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Effective stabilisation of α-helical structures in short peptides with acetylenic cross-linking agents†

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The peptides cross-linked by the acetylenic cross-linking agents showed high α-helical contents, and the α-helices thus formed survived up to substantially elevated temperature.

Secondary structures in proteins play a critical role in the vital functions for all living systems. Especially, α-helices seen in proteins are critically related in most biological recognition events.[1] If we are able to regulate α-helical structures in any short peptides at will, such short peptides might be utilised as biologically active molecular segments, *e.g.* peptide drugs.[1] Although particular amino acid sequences can exist as α-helical structures in proteins, they will almost always take random-coiled structures in their "isolated" short peptide states except for the cases of several special sequences.[2,3] Therefore, many efforts for stabilising α-helical structures in short peptides have been performed.[4] Recently, Verdine *et al.* reported a utilisation of Grubbs's ring closing metathesis (RCM) for stabilising α-helical structures in short peptides to yield ring-closed peptides with high α-helical contents.[5] This method, however, required unnatural amino acids having vinyl groups in their side chains for the RCM reactions. Most other methods for forming stable helical peptides also include the laborious preparation of unnatural amino acids.[4,6] To avoid the use of unnatural amino acids, one approach is the formation of pseudocyclic peptides for bridging between two side chains of natural amino acid residues such as by disulfide bonds and by metal chelates.[7–10] Although these methods were simple and straightforward, they had some fatal problems, *i.e.*, the formation of disulfide bonds is intrinsically reversible, and the metal chelates require toxic heavy metals and/or compete with various cations existing *in vivo*. Moreover, the positions of the amino acids (Cys, His, *etc.*) participating in the bridging reactions were restricted owing to the limitation of the short bridging distances. Taking into account the above points, covalent bonds would be suitable to fix α-helical structures for exploitation in pharmaceutical applications.[11] Thus, simple methods combining native peptides with artificial external bridging agents that can be prepared by standard organic reactions are preferable. Here we report a new class of cross-linking agents **1** and **2** possessing acetylenic cores and various lengths of iterative oxyethylene spacers. Since the new cross-linking agents have the right balance of rigidity and flexibility, they may be expected to realize a new method that brings up higher stabilisation of α-helical structures in short peptides than conventional covalent-type cross-linking agents composed of only alkyl or oxyethylene skeletons.[12,13]

Acetylenic cross-linking agents **1** and **2** were prepared from the treatment of the corresponding dicarboxylic acids with *N*,*N*′-disuccinimidyl carbonate.† Short peptides (16 residues) **A** and **B** for the cross-linking reactions were prepared with a peptide synthesiser (Fig. 1). These sequences contain two Lys residues at *i* and *i*+4 positions for **A** (or *i*+7 for **B**) and one Trp at the position not interfering with the cross-linking reactions. The former are for the cross-linking between their side chain amino groups with **1** and **2**, and the latter is for the determination of the peptide concentrations by UV spectra. To demonstrate the abilities of the new agents in detail, another two series of cross-linking agents **3** and **4** whose core units are only alkyl and oxyethylene skeletons were also prepared.

A mixture of a solution of each peptide (50 μL; 1.0×10^{-4} M in phosphate buffer) with a solution of the cross-linking agent (50 μL; 5.0×10^{-4} M in DMSO) was incubated at 25 °C in a thermo-mixer. A solution of excess Lys monomer was added to the reaction mixture after 15, 30, 45, and 60 min in order to quench the reaction. The reactivities of the cross-linking agents for the peptides were assessed by reverse-phase HPLC, and the cross-linked peptides were identified with ESI-MS. In all the reactions, no cross-linked dimers of the peptides were detected.†

The acetylenic cross-linking agents **1** and **2** reacted with both the peptides in good yields (≥70%; Fig. 2) and transformed the peptides into the cross-linked states within 60 min except for the combination of **2** and **A**. In this case, the yield of the cross-linked peptide was only 38%. The order of the cross-linking yields for **A** was **1b** > **1a**≫**2**, illustrating that the number of acetylene units apparently had an influence on the yields. A comparison of the results for **1a** and **1b** suggest that the number of oxyethylene units might also improve the yield. These mean that the cross-linking efficiency will be regulated by the number of acetylene and

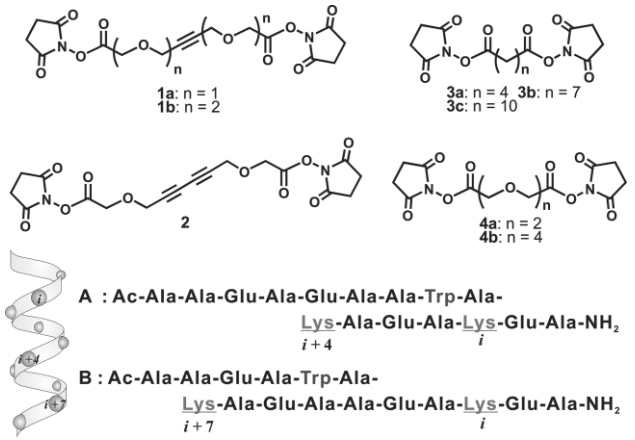

Fig. 1 Structures of the cross-linking agents (above) and the sequences of short peptides (below) in this study.

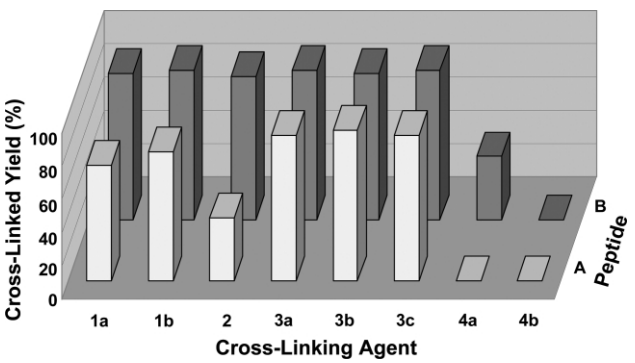

Fig. 2 The yields of the cross-linked peptides after 60 min.

