

Supporting information for:

**Ribosomal synthesis of dehydrobutyrine- and
methyllanthionine-containing peptides**

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Supplementary Text

More detailed observation of the isomerization of Vgl to Dhb in the model reactions.

When **1** was dissolved in D₂O buffered at pH 7.4 and its chemical behavior was monitored by ¹H-NMR, the α-proton was slowly deuterated at 37°C over 2.5 hours to yield **3a** (Fig. 1A, NMR1 & 2 in the Supporting Information). Upon heating **1** at 95°C for 0.5 hours, we found a new set of triplet appeared at 6.83 ppm, along with peaks assignable to acid **3b** generated by hydrolysis of the ester (Fig. 1A, NMR3). The observed triplet was assigned to the β-olefinic proton of Dhb, and one of the resulting γ-methyl protons was deuterated during the isomerization (**5**). The observed chemical shift of the triplet was similar to that of the β-olefinic proton of known dehydrobutyrines with (Z)-stereochemistry, which is the same configuration as naturally occurring Dhb in lantibiotics.¹⁻³ Although prolonged incubation at 95°C over 3 hours resulted in hydrolysis of the ester **5** giving Ac-Dhb-OH (**6**) (NMR4), no isomerization of **3b** was observed. We predicted that if Vgl were incorporated into the peptide chain the hydrolysis of the amide bond should be very sluggish; therefore, the isomerization of Vgl to Dhb would preferentially occur over the hydrolysis of the peptide at such an elevated temperature. Similarly, **2** was deuterated at 37°C to furnish **4**, but no isomerization was observed (Fig. 1B, and NMR 5 and 6). Based on the above results, it is expected that **2** can be charged onto tRNA without the olefin isomerization using a tRNA acylation ribozyme (referred to as flexizyme) that functions under very mild conditions, and the intact form of Vgl is likely incorporated into the peptide chain under the near neutral translation conditions at 37°C.⁴

As noted in the footnote of main text, although we also attempted the synthesis of N-acetyl-Vgl-NH-Et and the model reaction using it, we ended up isolating only N-acetyl-Dhb-NH-Et, an isomerized product. Presumably, the isomerization is readily

promoted under basic conditions.

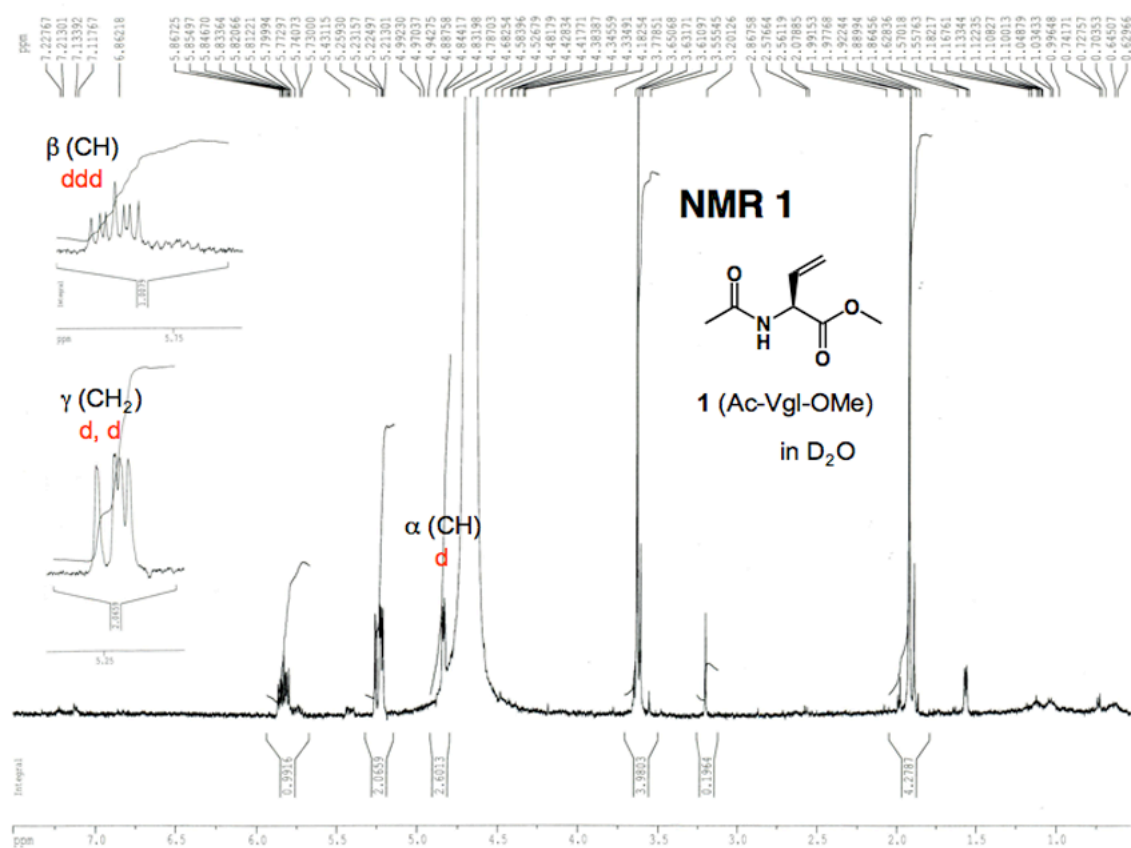
Attempts for the conversion of P-preMeLn1 to MeLn1 at 4°C and 37°C.

In addition to the heating conditions for the conversion of P-preMeLn1 to P-MeLn1, we examined the synthesis of P-MeLn1 at 4°C and 37°C. The experiments were performed using the similar procedure to the synthesis of MeLn-containing peptides, except that the P-preMeLn1 was incubated for 3 hours at 4°C or 37°C prior to CDAP treatment. As shown in Fig. S1B, the ms of the resulting peptide incubated at 4°C increase approximately 25 *m/z*, suggesting that the CN modification of Cys sidechain occurred. The same experiment at 37°C also gave the same result (data not shown). It should be noted that because the isomerization of Vgl to Dhb does not give any change in ms, it was unclear whether the isomerization took place. However, it is clear that the tandem isomerization and Michael addition at 4°C and 37°C does not take place under these conditions. On the other hand, as shown in Fig. S1C, after heating P-preMeLn1 for 3 hours at 95°C, the CDAP treatment gave no change in ms, indicating that the cyclization smoothly took place.

