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Versatile synthesis of fluorescent, cross-linked peptides as biological probes with the advantage of high helix content

Kazuhisa Fujimoto · Masaoki Kajino · Masahiko Inouye

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Abstract A variety of fluorophores were introduced at the N-termini of short peptides for use as biological-probes. The fluorescent peptides were cross-linked with a diacetylenic cross-linking agent between the amino acid side chains of ornithine (Orn) residues to produce peptides with high helix content.

Keywords Short helical peptide · Fluorophore · Fluorescence labeling · Biological probe

Introduction

Most intractable diseases involve abnormal protein expression. For instance, tumor cells overexpress specific proteins which elaborately interact with one another. Practical molecular probes that competitively bind to specific proteins are essential to enable cancer diagnosis at earlier stages. Such probes have to fulfill three requirements—low molecular weight, easy synthesis, and stability for metabolic enzymes. However, during the design of practical probes, low-molecular-weight molecules seem to barely cover the large area of the surfaces or the active sites of the protein–protein interactions targeted. It is also extremely difficult, de novo, to design and synthesize probes with a moderate molecular weight that can cover the large areas by organic synthesis only. Thus, peptide-based probes with stable

K. Fujimoto (✉)
Department of Applied Chemistry and Biochemistry, Kyushu Sangyo University, Fukuoka 813-8503, Japan
e-mail: kazuf@ip.kyusan-u.ac.jp

M. Kajino · M. Inouye (✉)
Graduate School of Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan
e-mail: inouye@pha.u-toyama.ac.jp

α-helical structures are regarded as powerful candidates for the aforementioned purpose because α-helices are of key importance in protein interactions.

Recently, we succeeded in stabilization of the helices of short peptides by bridging their amino acid side chains with cross-linking agents [1, 2].[1] DNA-binding cross-linked peptides were first designed and synthesized on the basis of the binding sites for protein–DNA interactions, and the peptides were then labeled at their N-termini with Tokyo green [6] to evaluate the binding efficiency of the peptides to DNAs [7]. The fluorescent, cross-linked peptides retained stable helical structures and had high binding affinity with substrate specificity for DNAs. The cross-linked peptides were found to be more stable for protease than non-cross-linked peptides, and their molecular weights were approximately 3,000. From different perspectives, fluorescent helical peptides have potential use as specific probes for specific proteins of clinical importance. Therefore, taking into account our previous findings, we prepared fluorescent, cross-linked peptides by using a variety of fluorophores and revealed their high helix content by CD analysis.

Experimental

Synthesis of 3-[4-(7-hydroxycoumarin-3-ylethynyl)phenyl]propanoic acid

To a THF (6 mL) solution of 3-bromo-7-hydroxycoumarin (120 mg, 0.5 mmol), $Pd(PPh_3)_4$ (29 mg, 0.025 mmol), and CuI (9.5 mg, 0.05 mmol,) at 70 °C under an Ar atmosphere, was added a solution of 3-(4-ethynylphenyl)propanoic acid [8] (120 mg, 0.85 mmol) in Et_3N (10 mL). The reaction mixture was heated under reflux for 6 h. After removal of the solvent by use of a rotary evaporator, the residue was dissolved in aqueous 1 M HCl and extracted with AcOEt. The AcOEt extract was evaporated by use of a rotary evaporator and chromatographed (silica gel; mobile phase from CH_2Cl_2 to CH_2Cl_2–CH_3OH 10:1 *v*/*v*) to give 3-[4-(7-hydroxycoumarin-3-ylethynyl)phenyl]propanoic acid: yield 57 % (95 mg); mp 231–233 °C; IR (KBr) $\nu = 3384, 1705, 1610, 1567, 1502, 1302, 1225, 1150, 1120\ cm^{-1}$; ^1H NMR (DMSO-d_6) $\delta = 2.57$ (t, $J = 7.6$ Hz, 2 H), 2.86 (t, $J = 7.6$ Hz), 6.76 (d, $J = 2.0$ Hz, 1 H), 6.84 (dd, $J = 2.4$, 8.8 Hz, 1 H), 7.20 (d, $J = 8.0$ Hz, 2 H), 7.44 (d, $J = 8.0$ Hz, 2 H), 7.57 (d, $J = 8.4$ Hz, 1 H), 8.29 (s, 1 H), 10.83 (s, 1 H), 12.17 (s, 1 H); ^{13}C NMR (DMSO-d_6) $\delta = 30.1, 34.6, 84.0, 93.0, 102.1, 106.3, 111.2, 113.7, 119.4, 128.7, 129.9, 131.2, 142.2, 146.4, 154.9, 159.0, 162.1, 173.5$; HRMS (ESI): *m*/*z*: calcd for $C_{20}H_{14}NaO_5$ $[M+Na]^+$ 357.0739; found 357.0732.