† Electronic supplementary information (ESI) available: all experimental procedures, HPLC profiles, and ESI-MS spectra. See http://www.rsc.org/suppdata/cc/b4/b403615h/

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oxyethylene units in acetylenic cross-linking agents and the Lys positions in short peptides. The alkyl type **3** could bridge both the peptides well (≥88%) within 60 min regardless of the alkyl spacer lengths and the positions of the two Lys residues. On the other hand, the oxyethylene type **4** showed much lower reactivities. The shorter **4a** reacted with **B** (*i*−*i*+7) in only poor yield (38%), while both the peptides remained unchanged over 60 min in the case of **4b** from the HPLC profiles.†

Fig. 3 shows CD spectra of the cross-linked peptides thus formed at 5 °C. The α-helical contents of the peptides were evaluated on the basis of the mean residue ellipticity at 222 nm.[5,14] The cross-linked peptides by the acetylenic **1** and **2** revealed higher α-helical contents for both the peptides than those by **3** and **4**. For peptide **A** (*i*−*i*+4), the monoacetylenic **1** stabilised the α-helical structure most effectively (≥35%), whereas ~15% of the native **A** folded up under the same conditions. The distance of the rigid spacer of the diacetylenic **2** might be longer than that of the short Lys–Lys interval in **A**, which could cause the peptide cross-linked by **2** to be relatively unstable. On the other hand, the peptide **B** revealed a different but clear tendency. Especially, in the combination of the diacetylenic **2** and the peptide **B** (*i*−*i*+7), characteristic Cotton effects were remarkably strong at 191–193, 208, and 222 nm, and a higher α-helical content (~65%) was observed than any other combination. Since this value was *ca.* 1.3 times that by the monoacethylenic **1**, the number of acetylene units is considered to contribute to the stabilisation of the α-helices. Although **3** and **4** stabilised the α-helical structures of both the peptides to a certain extent, the acetylenic **1** and **2** were found to be more effective stabilisers for these short peptides.

Noteworthy is that the α-helices thus formed survived up to substantially elevated temperature. Fig. 4 depicts the thermal-profiles of α-helical contents of the cross-linked peptides. As the temperature of a solution containing the native **A** was slowly raised, the secondary structure entirely turned into the unfolded (random-coiled) state at 35 °C, near human body temperature, while the corresponding ones cross-linked by **1** and **2** maintained *ca.* ~15% of the folded structures at the same temperature. At 5 °C, the stabilising abilities of the cross-linking agents decreased in the following order: **1b** > **1a** > **2**, but the difference became small to a negligible extent as the temperature was raised. In the case of the peptide **B**, the order of the α-helical contents changed to that of **2** > **1a** > **1b** over the entire temperature range. The peptide cross-linked by **2** kept *ca.* ~30% of α-helical structures at 35 °C. Although the peptides cross-linked by **3** and **4** almost became the unfolded states at 60 °C, the one cross-linked by the diacetylenic **2** still included *ca.* 15% of the folded structure even at 70 °C. Thus, the acetylenic cross-linking agents are superior to **3** and **4** for stabilising the α-helix structures of both the peptides within a temperature range covering most natural events and artificial experiments.

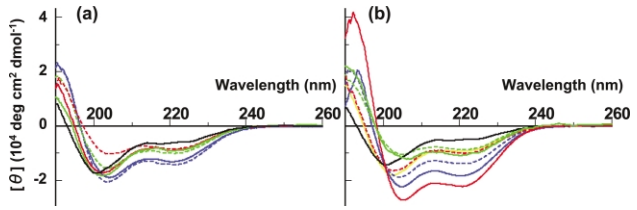

Fig. 3 CD spectra of the native and the cross-linked peptides dissolved in 2.5 × 10^{-3} M phosphate buffer (pH 7.0) at 5 °C, (a) for peptide **A** and (b) for peptide **B**: **native** (black), **1a** (blue), **1b** (blue dashed), **2** (red), **3a** (red dashed), **3b** (green), **3c** (green dashed), **4a** (yellow).

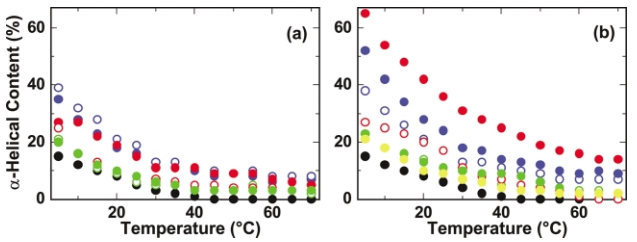

Fig. 4 Thermal-profiles of α-helical contents of the native and the cross-linked peptides at a temperature range from 5 to 70 °C, (a) for peptide **A** and (b) for peptide **B**: **native** (black closed circle), **1a** (blue closed circle), **1b** (blue open circle), **2** (red closed circle), **3a** (red open circle), **3b** (green closed circle), **3c** (green open circle), **4a** (yellow closed circle).

We have demonstrated a new class of cross-linking agents composed of actylenic cores for short peptides. The cross-linking agents can be prepared by standard organic reactions in good yields. The peptides cross-linked by the acetylenic agents showed higher α-helical contents and thermal-stabilities than conventionally cross-linked and native ones. In future investigations, these new cross-linking agents might be applied to many biological recognition events in which α-helical peptides participate.

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