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Analysis of the isomerization of diketopiperazine consisting of proline and aromatic amino acid residues using nuclear magnetic resonance

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Abstract

Under different concentrations of the base potassium deuteroxide KOD, the progress of reactions, such as enolization, D-substitution, isomerization, and conformational changes of diketopiperazine cyclo(L-Pro-L-Xxx) and cyclo(D-Pro-L-Xxx) (Xxx = Phe, Tyr) in D_2O solution, was investigated by ^1H nuclear magnetic resonance (NMR). Cyclo(L-Pro-L-Xxx) is mostly isomerized to cyclo(D-Pro-L-Xxx) in D_2O solution, whereas cyclo(D-Pro-L-Xxx) is only slightly isomerized to cyclo(L-Pro-L-Xxx) even under stronger basic conditions. After adding a deuterated organic solvent (CD_3COCD_3, CD_3SOCD_3 or CD_3OD) to a D_2O solution of cyclo(L-Pro-L-Xxx), cyclo(D-Pro-L-Xxx), or increasing the temperature of the D_2O solution, CH-π interaction between H_9 and the benzene ring of cyclo (D-Pro-L-Xxx) was stronger than that between $H_{8\alpha}$ and the benzene ring of cyclo(L-Pro-L-Xxx).

KEYWORDS
^1H NMR, aromatic amino acid, CH-π interaction, diketopiperazine, folded conformation, proline

1 | INTRODUCTION

Proline, which is an essential amino acid, is a cyclic α-secondary amino acid that has a characteristic structure that other α-primary amino acids do not. Thus, peptides and proteins containing proline residues often have a characteristic stereochemical structure and may exhibit unique functions.[1,2]

We synthesized cyclic octapeptides with proline residues and analyzed their conformations in solution and developed higher order functions.

Based on the ^{13}CNMR spectrum, cyclo(L-Phe-L-Pro-Gly-L-Pro)$_2$ took a C_2 symmetric conformation in which the peptide bond between L-Phe-L-Pro consisted of both cis- and trans- types in $CDCl_3$ solution. By forming a complex with caesium thiocyanate CsSCN or L-phenylalanine methyl ester hydrochloride L-PheOMe•HCl, a C_2 symmetric conformation with only a trans-type peptide bond was observed.[3,4]

Cyclo(L-Phe-L-Pro)$_4$ and cyclo (L-Try-L-Pro)$_4$ have a rigid C_2 symmetric conformation with two 1,4-hydrogen bonds in $CDCl_3$ and CD_3OD solution, and formed a 1:1 complex with phenylalanine methyl ester hydrochloride PheOMe•HCl or noradrenaline hydrochloride, and recognized their asymmetry.[5–8]

However, as most of these cyclic octapeptides containing proline residues are insoluble in water, in order to develop functions under conditions closer to those in vivo, we investigated water-soluble diketopiperazine (cyclic dipeptide) containing proline residues. Then, we examined the behaviour of cyclo(Pro-Xxx) (Xxx = Phe, Tyr) in D_2O using nuclear magnetic resonance (NMR) (Figure 1). We found that cyclo

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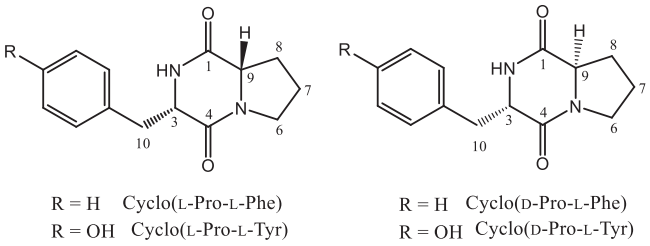

FIGURE 1 Cyclo(Pro-Xxx) (Xxx = Phe, Tyr)

(L-Pro-L-Xxx) and cyclo (L-Pro-D-Xxx) had a folded conformation, and under basic conditions, cyclo (L-Pro-L-Xxx) isomerized to its diastereomer cyclo(D-Pro-L-Xxx).[9]

Under different concentrations of the base KOD, the progress of reactions, such as enolization, D-substitution, isomerization, and conformational changes of cyclo(L-Pro-L-Xxx) and cyclo(D-Pro-L-Xxx) in D_2O solution, were investigated in detail by NMR.

2 | RESULTS AND DISCUSSION

2.1 | Isomerization of diketopiperazine cyclo(Pro-Yyy) (Phe, Tyr, Trp) under basic conditions

2.1.1 | Cyclo(Pro-Phe)

^{1}H NMR spectra of cyclo(L-Pro-L-Phe) were measured in D_2O solution or D_2O solution with KOD concentrations of 1.0×10^{-6}, 1.0×10^{-5}, 1.0×10^{-4}, 1.0×10^{-3}, 1.0×10^{-2}, 1.0×10^{-1}, and 1.0×10^{0} M (Figure 2).

