Ribosomal synthesis of dehydrobutyrine- and methyllanthionine-containing peptides†

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We report here the ribosomal synthesis of methyllanthionine-containing cyclic peptides involving a site-specific incorporation of vinylglycine under the reprogrammed genetic code, followed by the isomerization of the vinylglycine to dehydrobutyrine, and the subsequent intramolecular Michael addition of a cysteine residue placed at a downstream position of the vinylglycine.

Dehydrobutyrine (Dhb), one of the family of dehydroamino acids, is frequently found in naturally occurring peptides.[1] For instance, lantibiotics, a class of bacteriocin, have Dhb residues that are generated by enzymatic dehydration of a threonine residue in the precursor peptides expressed by the translation machinery.[2] In addition, lantibiotics also contain thioether-bridged cyclic residues, referred to as methyllanthionine (MeLn). These unique residues are generated from Dhb residues by nucleophilic attack of the sulfhydryl group in cysteine (Cys) residues *via* enzymatic intramolecular Michael addition, forming cyclic structures.[2] The thioether bonds formed at MeLn residues are non-reducible, thereby providing physiological stability of their active structures. This suggests that MeLn-bridged cyclic structures provide excellent peptidic frameworks for the discovery of new drug candidates.[3] However, since the formation of Dhb and MeLn residues from the precursor peptides relies on the actions of specific enzymes,[2] the biosynthetic method would not be synthetically versatile enough to generate diverse kinds of such peptides. On the other hand, direct incorporation of Dhb into the nascent peptide chain is very likely intractable due to the hydrolytic instability of the 2-amino group on Dhb itself (decomposing to the 2-oxoacyl group) and its inefficient elongation by the translation machinery. Hence, a generic and simple method that facilitates the synthesis of Dhb- and MeLn-containing peptides, particularly which enables the construction of a library is needed.

We report here a novel methodology for the ribosomal synthesis of Dhb-containing peptides and MeLn-containing cyclic peptides without enzymatic assistance by expressing the precursor peptides bearing vinylglycine (Vgl) residue. We conceived that Vgl could be useful as a key non-proteinogenic amino acid based on the following considerations: (1) Vgl could be site-specifically incorporated into the desired peptide sequence by means of the genetic code reprogramming,[4] and Cys could also be incorporated into a downstream position of the Vgl residue in the peptide chain. (2) Heating of the Vgl-containing peptide at nearly neutral pH could induce isomerization from Vgl to Dhb, and simultaneously, the resulting Dhb residue could accept the intramolecular Michael addition by Cys side-chains. Our report herein includes the ribosomal synthesis of MeLn-containing cyclic peptides that mimic the B- and C-ring segments of nisin,[2c] one of the members of lantibiotics.

Although it was known that treatment of *N*-benzoyl-Vgl esters under acidic or basic conditions in non-aqueous media, resulted in isomerization from Vgl to Dhb residue,[5] the chemical behaviour of Vgl at neutral or near-neutral pH in aqueous media was unknown in the literature. Therefore, at the first stage of this series of experiments, we synthesized two esters, *N*-acetyl-Vgl methyl ester and Vgl 3,5-dinitrobenzyl ester (**1** and **2** in Fig. 1A and B, respectively), and their chemical behaviours in D_2O buffered at pH 7.4 were monitored by ^{1}H-NMR. It should be noted that **1** was a mimic of Vgl in a peptidic structure[6] and **2** was a substrate for our acylation RNA catalyst (flexible tRNA acylation ribozyme; flexizyme)[4c] that would be used in the subsequent experiment. The summary of the observations was as follows (see more detailed observations and discussions in the ESI†): (1) The α-proton of **1** and **2** was deuterated at 37 °C over 2.5 h to yield **3a** and **4**, respectively, but no isomerization to Dhb was observed. (2) Upon heating **1** at 95 °C for 0.5 h, the isomerization of **1** occurred to yield **5** with a single deuteration on the γ-methyl group, along with a byproduct **3b** generated through the ester hydrolysis. The characteristic triplet at δ 6.83 ppm assigned to the β-olefinic proton of Dhb has a similar chemical shift to that of (*Z*)-Dhb found in lantibiotics.[7] Based on the above results, we predicted that **2** would

