# The Building of Antiparallel Four α-Helix Bundle Structure with a Pair of Pyrenylalanines on a Cyclic Pseudo-peptide Template

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The 4α-helix bundle structure bearing a pair of pyrenylalanines as a fluorescence probe was built on a cyclic pseudo-peptide template and characterized by the pyrene excimer emission.

The adequate linking of the secondary structure of polypeptide such as α-helices and β-sheets, constructs the three-dimensional structure of protein. The mimicking of such complex proteins using the *de novo* design strategy, has yet some difficulty in the modeling of the flexible turn structures. The TASP (template assembled synthetic protein) concept first proposed by Mutter *et al.*, utilizes the synthetic templates to connect peptides.[1] The folding of the high molecular weight proteins may be mimicked by using the TASP concept. However, few have succeeded in the introduction of different peptide segments into the template.[2] The interactions between the helices combined to the template have been also limittedly evaluated. Therefore, we attempted to make use of a cyclic pseudo-peptide template, which can tether two sets of peptide segments in an antiparallel mode through the amide bonds. By incorporating pyrenylalanines to one set of peptide segments as a fluorescent probe,[3] this cyclic pseudo-peptide template is demonstrated to be a promising candidate for the polypeptide *de novo* design.

The cyclic pseudo-octapeptide *cyclo*(-Lys-Abz-Api-Abz-)$_2$ (**1**, Abz; *m*-aminobenzoic acid, Api; L-α-aminopimelic acid) was synthesized as a pseudo-peptide template (Figure 1a). The cyclic structure seemed to be necessary to control the distance between the peptide segments. The introduction of *m*-aminobenzoic acid would make the ring rigid.[4] The use of Lys and Api for anchoring the peptide segments, offered long spacer units (four methylene units) between the template and the peptide segments. The protected linear pseudo-octapeptide precursor, Boc-(-Lys(Z)-Abz-Api(OBn)-Abz-)$_2$-$OCH_2COPh$, was synthesized through the repeated solution-phase couplings of the dipeptide fragments. After the deprotection of N- and C- termini, the cyclization of the linear pseudo-peptide was carried out by the addition of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate and 1-hydroxybenzotriazole in DMF for 30 min (70% yield after silica gel chromatography). FAB-MS: *m/z* 1500 ($M+H^+$). $^1$H-NMR spectra of the protected **1** and the deprotected **1** in DMSO-$d_6$ indicated that these cyclic pseudo-peptides are of $C_2$ symmetry.

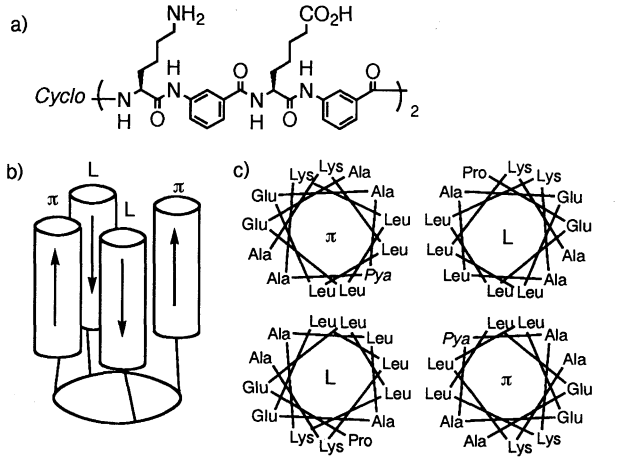


**Figure 1.** (a) Cyclic pseudo-octapeptide template, *cyclo*(-Lys-Abz-Api-Abz-)$_2$ (**1**). Abz; *m*-aminobenzoic acid, Api; L-α-aminopimelic acid. b) Side view of four α-helix bundle structure of **2** built on **1**. π-chain = -Ala-Glu-Leu-Leu-Lys-Ala-*Pya*-Ala-Glu-Leu-Leu-Lys-Ala-ethanolamide. L-chain = L-lactyl-Pro-Glu-Leu-Leu-Lys-Ala-Leu-Ala-Glu-Leu-Leu-Lys-Ala-. *Pya*; 1-pyrenylalanine. (c) Upper view of **2**.