Synthesis of fluorescent, cross-linked peptides

The extending reaction of amino acid residues were performed with an automated peptide synthesizer on an Fmoc-NH-SAL resin (capacity 0.59 mmol/g). After the automated synthesis, the N-terminal amino groups of the peptides were

[1] Other helical peptides and helical mimetics are discussed in Refs. [3–5].

fluorescence-labeled on the resin by treating with fluorophore-based carboxylic acids[2] [8] (5 equiv), HBTU (10 equiv), HOBt (10 equiv), and *N*,*N*′-diisopropylethyl amine (10 % *v*/*v*) in a mixture of DMF and DMSO (1:1) over 8 h at room temperature. Dde (= 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl) groups on the ornithine (Orn) residues of the peptides were selectively removed by use of hydrazine monohydrate (3 % *v*/*v*) in DMF in 1 h × 2 at room temperature. The deprotected peptides were cross-linked with the cross-linking agent [3–5] (3 equiv) over 12 h at room temperature on the resin. Peptide cleavage and side chain deprotection of amino acids were achieved by treatment under suitable conditions: TFA–ethanedithiol–thioanisole 18:1:1 for 1.5 h at room temperature. All of the peptides were purified by reversed-phase HPLC (Cosmosil $5C_{18}$-MS-2 column, 10 × 150 mm^2, Nacalai Tesque) and eluted with a mixture of 0.1 % TFA buffer and CH_3CN including 0.1 % TFA by use of the following linear CH_3CN gradients at a flow rate of 2.0 mL min^{-1}: 5–45 % (0–40 min) for **cA**, 15–55 % (0–40 min) for **cA**·**1** and **cA**·**2**, 20–60 % (0–40 min) for **cA**·**3**, 20–60 % (0–40 min) for **cA**·**4**, and 10–50 % (0–40 min) for **cA**·**5**. The peptide fractions were monitored at 220 nm with a UV detector, and were identified by MALDI TOF MS.

Mass spectral data for peptides

A·**1**: *m*/*z*: calcd for $[M + H]^+$; $C_{124}H_{206}N_{57}O_{22}$: 2845.7; found: 2846.2
cA·**1**: *m*/*z*: calcd for $[M + H]^+$; $C_{134}H_{211}N_{57}O_{26}$: 3035.7; found: 3035.4
A·**2**: *m*/*z*: calcd for $[M + H]^+$; $C_{126}H_{210}N_{57}O_{22}$: 2873.7; found: 2873.7
cA·**2**: *m*/*z*: calcd for $[M + H]^+$; $C_{136}H_{215}N_{57}O_{26}$: 3063.7; found: 3063.1
A·**3**: *m*/*z*: calcd for $[M + H]^+$; $C_{119}H_{208}N_{57}O_{22}$: 2787.7; found: 2787.2
cA·**3**: *m*/*z*: calcd for $[M + H]^+$; $C_{129}H_{213}N_{57}O_{26}$: 2977.7; found: 2977.7
A·**4**: *m*/*z*: calcd for $[M + H]^+$; $C_{123}H_{210}N_{57}O_{22}$: 2837.7; found: 2837.2
cA·**4**: *m*/*z*: calcd for $[M + H]^+$; $C_{133}H_{216}N_{57}O_{26}$: 3027.7; found: 3028.4
A·**5**: *m*/*z*: calcd for $[M + H]^+$; $C_{119}H_{206}N_{57}O_{25}$: 2833.7; found: 2833.8
cA·**5**: *m*/*z*: calcd for $[M + H]^+$; $C_{129}H_{212}N_{57}O_{29}$: 3023.7; found: 3023.2.