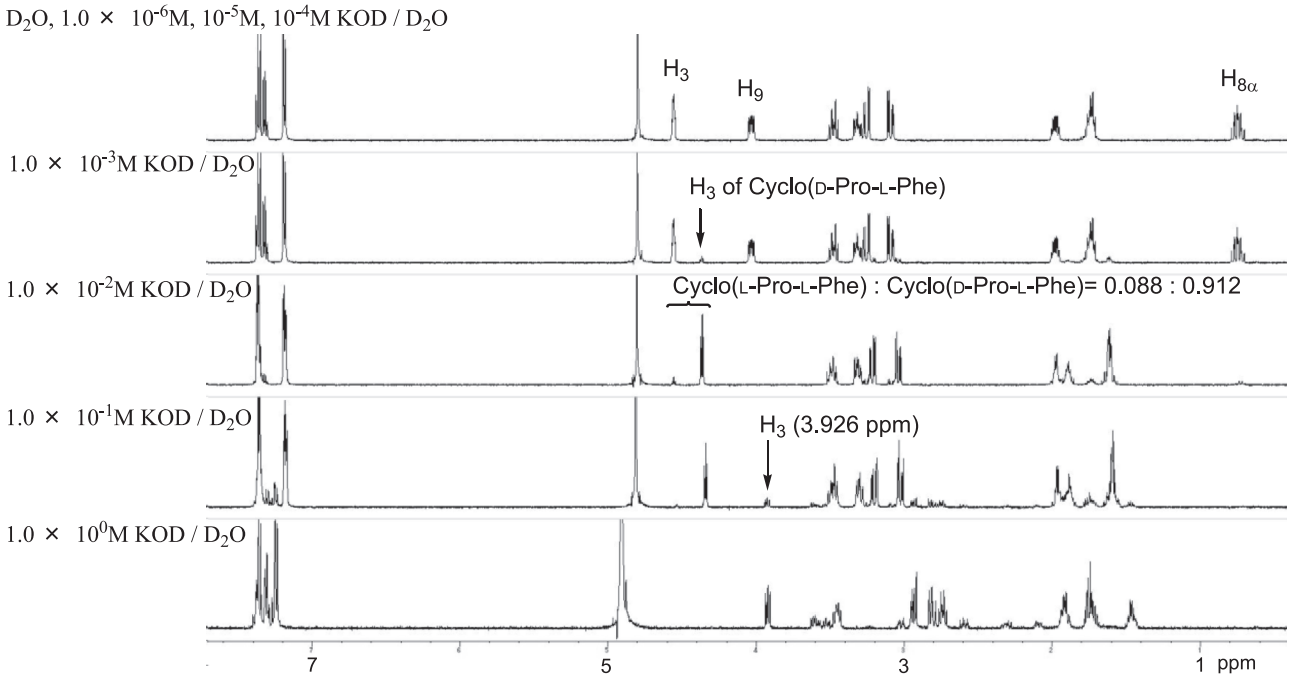

FIGURE 2 ^{1}H nuclear magnetic resonance (NMR) spectrum of cyclo(L-Pro-L-Phe) in D_2O solution under basic conditions

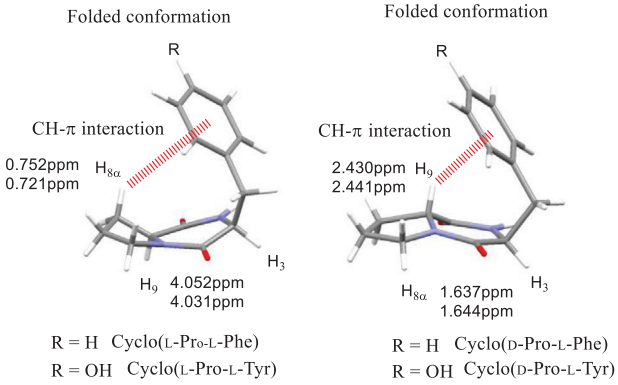

FIGURE 3 Folded conformation of cyclo(Pro-Xxx) (Xxx = Phe, Tyr) with CH-π interaction

In the ^{1}H NMR spectrum in D_2O, the proton signal for $H_{8\alpha}$ of cyclo(L-Pro-L-Xxx) (Xxx = Phe, Tyr) at 0.752 and 0.721 ppm was in a higher magnetic field than that of cyclo (D-Pro-L-Xxx) at 1.637 and 1.644 ppm. This was due to the magnetic anisotropic shielding effects of the ring current from the benzene ring. CH-π interactions were formed between the $H_{8\alpha}$ of cyclo(L-Pro-L-Xxx) and their benzene rings; cyclo(L-Pro-L-Xxx) took a folded conformation in D_2O (Figure 3).[10–12]

When the basicity was increased, the ^{1}H NMR spectra of cyclo(L-Pro-L-Phe) did not differ in the solution from 1.0×10^{-6} to 1.0×10^{-4} M KOD/D_2O, but the integrated value of the H_9 proton signal decreased in the ^{1}H NMR spectrum of 1.0×10^{-3} M KOD/D_2O solution and disappeared completely in that of the 1.0×10^{-2} M KOD/D_2O solution.

We considered that enolization of the O_1-C_1-C_9-H_9 moiety occurred and the H_9 proton signal disappeared by D-substitution. On

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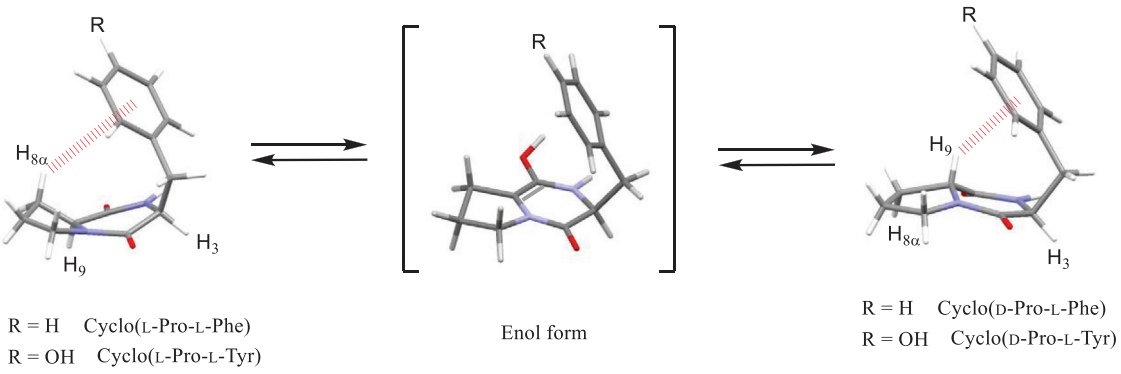

FIGURE 4 Isomerization of cyclo(Pro-Phe) (Xxx = Phe, Tyr) via the enol form

the other hand, enolization of the O_4-C_4-C_3-H_3 moiety did not occur (Figure 4). D-substitution of H_9 was thought to occur due to the reason that H_9 was in an axial conformation at the bridgehead position. In addition, the integrated value of the proton signal for H_3 of cyclo(L-Pro-L-Phe) decreased from that in the ^{1}H NMR spectrum of 1.0×10^{-3} M KOD/D_2O solution, whereas that of the isomerized diastereomer cyclo(D-Pro-L-Phe) increased. Moreover, the integrated value of the $H_{8\alpha}$ proton signal observed at a high magnetic field shift decreased. The ratio of the integrated value of proton signal for H_3 of cyclo(L-Pro-L-Phe) and that of cyclo(D-Pro-L-Phe) in the ^{1}H NMR spectrum of a 1.0×10^{-2} M KOD/D_2O solution, in which the proton signal of H_9 disappeared and isomerization was completed, was 0.088:0.912, and cyclo(D-Pro-L-Phe) was markedly more predominant than cyclo(L-Pro-L-Phe).

Furthermore, in the ^{1}H NMR spectrum of the 1.0×10^{-1} M KOD/D_2O solution with higher basicity, a new proton signal was observed at 3.926 ppm. We considered that H_9 (D_9) was separated as H^+ (D^+) from cyclo(L-Pro-L-Phe) and cyclo(D-Pro-L-Phe), and an anionic form was generated. Therefore, the signal was thought to be derived from H_3 of the anionic form. However, the generation of the anionic forms has not yet been confirmed by spectral analysis. In the ^{1}H NMR spectrum of 1.0×10^0 M KOD/D_2O solution, the H_3 proton signals of cyclo(L-Pro-L-Phe) and cyclo(D-Pro-L-Phe) disappeared, and only the H_3 proton signals considered to be from the anion forms were observed.

In the same manner, ^{1}H NMR spectra of cyclo(D-Pro-L-Phe) were measured in D_2O solution or D_2O solution with KOD concentrations of 1.0×10^{-6}, 1.0×10^{-5}, 1.0×10^{-4}, 1.0×10^{-3}, 1.0×10^{-2}, 1.0×10^{-1} and 1.0×10^0 M (Figure 5).