The side-chain protecting groups of Lys(Z) and Api(OBn) were removed by hydrogenolysis, then the ε-amino groups of Lys residues were re-protected by Boc. The amphiphilic peptide segment (π- chains, Figure 1c) were synthesized by the solid-phase syntheses on Kaiser's oxime resin and the solution-phase fragment couplings.[5] The π-chain contains a 1-pyrenylalanine residue in the center of the hydrophobic region. The C-termini of the π-chains were ended as ethanolamide. The protected π-chains were condensed to the ω-carboxyl groups of the Api residues of the template with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride) and 1-benzotriazole, yielding template-(π-chain)$_2$. The Lys(Boc) protecting groups of the template-(π-chain)$_2$ were cleaved by TFA. Then, the protected L-chains, in which the N-termini were protected with Boc, were combined to the template-(π-chain)$_2$. These Boc groups of the L-chains were cleaved after connecting to the template and the N-termini were again capped with L-lactyl groups. The obtained template combined with 4α-helix peptide segments were treated with anhydrous HF and purified by size exclusion chromatography (Sephadex G-50, eluted with 40% acetic acid), in which the elution volume showed that the product was of appropriate molecular weight. The total yield of **2** was 15% based on **1**. The amino acid analysis of **2** indicated that the four peptide segments were successfully incorporated into the template: Ala (14) 14.00, Leu (18) 19.71, Glu (8), 7.34, Lys (10) 10.41, Pro (2) 1.85, Api (2) 2.04. Pya was not eluted during the standard analyses. The peptide segments (π- and L-chains) are of amphiphilic structures and thus **2** was expected to take the 4α-helix bundle structure in water, the hydrophilic residues being outside and the hydrophobic residues inside (Figure 1).

In $H_2O$ (5% 2,2,2-trifluoroethanol), **2** (30 μmol·dm$^{-3}$) showed the typical CD spectral profile of α-helix peptide (double minimum at 208 and 222 nm). The α-helix content was calculated from the [θ] value at 222 nm considering 52 amino acid residues ($[\theta]^{MRW}_{222}$ = -26700 deg·cm$^2$·dmol$^{-1}$), which corresponds to 74% α-helicity.[6] Such high α-helicity of **2** is contrast to the 43% α-helicity of the π-chain segment, which is not connected to the template. Moreover, **2** was not denaturated by the addition of 7.0 mol·dm$^{-3}$ guanidine hydrochloride, though the α-helicity of the simple π-chain segment fallen to 30% with 4.0 mol·dm$^{-3}$ guanidine hydrochloride. Thus, the peptide seg-

