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# Development of a Series of Cross-Linking Agents that Effectively Stabilize α-Helical Structures in Various Short Peptides

**Kazuhisa Fujimoto,* Masaoki Kajino, and Masahiko Inouye*[a]**

**Abstract:** A series of cross-linking agents of varying rigidity and length were designed to stabilize helical structures in short peptides and were then synthesized. The sequences of the short peptides employed in this study each include two X residues (X = Dap, Dab, Orn, and Lys) at the *i*/*i*+4, *i*/*i*+7, or *i*/*i*+11 positions to provide the sites for cross-linking. These peptides were subjected to reaction with the synthesized cross-linking agents, and the helical content of the resulting cross-linked peptides were analyzed in detail by circular dichroism. For each of the peptide classes we found combinations with the cross-linking agents suitable for the construction of stable helical structures up to >95% helicity at 5 °C. Our method could also be applied to biologically related sequences seen in native proteins such as Rev.

**Keywords:** alpha helices · cross-linking agents · helical structures · peptides

## Introduction

Because particular amino acid sequences that exist as helices in proteins usually adopt random-coiled structures in their isolated short-peptide forms, effective stabilization of the helices requires artificial efforts[1] such as the utilization of hydrogen bond surrogates,[2] the introduction of non-natural amino acids,[3] the use of specific sequences,[4] or the cross-linking of two side chains in the residues.[5] In our previous paper, we were able to stabilize α-helices of short peptides with acetylenic cross-linking agents (Figure 1).[6] In these peptides, Lys residues were located at the *i*/*i*+4 and *i*/*i*+7 positions for the cross-linking reactions, and the cross-linked peptides showed moderate α-helical content of up to 35% (*i*/*i*+4) or 65% (*i*/*i*+7) at 5 °C. However, the cross-linked peptides probably need to display much higher helical content and greater thermal stabilities at room temperature for practical uses. Moreover, there was no reason to believe that the combination of Lys residues and the acetylenes represented the optimal linkage. It was thus decided that a variety of cross-linked patterns should be developed for investigation of the application of these peptides to life sciences, especially to peptide inhibitors and peptide drugs.[7] Here we report the results obtained for a series of new cross-linking agents that effectively stabilize α-helical structures in various short peptides, as well as further information on the acetylenic ones.

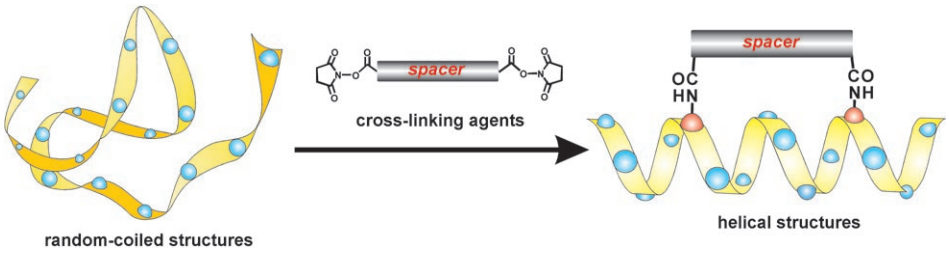


Figure 1. A schematic representation of the stabilization of helical structures in short peptides by use of cross-linking agents.

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## Results and Discussion

**Short peptides used in this study**: One turn in an α-helix consists of 3.6 amino acid residues, so the two side chains of

  

the residues at positions *i* and *i*+4 (one turn: 0.5 nm from *i*), *i* and *i*+7 (two turns: 1.1 nm from *i*), or *i* and *i*+11 (three turns: 1.6 nm from *i*) in the helices are oriented in almost the same direction. These distances in the helices help us to identify favorable cross-linking agents for stabilizing helical structures in short peptides. As shown in Figure 2a, two "X"

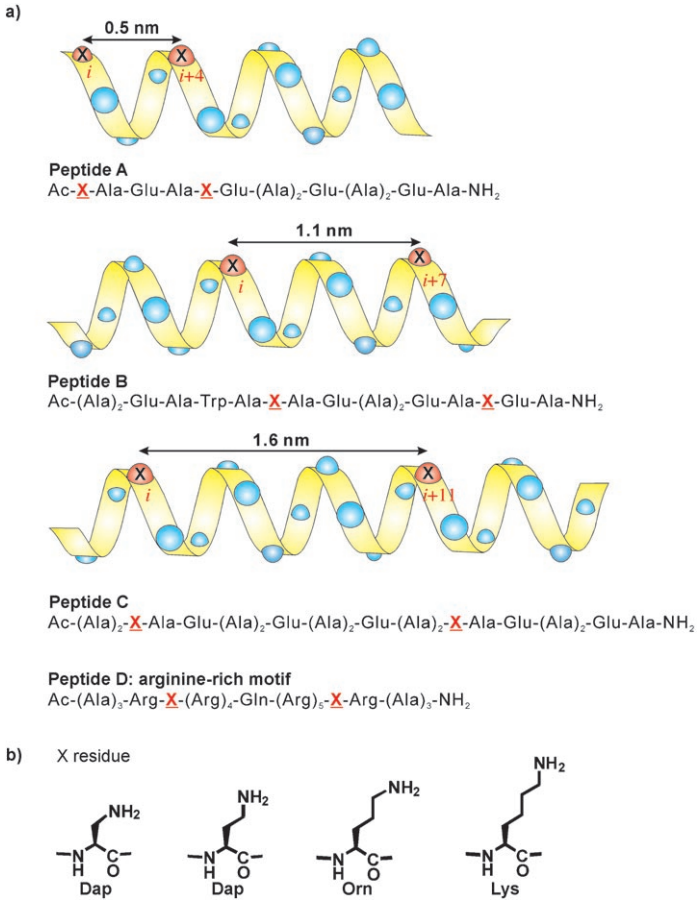


Figure 2. a) Helical structures and sequences of short peptides used in this study: peptide classes **A** (*i*/*i*+4), **B** (*i*/*i*+7), **C** (*i*/*i*+11), and **D** (*i*/*i*+11). b) Amino acids "X" (Dap, Dab, Orn, and Lys) as cross-linking agents in short peptides.