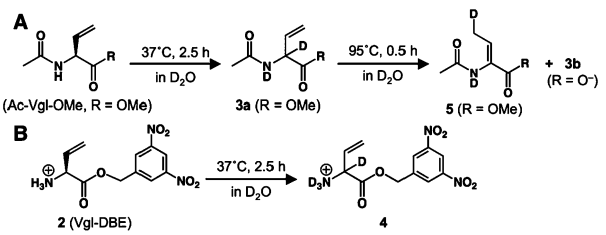

Fig. 1 Model reactions for the isomerization of Vgl to Dhb. Each reaction was monitored by ^{1}H-NMR. See ESI for more details.†

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remain intact under mild conditions, *i.e.* Vgl would be likely intact during flexizyme-catalyzing aminoacylation without isomerization to Dhb, while at elevated temperatures Vgl in peptide chain would isomerize to (*Z*)-Dhb.

At the second stage, the ribosomal synthesis of a model peptide containing Vgl at a specific site was performed (Fig. 2A, P-vgl). We used the genetic code reprogramming by means of a withdrawn PURE (protein translation using recombinant elements)[8] (*w*PURE) system integrated with the flexizyme.[4c–e] In this particular experiment, a *w*PURE system lacking tryptophan (Trp) was prepared to make the cognate codon (UGG) vacant; Vgl-tRNA$^{\mathrm{AsnE\text{-}2}}_{\mathrm{CCA}}$[4d] was prepared by the flexizyme and added to the *w*PURE system for the reassignment of UGG to Vgl. Tricine-SDS-PAGE analysis of the peptide expressed under three different conditions (Fig. 2B) revealed that P-vgl was expressed only in the presence of Vgl-tRNA$^{\mathrm{AsnE-2}}_{\mathrm{CCA}}$ in the *w*PURE system (lanes 2 *vs.* 3) and its expression level was 44% relative to the wildtype peptide (P-wt) expressed in the *w*PURE system plus Trp (lanes 1 *vs.* 3). MALDI-TOF mass spectrometry of P-vgl showed a single major peak consistent with the desired molecular mass (Fig. 2C). Upon heating P-vgl at 95 °C, we expected that the Vgl would isomerize to Dhb residue. To confirm this Vgl → Dhb isomerization, P-vgl was incubated with an excess amount of 2-mercaptoethanol (ME), by which the generated Dhb was trapped to yield the corresponding Michael-adduct (P-vgl-ME). In fact, the molecular weight of the resulting peptide increased by 78 Da that was consistent with that of ME (Fig. 2D). This result clearly demonstrates that the isomerization of Vgl to Dhb occurs under heating and the resulting Dhb residue is readily reactive with a thiol *via* intermolecular Michael addition to afford thioethers.

At the final stage of our experiments, we synthesized MeLn-containing cyclic peptides. We chose two cyclic

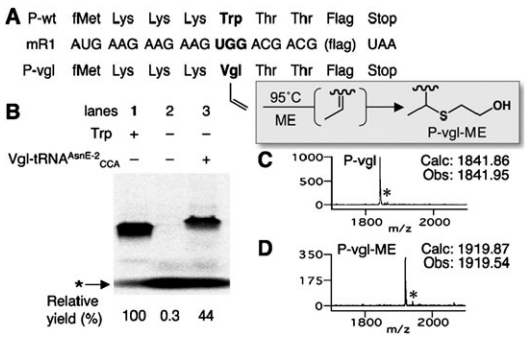

Fig. 2 Ribosomal synthesis of a Dhb-containing peptide. (A) mRNA and the expressed peptide sequences used in this study. Conversions from Vgl to Dhb, and to the ME adduct was illustrated in the grey box. Flag in parentheses indicates the RNA sequence encoding a Flag peptide (DYKDDDDK). (B) Tricine-SDS PAGE analysis of the translation products. Lane 1, wild type expression in *w*PURE system plus Trp; lane 2, *w*PURE system with tRNA$^{\mathrm{AsnE\text{-}2}}_{\mathrm{CCA}}$, lanes 3, *w*PURE system with Vgl-tRNA$^{\mathrm{AsnE\text{-}2}}_{\mathrm{CCA}}$. The band indicated by the asterisk corresponds to the remaining [^{14}C]-Asp that was not incorporated into the peptide. (C) MALDI-TOF mass spectrum of the expressed peptide in *w*PURE system before ME treatment, and (D) that after. The calculated (Calc) and observed (Obs) mass values are shown in each spectrum. Each peak labelled with asterisk denotes a sodium adduct of the major product.