Stereochemistry of the MeLn residue in P-MeLn1 and P-MeLn2.

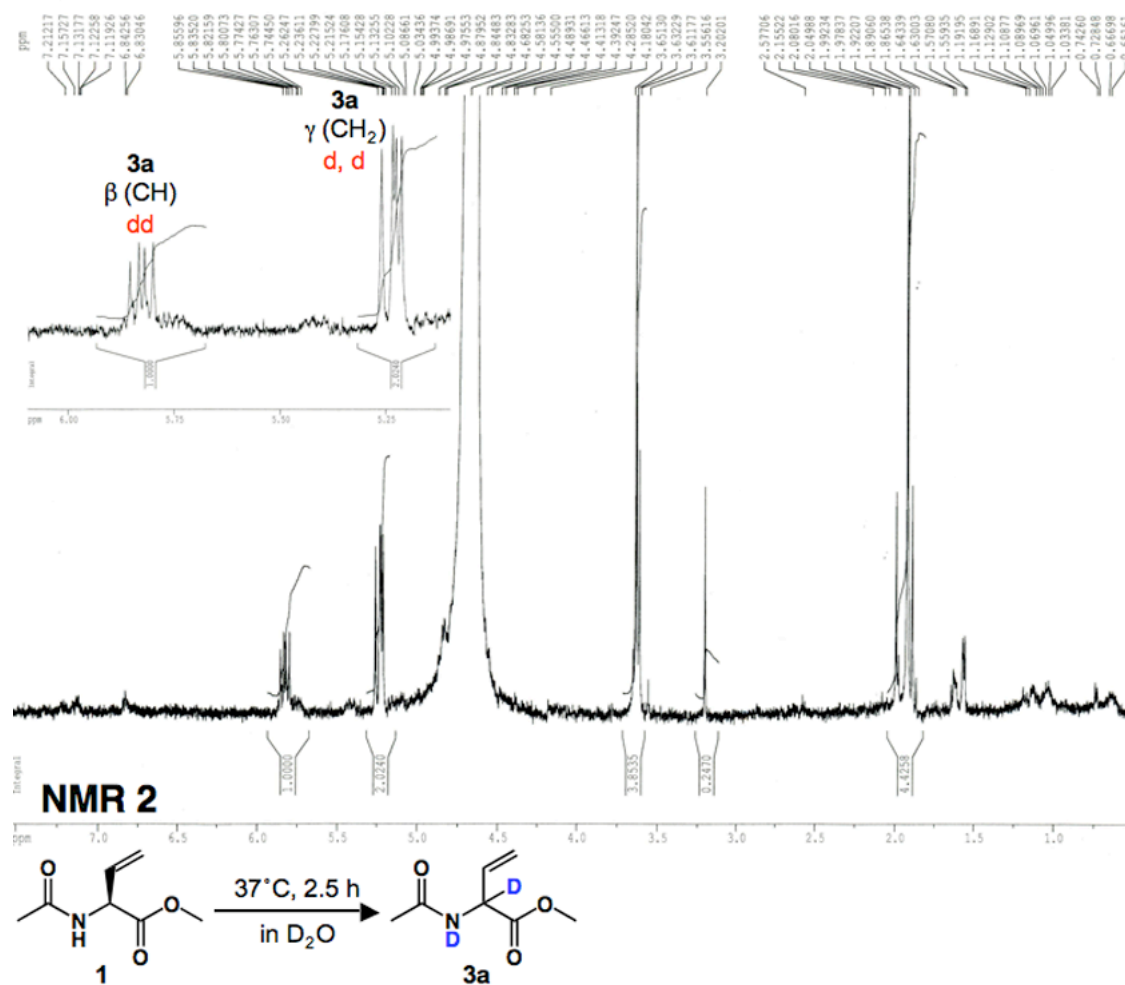
Since the cyclization of P-preMeLn yields two new stereogenic centers, four stereoisomers can be potentially produced. Although the absolute configuration of the resulting MeLn-containing cyclic peptide was unable to be determined due to the insufficient quantity available by the ribosomal expression, P-preMeLn1 likely contains a (*Z*)-Dhb based on our earlier study using **1**. Zhou *et al.* reported that a chemically prepared (*Z*)-Dhb-containing pre-B-ring peptide of nisin was cyclized non-enzymatically to yield the MeLn-containing cyclic peptide bearing (2*S*, 3*S*, 6*R*)-absolute configuration consistent with the natural product.^{2,3} Thus, it is likely that P-MeLn1 also bears the same stereo-configuration, albeit no data is available on the configuration of P-MeLn2 at present.