residues are placed at the *i*/*i*+4, *i*/*i*+7, and *i*/*i*+11 positions as cross-linking sites for peptide classes **A** (13 residues), **B** (16 residues), and **C** (20 residues), respectively. Peptide class **D** (20 residues) also has the *i*/*i*+11 relationship; its sequence is extracted from the arginine-rich region seen in natural Rev, a HIV-1 regulatory protein.[8,9]

**Locations of cross-linking sites in the short peptides**: The peptide class **A** was designed to have its cross-linking site X at the N terminus, in view of our previous results. In that study, a photochromic cross-linking agent bearing a spiropyran skeleton was introduced between two Lys residues in the N-terminal region in the *i*/*i*+7 positions.[10] The local helix thus formed by the cross-linkage at the terminal was able to provide total structural regulation of the target peptides, which we called a "*trigger effect*". If such regulation could also be achieved by linking shorter distances (*i*/*i*+4) in peptides, this strategy should be fruitful for regulating various interactions, such as in peptide–protein and peptide–DNA complexes. Therefore, if the peptide possesses a sequence relating to biological recognition events at its C terminus, a cross-linked region at the N terminus can be utilized as a control unit for the interactions.

In the cases of the peptides **B**–**D**, the cross-linking regions are positioned around the centers of the sequences. This situation causes one side of such a cross-linked peptide to be broadly masked with the cross-linking agents, leaving the opposite side untouched. When the sequence of the amino acid residues protruding at the opposite side is designed to interact side by side with other biomolecules, this opposite region could be used as a novel recognition motif with several functional groups lined up linearly. Indeed, in the peptides **D**, the positions of X were decided upon so as not to hamper the arginine-rich side of the peptide from binding with RRE RNA, on the basis of X-ray crystallographic results for the Rev–RRE complex.[11,12]

**Type of X residue for the cross-linking sites**: In the previous paper, we chose Lys as a cross-linking residue because of its natural presence in the peptides of interest. In the cases of short peptides, however, solid-phase synthesis allows incorporation of any type of natural or non-natural amino acid into the sequences, which can be applied to biological recognition events at will. Moreover, cross-linked Lys residues no longer maintain their inherent cationic properties for contributing to interactions with other species. These features suggest that the cross-linking site X should be variable with each individual experiment for stabilizing α-helices. Therefore, we additionally introduced diaminopropionic acid (Dap), diaminobutyric acid (Dab), and ornithine (Orn) into the peptide sequences as cross-linking sites X for extending our strategy (Figure 2b). These four amino acids have various lengths of methylene spacers between the terminal amino group of the side chain and the α-carbon: Dap, Dab, Orn, and Lys contain one, two, three, and four carbons, respectively, for the spacers.

**Cross-linking agents**: Ten cross-linking agents have been newly tested, in addition to the acetylenic **1** and **2**, as shown in Figure 3.[6] In the new cross-linking agents, the spacers are made up of short alkane, benzene, naphthalene, biphenyl, phenanthrene, and fluorene cores without flexible alkoxy chains, unlike in **1** and **2**. The alkyl side chains of the cross-linked amino acids X thus have a role for varying the overall length of the resulting cross-linker, meaning that cross-linking agents of slightly shorter length than a targeted pitch might be advantageous for formation of stable helices. With this in mind, short alkylene-based **3** and **4** would be expected to be suitable for stabilizing α-helices in the peptide class **A**. Benzene-based **5** and **6**, of moderate spacer length, should be potent candidates for the peptides **B**, while the longer ones of the biphenylene-based **7**–**9** and the highly

  

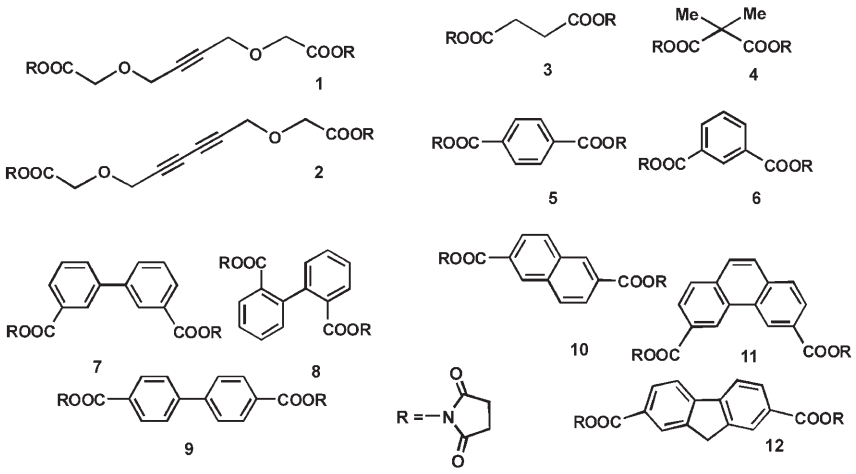


Figure 3. Cross-linking agents: acetylenic **1** and **2**, short alkylene-based **3** and **4**, benzene-based **5** and **6**, biphenylene-based **7–9**, and highly rigid **10–12**.

rigid **10–12** should serve for the peptides **C**. Furthermore, biphenyl, naphthalene, phenanthrene, and fluorene cores are fluorescent, so that peptides cross-linked with **7–12** could be useful for analyzing biological recognition events such as peptide–DNA interactions through their fluorescence.[13]

**Preparation and CD spectra of cross-linked peptides**: All of the cross-linking agents were synthesized from the corresponding dicarboxylic acids and *N,N′*-disuccinimidyl carbonate. The dicarboxylic acids are commercially available or easily synthesized. Short peptides **A–D** were prepared with a peptide synthesizer. The cross-linking reactions and purification were performed by the previously reported procedures,[6] and the isolation yields were about 90–30% except in a few cases. To elucidate the helical content of the cross-linked peptides accurately, CD measurements must be carried out below this concentration, thus at a concentration sufficiently low that intermolecular association of the cross-linked peptides is negligible. Therefore, the intermolecular association tendencies of the cross-linked peptides were studied in advance with **C·12** with X = Lys, as the fluorene core of **12** is one of the most hydrophobic of the cross-linking agents tested and can self-associate in water. The UV and CD spectra of **C·12** with X = Lys were measured in phosphate buffer (100 mM, pH 6.6) at 5 °C. The shapes of the spectra are not sensitive to their concentrations, and at $\leq 5.0\times10^{-4}$ M the absorbances and ellipticities fit in proportion to the concentration, obeying Beer's law (Figure S1). Contribution of intermolecular association of the cross-linked peptides can thus be ruled out below that concentration. Taking this into account, all of the following measurements were conducted at ca. $1.0\times10^{-4}$ M concentrations of the cross-linked peptides in phosphate buffer (100 mM, pH 6.6) at 5 and 25 °C.