In the ^{1}H NMR spectrum in D_2O, the proton signal for H_9 of cyclo(D-Pro-L-Xxx) (Xxx = Phe, Tyr) at 2.430 and 2.441 ppm was observed in a higher magnetic field than that of cyclo(D-Pro-L-Xxx) at 4.052 and 4.031 ppm. This was due to the magnetic anisotropic shielding effects of the ring current from the benzene ring, and CH-π interactions were formed between the H_9 of cyclo(D-Pro-L-Xxx) and their benzene rings, and cyclo(D-Pro-L-Xxx) took a folded conformation in D_2O (Figure 3).

When the basicity was increased, the ^{1}H NMR spectra of cyclo(D-Pro-L-Phe) did not differ in solution from 1.0×10^{-6} to 1.0×10^{-4} M KOD/D_2O, but the H_9 proton signal decreased from the ^{1}H NMR spectrum of 1.0×10^{-2} M KOD/D_2O solution and disappeared completely in that of 1.0×10^{-1} M KOD/D_2O solution. This was because the enolization of the O_1-C_1-C_9-H_9 moiety progressed and H_9 was substituted by the D atom of D_2O. In addition, a slight proton signal for H_3, derived from the isomerization of the diastereomer cyclo(L-Pro-L-Phe) was observed in the ^{1}H NMR spectrum of 1.0×10^{-2} M KOD/D_2O solution. In the ^{1}H NMR spectrum of 1.0×10^{-1} M KOD/D_2O solution, the proton signal for H_9 disappeared completely. Based on the integrated value of the proton signal for H_3, the ratio of cyclo(L-Pro-L-Phe) and cyclo(D-Pro-L-Phe) was 0.044:0.956, and cyclo(D-Pro-L-Phe) was markedly more predominant than cyclo(L-Pro-L-Phe). Furthermore, in the ^{1}H NMR spectrum of 1.0×10^{-1} M KOD/D_2O solution, the proton signal thought to be derived from H_3 of the anionic form was observed at 3.926 ppm.

2.1.2 | Cyclo(Pro-Tyr)

^{1}H NMR spectra of cyclo(L-Pro-L-Tyr) and cyclo(D-Pro-L-Tyr) were measured in D_2O solution or D_2O solution with KOD concentrations of 1.0×10^{-6}, 1.0×10^{-5}, 1.0×10^{-4}, 1.0×10^{-3}, 1.0×10^{-2}, 1.0×10^{-1} and 1.0×10^0 M (Figures 6 and 7).

After increasing the basicity, the ^{1}H NMR spectra of cyclo(L-Pro-L-Tyr) did not differ in solution from the ^{1}H NMR spectra of 10^{-6}–1.0×10^{-4} M KOD/D_2O, but the integrated value of H_9 proton signal decreased in the ^{1}H NMR spectrum of 1.0×10^{-3} M KOD/D_2O solution. In addition, the proton signal for H_9 disappeared completely in that of the 1.0×10^{-1} M KOD/D_2O solution due to the enolization of the O_1-C_1-C_9-H_9 moiety of cyclo(L-Pro-L-Tyr) and D-substitution of H_9. The basicity of which the enolization and D-substitution were completed was stronger than that for cyclo(L-Pro-L-Phe). This was considered to be due to the hydroxyl group of the Tyr residue. As cyclo(L-Pro-L-Tyr) became a potassium salt in the 1.0×10^{-1} M KOD/D_2O solution, two doublets derived from hydrogen atoms bonded to the benzene ring were observed with a high magnetic field shift. At that time, the ratio of cyclo(L-Pro-L-Tyr) and cyclo(D-Pro-L-Tyr) was 0.090:0.910, and cyclo(D-Pro-L-Tyr) was markedly more predominant than cyclo(L-Pro-L-Tyr). Furthermore, based on the ^{1}H NMR spectrum of 1.0

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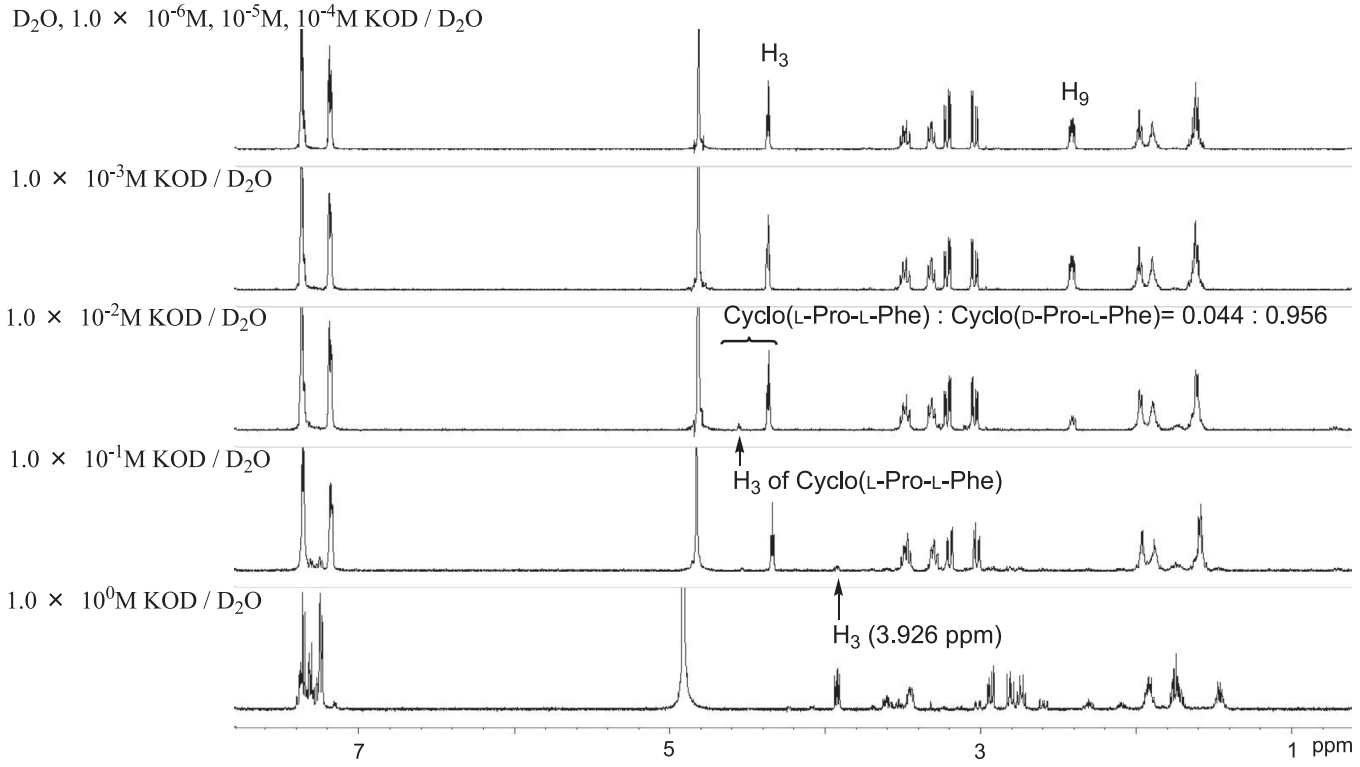