Results and discussion

Synthesis of fluorescent, cross-linked peptides

Figure 1 shows the amino acid sequence of the peptides used in this study and the chemical structures of fluorophores tethered at the N-termini of the peptides: the resulting fluorescent peptides are denoted **A**·**1**–**A**·**5**. The amino acid sequence, designed according to that of the Rev peptide binding to RRE RNA, includes two Orn residues as cross-linked moieties. Five fluorophores were introduced at the N-termini of the Rev-arranged peptide: phenylethynylpyrenes **1** and **2** [8], pyrene **3**, perylene **4**, and phenylethynylcoumarin **5**. To examine the effect on helix content of

[2] Synthesis of the fluorophore-based carboxylic acids of **A**·**2** and **A**·**4** is discussed elsewhere [9, 10].

spacer length between the N-termini and fluorophores, two types of phenylethynylpyrene-based fluorophore (**1** and **2**) were prepared.

All of the fluorescent peptides **A·1**–**A·5** were synthesized in accordance with a previously published method [7]. The extending reaction of amino acid residues, fluorophore-labeling reaction, and cross-linking reaction were performed on the synthetic resin. In the labeling reactions, the fluorophore-based carboxylic acids were reacted with the deprotected N-termini of the synthesizing peptides in the presence of HBTU, HOBt, and *N*,*N*′-diisopropylamine. When chemical modification of the peptides on the synthetic resin was complete, the peptides were released from the resin and purified by HPLC. Fluorescent peptides, **A·1**–**A·5**, were cross-linked with a diacetylenic cross-linking agent to yield **cA·1**–**cA·5**. The combination of the diacetylenic agent and orn was the most suitable for stabilizing the helical structures of such Arg-rich peptides at the $i/i + 11$ position for the cross-linking reaction [1, 2].

UV–visible absorption and excitation/emission spectra of fluorescent peptides

The UV–visible absorption spectra of the fluorescent, cross-linked peptides **cA·1**–**cA·5** are displayed in Fig. 2. These spectra were measured at concentrations ranging from 33 to 82 μM. The λ_{max} and ε values of the peptides are shown in each spectrum. The λ_{max} values of **cA·1** and **cA·2** were found to be shorter than that of the phenylethynylpyrene fluorophore, which is reportedly ca 380 nm. Furthermore, their ε values were almost half that of **cA·3**, in which native pyrene was used as the fluorophore. These findings suggest that, at UV–visible-measurement concentrations, the phenylethynylpyrene moieties of **cA·1** and **cA·2** self-associate to form H-aggregates [11]. It is plausible that, during aggregation, the peptide moieties align

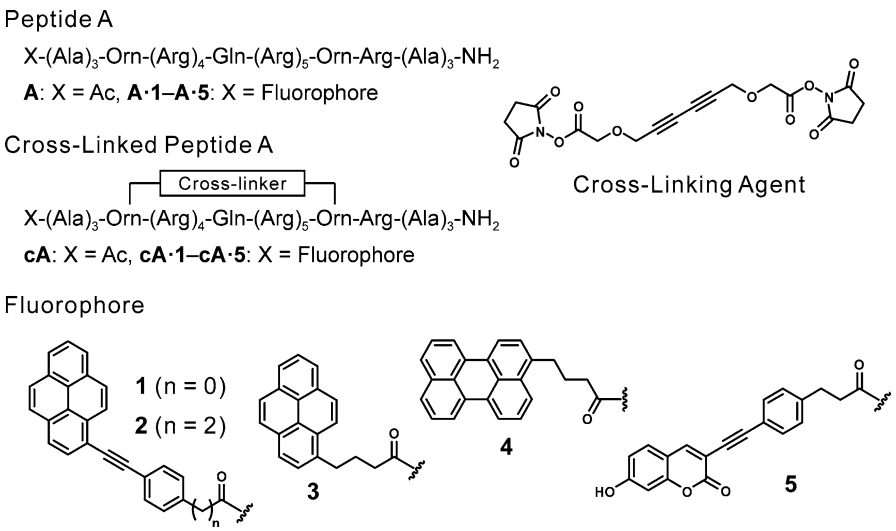

Fig. 1 Amino acid sequence of peptides, and chemical structures of the cross-linking agent and fluorophores **1**–**5** in this study

alternately, as shown in Fig. 2, to minimize the electrostatic repulsion of the Arg residues.