NMR spectra



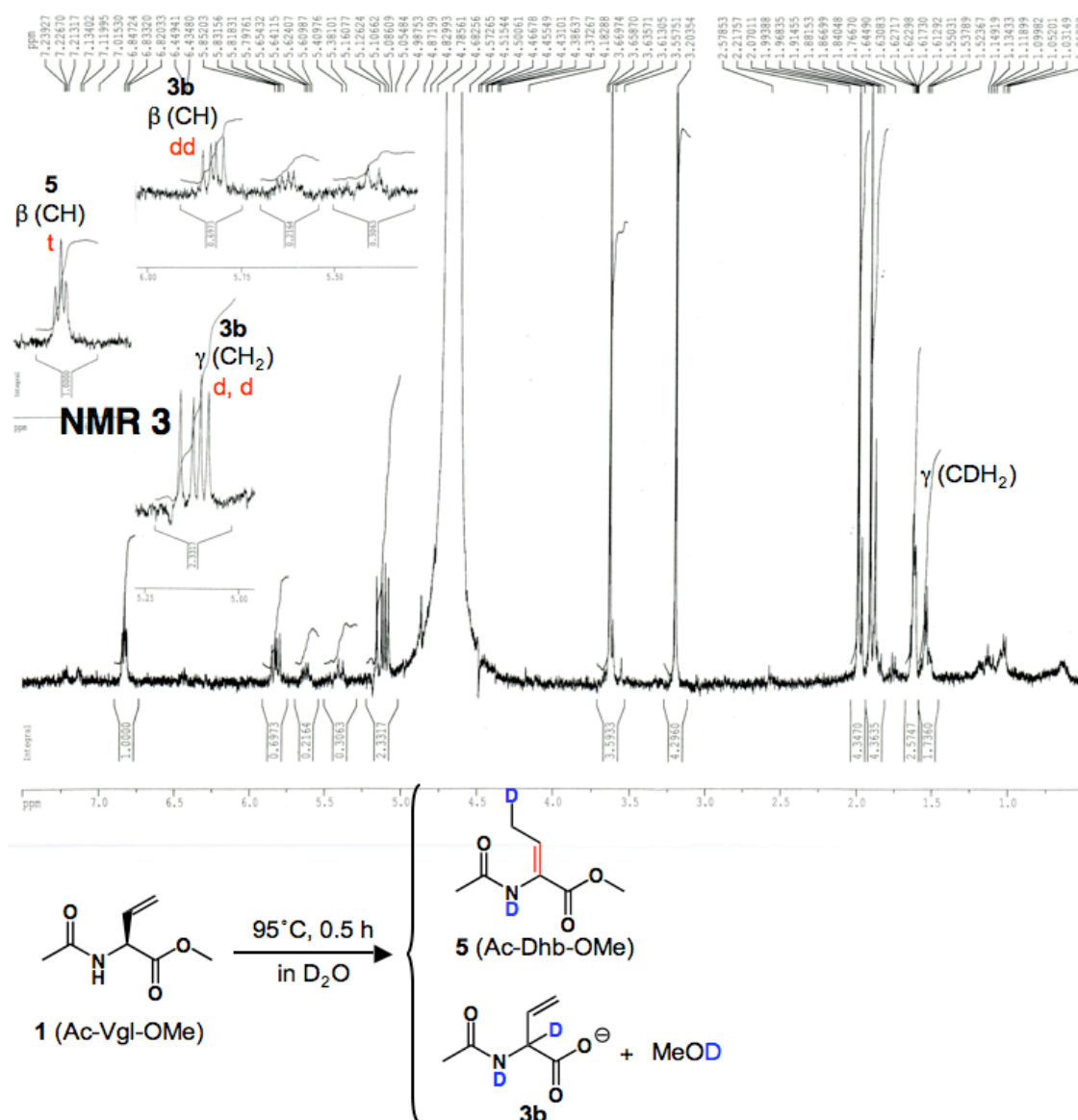
NMR 1

¹H NMR spectrum of **1** in D₂O. The identification and coupling pattern of each peak was shown in the figure as follows; (d = doublet, t = triplet, dd = double-doublet, and ddd = double-double-doublet).