FIGURE 5 ^{1}H nuclear magnetic resonance (NMR) spectrum of cyclo(D-Pro-L-Phe) in D_2O solution under basic conditions

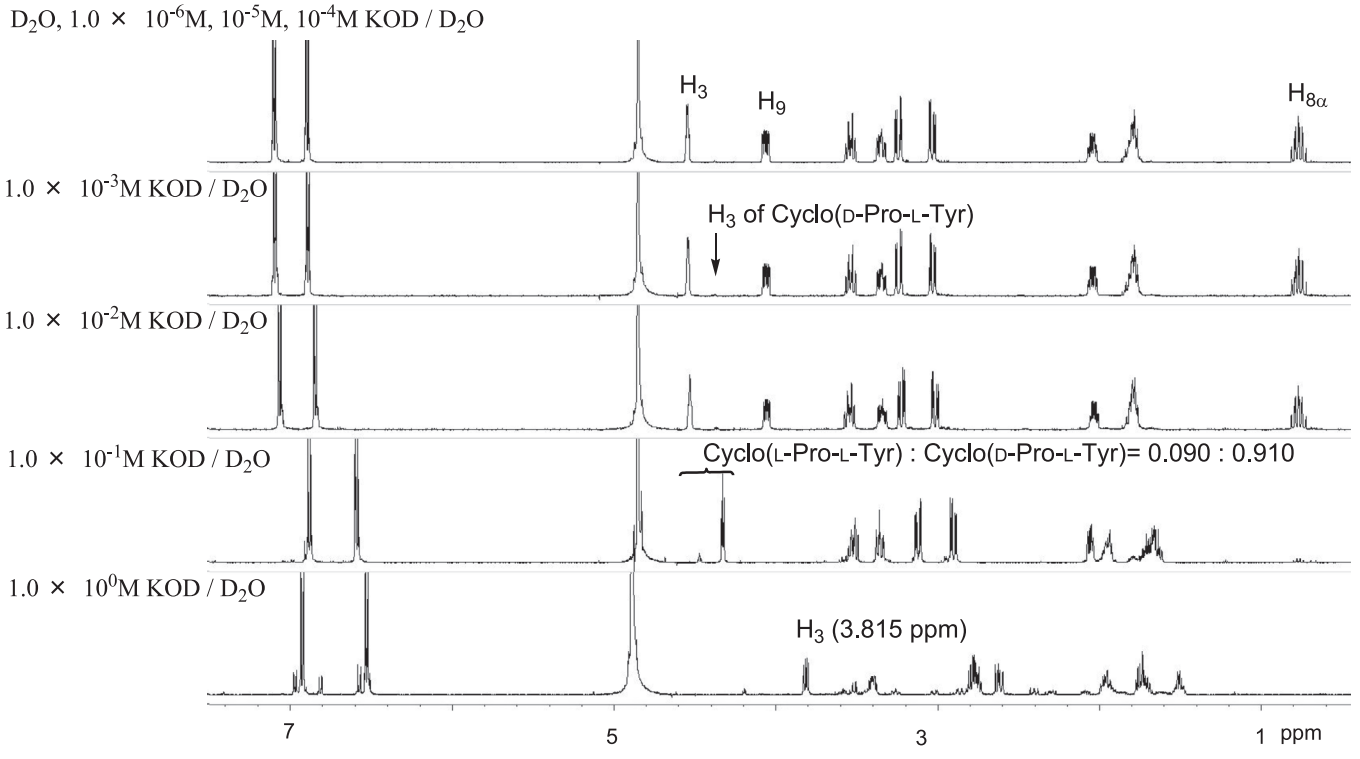

FIGURE 6 ^{1}H nuclear magnetic resonance (NMR) spectrum of cyclo(L-Pro-L-Tyr) in D_2O solution under basic conditions

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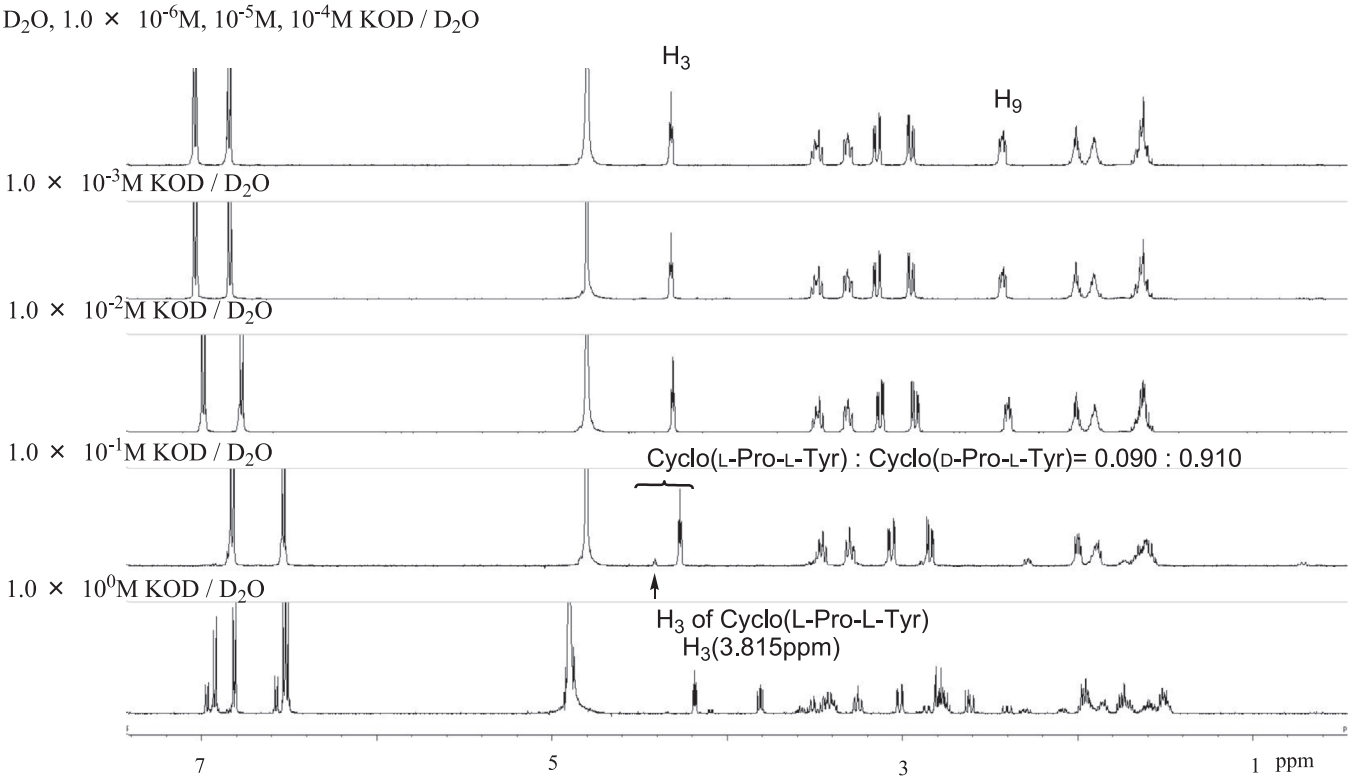