In the excitation/emission spectra of the fluorescent, cross-linked peptides at 3.8–8.2 μM, excluding **cA·1** (Fig. 3), each emission spectrum was almost consistent with the reflection of the corresponding excitation spectrum in a mirror. The mirror relationship indicates that the fluorophores on **cA·2–cA·5** scarcely aggregate at the measurement concentrations. The alkyl-spacer of **cA·2** retained the mirror relationship, whereas the relationship was deformed in the spectra of **cA·1**, with no spacer. The intensity of vibronic band I was smaller than that of vibronic band III in the excitation spectrum of **cA·1**, with no spacer, meaning that the attached

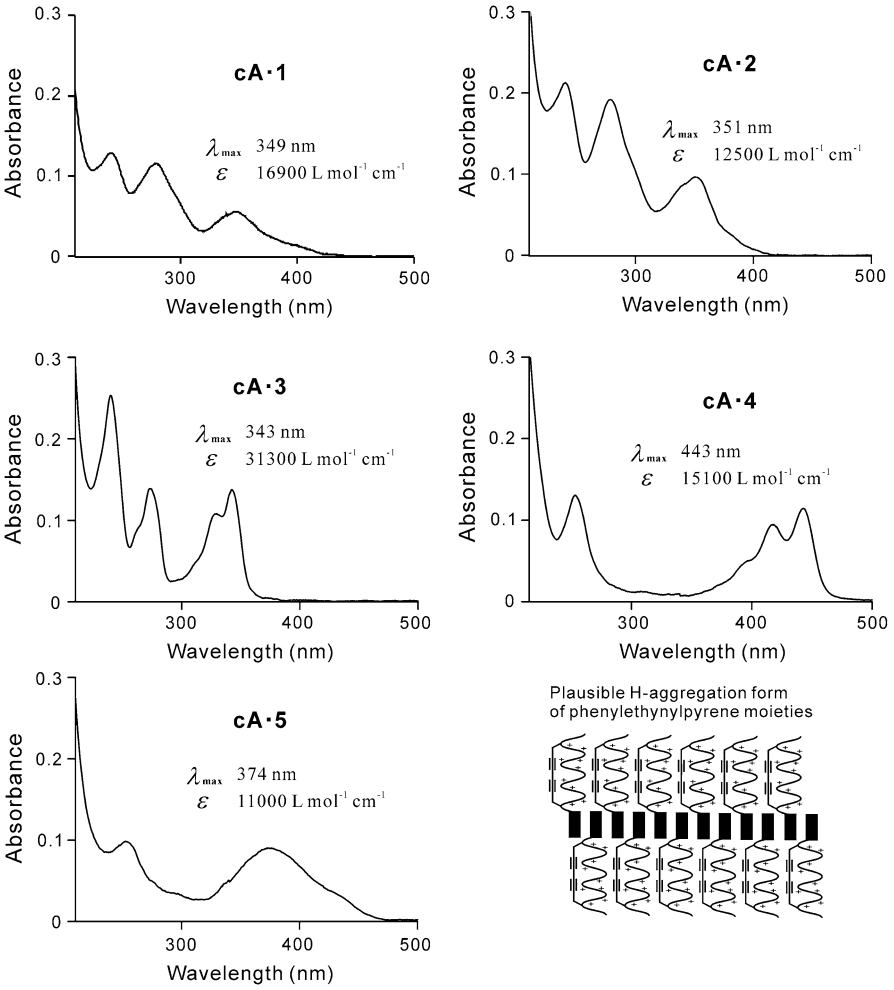

Fig. 2 UV–visible absorption spectra of the fluorescent, cross-linked peptides **cA·1–cA·5** dissolved in a 100 mM phosphate buffer (pH 6.6) at 25 °C: [**cA·1**] = 33 μM, [**cA·2**] = 77 μM, [**cA·3**] = 44 μM, [**cA·4**] = 76 μM, and [**cA·5**] = 82 μM. A plausible H-aggregation form of the phenylethynylpyrene moieties shown as a *black rectangle* (*lower right*)

fluorophore exists in a hydrophobic environment [12]. Moreover, the emission spectrum of **cA·1** was broadened and shifted to longer wavelength than that of alkyl-spacer-type **cA·2**. These findings imply that **cA·1** self-assembles more easily than **cA·2** to form H-aggregates.