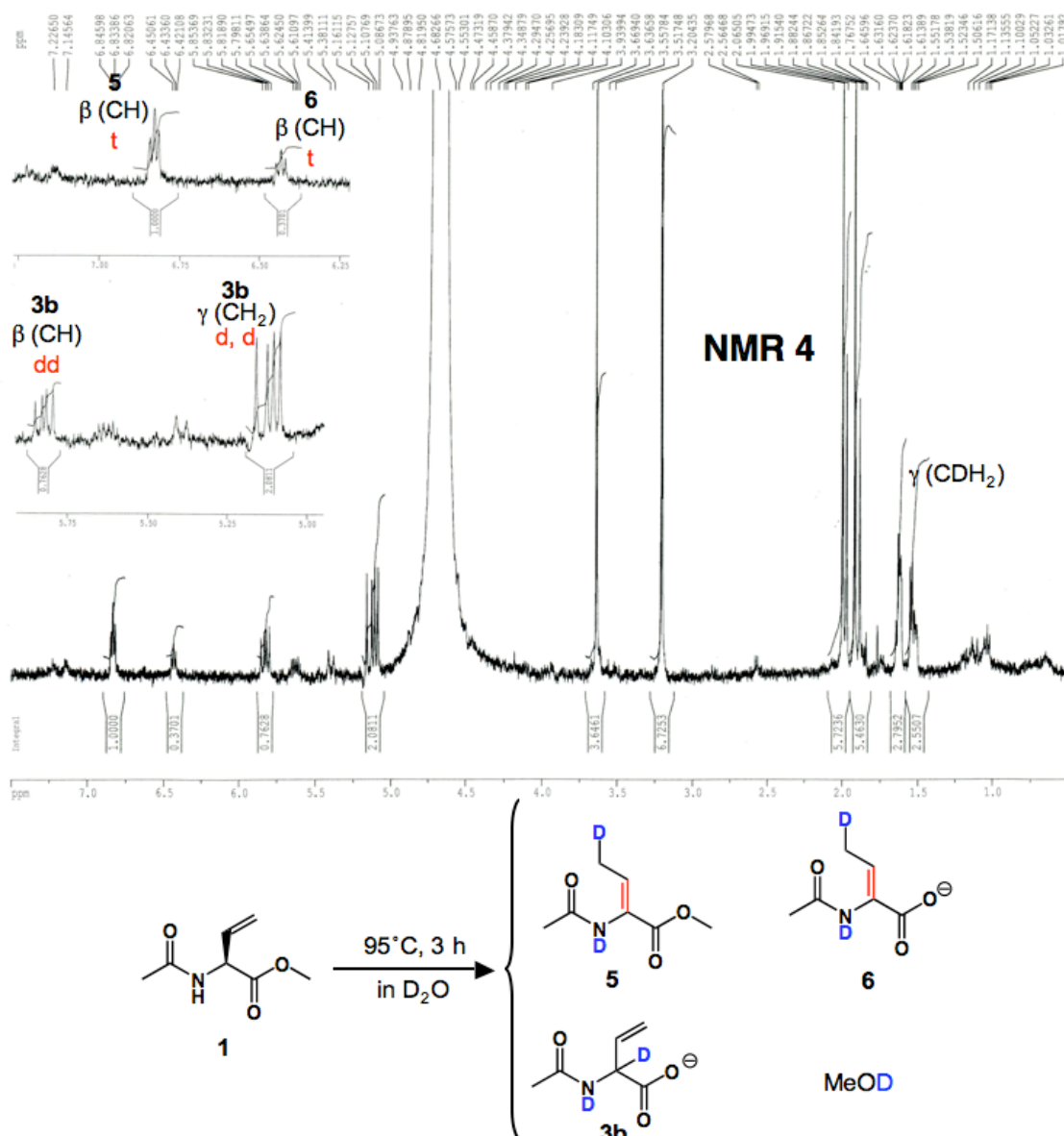
NMR 2

¹H NMR spectrum of **1** after incubation at 37°C in D₂O for 2.5 hours. The identification and coupling pattern of each peak was shown in the figure. The position of the peak corresponding to α-proton of **1** in NMR 1 was shown as [α (CD)]. In this spectrum, the peak corresponding to the α-proton disappeared due to its deuteration upon incubation.



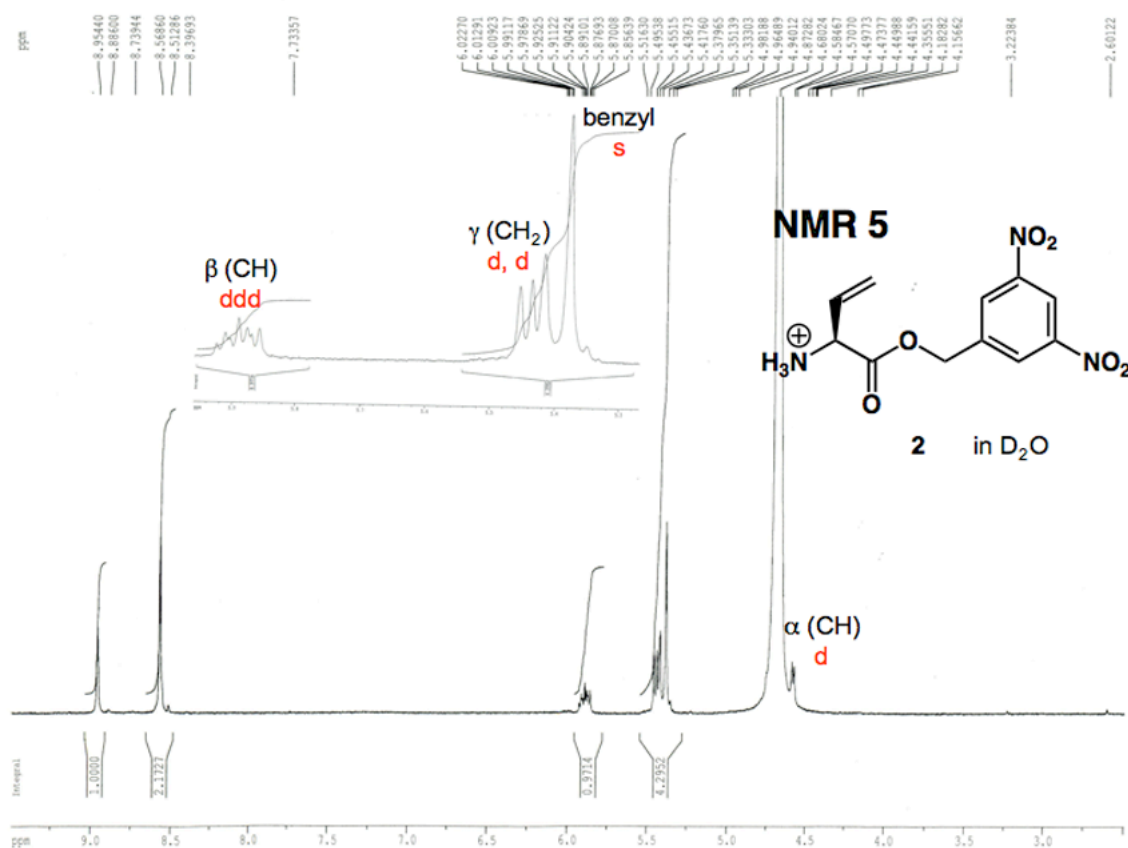
NMR 3

^1H NMR spectrum of **1** after incubation at 95°C in D_2O for 0.5 hours. The identification and coupling pattern of each peak was shown in the figure. The peak corresponding to γ -protons of **5** could not be clearly observed in the spectrum.