FIGURE 7 ^{1}H nuclear magnetic resonance (NMR) spectrum of cyclo(D-Pro-L-Tyr) in D_2O solution under basic conditions

$\times 10^{-1}$ M KOD/D_2O solution, the integrated value of the proton signal for $H_{8\alpha}$ of cyclo(L-Pro-L-Tyr) decreased until the proton signal was only slightly observed. However, a new signal thought to be derived from H_3 of the anionic form was observed at 3.815 ppm in the ^{1}H NMR spectrum of 1.0×10^0 M KOD/D_2O solution.

After increasing the basicity, the proton signal for H_9 of cyclo(D-Pro-L-Tyr) did not disappear even in the 1.0×10^0 M KOD/D_2O solution. Then the ratio of cyclo(L-Pro-L-Tyr) and cyclo(D-Pro-L-Tyr) was estimated using the integrated value of the proton signal for H_3 in the ^{1}H NMR spectrum of 1.0×10^{-1} M KOD/D_2O solution, in which D-substitution of H_9 of cyclo(L-Pro-L-Tyr) and disappearance of its H_9 were completed. The ratio was 0.090:0.910, with cyclo(D-Pro-L-Tyr) predominating. A proton signal thought to be derived from H_3 of the anionic form was newly observed at 3.815 ppm in ^{1}H NMR spectrum of 1.0×10^0 M KOD/D_2O solution.

2.1.3 | Cyclo(Pro-Trp)

As cyclo(L-Pro-L-Trp) and cyclo(D-Pro-L-Trp) (Figure 8) were insoluble in D_2O, their ^{1}H NMR spectra were observed using CD_3OD as the measurement solvent. ^{1}H NMR spectra of cyclo(L-Pro-L-Trp) and cyclo(D-Pro-L-Trp) were measured in CD_3OD solution or CD_3OD solution with KOD concentrations of 1.0×10^{-6}, 1.0×10^{-5}, 1.0×10^{-4}, 1.0×10^{-3}, 1.0×10^{-2}, 1.0×10^{-1} and 1.0×10^0 M (Figures 9 and 11).

As shown in Figure 10, two proton signals for H_{8a} of cyclo(L-Pro-L-Trp) in the ^{1}H NMR spectrum in CD_3OD solution were observed.

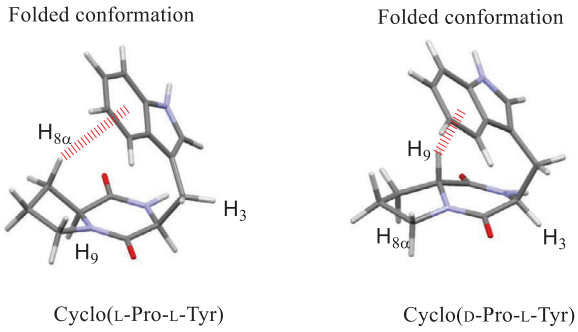

FIGURE 8 Folded conformation of cyclo(Pro-Trp) wth CH-π interaction

In addition to the folded conformation due to CH-π interaction between $H_{8\alpha}$ and its indole skeleton, an extended conformation was slightly present. The ratio of folded to extended conformations was 0.993:0.007, based on the integrated value of the proton signal for $H_{8\alpha}$. On the other hand, cyclo(D-Pro-L-Trp) in CD_3OD solution only took a folded conformation due to CH-π interaction between H_9 and its indole skeleton.

Based on the integrated value of the H_3 proton signal in the ^{1}H NMR spectrum of 1.0×10^{-1} M KOD/CD_3OD where the H_9 proton signal of cyclo(L-Pro-L-Trp) disappeared and isomerization was completed, the ratio of cyclo(L-Pro-L-Trp) to cyclo(D-Pro-L-Trp) was 0.063:0.937, and cyclo(D-Pro-L-Trp) production was markedly dominant over that of cyclo(L-Pro-L-Trp).

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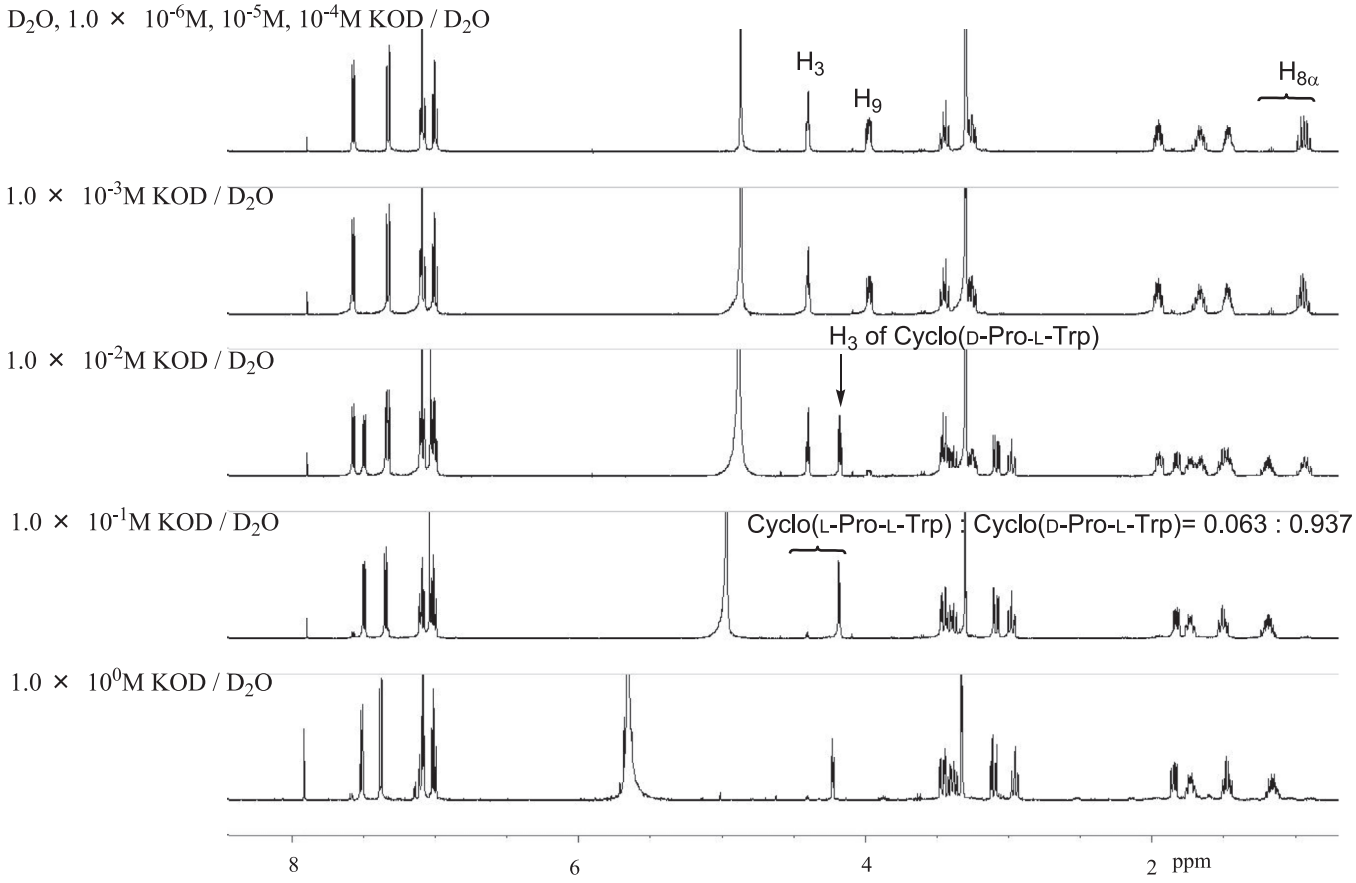