To identify effective cross-linking agents in each of the peptide classes **A–C**, we measured the CD spectra of the cross-linked peptides formed by various combinations between **A–C** and **1–12**. Figure 4, for instance, shows the CD spectra of native **B** (X = Orn) and its cross-linked **B·5**. All of the native peptides **A–C** adopt almost random-coiled structures (≤20% helicities) as judged from their CD spectra at 5 °C. On the other hand, the cross-linked peptides turned into stable helices when the combination was preferable, as in the case of **B·5** (see Figures S2–S22 in the Supporting Information for the spectra of other cross-linked peptides). Most of the cross-linked peptides include chromophores absorbing at ≥190 nm, the absorption bands of which overlap with the CD

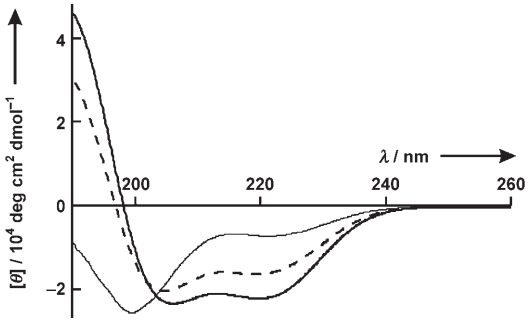


Figure 4. CD spectra of native and the cross-linked **B** (X = Orn) with **5** dissolved in phosphate buffer (100 mM, pH 6.6) at 5 and 25 °C: native **B** (——) at 5 °C, **B·5** at 5 °C (——), and **B·5** at 25 °C (-----).

active region arising from peptide backbones. Nevertheless, isodichroic points were observed at 202 nm in the spectra of the cross-linked peptides on varying the temperature from 5 to 60 °C, indicating that the chromophores do not interfere with the CD region (see Figure S23 in the Supporting Information).

**Evaluation of helical content of the cross-linked peptides**: We evaluated the helical content of the cross-linked peptides by means of their CD spectra. Helical content in representative cross-linked peptides were calculated from the mean residue ellipticity at 222 nm,[5d] the concentrations of the solutions being determined by directly weighing the samples on a microbalance. Table 1 shows the helical content (%) of the cross-linked peptides that showed outstanding α-helicities (≥60% except for **A·4** with X = Orn). Other combinations were roughly estimated for their α-helicities by comparison of their CD spectra with those of the strictly calculated ones and are marked as H (high), M (middle), and L (low), corresponding to 60–40%, 40–20%, and <20% helical content, respectively.

  

Table 1. Helical content of peptides cross-linked with various cross-linking agents at 5 and 25 °C (in brackets).[a]

| Cross-linking agents | Helical content | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Peptides **A** (*i*/*i*+4: 0.5 nm) **X**= | | | | Peptides **B** (*i*/*i*+7: 1.1 nm) **X**= | | | | Peptides **C** (*i*/*i*+11: 1.6 nm) **X**= | | | |
| | Dap | Dab | Orn | Lys | Dap | Dab | Orn | Lys | Dap | Dab | Orn | Lys |
| **1** | – | – | – | – | – | – | – | [b] | – | – | – | – |
| **2** | – | – | – | – | – | – | – | [b] | – | >95 (70) | 90 (60) | 90 (55) |
| **3** | M (L) | L (L) | 60 (40) | M (L) | – | L (L) | H (M) | M (L) | – | – | – | |
| **4** | M (M) | L (L) | 50 (35) | M (L) | – | L (L) | L (L) | M (M) | – | – | – | – |
| **5** | L (L) | L (L) | L (L) | L (L) | L (L) | M (L) | 80 (60) | H (M) | – | – | – | – |
| **6** | L (L) | L (L) | H (L) | L (L) | – | 90 (60) | 85 (60) | H (M) | – | – | – | – |
| **7** | – | – | – | – | – | H (M) | H (M) | H (M) | – | – | – | – |
| **8** | – | – | – | – | – | M (L) | M (M) | H (M) | – | – | – | – |
| **9** | – | – | – | – | – | L (L) | L (L) | L (L) | – | M (M) | H (H) | H (H) |
| **10** | – | – | – | – | – | L (L) | M (L) | M (L) | – | H (M) | 90 (70) | >95 (75) |
| **11** | – | – | – | – | – | M (L) | M (M) | H (M) | – | M (L) | M (M) | H (M) |
| **12** | – | – | – | – | – | L (L) | L (L) | L (L) | – | M (M) | 85 (70) | 95 (70) |

[a] Standard deviation is <6% for the number noted. "–": No change or not measured. [b] See ref. [6].

**i) Peptide class A (*i*/*i*+4—distance of 0.5 nm)**: The most effective cross-linking agent for the peptide class **A** was ethylene-based **3**, with an appropriate spacer length (ca. 0.3–0.4 nm) between the two $^{-}$*C*OON$^{-}$ groups. In particular, the cross-linked **A·3** with X = Orn displayed a higher helical content—of 60%—than any other combination at 5 °C and kept ca. 40% of its helical structure even at 25 °C. This observation confirms that the whole helical structure in the peptide of 13 residues would be influenced by the local structural change at the N terminus through the trigger effect described above. While other short cross-linking agents—methylene-based **4** and *m*-phenylene-based **6**—were able to stabilize the helices somewhat, the cross-linking agents with longer spacers never contributed to the stabilization of the helices.