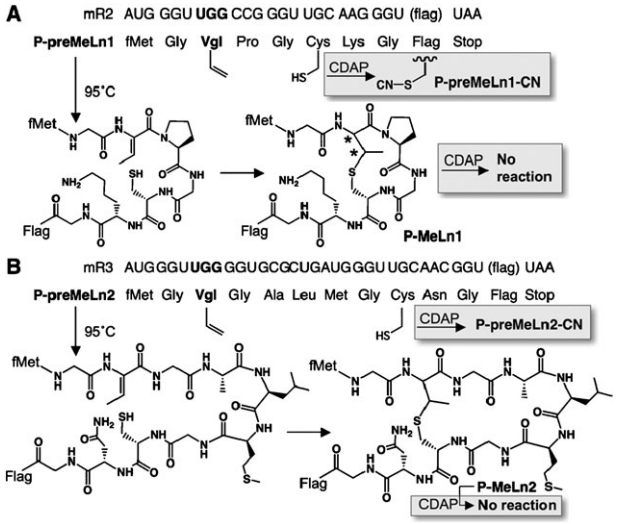

Fig. 3 Synthesis of B-ring (A) and C-ring (B) segments of nisin. CDAP reaction of each peptide is shown in the grey box. Asterisks indicate stereogenic centers newly produced by the intramolecular Michael addition. See ESI for the MALDI-TOF mass spectra of the respective peptides and the stereochemistry.†

peptides as synthetic targets based on the sequences of B- and C-ring segments of nisin (Fig. 3A and B, P-MeLn1 and P-MeLn2). We designed two mRNAs encoding the MeLn-containing cyclic peptide sequences along with the C-terminal Flag-tag appendix, in which the MeLn residue was replaced with Vgl and Cys assigned by UGG and UGC codons (mR2 and mR3). MALDI-TOF mass spectrometry of the expressed peptides showed the consistent mass values with those of Vgl-containing peptides (P-preMeLn1 and P-preMeLn2, respectively; ESI Fig. S1A and E).† We then performed thermal isomerization of the Vgl to Dhb residue and simultaneous intramolecular Michael addition of the Cys thiol. Unlike the intermolecular reaction demonstrated in Fig. 2, the molecular weight of the peptide before and after the tandem isomerization-cyclization would not change; therefore we verified if the Cys thiol in the products reacted with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP), which is known as a thiol-selective cyanylation agent[9] suitable for monitoring a loss of the free thiol group. As expected, when both precursor peptides were treated with CDAP, their respective mass values increased by 25 Da corresponding to the cyano group (ESI Fig. S1B and F).† In contrast, upon the CDAP treatment of the heated peptides (MnLn1 and MnLn2), their mass values did not change (ESI Fig. S1D and H).† This concluded that the desired cyclization took place smoothly to afford the MeLn-containing cyclic peptides.[10,11]

Here we have reported a novel methodology for the ribosomal synthesis of MeLn-containing cyclic peptides without assistance of modification enzymes. The key chemistry utilized in this method is the incorporation of Vgl into a specific site designated by a reprogrammed genetic code and the thermal isomerization of the Vgl residue to Dhb followed by simultaneous intramolecular Michael addition of the sulfhydryl group of a Cys residue present in the peptide chain. The virtue of this method requires no reagents that potentially damage the amino acid residues after the incorporation of Vgl.

Moreover, the peptide sequence is readily programmable by the mRNA template sequence. Seebeck *et al*. recently reported a method to generate dehydroalanine-containing peptides from the selenalysine-containing precursor peptides expressed by translation.[12] This elegant method relies on oxidative elimination of selenium by hydrogen peroxide to afford a dehydroalanine residue. However, strong oxidative reagents such as hydrogen peroxide could also oxidize the thiol in Cys (as well as the sulfide group in methionine) exisiting in the peptide chain, so that Cys may be no longer reactive with Dha as a Michael donor. In contrast, the method reported here resulted in a clean formation of peptides bearing intact Dhb and Cys residues, which can readily be transformed into MeLn residues by heating. Hence, a coupling of ribosomal peptide library synthesis with this methodology opens a new avenue for the discovery of novel MeLn-containing cyclic peptides with not only antimicrobial but also other biological activities when it is integrated with an appropriate screening technique.

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