FIGURE 9 ^{1}H nuclear magnetic resonance (NMR) spectrum of cyclo(L-Pro-L-Trp) in D_2O solution under basic conditions

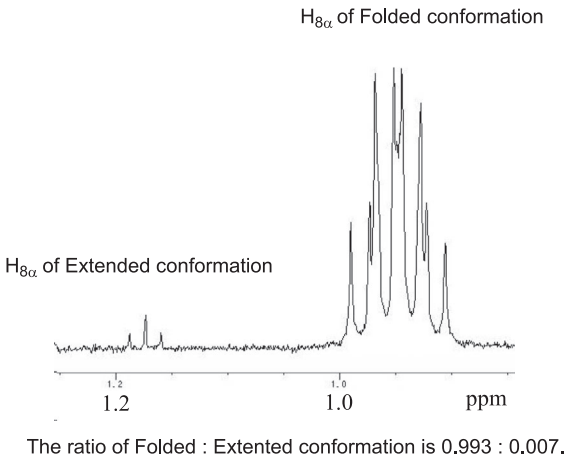

FIGURE 10 Proton signal for $H_{8\alpha}$ of cyclo(L-Pro-L-Trp) in ^{1}H nuclear magnetic resonance (NMR) spectrum of CD_3OD solution

On the other hand, the proton signal of H_9 of cyclo(D-Pro-L-Trp) disappeared in the ^{1}H NMR spectrum of the 1.0×10^{0} M KOD/CD_3OD solution, therefore, the ratio of cyclo(L-Pro-L-Trp) to cyclo(D-Pro-L-Trp) was estimated using the integrated value of the proton signal for H_3. The ratio was 0.043:0.957, with cyclo(D-Pro-L-Trp) being predominant.

3 | CONFORMATION CHANGE OF DIKETOPIPERAZINE CYCLO(PRO-Xxx) (XXX = PHE, TYR)

3.1 | Addition of organic solvent to a D_2O solution of Cyclo(Pro-Xxx)

In order to investigate the strength of CH-π interactions, changes in the chemical shift value of the proton signal for $H_{8\alpha}$ of cyclo(L-Pro-L-Xxx) (Xxx = Phe, Tyr), and that of H_9 of cyclo(D-Pro-L-Xxx) in D_2O solution were measured by adding a water-soluble deuterated organic solvent (CD_3COCD_3, CD_3SOCD_3 or CD_3OD). The addition of an organic solvent was considered to have weakened the CH-π interaction derived from the hydrophobic effect, resulting in a conformational change from folded to extended (Figure 12).

The chemical shift values of the proton signal for $H_{8\alpha}$ of cyclo(L-Pro-L-Xxx) in D_2O taking a folded conformation due to CH-π interaction between $H_{8\alpha}$ and its benzene ring were 0.752 and 0.721 ppm. The chemical shift values of the proton signal for $H_{8\alpha}$ of cyclo(D-Pro-L-Xxx) in D_2O were 1.637 and 1.644 ppm, which were regarded as the chemical shift values of the proton signal for $H_{8\alpha}$ of cyclo(L-Pro-L-Xxx) taking only the extended conformation without CH-π interaction.