**ii) Peptide class B (*i*/*i*+7—distance of 1.1 nm)**: We have reported the use of the acetylenic cross-linking agents **1** and **2** for stabilizing α-helical structures of the peptide **B** (X = Lys).[6] To explore the influence of the spacer lengths on the helical content in detail, we examined all of the cross-linking agents for peptide class **B**. Among them, *p*-phenylene-based **5** (spacer length: ca. 0.6 nm) and *m*-phenylene-based **6** (spacer length: ca. 0.5 nm) formed extremely stable helices both at 5 °C (80–90%) and at 25 °C (60%). These values for the helical content are rather higher than those reported previously with use of the acetylenic **1** and **2**. Although biphenylene-based **9** (spacer length: ca. 1.0 nm) and fluorene-based **12** (spacer length: ca. 0.9 nm) are approximately fitted for bridging the *i*/*i*+7 pitch, the lengths of the alkyl side chains in the cross-linked amino acids X should be considered as described above. For **9** and **12**, the extra lengths of the alkyl side chains might cause the cross-linked peptides to be flexible, which should result in the formation of relatively unstable helices. On the other hand, the phenylene-based **5** and **6** are likely more suitable in terms of their slightly shorter and rigid spacers. Even in the case of **B·5**, the alkyl side chain of Dap is too short to form a stable cross-linked peptide: the peptide skeleton is too close to the rigid benzene core of Dap.

**iii) Peptide class C (*i*/*i*+11—distance of 1.6 nm)**: Diacetylenic **2** (spacer length: ca. 0.8–1.3 nm), naphthalene-based **10** (spacer length: ca. 0.8 nm), and fluorene-based **12** (spacer length: ca. 0.9 nm) provided much higher helical content (>80%) in the cross-linked peptides **C** at 5 °C. In particular, **C·2** (X = Dab) and **C·10** (X = Lys) were confirmed to exist in almost fully helical states (>95%), as if the sequences of the peptides had been incorporated into naturally occurring proteins. We succeeded in constructing almost complete helices from the short peptides, which comprise only ≈20% helix in their native forms, with our cross-linking agents. Even when the temperature was raised to 25 °C, the cross-linked **C·10** with X = Lys still sustained a high helical content (75%), offering promise that our method can be used for biological application in vivo.

As speculated in the section on cross-linking agents, cross-linkers that were rather short, in relation to the target distances between the two X residues, were found to be favorable for effective stabilization of α-helices in the three short peptides. On the basis of the data discussed above, the helical structures of the cross-linked peptides could roughly be optimized in the cases in which the lengths of the cross-linkers were about 50–60% of the target pitches.

**Application to biologically important sequences**: Finally, we applied our strategy to biologically related sequences directed toward natural recognition events. As a target we selected an arginine-rich sequence existing in the Rev protein that binds to Rev responsive element (RRE) RNA in HIV replication. In view of the above observations with the peptides **C**, the two arginine-rich sequences of **D** (X = Orn and Lys) were cross-linked with **2** and **10**. Figure 5 displays the greatly increased helical content after the cross-linking reaction in the case of **D** (X = Orn) with the cross-linking agent **2**. Thus, only ≈20% of native **D** fold up at 5 °C, whereas the cross-

  

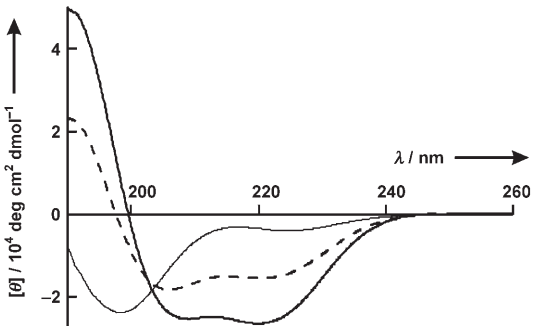


Figure 5. CD spectra of the native and the cross-linked **D** (X=Orn) with **2** dissolved in phosphate buffer (100 mM, pH 6.6) at 5 and 25 °C: native **D** (——) at 5 °C, **D·2** at 5 °C (——), and **D·2** at 25 °C (-----).

linked **D·2** was found to exist 95 % in the helical state at the same temperature. The cross-linked **D·2** still kept 60 % α-helical structure at 25 °C, close to the temperature range covering artificial experiments. This finding implies that the cross-linked **D·2** might be applicable in artificial segments with affinity for naturally occurring RRE RNA.

## Conclusion

We have developed a series of cross-linking agents and have found combinations of cross-linking agents and short peptides suitable for effective stabilization of helical structures in the cross-linked states. The best combinations were ethylene-based **3** for peptide class **A** (*i*/*i*+4; X=Orn), *m*-phenylene-based **6** for **B** (*i*/*i*+7; X=Dab), and naphthalene-based **10** for **C** (*i*/*i*+11; X=Lys) at both 5 and 25 °C. In particular, the cross-linked **C·10** showed >95 % helical content at 5 °C and 75 % even at 25 °C. Furthermore, we were able to obtain stable helical structures in peptide **D** (*i*/*i*+11; X=Orn and Lys), an arginine-rich motif from natural Rev. In future investigations, the cross-linking agents identified in this study will be widely applied to regulation and inhibition of biological recognition events in which α-helices participate.

## Experimental Section

**Materials and general procedures**: The cross-linking agents **3** and **5** were commercially available, while **4**,[14] **6**,[15] and **9**[16] had been reported previously. Other cross-linking agents were prepared from their corresponding dicarboxylic acids by the same procedure as described for **1** and **2**.[6] The dicarboxylic acid precursors for **7**, **8**, and **10** were commercially available, and those for **11** and **12** were synthesized by published procedures.[17,18] NMR spectra were recorded on a JEOL FX-270 or a Varian Gemini 300 spectrometer. IR spectra were measured on a JASCO-FT/IR-460 plus spectrometer. MALDI TOF-MS spectra were obtained by use of a Brüker Autoflex mass spectrometer. ESI-HRMS analyses were carried out on a JEOL JMS-T100 LC mass spectrometer. Melting points were determined with a Yanako MP-500D instrument and were not corrected.