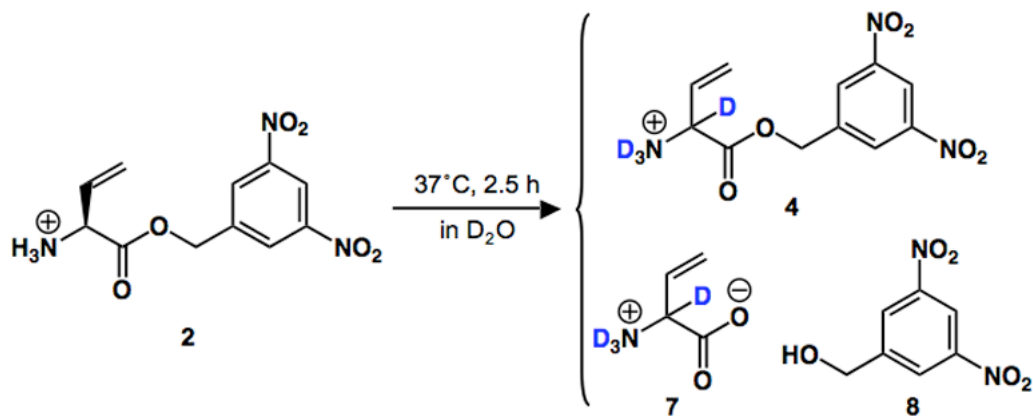
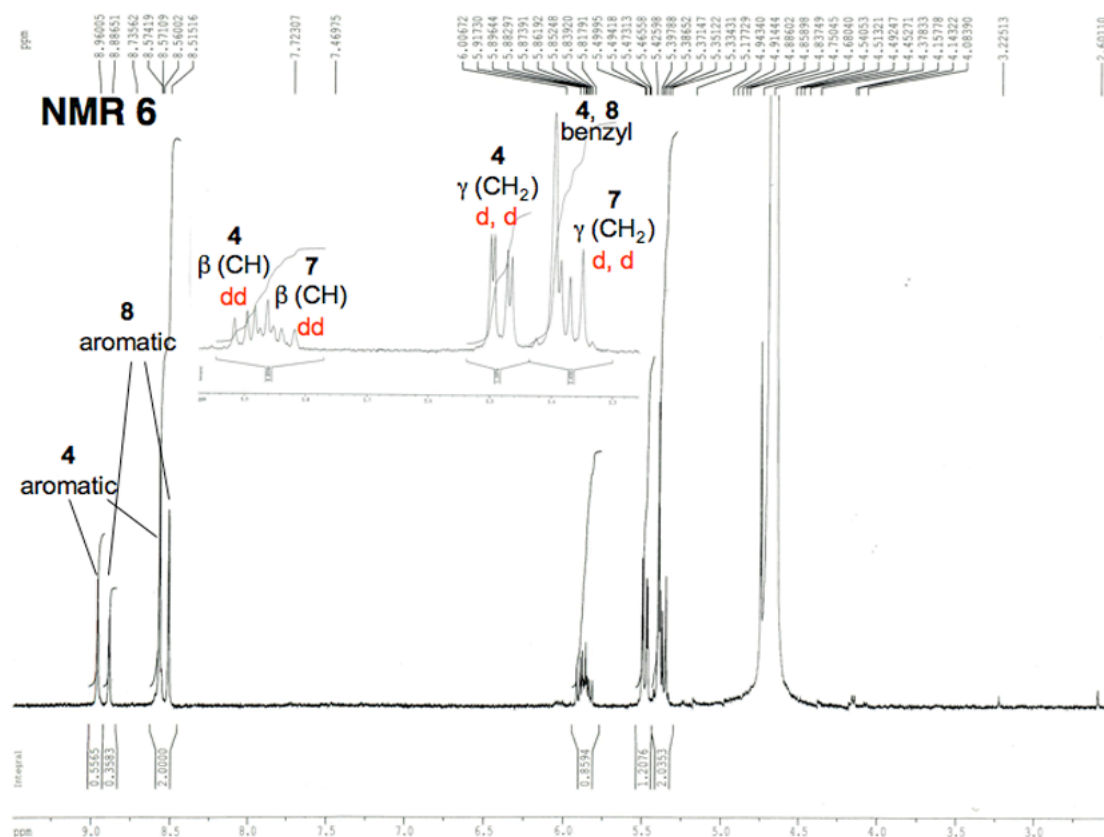
NMR4

^1H NMR spectrum of **1** after incubation at 95°C in D_2O for 3 hours. The identification and coupling pattern of each peak was shown in the figure. The peak corresponding to γ -protons of **5** and **6** could not be clearly observed in the spectrum.



NMR 5

¹H NMR spectrum of **2** in D₂O. The identification and coupling pattern of each peak was shown in the figure.



NMR 6

1H NMR spectrum of **2** after incubation at $37^\circ C$ in D_2O for 2.5 hours. The position of the peak corresponding to α -proton of **2** in NMR 5 was shown as [α (CD)]. In this spectrum, the peak corresponding to the α -proton disappeared due to its deuteration upon incubation.

