined these parameters for **14** under similar conditions to which the peptides were monitored. These values were determined to be 1.54×10^4 cm^{-1} M^{-1} and 0.54 at pH 8.0 in 250 mM borate buffer. The above data encouraged us to perform the same chemistry in intramolecular fashion and apply it to the directed cyclization of peptides.

We synthesized a phenylalanine derivative bearing a benzylamine group on the α-amino group (Scheme 1A, **2c**) and charged it onto $tRNA^{fMet}_{CAU}$ by means of flexizyme.[7,8,19,20] A *w*PURE system lacking Met and Ile was prepared, to which F^{bza}-$tRNA^{fMet}_{CAU}$ and W^{OH}-$tRNA^{Asn\text{-}E1}_{GAU}$ were added in order to initiate the translation with F^{bza} instead of *N*-formylmethionine (fMet); this was followed by elongation with the corresponding amino acid sequence including W^{OH}11 (Figure 1A, **4**). Oxidation of dodecamer **4** in the presence of $K_3Fe(CN)_6$ resulted in a decrease in molecular weight that corresponded to a loss of six protons observed in MALDI-TOF analysis of the product; this suggested that **5** was being produced (Figure 1B and C). The excitation and emission profiles of **5** showed the respective maxima at 345 and 460 nm, whereas such a profile was not observed for **4** (Figure 1D). The fluorescent profile of **5** was similar to that of **14** as well as the modified peptides **3a** and **3b**. This result solidified the idea that the linear peptide **4** was cyclized to afford **5** with the expected eleven-membered ring structure closed by the fluorescent linker (Figure 1A).

To survey the orthogonality of the oxidative coupling reaction with various sidechains of proteinogenic amino acids, we designed peptide sequences containing F^{bza}, W^{OH} and those amino acids that are potentially reactive under oxidative conditions, such as Lys, Cys, Tyr and Trp (as opposed to an unreactive Leu) at position 13 (Figure 2A, **6**–**10**). Expression of peptides **7**–**10** took place smoothly with efficiencies of the range of 51–75% relative to the control Leu13-containing peptide **6** (Figure 2B, lanes 1–5). MALDI-TOF analysis of the resulting peptides indicated that all were converted to the desired cyclic peptides based on the observation of the same mass change corresponding to the loss of six protons (Figure S6A–E) with one exception; peptide **8**, which contains Cys13, seemed to have an extra set of peaks with an average of −8.07 mass shift along with the set of peaks with a −6.04 shift (Figure S6C, **8**). To the best of our knowledge, the sulfhydryl group of glutathione intermolecularly reacts with a dione derived from the oxidation of W^{OH}; this results in a 7-*S*-glutathionyl-tryptophan-4,5-dione adduct.[21] Thus, we attributed the above observation to a similar side-reaction between Cys13 and W^{OH}7 that competed with the reaction between F^{bza} and W^{OH}7. To further confirm the oxidative coupling between F^{bza} and W^{OH}7, we mea-

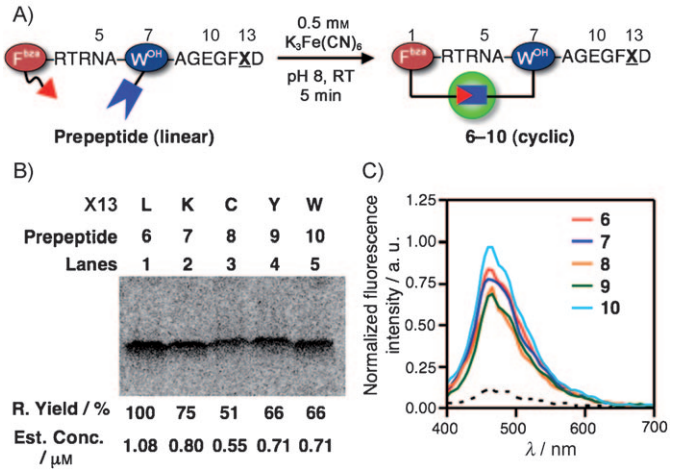

Figure 2. Competitive reaction between W^{OH} and potentially reactive proteinogenic amino acids against W^{OH} and F^{bza}. A) Peptide sequences used for the verification. The proteinogenic residue X13 was altered to L, K, C, Y, and W. B) Tricine SDS-PAGE analysis of the linear peptide labeled with [^{14}C]-Asp. The band of peptide was quantified by radio-autoradiography. Lane 1, **Pre-6** (X13 = L); lane 2, **Pre-7** (K); lane 3, **Pre-8** (C); lane 4, **Pre-9** (Y); lane 5, **Pre-10** (W). The peptide concentration was estimated by our standard quantification method in which the radioisotope (RI) counts of [^{14}C]-Asp were plotted against its known concentration to determine the calibration line. Then, the RI counts of the expressed peptides were fitted to the calibration line to estimate their respective concentration. R. Yield and Est. Conc. denote relative yield and estimated concentration. C) Emission spectra of cyclic **6**–**10**. The spectra were recorded in 250 mM borate buffer and DMSO 10% (*v*/*v*) adjusted pH at 8.0. Solid lines show the emission profiles at an excitation wavelength of 345 nm. The dashed line shows a blank profile obtained in translation without DNA template, while red, blue, orange, green and cyan lines indicate those of **6**, **7**, **8**, **9**, and **10**, respectively.