**Physical and spectroscopic data for cross-linking agents**

**Biphenyl-3,3′-dicarboxylic acid bis(2,5-dioxopyrrolidin-1-yl) ester (7)**: Yield 71 % (15 mg); m.p. >165 °C (decomp); $^1$H NMR (270 MHz, $CDCl_3$): $\delta$=2.93 (s, 8H), 7.64 (t, $J$=7.3 Hz, 2H), 7.93 (m, 2H), 8.18 (m, 2H), 8.38 ppm (t, $J$=1.6 Hz, 2H); $^{13}$C NMR (67.5 MHz, $CDCl_3$): $\delta$=169.1, 161.6, 140.4, 133.6, 130.1, 129.7, 129.2, 126.1, 25.7 ppm; IR (KBr): $\tilde{\nu}$=1774, 1736 cm$^{-1}$; HRMS (ESI): *m*/*z*: calcd for $C_{22}H_{16}N_2NaO_8$: 459.0804; found: 459.0803 $[M+Na]^+$.

**Biphenyl-2,2′-dicarboxylic acid bis(2,5-dioxopyrrolidin-1-yl) ester (8)**: Yield 96 % (420 mg); m.p. 225–228 °C; $^1$H NMR (270 MHz, $CDCl_3$): $\delta$=2.77 (s, 8H), 7.33 (dd, $J$=1.4, 7.6 Hz, 2H), 7.52 (dt, $J$=1.1, 8.0 Hz, 2H), 7.66 (dt, $J$=1.4, 7.6 Hz, 2H), 8.21 ppm (dd, $J$=1.4, 7.8 Hz, 2H); $^{13}$C NMR (67.5 MHz, $CDCl_3$): $\delta$=168.5, 161.1, 142.7, 133.3, 130.7, 130. 5, 127.8, 123.0, 25.5 ppm; IR (KBr): $\tilde{\nu}$=1774, 1732 cm$^{-1}$; HRMS (ESI): *m*/*z*: calcd for $C_{22}H_{16}N_2NaO_8$: 459.0804; found: 459.0801 $[M+Na]^+$.

**Naphthalene-2,6-dicarboxylic acid bis(2,5-dioxopyrrolidin-1-yl) ester (10)**: Yield 19 % (38 mg); m.p. >295 °C (decomp); $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$=2.95 (s, 8H), 8.20 (d, $J$=8.4 Hz, 2H), 8.51 (d, $J$=8.4 Hz, 2H), 9.03 ppm (s, 2H); $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta$=169.8, 161.2, 134.5, 131.8, 131.0, 125.5, 124.5, 25.5 ppm; IR (KBr): $\tilde{\nu}$=1771, 1738 cm$^{-1}$; HRMS (ESI): *m*/*z*: calcd for $C_{20}H_{14}N_2NaO_8$: 433.0648; found: 433.0646 $[M+Na]^+$.

**Phenanthrene-3,6-dicarboxylic acid bis(2,5-dioxopyrrolidin-1-yl) ester (11)**: Yield 26 % (12 mg); m.p. 121–124 °C; $^1$H NMR (300 MHz, $CDCl_3$): $\delta$=2.98 (s, 8H), 7.96 (s, 2H), 8.04 (d, $J$=8.4 Hz, 2H), 8.31 (dd, $J$=1.5, 8.4 Hz, 2H), 9.49 ppm (d, $J$=1.5 Hz, 2H); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$=25.7, 123.5, 126.7, 127.5, 129.3 129.5, 129.6, 135.8, 161.6, 168.3, 168.9 ppm; IR (KBr): $\tilde{\nu}$=1770, 1736 cm$^{-1}$; HRMS (ESI): *m*/*z*: calcd for $C_{24}H_{16}N_2NaO_8$: 483.0804; found: 483.0803 $[M+Na]^+$.

**Fluorene-2,7-dicarboxylic acid bis(2,5-dioxopyrrolidin-1-yl) ester (12)**: Yield 98 % (47 mg); m.p. >274 °C (dec); $^1$H NMR (270 MHz, $CDCl_3$): $\delta$=2.94 (s, 8H), 4.08 (s, 2H), 7.99 (d, $J$=4.2 Hz, 2H), 8.24 (d, $J$=4.2 Hz, 2H), 8.38 ppm (s, 2H); $^{13}$C NMR (67.5 MHz, $CDCl_3$): $\delta$=169.2, 161.9, 148.0, 144.7, 130.1, 127.5, 124.7, 121.4, 36.9, 25.7 ppm; IR (KBr): $\tilde{\nu}$=1774, 1736 cm$^{-1}$; HRMS (ESI): *m*/*z*: calcd for $C_{23}H_{16}N_2NaO_8$: 471.0804; found: 471.0805 $[M+Na]^+$.

**Solid-phase peptide synthesis (SPPS)**: All of the peptides were synthesized with an automated peptide synthesizer by standard Fmoc chemistry. Peptides were constructed on an Fmoc-NH-SAL resin (capacity 0.59 mmol g$^{-1}$). After the automated SPPS, N-terminal amino groups were acetylated with $Ac_2O$ (5 %) in NMP (or 2 % *N*,*N*-diisopropylamine, 9 % $Ac_2O$, and 1.9 % HOBt·$H_2O$ in DMF for peptides **D**) over 12 min (30 min for peptides **D**) at room temperature. Peptide cleavage and side chain deprotection of amino acids were carried out by treatment under suitable conditions: TFA/ethanedithiol/thioanisole 18:1:1 for peptides **A** and **C**, TFA/ethanedithiol/thioanisole/2-methylindole 90:5:5:0.1 for peptides **B**, and TFA/ethanedithiol/thioanisole/thiophenol/DMSO/$H_2O$ 83:2.5:5:2:3 for peptides **D** over 2 h (12 h for peptides **D**) at room temperature.

**Peptide purification**: Peptides **A**–**D** were purified by reversed-phase HPLC (column; COSMOSIL 5$C_{18}$-AR-300 nacalai tesque, 10×150 mm) and eluted with TFA buffer (0.1 %) and the following $CH_3CN$ (including 0.1 % TFA) linear gradients at a flow rate of 2.0 mL min$^{-1}$; 5–45 % (0–40 min) for peptides **A**, 10–50 % (0–40 min) for peptides **B**, 15–55 % (0–40 min) for peptides **C**, and 5–45 % (0–40 min) for peptides **D**. The fractions of the peptides were monitored at 220 nm with a UV detector, and were identified by ESI-MS and MALDI TOF MS.