Supplemental Figure

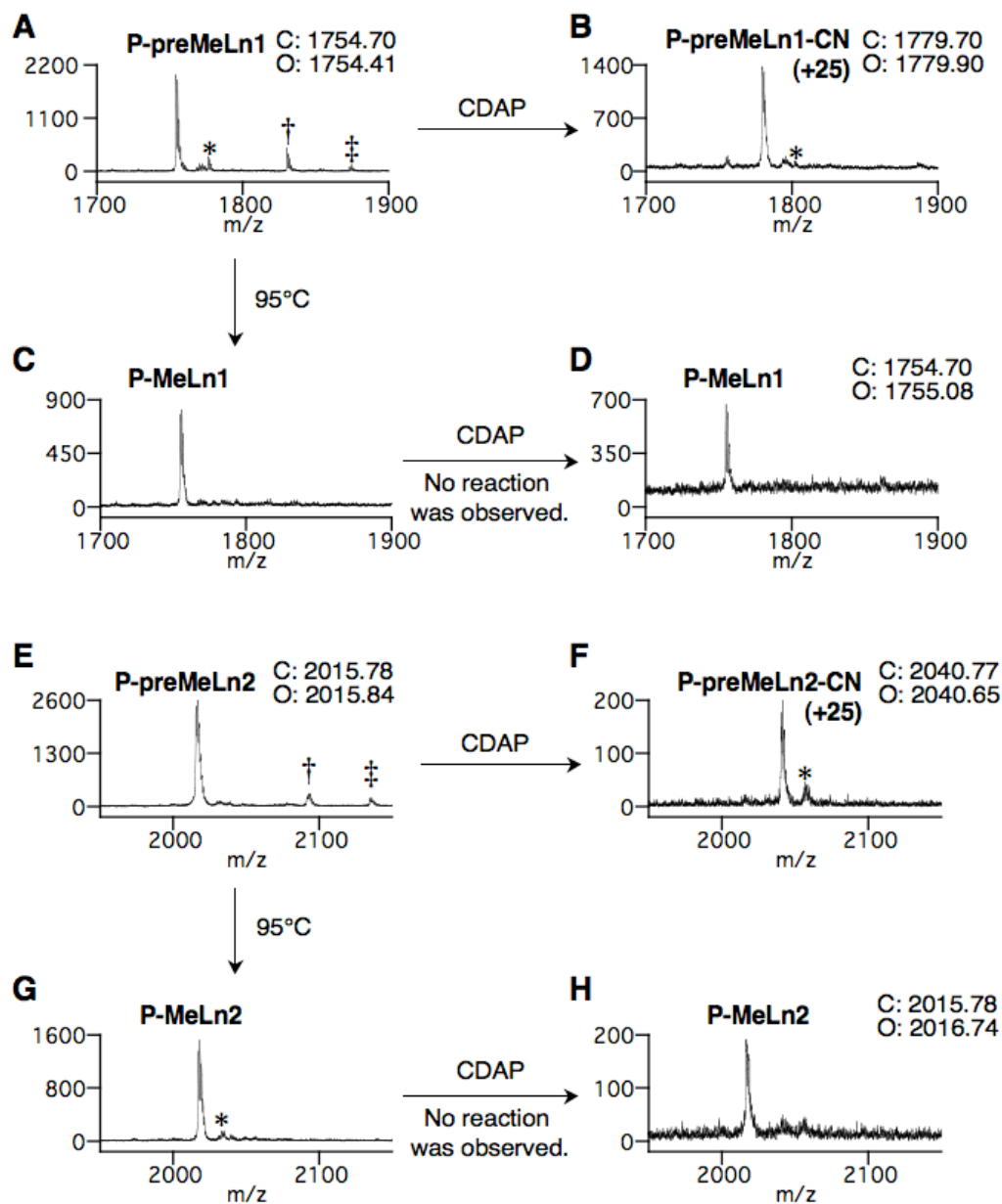


Fig. S1

MeLn-formation from Dhb via the isomerization of Vgl. (A) Mass spectrum of the expressed P-preMeLn1. (B) Mass spectrum of P-preMeLn1 after 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) treatment. (C) Mass spectrum of P-preMeLn1 after heat manipulation (P-MeLn1). (D) Mass spectrum of P-preMeLn1 after heat manipulation followed by CDAP treatment (P-MeLn1). (E) Mass spectrum of the expressed P-preMeLn2. (F) Mass spectrum of P-preMeLn2 after CDAP treatment. (G) Mass spectrum of P-preMeLn2 after heat manipulation

(P-MeLn2). (H) Mass spectrum of P-preMeLn2 after heat manipulation followed by CDAP treatment (P-MeLn2).⁵ The peaks labeled with * correspond to sodium adduct ion of the corresponding major product. The minor peaks labeled with † and ‡ indicate the 2-ME adducts and Cys adducts onto the thiol of Cys in the major products. Note that these minor peaks derived from disulfide adducts disappeared after CDAP treatment because of tris(2-carboxyethyl)phosphine (TCEP) existing in the reaction mixture. The difference in the observed m/z value by 1 mass unit in Fig. S1H is possibly due to hydrolysis of the amide bond in asparagine sidechain occurred during the heat-induced cyclization process.

Supplementary methods

Materials

Chemicals were purchased from Watanabe Chemical Industries, Nacalai Tesque, Kanto Chemical, Sigma-Aldrich Japan, or Wako Pure Chemical Industries unless noted otherwise and used without further purification. *N*-acetyl-Vgl methyl ester **1** was synthesized according to the known procedure.⁶ All oligonucleotides were purchased from Operon Biotechnologies. Flexizyme and tRNA molecules were synthesized using the same procedure as previously described.^{7,8}

Synthesis of vinylglycine 3,5-dinitrobenzyl ester hydrochloride (Vgl-DBE) **2**.

1,3-Dicyclohexylcarbodiimide (92 mg, 0.45 mmol) in 0.6 ml of AcOEt was added to a mixture of Boc-Vgl-OH^{9,10} (82 mg, 0.41 mmol) and 3,5-dinitrobenzyl alcohol (97 mg, 0.49 mmol) in 0.4 mL of 1,4-dioxane. The reaction mixture was stirred at room temperature for 12 h. After the reaction, the precipitate was removed by filtration, and the filtrate was evaporated under reduced pressure and then roughly purified by silica gel column chromatography. The resulting residue was dissolved in CH₂Cl₂ (1 mL) and then added AcOEt containing 4 M HCl (2 mL). The reaction mixture was stirred at room temperature for 15 min. The solvent was evaporated to dryness and the remained HCl was removed by repeating the addition of Et₂O (5 mL) and concentration under reduced pressure three times. The residue was dissolved in MeCN (0.3 mL), and then the product was precipitated by the addition of Et₂O (3 mL). The precipitant was dried *in vacuo* to give **2**: ¹H NMR (DMSO-*d*⁶, 500 MHz) δ 8.82 (s, 1H), 8.67 (s, 1H), 8.52 (br, 3H), 5.93 (m, 1H), 5.74, (s, 3H), 5.6–5.52 (m, 4H), 4.86 (br, 1H); ¹³C NMR (DMSO-*d*⁶, 150 MHz) δ 168.4, 149.0 (2C), 140.4, 129.6, 129.1 (2C), 123.6, 119.3, 66.0, 54.9; ESI-MS *m/z* 282 [(M–Cl)⁺].

Model reaction of isomerization from Vgl to Dhb.

As an NMR solvent, phosphate-buffered deuterium oxide was prepared as follows; phosphate buffer purchased from Nacalai tesque Inc. (pH 7.4, 1 mL) was lyophilized to give pellet which was then dissolved in deuterium oxide (D₂O). The resulting solution was subjected to an iterative procedure consisting of lyophilization and substitution into D₂O. After the third lyophilization, the pellet was dissolved in D₂O (1 mL) to afford phosphate-buffered deuterium oxide. A solution of acetyl vinylglycine methyl ester **1**

(0.4 mg, 3 μ mol) in phosphate buffered deuterium oxide (pH 7.4, 500 μ L) was incubated at each temperature (rt, 37 $^{\circ}$ C, or 95 $^{\circ}$ C) in an NMR tube and the isomerization reaction was monitored by 1 H-NMR spectroscopy (AMX-500, Bruker) at the following time points; after 2.5 hours, and 5 hours at 37 $^{\circ}$ C, and after 0.5 hours, 1 hour, and 3 hours at 95 $^{\circ}$ C. A solution of **2** (2 mg, 6 μ mol) in phosphate buffered deuterium oxide (pH 7.4, 500 μ L) was also incubated at each temperature (rt, 37 $^{\circ}$ C, or 95 $^{\circ}$ C) in an NMR tube and the 1 H-NMR spectra were measured at the following time points; after 1 hours, 2.5 hour, and 3 hours at 95 $^{\circ}$ C.