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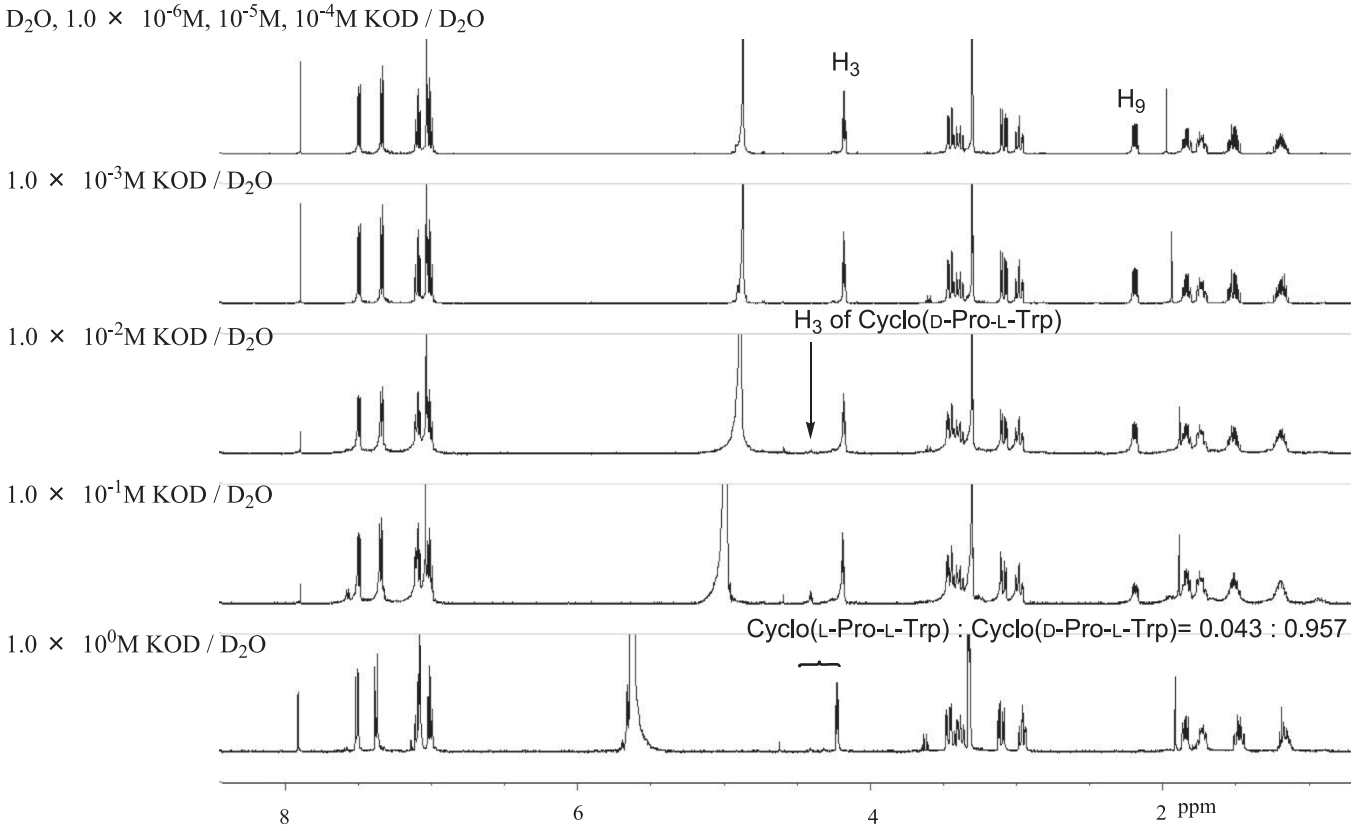
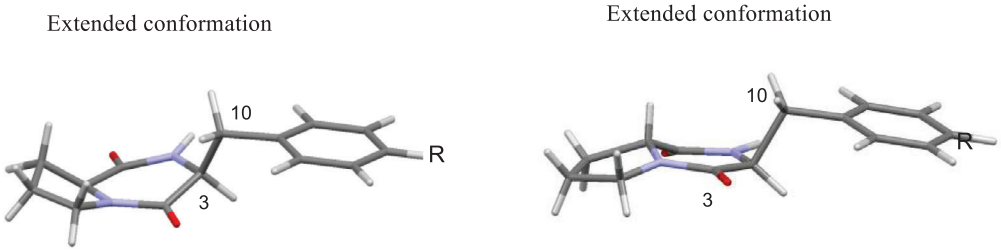


FIGURE 11 ^{1}H nuclear magnetic resonance (NMR) spectrum of cyclo(D-Pro-L-Trp) in D_2O solution under basic conditions



R = H Cyclo(L-Pro-L-Phe)
R = OH Cyclo(L-Pro-L-Tyr)

R = H Cyclo(D-Pro-L-Phe)
R = OH Cyclo(D-Pro-L-Tyr)

FIGURE 12 Extended conformation of cyclo(Pro-Xxx) (Xxx = Phe, Tyr)

Similarly, the chemical shift values of the proton signal for H_9 of cyclo(D-Pro-L-Xxx) taking a folded conformation due to CH-π interaction between H_9 and its benzene ring were 2.430 and 2.441 ppm. The chemical shift values of the proton signal for H_9 of cyclo(L-Pro-L-Xxx) were 4.052 and 4.031 ppm, which were regarded as the chemical shift values of the proton signal for H_9 of cyclo(D-Pro-L-Xxx) taking only the extended conformation without the CH-π interaction.

The ratio of deuterated organic solvent (CD_3COCD_3, CD_3SOCD_3 or CD_3OD) is shown on the horizontal axis, and the ratio of the extended conformation is shown on the vertical axis in Figure 13A–C. The ratio of the extended conformation of cyclo(L-Pro-L-Xxx) increased more than that of cyclo(D-Pro-L-Xxx) with increasing concentrations of deuterated organic solvent. As shown in Figure 11A, in CD_3COCD_3, cyclo(L-Pro-L-Xxx) (Xxx = Phe, Tyr) took an extended conformation at a ratio of 100, 100%, whereas, cyclo(D-Pro-L-Xxx) (Xxx = Phe, Tyr) took an extended conformation at a ratio of only 28.5, 27.4%. CH-π interaction in cyclo(D-Pro-L-Xxx) was stronger than that in cyclo(L-Pro-L-Xxx).

Furthermore, it was found that as the chemical shift value of the proton signal for H_3 change was hardly affected by the addition of a deuterated organic solvent to a D_2O solution of cyclo(L-Pro-L-Xxx) and cyclo(D-Pro-L-Xxx), the change from a folded to extended conformation was due to rotation of the C_3-C_{10} bond.

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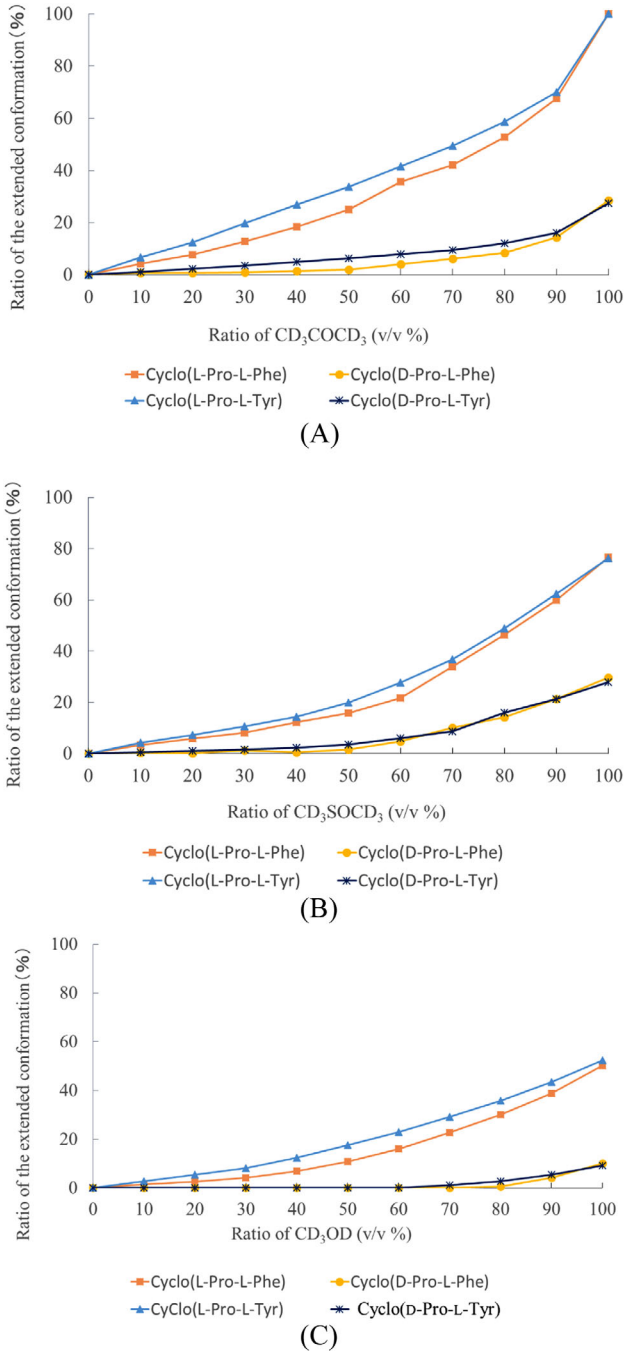