*Peptides* ***A***: X=Dap: *m*/*z*: calcd for $C_{49}H_{81}N_{16}O_{22}$: 1245.6; found: 1245.1 $[M+H]^+$ (ESI); X=Dab: *m*/*z*: calcd for $C_{51}H_{85}N_{16}O_{22}$: 1273.6; found: 1273.5 $[M+H]^+$ (MALDI); X=Orn: *m*/*z*: calcd for $C_{53}H_{89}N_{16}O_{22}$: 1301.6; found: 1301.5 $[M+H]^+$ (MALDI); X=Lys: *m*/*z*: calcd for $C_{55}H_{93}N_{16}O_{22}$: 1329.7; found: 1329.5 $[M+H]^+$ (MALDI).

*Peptides* ***B***: X=Dap: *m*/*z*: calcd for $C_{66}H_{102}N_{20}O_{25}$: 787.4; found: 787.2 $[M+2H]^{2+}$ (ESI); X=Dab: *m*/*z*: calcd for $C_{68}H_{106}N_{20}O_{25}$: 801.4; found: 801.3 $[M+2H]^{2+}$ (ESI); X=Orn: *m*/*z*: calcd for $C_{70}H_{110}N_{20}O_{25}$: 815.4; found: 815.1 $[M+2H]^{2+}$ (ESI); X=Lys: *m*/*z*: calcd for $C_{72}H_{114}N_{20}O_{25}$: 829.4; found: 829.2 $[M+2H]^{2+}$ (ESI).

*Peptides* ***C***: X=Dab: *m*/*z*: calcd for $C_{74}H_{123}N_{23}O_{31}$: 914.9; found: 914.6 $[M+2H]^{2+}$ (ESI); X=Orn: *m*/*z*: calcd for $C_{76}H_{126}N_{23}O_{31}$: 1856.9; found:

  

1856.9 $[M+H]^+$ (MALDI); X=Lys, calcd for $C_{78}H_{130}N_{23}O_{31}$: 1884.9; found: 1884.9 $[M+H]^+$ (MALDI).

*Peptides* ***D***: X=Orn: *m/z*: calcd for $C_{101}H_{196}N_{57}O_{22}$: 2559.6; found: 2559.3 $[M+H]^+$ (MALDI); X=Lys, calcd for $C_{103}H_{201}N_{57}O_{22}$: 1294.3; found: 1294.0 $[M+2H]^{2+}$ (ESI).

**Cross-linking reactions—Peptides A–C**: A cross-linking agent solution in DMSO (0.5 mL, $5.0\times10^{-4}$ M) was added to a peptide solution (0.5 mL, $1.0\times10^{-4}$ M) in phosphate buffer (100 mM, pH 6.6). The reaction mixture was incubated at 25 °C in a thermo-mixer for 0.5–2 h. The cross-linked peptides **A–C** were purified by use of the following $CH_3CN$ (including 0.1% TFA) linear gradients (0–40 min) at a flow rate of 1.0 mL min$^{-1}$; 5–65% for cross-linked peptides **A** and 10–70% for cross-linked peptides **B** and **C**.

**Peptides D**: A cross-linking agent solution in DMSO (0.4 mL, $4.17\times10^{-4}$ M) was added to an EtOH solution (0.6 mL) of a native peptide **D** ($1.25\times10^{-4}$ M). The reaction mixture was stirred at 30 °C in a thermo-mixer for 12 h. The cross-linked peptides **D** were isolated under conditions similar to those described above, except for use of a 0–60% $CH_3CN$ (including 0.1% TFA) linear gradient. All of the cross-linked peptides were identified by ESI-MS and MALDI TOF-MS.

**Yields of cross-linked peptides**: The reaction yields of the cross-linked peptides were estimated by comparison of the peak areas of the remaining native peptides with those of the cross-linked peptides in the HPLC charts of the reaction mixtures. The molar ratios for the unreacted and the cross-linked peptides were calculated by calibration of their molar extinction coefficients ($\varepsilon$) at 220 nm. The $\varepsilon$ values of the cross-linked peptides were each defined to be the sum of the values for the native peptide and the cross-linking agent at 220 nm.

**Cross-linked peptides**: **A·3**: X=Dap, 85%; X=Dab, 70%; X=Orn, 75%; X=Lys, 85%. **A·4**: X=Dap, 10%; X=Dab, 70%; X=Orn, 55%; X=Lys, 55%. **A·5**: X=Dap, 20%; X=Dab, 45%; X=Orn, 60%; X=Lys, 75%. **A·6**: X=Dap, 50%; X=Dab, 75%; X=Orn, 75%; X=Lys, 70%. **B·3**: X=Dab, 85%; X=Orn, 85%; X=Lys, 90%. **B·4**: X=Dab, 40%; X=Orn, 45%; X=Lys, 35%. **B·5**: X=Dap, 15%; X=Dab, 45%; X=Orn, 50%; X=Lys, 40%. **B·6**: X=Dab, 75%; X=Orn, 80%; X=Lys, 85%. **B·7**: X=Dab, 40%; X=Orn, 40%; X=Lys, 40%. **B·8**: X=Dab, 30%; X=Orn, 15%; X=Lys, 30%. **B·9**: X=Dab, 15%; X=Orn, 20%; X=Lys, 15%. **B·10**: X=Dab, 20%; X=Orn, 30%; X=Lys, 30%. **B·11**: X=Dab, 55%; X=Orn, 45%; X=Lys, 55%. **B·12**: X=Dab, 55%; X=Orn, 70%; X=Lys, 40%. **C·2**: X=Dab, 85%; X=Orn, 65%; X=Lys, 60%. **C·9**: X=Dab, 65%; X=Orn, 45%; X=Lys, 30%. **C·10**: X=Dab, 60%; X=Orn, 35%; X=Lys, 35%. **C·11**: X=Dab, 65%; X=Orn, 55%; X=Lys, 65%. **C·12**: X=Dab, >95%; X=Orn, 90%; X=Lys, >95%. **D·2**: X=Orn, 15%; X=Lys, 30%. **D·10**: X=Orn, <5%; X=Lys, <5%.