Preparation of mDNA for peptide synthesis.

Three types of DNA templates were used in this study for in vitro coupled transcription and translation:

mDNA1: 5'-GCATA TGTAAC TACGA CTCAC TATAG GGTAA ACTTT AATAA GGAGA AAAAC ATGAA GAAGA AGTGG ACGAC GGACT ACAAG GACGA CGACG ACAAG TAAGC TTCG-3' / 5'-CGAAG CTTAC TTGTC GTCGT CGTCC TTGTA GTCCG TCGTC CACTT CTTCT TCATG TTTT CTCCT TATTA AAGTT AACCC TATAG TGAGT CGTAT TACAT ATGC-3'

mDNA2: 5'-GCATA TGTAAC TACGA CTCAC TATAG GGTAA ACTTT AATAA GGAGA AAAAC ATGGG TTGGC CGGGT TGCAA GGGTG ACTAC AAGGA CGACG ACGAC AAGTA AGCTT CG-3' / 5'-CGAAG CTTAC TTGTC GTCGT CGTCC TTGTA GTCAC CCTTG CAACC CGGCC AACCC ATGTT TTTCT CCTTA TTAAA GTTAA CCCTA TAGTG AGTCG TATTA CATAT GC-3'

mDNA3: 5'-GCATA TGTAAC TACGA CTCAC TATAG GGTAA ACTTT AATAA GGAGA AAAAC ATGGG TTGGG GTGCG CTGAT GGGTT GCAAC GGGAC TACAA GGACG ACGAC GACAA GTAAA GCTTC G-3' / 5'-CGTAT ACATT ATGCT GAGTG ATATC CCAAT TGAAA TTATT CCTCT TTTTG TACCC AACCC CACGC GACTA CCCAA CGTTG CCCTG ATGTT CCTGC TGCTG CTGTT CATT CGAAG C-3'

DNA primers used for the synthesis of DNA templates listed below;

P1: 5'-TAATA CGACT CACTA TAGGG TTAAC TTAA CAAGG AGAAA AAC-3'

P2: 5'-GGTGG TCCAC TTCTT CTTCA TGTTT TTCTC CTTGT TAAA-3'

P3: 5'-GCATA TGTAAC TACGA CTCAC TATAG-3'

P4: 5'-GTCGT CGTCC TTGTA GTCGG TGGTC CACTT CTTCT T-3'

P5: 5'-CGAAG CTTAC TTGTC GTCGT CGTCC TTGTA GTC-3'

P6: 5'-GTCAC CCTTG CAACC CGGCC AACCC ATGTT TTTCT CCTTG
TTAAA-3'

P7: 5'-GTCGT CGTCC TTGTA GTCAC CCTTG CAACC CGG-3'

P8: 5'-GCAAC CCATC AGCGC ACCCC AACCC ATGTT TTTCT CCTTG
TTAAA-3'

P9: 5'- CGAAG CTTAC TTGTC GTCGT CGTCC TTGTA GTC-3'

mDNA1 was prepared by the following procedure. P1 was annealed with P2 and extended by Taq DNA polymerase. The resulting product was diluted 100 times with PCR reaction mixture and amplified using P3 and P4 as 5'- and 3'-primers, respectively. Furthermore, the resulting product was diluted 200 times with PCR reaction mixture and amplified using P3 and P5 as 5'- and 3'-primers, respectively. The PCR product was purified by phenol-chloroform extraction and ethanol precipitation. The DNA was dissolved in water and its concentration was adjusted to 0.4 μM . mRNA2 was prepared with the same procedure using P1, P6, P3, P7 and P5 as primers. mRNA3 was also prepared with the same procedure using P1, P8, P3, P9 and P5 as primers.

Preparation of Vgl-tRNA^{AsnE-2}_{CCA}¹¹

Vgl-tRNA^{AsnE-2}_{CCA} was prepared by the following procedure. 40 μM tRNA^{AsnE-2}_{CCA} in 0.2 M HEPES-K buffer pH 7.5, 0.2 M KCl (7.5 μL) was heated at 95 °C for 3 min and cooled to 25 °C for 5 min. MgCl₂ (3 M; 3 μL) and flexizyme (200 μM ; 1.5 μL) were added and the mixture was incubated at 25 °C for 5 min. The reaction was initiated by addition of 3 μL of 25 mM **2** (Vgl-DBE) in DMSO and incubated on ice for 2 hours. After acylation, the reaction was stopped by addition of 45 μL of 0.6 M sodium acetate at pH 5, and the RNA was recovered by ethanol precipitation. The pellet was rinsed twice with 100 μL of 70 % ethanol with 0.1 M sodium acetate pH 5.0, and once with 30 μL of 70 % ethanol. The Vgl-tRNA^{AsnE-2}_{CCA} was dissolved in 0.5 μL of 1 mM sodium acetate just before adding to translation mixture.

Ribosomal synthesis of Dhb-containing peptides.