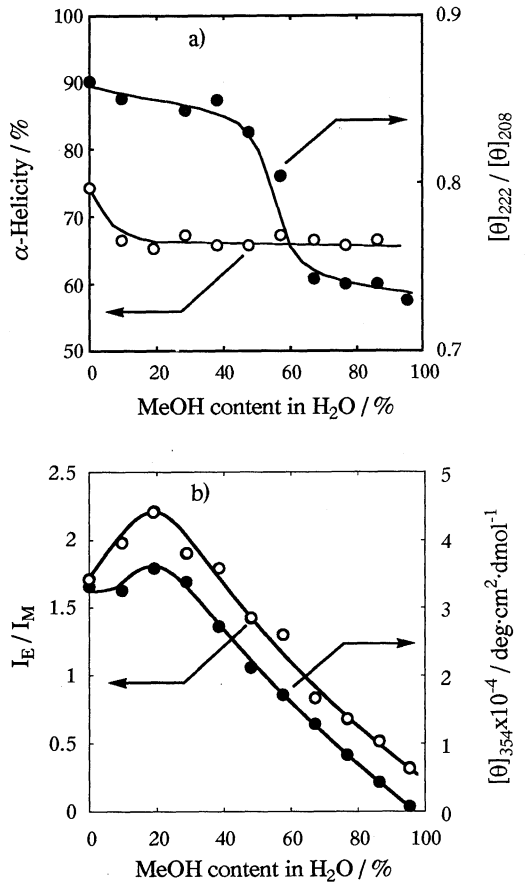


**Figure 2.** a) α-Helicity (O) and $[\theta]_{222}/[\theta]_{208}$ ratio (●) of **2** (30 μmol·dm$^{-3}$) in MeOH-$H_2O$. b) $I_E/I_M$ ratio (O) and $[\theta]_{354}$, the induced Cotton effect at the pyrene absorption region per pya residue (●).

ments of **2** were highly α-helical in $H_2O$, probably owing to the formation of the 4α-helix bundle structure through the hydrophobic interactions. The intensity of the CD band of **2** at 222 nm did not differ much by the addition of MeOH (5-95%) (Figure 2a). The α-helix contents of **2** were almost the same in $H_2O$ and in MeOH. However, $\theta_{222}/\theta_{208}$ value, which is known to indicate the compactness of the helices, decreased with the addition of MeOH.[7] The 4α-helix bundle structure of **2** formed in $H_2O$ loosened into four single α-helices with the addition of MeOH.

The formation and deformation of the 4α-helix bundle structure were unambiguously evaluated by the fluorescence spectra as follows. In $H_2O$ (5% 2,2,2-trifluoroethanol), **2** (30 μmol·dm$^{-3}$) showed the strong pyrene excimer emission at 466 nm (excited at 342 nm), suggesting that the pyrene rings on the different α-helix segments of **2** were located in close proximity.[3] This result is in consistent with the formation of the 4α-helix bundle structure through the hydrophobic interactions on the pseudo-peptide template (see Figure 1). The pyrene excimer emission ($I_E$, 466 to 476 nm depending on the solvent system) increased in its intensity with the addition of MeOH (0 to 20%), then substantially decreased by the further addition of MeOH (Figure 2b). However, the emission from the pyrene monomer ($I_M$, 397 to 401 nm) was not so much different. The addition of large amount of MeOH should weaken the hydrophobic interaction between the side chains of peptides, to form four single α-helices as described above. In such circumstances, the pyrene rings (pyrenylalanine residues) would be located distantly, and the intensity of the pyrene excimer emission decreases. The reason why pyrene excimer is formed most efficiently in 20% MeOH, but not without MeOH was yet unclear. In this connection, it is very interesting that the induced Cotton effect at 354 nm (the region of the absorption of the pyrenylalanine) was the strongest in intensity in 20% MeOH, but not without MeOH (Figure 2b). The induced CD depends on the circumstance of the pyrene rings. The pyrenylalanine residues might locate in more well-organized circumstance in 20% MeOH by some reasons. For instance, the fluidity of the peptide segments with a small amount of MeOH might be one reason.

It is noteworthy to compare the stability of the 4α-helix bundle structure of **2** and that of the single chain 53-peptide, which we have reported earlier.[3a] The 4α-helix bundle structure of the single chain 53-peptide loosened with the addition of MeOH, and no hydrophobic interaction between helices was observed in 60% MeOH by both CD and the fluorescence measurements. However, the hydrophobic interaction between helices of **2** remained even in 80% MeOH (Figure 2b). The α-helix segments of the 53-peptide were connected by the tight turn units consisting of D-Ala-Pro sequence. The flexible connection units of **2** seem to stabilize its 4α-helix bundle structure more effectively than the D-Ala-Pro turn units of the 53-peptide.

In conclusion, the 4α-helix bundle structure was built on the cyclic pseudo-peptide template in aqueous solution. The addition of MeOH loosened the bundle structure to give single α-helices, which was clearly observed by the decrease of the pyrene excimer. To our knowledge, this is the first example that the 4α-helix bundle structure on the template was observed with direct evidence involving the fluorescence incident. On the basis of this finding, syntheses of more functional TASPs using **1** are now in progress.

## References and Notes

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