**Mass spectral data for cross-linked peptides**

**Cross-linked peptide A·3**: X=Dap: *m/z*: calcd for $C_{53}H_{83}N_{16}O_{24}$: 1327.6; found: 1327.8 $[M+H]^+$ (MALDI); X=Dab: *m/z*: calcd for $C_{55}H_{86}N_{16}NaO_{24}$: 1377.6; found: 1377.8 $[M+Na]^+$ (MALDI); X=Orn: *m/z*: calcd for $C_{57}H_{90}N_{16}NaO_{24}$: 1405.6; found: 1405.1 $[M+Na]^+$ (MALDI); X=Lys: *m/z*: calcd for $C_{59}H_{94}N_{16}NaO_{24}$: 1433.7; found: 1433.1 $[M+Na]^+$ (MALDI).

**Cross-linked peptide A·4**: X=Dap: *m/z*: calcd for $C_{54}H_{84}N_{16}NaO_{24}$: 1363.6; found: 1363.8 $[M+Na]^+$ (MALDI); X=Dab: *m/z*: calcd for $C_{56}H_{88}N_{16}NaO_{24}$: 1391.6; found: 1391.6 $[M+Na]^+$ (MALDI); X=Orn: *m/z*: calcd for $C_{58}H_{92}N_{16}NaO_{24}$: 1419.6; found: 1419.4 $[M+Na]^+$ (MALDI); X=Lys: *m/z*: calcd for $C_{60}H_{96}N_{16}NaO_{24}$: 1447.7; found: 1447.3 $[M+Na]^+$ (MALDI).

**Cross-linked peptide A·5**: X=Dap: *m/z*: calcd for $C_{57}H_{83}N_{16}O_{24}$: 1375.6; found: 1375.4 $[M+H]^+$ (ESI); X=Dab: *m/z*: calcd for $C_{59}H_{86}N_{16}NaO_{24}$: 1425.6; found: 1425.6 $[M+Na]^+$ (MALDI); X=Orn: *m/z*: calcd for $C_{61}H_{90}N_{16}NaO_{24}$: 1453.6; found: 1453.6 $[M+Na]^+$ (MALDI); X=Lys: *m/z*: calcd for $C_{63}H_{94}N_{16}NaO_{24}$: 1481.7; found: 1481.7 $[M+Na]^+$ (MALDI).

**Cross-linked peptide A·6**: X=Dap: *m/z*: calcd for $C_{57}H_{83}N_{16}O_{24}$: 1375.6; found: 1375.4 $[M+H]^+$ (ESI); X=Dab: *m/z*: calcd for $C_{59}H_{86}N_{16}NaO_{24}$: 1425.6; found: 1425.4 $[M+Na]^+$ (MALDI); X=Orn: *m/z*: calcd for $C_{61}H_{90}N_{16}NaO_{24}$: 1453.6; found: 1453.1 $[M+Na]^+$ (MALDI); X=Lys: *m/z*: calcd for $C_{63}H_{94}N_{16}NaO_{24}$: 1481.7; found: 1481.1 $[M+Na]^+$ (MALDI).

**Cross-linked peptide B·3**: X=Dab: *m/z*: calcd for $C_{72}H_{108}N_{20}O_{27}$: 842.4; found: 842.3 $[M+2H]^{2+}$ (ESI); X=Orn: *m/z*: calcd for $C_{74}H_{111}N_{20}O_{27}$: 1711.8; found: 1711.1 $[M+H]^+$ (MALDI); X=Lys: *m/z*: calcd for $C_{76}H_{116}N_{20}O_{27}$: 870.4; found: 870.0 $[M+2H]^{2+}$ (ESI).

**Cross-linked peptide B·4**: X=Dab: *m/z*: calcd for $C_{73}H_{110}N_{20}O_{27}$: 849.4; found: 849.1 $[M+2H]^{2+}$ (ESI); X=Orn: *m/z*: calcd for $C_{75}H_{113}N_{20}O_{27}$: 1725.8; found: 1725.7 $[M+H]^+$ (MALDI); X=Lys: *m/z*: calcd for $C_{77}H_{116}N_{20}NaO_{27}$: 1775.8; found: 1775.4 $[M+Na]^+$ (MALDI).

**Cross-linked peptide B·5**: X=Dap: *m/z*: calcd for $C_{74}H_{104}N_{20}O_{27}$: 852.4; found: 852.0 $[M+2H]^{2+}$ (ESI); X=Dab: *m/z*: calcd for $C_{76}H_{108}N_{20}O_{27}$: 866.4; found: 866.2 $[M+2H]^{2+}$ (ESI); X=Orn: *m/z*: calcd for $C_{78}H_{112}N_{20}O_{27}$: 880.4; found: 880.6 $[M+2H]^{2+}$ (ESI); X=Lys: *m/z*: calcd for $C_{80}H_{114}N_{20}NaO_{27}$: 1809.8; found: 1809.6 $[M+Na]^+$ (MALDI).

**Cross-linked peptide B·6**: X=Dab: *m/z*: calcd for $C_{76}H_{108}N_{20}O_{27}$: 866.4; found: 866.2 $[M+2H]^{2+}$ (ESI); X=Orn: *m/z*: calcd for $C_{78}H_{112}N_{20}O_{27}$: 880.4; found: 880.6 $[M+2H]^{2+}$ (ESI); X=Lys: *m/z*: calcd for $C_{80}H_{116}N_{20}O_{27}$: 894.4; found: 894.0 $[M+2H]^{2+}$ (ESI).

**Cross-linked peptide B·7**: X=Dab: *m/z*: calcd for $C_{82}H_{112}N_{20}O_{27}$: 904.4; found: 904.1 $[M+2H]^{2+}$ (ESI); X=Orn: *m/z*: calcd for $C_{84}H_{116}N_{20}O_{27}$: 918.4; found: 918.0 $[M+2H]^{2+}$ (ESI); X=Lys: *m/z*: calcd for $C_{86}H_{120}N_{20}O_{27}$: 932.4; found: 932.0 $[M+2H]^{2+}$ (ESI).