sured the fluorescence spectra of the product (Figure 2C). Fluorescence intensities were normalized according to the concentration of the respective linear peptides **7**–**10** against the control peptide **6** and quantified by tricine SDS-PAGE analysis (Figure 2B). All peptides exhibited similar fluorescent properties; this is consistent with the idea that they were cyclized. It should be noted that even though production of peptide **8** was accompanied by side-product formation, its fluorescent intensity was similar to that of **6** (Figure 2C, spectrum **8**). Because we expected that the side-product would not show a similar fluorescent property, we concluded that the side-reaction between Cys13 and $W^{OH}7$ was fairly minor; this provided evidence that the intramolecular coupling reaction between F^{bza} and $W^{OH}7$ took place in a fairly selective manner.

Lastly, we verified that this cyclization method would be applicable to the synthesis of peptides with various ring sizes (Figure 3). We prepared three different mRNAs capable of expressing decamer, octamer, and hexamer peptides in the presence of F^{bza}-tRNA$^{fMet}_{CAU}$ and W^{OH}-tRNA$^{Asn-E1}_{GAU}$ in the Met/Ile-lacking *w*PURE system. Treatment of the expressed peptides with $K_3Fe(CN)_6$ was expected to produce the corresponding cyclic peptides rings containing W^{OH} at positions 9, 7, and 5 in peptides **11**–**13**, respectively (Figure 3A–C). In all cases, the linear peptides were cleanly converted to the cyclic peptides with the expected loss of six protons detected by MALDI-TOF analysis (Figure 3A–C, ms data in the middle panel). Moreover, all of the cyclic peptides **11**–**13** exhibited nearly identical fluorescent profiles to **14**, whereas the linear ones showed no fluorescence (Figure 3A–C, the bottom panel). These data clearly demonstrate that this method is applicable to the cyclization of peptides with a wide range of ring sizes (from five- to eleven-membered rings).

In conclusion, we have developed a new method for the cyclization of peptides that involves oxidative coupling of W^{OH} and F^{bza} attached to their N terminus by means of reprogramming the genetic code. The selective coupling of the above pairs takes place rapidly upon addition of $K_3Fe(CN)_6$ and generates the desired fluorescent heterocyclic linkage. This chemistry enables us to convert nonfluorescent linear peptides to fluorescent cyclic peptides. Notably, the reaction takes place independently from the forming ring sizes, at least in the range of five- to eleven-membered rings as demonstrated in the current study. The chemistry demonstrated here by using the *w*PURE system is readily applicable to the platform of solid-phase or

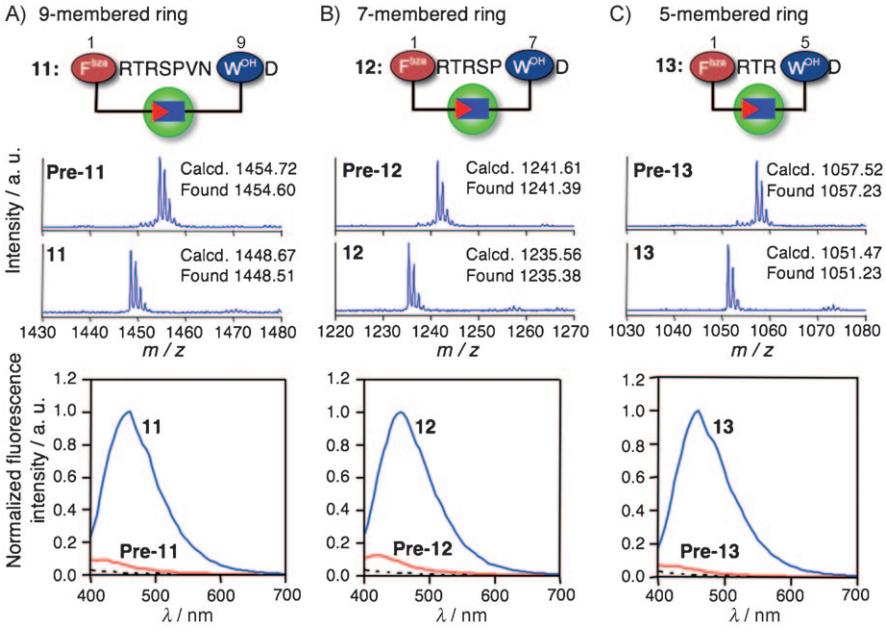

Figure 3. Synthesis of cyclic peptides with various ring sizes. Peptides **11**–**13** consist of A) 9, B) 7, and C) 5-membered rings. The upper panel of each Figure shows the structure of cyclic peptide, the middle panel exhibits MALDI-TOF spectra of precursor (**Pre-11**–**13**) and cyclic peptides (**11**–**13**), and bottom panel shows the excitation and emission profiles of **Pre-11**–**13** and **11**–**13**. The dashed line shows a blank profile. The conditions and designations of lines are the same as Figure 1.

solution-phase chemical synthesis, and thereby allows us to access to the large-scale production of the fluorescent cyclic peptide. On the other hand, the ribosomal expression of the peptides in the mRNA-encoding manner enables us to couple with an appropriate in vitro display technique that facilitates the screening of a large sequence space of cyclic peptides. Thus, the synthetic methodology reported herein offers us a new means for the discovery of cyclic peptide probes containing a unique fluorescent heterocyclic structure against various biological targets.