The wPURE system^{7,8} was prepared without 20 standard amino acids, and to this was added the necessary standard amino acids as noted below: Translation reaction was performed by the following procedure. 0.4 mM mDNA1 (0.25 μL), amino acid mixture

(200 μ M Met, 200 μ M Lys, 200 μ M Thr, and 200 mM Tyr; 0.25 μ L), and 585 μ M [14 C]-Asp (0.21 μ L) were added to wPURE system mixture (1.29 μ L) on ice. The precipitated Vgl-tRNA^{AsnE-2}_{CCA} was dissolved in 0.5 μ L of 1 mM sodium acetate and added to the wPURE mixture. Then, the translation mixture (2.5 μ L) was incubated at 37 °C for 1 hour. The products were analyzed by Tricine-SDS-PAGE and autoradiography (FLA-5100, Fuji). Each band of peptide product was quantified with Multi Gauge software (Fuji) and the ratio of [14 C]-Asp incorporated into full-length peptide to [14 C]-Asp added into the translation reaction was calculated. Based on these values, the yields of P-MeLn1 and P-MeLn2 were determined to be 1.1 and 0.8 pmol/ μ L, respectively.

For the mass spectrometry analysis, translation reaction was carried out with 5 μ L scale using non-labeled Asp instead of [14 C]-Asp. The translation product was immobilized with FLAG-M2 agarose (Sigma). After washing the resin with 30 μ L of W buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl), the immobilized peptides were eluted with 10 μ L of 0.2 % TFA. The purified peptide (P-vgl) was desalted with ZipTip $_{\mu$ -C18 (Millipore), and eluted with 1 μ L of a 50% MeCN, 0.1% TFA solution saturated with the matrix (*R*)-cyano-4-hydroxycinnamic acid onto MTP 384 target plate ground steel T F (BRUKER DALTONICS). MALDI mass measurement was performed using autoflex II TOF/TOF under the linear/positive mode and externally calibrated with peptide calibration standard II (BRUKER DALTONICS).

For the isomerization of Vgl to Dhb and subsequent modification with 2-mercaptoethanol (ME), 1.5 μ L of 1 M Bicine-K buffer (pH 9.0) and 1.2 μ L of 1 M ME were added to 10 μ L of the Flag-purified P-vgl. The mixture was incubated at 95°C for 0.5 hours. After incubation, the solution was desalted with ZipTip $_{\mu$ -C18 and analyzed by MALDI mass spectrometry.

Ribosomal synthesis of MeLn-containing peptides (P-MeLn1/P-MeLn2).

0.4 mM mDNA2 or mDNA3 (0.5 μ L) and amino acid mixture (200 μ M Met, 200 μ M Gly, 200 μ M Pro, 200 μ M Cys, 200 μ M Asp, 200 μ M Tyr, and 200 μ M Lys for mDNA2, 200 μ M Met, 200 μ M Gly, 200 μ M Ala, 200 μ M Leu, 200 μ M Cys, 200 μ M Asp, 200 μ M Tyr, and 200 μ M Lys for mDNA3; 0.5 μ L) were added to wPURE system mixture (3 μ L) on ice. The precipitated Vgl-tRNA^{AsnE-2}_{CCA} was dissolved in 1 μ L of 1 mM sodium acetate and added to the wPURE mixture. The translation mixture (5 μ L)

was incubated at 37 °C for 1 hour then purified with FLAG-M2 agarose. Then, 1.5 μ L of 1 M Bicine-K buffer (pH 9.0) and 1 μ L of 200 mM tris(2-carboxyethyl)phosphine (TCEP) were added to 10 μ L of the purified peptide (P-preMeLn1 or P-preMeLn2). The mixture was incubated at 95°C for 0.5 hours to yield the MeLn-containing peptide (P-MeLn1 or P-MeLn2).

For the CDAP treatment, to a peptide solution (P-preMeLn1, P-preMeLn2, P-MeLn1 or P-MeLn2; 10 μ L) was added 200 mM CDAP in reaction buffer [100 mM citrate-Na buffer (pH 3.0), 4M guanidine hydrochloride] (1 μ L) and 500 mM citrate-Na buffer (pH 3.0; 5 μ L), and the reaction mixture was incubated at 37°C for 15 min. The resulting solution was acidified with 4% TFA (8 μ L) and desalted with ZipTip $_{\mu$ -C18, and then analyzed by MALDI mass spectrometry.

References and footnotes

- (1) Slijper, M.; Hilbers, C. W.; Konings, R. N. H.; van de Ven F. J. M. *FEBS Lett.* **1989**, *252*, 22–28.
- (2) Zhou, H.; van der Donk, W. A. *Org. Lett.* **2002**, *4*, 1335–1338.
- (3) Zhu, Y.; Gieselman, M. D.; Zhou, H.; Averin, O.; van der Donk, W. A. *Org. Biomol. Chem.* **2003**, *1*, 3304–3315.
- (4) We expect that the charged Vgl on tRNA is possibly racemized under the mild conditions where **2** is incubated with the tRNA in the presence of flexizyme on ice. However, since the translation system generally incorporates the *L*-amino acid more efficiently than the *D*-form, it can be anticipated that *L*-Vgl is preferentially incorporated into the peptide chain.
- (5) Minor peaks of two unknown side products (2181 Da and 2292 Da) were observed in the spectrum of P-MeLn2 after CDAP treatment. These peaks were not seen in the spectrum of P-preMeLn2, and thus these peaks appeared under heating conditions. Unfortunately, we were unable to assign these ms values to plausible peptide products. Therefore, it is possible that these are not necessarily originating from P-preMeLn2.
- (6) Davis, B. G.; Kramer, H. B. R. PCT Int. Appl. WO 2005000873, 2005.
- (7) Murakami, H.; Ohta, A.; Ashigai, H.; Suga, H. *Nat. Methods* **2006**, *3*, 357–359.
- (8) Ohta, A.; Murakami, H.; Higashimura, E.; Suga, H. *Chem. Biol.* **2007**, *14*, 1315–1322.
- (9) Hanessian, S.; Sahoo, S. P. *Tetrahedron Lett.* **1984**, *25*, 1425–1428.
- (10) Nudelman, A.; Marcovici-Mizrahi, D.; Nudelman, A.; Flint, D.; Wittenbach, V. *Tetrahedron Lett.* **2004**, *60*, 1731–1348.
- (11) tRNA^{AsnE-2} is an engineered elongator tRNA developed in our group; see also the references for the detailed description⁸.