Helix content of fluorescent peptides

The helix content of the fluorescent, cross-linked peptides **cA·1–cA·5** were estimated on the basis of their CD spectra recorded at 5 °C in accordance with a

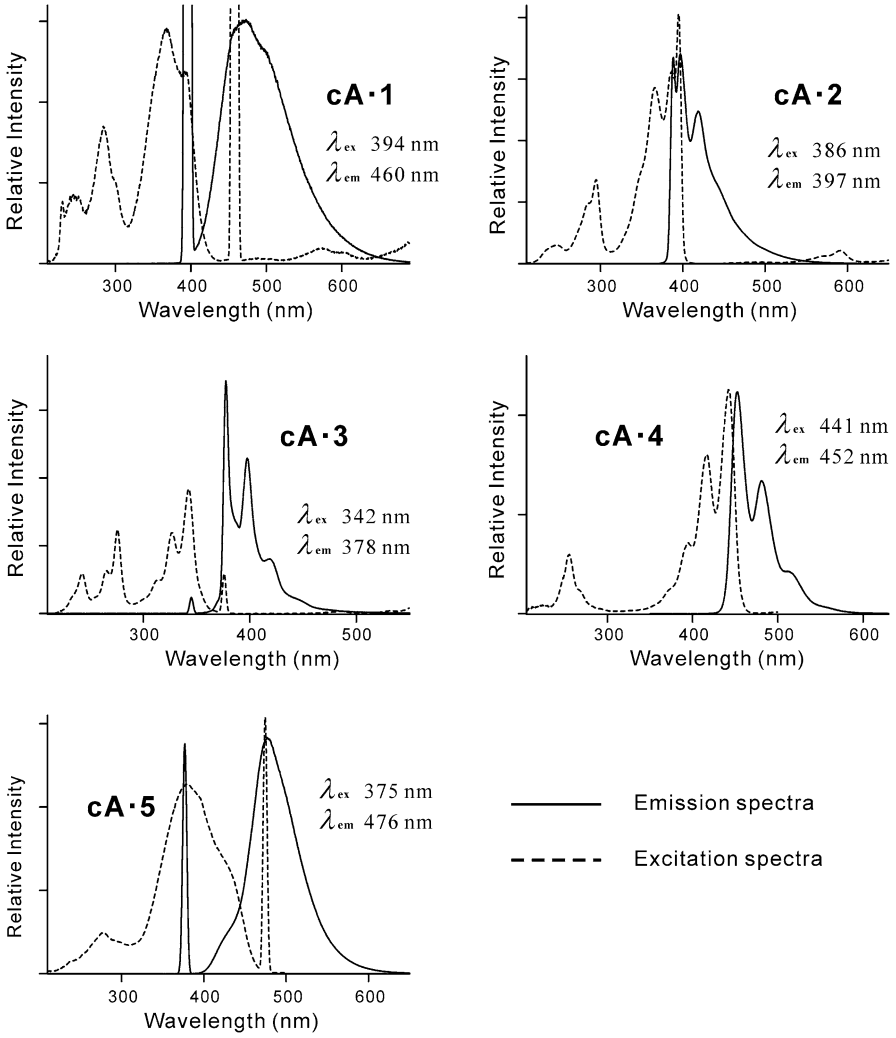

Fig. 3 Excitation/emission spectra of fluorescent, cross-linked peptides **cA·1–cA·5** dissolved in a 100 mM phosphate buffer (pH 6.6) at 25 °C: [**cA·1**] = 5.0 μM, [**cA·2**] = 5.0 μM, [**cA·3**] = 4.4 μM, [**cA·4**] = 3.8 μM, and [**cA·5**] = 8.2 μM