Experimental Section

Chemical synthesis of the amino acid derivatives, biotinyl benzylamine, and the benzoxazole fluorophore is described in the Supporting Information. Flexizyme, $tRNA^{Asn-E1}_{GAU}$, $tRNA^{fMet}_{CAU}$, and DNA templates coding peptides were prepared as previously reported.[13] Aminoacyl-tRNAs (W^{OH}-$tRNA^{Asn-E1}_{GAU}$ and F^{bza}-$tRNA^{fMet}_{CAU}$) were prepared by using the flexizyme system. The *w*PURE system was reconstituted with the same components as the PURE system except that the necessary proteinogenic amino acids were added. Peptide **3**, which contained W^{OH}, was synthesized by using W^{OH}-$tRNA^{Asn-E1}_{GAU}$ and the *w*PURE system without Ile. Linear peptides **4**, and **6**–**13** containing W^{OH} and F^{bza} were synthesized by using W^{OH}-$tRNA^{Asn-E1}_{GAU}$, F^{bza}-$tRNA^{fMet}_{CAU}$ and the *w*PURE system without Ile and Met. Translation mixtures were incubated at 37 °C for 30 min. For MALDI-TOF analysis, 5.0 μL of reaction mixture was acidified with TFA (1% (*v*/*v*)) in H_2O and purified by using a PerfectPure C-18 Tip (Eppendorf, Hamburg, Germany). Peptide product on the resin was eluted with an acetonitrile (50% (*v*/*v*))/TFA (0.1% (*v*/*v*)) solution (2.0 μL total) saturated with the matrix α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Bremen, Germany).

For MALDI-TOF analysis of oxidation coupling reactions, modification was performed as follows: borate buffer (1.0 M, 2.5 μL, pH 8.0), **2a** or **2b** in DMSO (100 mM, 1.0 μL), and $K_3Fe(CN)_6$ in H_2O (13.3 mM, 1.5 μL) were added to the translation mixture (5.0 μL) containing **3** (0.46 μM). Components and concentrations of translation mixture were described in elsewhere.[22] On the other hand, cyclization was carried out as follows: borate buffer (500 mM, 2.5 μL, pH 8.0), and $K_3Fe(CN)_6$ in H_2O (2.5 mM, 2.5 μL) were added to the translation mixture (5.0 μL). The resulting mixture was left for 5 min at room temperature. The translation mixture was acidified with TFA in H_2O (1% (*v*/*v*)). The product was purified by using a C-18 Tip and eluted with acetonitrile (50% (*v*/*v*))/ TFA (0.1% (*v*/*v*)) solution (2.0 μL total) saturated with the matrix α-cyano-4-hydroxycinnamic acid.

For fluorescence analysis of oxidation coupling reactions, each peptide was purified by using a C-18 Tip from translation mixture (5.0 μL), and eluted with an acetonitrile (50% (*v*/*v*))/TFA (0.1% (*v*/*v*)) solution (10 μL total). The eluent was evaporated for 30 min at room temperature, and the resulting residue was dissolved in H_2O (1.25 μL). Borate buffer (1.0 M, 1.25 μL, pH 8.0), **2a** or **2b** in DMSO (100 mM, 0.5 μL) were added to modified peptide **3**, and then $K_3Fe(CN)_6$ in H_2O (5 mM, 2.0 μL) was added. To generate cyclic peptide **5** and **6**–**13**, the peptide residue was dissolved in H_2O (1.25 μL), borate buffer (1.0 M, 1.25 μL, pH 8.0), and DMSO (0.5 μL), and then added to $K_3Fe(CN)_6$ in H_2O (1.25 mM, 2.0 μL). The resulting mixture was incubated for 5 min at room temperature. The emission and excitation spectra were measured by using a 1536-well micro plate (BD Falcon, Franklin Lakes, USA) at 25 °C. Excitation and emission slit-widths were kept constant at 5.0 nm.

Acknowledgements

We thank Dr. M. Komiyama for the use of UV-Vis spectrophotometer and spectrofluorometer and Dr. P. C. Reid for critical proofreading. We thank Drs. I. Hirao and T. Mitsui in the RIKEN Genomic Sciences Center for the use of a FAB mass spectrometer. This work was supported by grants of Japan Society for the Promotion of Science Grants-in-Aid for Scientific Research (S) (16101007) and a research and development projects of the Industrial Science and Technology Program in the New Energy and Industrial Technology Development Organization (NEDO) to H.S.

Keywords: cyclization · fluorescent probes · oxidation · peptides · protein modifications

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Received: January 12, 2009
Published online on May 26, 2009