FIGURE 13 Ratio of the extended conformations of cyclo(L-Pro-L-Xxx) and cyclo(D-Pro-L-Xxx) (Xxx = Phe, Tyr) with an increase in (A) CD_3COCD_3, (B) CD_3SOCD_3 or (C) CD_3OD

3.2 | Increase in temperature of a D_2O solution of cyclo(Pro-Xxx)

In order to investigate the strength of CH-π interaction, changes in the chemical shift values of the proton signal for $H_{8\alpha}$ of cyclo(L-Pro-L-Xxx) (Xxx = Phe, Tyr) and for H_9 of cyclo(D-Pro-L-Xxx) in D_2O solution were measured by increasing the temperature from 25 to 80°C. An increase in temperature was considered to weaken the CH-π interaction, resulting in a conformational change from folded to extended.

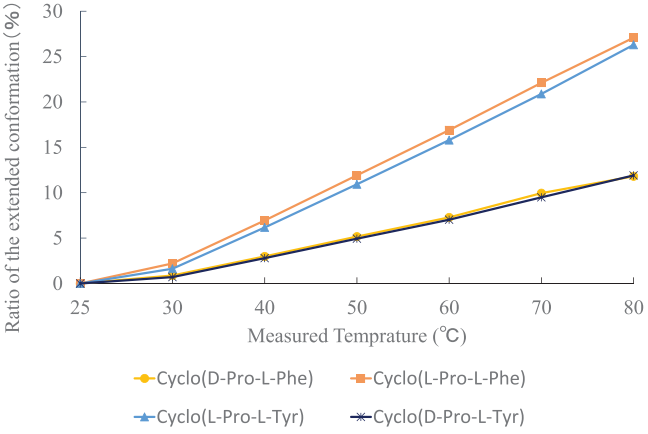

FIGURE 14 Ratio of the extended conformations of cyclo(L-Pro-L-Xxx) and cyclo(D-Pro-L-Xxx) (Xxx = Phe, Tyr) with increasing temperature

The ratio of the extended conformations of cyclo(L-Pro-L-Xxx) and cyclo(D-Pro-L-Xxx) with a change from 25 to 80°C is shown in Figure 14. The ratio of the extended conformation of cyclo(L-Pro-L-Xxx) increased more than that of cyclo(D-Pro-L-Xxx) with increasing temperature, suggesting that CH-π interactions in cyclo(D-Pro-L-Xxx) were stronger than those in cyclo(L-Pro-L-Xxx).

4 | CONCLUSION

Diketopiperazine cyclo(L-Pro-L-Xxx) (Xxx = Phe, Tyr) isomerized via the enol form under basic conditions and produced remarkedly more cyclo(D-Pro-L-Xxx) than cyclo(L-Pro-L-Xxx) (Figure 4). In addition, enolization of cyclo(D-Pro-L-Xxx) required stronger basic conditions than cyclo(L-Pro-L-Xxx), resulting in almost only cyclo(D-Pro-L-Xxx).

Change from a folded to extended conformation due to elimination of CH-π interactions in cyclo(L-Pro-L-Xxx) and cyclo(D-Pro-L-Xxx) was investigated by the addition of a deuterated organic solvent (CD_3COCD_3, CD_3SOCD_3 or CD_3OD), and increase in temperature. These experiments demonstrated that the CH-π interactions in cyclo(D-Pro-L-Xxx) are stronger than those in cyclo(L-Pro-L-Xxx).

Therefore, under basic conditions, diketopiperazine cyclo(L-Pro-L-Xxx) (Xxx = Phe, Tyr) produced more cyclo(D-Pro-L-Xxx) than cyclo(L-Pro-L-Xxx).

5 | EXPERIMENTAL SECTION

5.1 | Preparation of cyclo(L-Pro-L-Yyy) and cyclo(D-Pro-L-Yyy) (Yyy = Phe, Tyr, Trp)

tert-Butoxycarbonyl (Boc)-L-Pro-OH was purchased from Peptide Institute Ltd., and Boc-D-Pro-OH, H-L-Phe-OBzl•*p*-tosylate,

H-L-Tyr-OBzl•*p*-tosylate, and H-L-Trp-OBzl•HCl were purchased from Kokusan Kagaku Co. Ltd.

The detailed method for synthesizing diketopiperazine cyclo (Pro-Yyy) (Yyy = Phe, Tyr, Trp) was described in our previous report.[13]

The 3D structure of cyclo(Pro-Xxx)(Xxx = Phe, Tyr, Trp) was constructed using ChemBio3D Ultra 14.0 (PerkinElmer), and then optimized by molecular dynamics.

5.2 | NMR experiments

^{1}H NMR spectra were recorded at 30°C on a JEOL JNM-ECZ400R (Tokyo, Japan) operating at 400 MHz using a 5 mm ϕ sample tube. In general, ^{1}H NMR experiments were performed with 32 K data points covering a spectral width of 10,000 Hz with ca. 3.7 s pulse delay time and 16 scan times. Deuterated water D_2O (99.9 atom % D; Fuji Film Wako Pure Chemical Industries Ltd.) was used as a measurement solvent. Chemical shift values are expressed in ppm downfield using deuterated sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS-d_6; Fiji Film Wako Pure Chemical Industries Ltd.) as an internal standard.

Deuterated acetone CD_3COCD_3 was purchased from Fuji Film Wako Pure Chemical Industries Ltd., deuterated dimethylsulfoxide CD_3SOCD_3 was purchased from Eurosotop, and deuterated methanol CD_3OD was purchased from Cambridge Isotope Laboratories Inc.

The concentration of the sample cyclo(Pro-Yyy) (Yyy = Phe, Tyr, Trp) in the NMR measurement was set to 40 mmol and the basic conditions were adjusted using a 40 wt% potassium deuteroxide KOD/D_2O solution (Sigma-Aldrich).

In general, nuclear Overhauser effect spectroscopy was conducted with 32 K data points covering a spectral width of 10,000 Hz and a ca. 5 s presaturation time at 30°C, with the other parameters the same as for the ^{1}H NMR spectrum.

Quantitative ^{1}H NMR was performed using the following optimized parameters: probe temperature, 30°C; spinning, off; number of scans, 8; spectral width, 20 ppm; relaxation delay, 64 s and pulse angle, 90°.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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