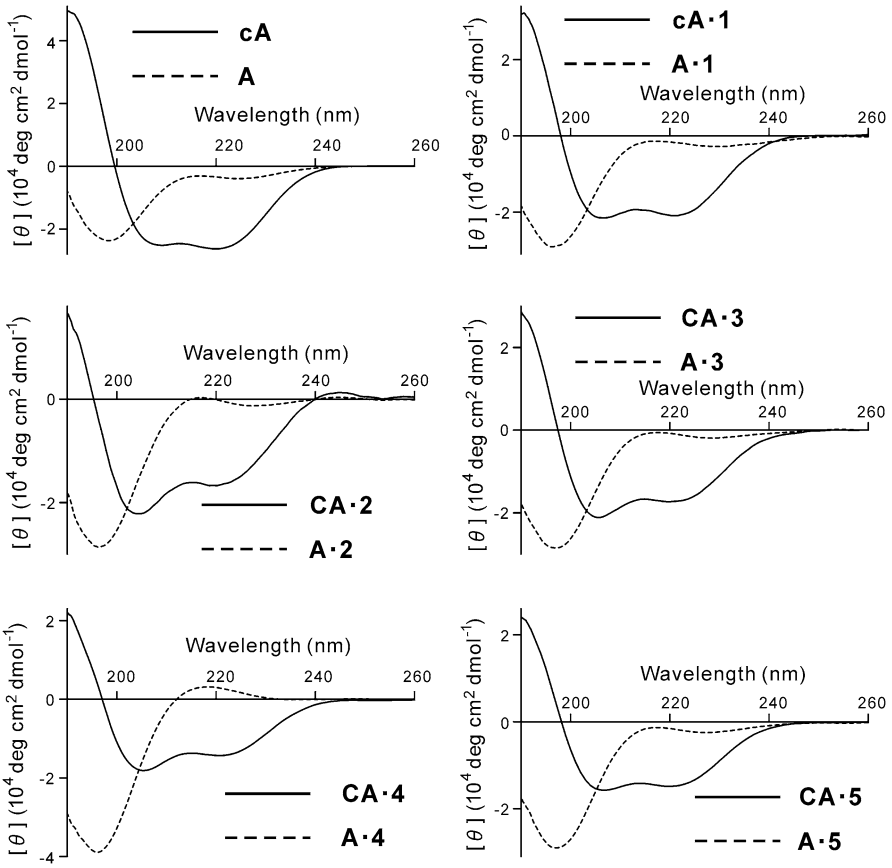

Fig. 4 CD spectra of peptides **A**, **A·1**–**A·5**, **cA**, and **cA·1**–**cA·5** dissolved in a 100 mM phosphate buffer (pH 6.6) at 5 °C: [**A**] = 75 μM, [**A·1**] = 49 μM, [**A·2**] = 150 μM, [**A·3**] = 81 μM, [**A·4**] = 75 μM, [**A·5**] = 75 μM, [**cA**] = 82 μM, [**cA·1**] = 33 μM, [**cA·2**] = 77 μM, [**cA·3**] = 44 μM, [**cA·4**] = 76 μM, and [**cA·5**] = 82 μM

published procedure [1, 2] (Fig. 4; Table 1). The CD spectra of the non-cross-linked peptides **A** and **A·1**–**A·5** were also recorded to examine the effect of the cross-linker on helix stabilization in the presence of the fluorophores. The helix content of the non-cross-linked **A** was 20 %, and that of **A·1**–**A·5** was below 14 %. The cross-linked **cA** without a fluorophore folded into a stable α-helix, as reported in our previous study; its helix content was 93 % [1, 2]. Values for the fluorescent, cross-linked peptides ranged from 55 to 76 %, which were smaller than that of **cA**. The fluorophores at the N-termini of the cross-linked peptides **cA·1**–**cA·5** can be regarded as affecting the helical structures. Nevertheless, the helix content of the cross-linked peptides was much higher than that of their non-cross-linked counterparts. Comparing the CD spectra of **cA·1** and **cA·2**, we could speculate that the alkyl spacer between the N-termini and the phenylethynyl groups affect their higher-order structures. Although fluorophores should be selected with care to

Table 1 Helix content of peptides **A**–**A·5** and **cA**–**cA·5** at 5 °C

Helix content (%) of non-cross-linked peptides					
A	**A·1**	**A·2**	**A·3**	**A·4**	**A·5**
20	14	10	11	<5	14
Helix content (%) of cross-linked peptides					
cA	**cA·1**	**cA·2**	**cA·3**	**cA·4**	**cA·5**
93	76	61	64	55	56

avoid such aggregation, the high helix content observed here confirms that our fluorescent, cross-linked peptides can be potent peptide-based probes for biological analysis.

Conclusion

We have synthesized fluorescent, cross-linked peptides with high helix content. A variety of fluorophores could be easily attached to the N-termini of helical peptides by use of fluorophore-based carboxylic acids. In the future, fluorescent, cross-linked peptides that bind strongly to a specific protein overexpressed in a tumor cell are expected to be developed.

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