**Cross-linked peptide B·8**: X=Dab: *m/z*: calcd for $C_{82}H_{111}N_{20}O_{27}$: 1807.8; found: 1807.5 $[M+H]^+$ (MALDI); X=Orn: *m/z*: calcd for $C_{84}H_{116}N_{20}O_{27}$: 918.4; found: 918.0 $[M+2H]^{2+}$ (ESI); X=Lys: *m/z*: calcd for $C_{86}H_{120}N_{20}O_{27}$: 932.4; found: 932.0 $[M+2H]^{2+}$ (ESI).

**Cross-linked peptide B·9**: X=Dab: *m/z*: calcd for $C_{82}H_{111}N_{20}O_{27}$: 1807.8; found: 1807.6 $[M+H]^+$ (MALDI); X=Orn: *m/z*: calcd for $C_{84}H_{116}N_{20}O_{27}$: 918.4; found: 918.0 $[M+2H]^{2+}$ (ESI); X=Lys: *m/z*: calcd for $C_{86}H_{120}N_{20}O_{27}$: 932.4; found: 932.0 $[M+2H]^{2+}$ (ESI).

**Cross-linked peptide B·10**: X=Dab: *m/z*: calcd for $C_{80}H_{110}N_{20}O_{27}$: 891.4; found: 891.2 $[M+2H]^{2+}$ (ESI); X=Orn: *m/z*: calcd for $C_{82}H_{114}N_{20}O_{27}$: 905.4; found: 905.2 $[M+2H]^{2+}$ (ESI); X=Lys: *m/z*: calcd for $C_{84}H_{118}N_{20}O_{27}$: 919.4; found: 919.1 $[M+2H]^{2+}$ (ESI).

**Cross-linked peptide B·11**: X=Dab: *m/z*: calcd for $C_{84}H_{111}N_{20}O_{27}$: 1831.8; found: 1831.5 $[M+H]^+$ (MALDI); X=Orn: *m/z*: calcd for $C_{86}H_{114}N_{20}NaO_{27}$: 1881.8; found: 1881.7 $[M+Na]^+$ (MALDI); X=Lys: *m/z*: calcd for $C_{88}H_{118}N_{20}NaO_{27}$: 1909.8; found: 1909.7 $[M+Na]^+$ (MALDI).

**Cross-linked peptide B·12**: X=Dab: *m/z*: calcd for $C_{83}H_{111}N_{20}O_{27}$: 1819.8; found: 1819.4 $[M+H]^+$ (MALDI); X=Orn: *m/z*: calcd for $C_{85}H_{115}N_{20}O_{27}$: 1847.8; found: 1847.5 $[M+H]^+$ (MALDI); X=Lys: *m/z*: calcd for $C_{87}H_{118}N_{20}NaO_{27}$: 1897.8; found: 1897.4 $[M+Na]^+$ (MALDI).

**Cross-linked peptide C·2**: X=Dab: *m/z*: calcd for $C_{84}H_{129}N_{23}O_{35}$: 1010.0; found: 1009.7 $[M+2H]^{2+}$ (ESI); X=Orn: *m/z*: calcd for $C_{86}H_{132}N_{23}O_{35}$: 2046.9; found: 2047.1 $[M+H]^+$ (MALDI); X=Lys: *m/z*: calcd for $C_{88}H_{136}N_{23}O_{35}$: 2075.0; found: 2075.0 $[M+H]^+$ (MALDI).

**Cross-linked peptide C·9**: X=Dab: *m/z*: calcd for $C_{88}H_{127}N_{23}NaO_{33}$: 2056.9; found: 2057.2 $[M+Na]^+$ (MALDI); X=Orn: *m/z*: calcd for $C_{90}H_{132}N_{23}O_{33}$: 2062.9; found: 2062.5 $[M+H]^+$ (MALDI); X=Lys: *m/z*: calcd for $C_{92}H_{135}N_{23}NaO_{35}$: 2112.9; found: 2112.8 $[M+Na]^+$ (MALDI).

**Cross-linked peptide C·10**: X=Dab: *m/z*: calcd for $C_{86}H_{127}N_{23}O_{33}$: 1004.9; found: 1004.7 $[M+2H]^{2+}$ (ESI); X=Orn: *m/z*: calcd for $C_{88}H_{129}N_{23}NaO_{33}$: 2058.9; found: 2059.2 $[M+Na]^+$ (MALDI); X=Lys: *m/z*: calcd for $C_{90}H_{134}N_{23}O_{35}$: 2065.0; found: 2064.6 $[M+H]^+$ (MALDI).

**Cross-linked peptide C·11**: X=Dab: *m/z*: calcd for $C_{90}H_{128}N_{23}O_{33}$: 2058.9; found: 2058.8 $[M+H]^+$ (MALDI); X=Orn: *m/z*: calcd for $C_{92}H_{132}N_{23}O_{33}$: 2086.9; found: 2086.7 $[M+H]^+$ (MALDI); X=Lys: *m/z*: calcd for $C_{94}H_{136}N_{23}O_{33}$: 2115.0; found: 2115.2 $[M+H]^+$ (MALDI).

**Cross-linked peptide C·12**: X=Dab: *m/z*: calcd for $C_{89}H_{127}N_{23}NaO_{33}$: 2068.9; found: 2068.8 $[M+Na]^+$ (MALDI); X=Orn: *m/z*: calcd for $C_{91}H_{131}N_{23}NaO_{33}$: 2096.9; found: 2096.6 $[M+Na]^+$ (MALDI); X=Lys: *m/z*: calcd for $C_{93}H_{136}N_{23}O_{33}$: 2103.0; found: 2102.6 $[M+H]^+$ (MALDI).

  

**Cross-linked peptide D·2**: X = Orn: *m/z*: calcd for $C_{111}H_{202}N_{57}O_{26}$: 2749.6; found: 2749.2 $[M+H]^+$ (MALDI); X = Lys: *m/z*: calcd for $C_{113}H_{206}N_{57}O_{26}$: 2777.7; found: 2778.2 $[M+H]^+$ (MALDI).

**Cross-linked peptide D·10**: X = Orn: *m/z*: calcd for $C_{113}H_{200}N_{57}O_{24}$: 2739.6; found: 2739.2 $[M+H]^+$ (MALDI); X = Lys: *m/z*: calcd for $C_{115}H_{204}N_{57}O_{24}$: 2767.6; found: 2767.6 $[M+H]^+$ (MALDI).

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