A pair of pyrene groups as a conformational probe for antiparallel β-sheet structure formed in cyclic peptides

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We have developed the utility of a pair of pyrene groups on L-1-pyrenylalanines (Pya) as a conformational probe for designed peptides composed of α-helices. Here, we expand the usefulness of the probing method to the antiparallel β-sheet structure formed in cyclic peptides. A pair of Pya residues were introduced into cyclic decapeptides, gramicidin S and its analogous peptide, at various positions which were involved in antiparallel β-sheet structures with amphiphilic character. When the two Pya residues were deployed on different β-strands, exciton interaction in circular dichroism spectra showed that the two pyrene rings were arranged with a left-handed twist. They were orientated in a right-handed sense on the same strand. The pyrene rings showed strong excimer emission. These results demonstrate that the behaviour of the pyrene probe is coincident with the left-handed orientation of two β-strands and the right-handed twist of a single β-strand in natural protein structures.

Artificial proteins composed of an α-helix structure,[1–9] a β-structure,[10–16] and an α/β mixed structure[17,18] have been extensively constructed using *de novo* design. Information on 3D structures of peptides and proteins including side chains is difficult to elucidate except by detailed analyses with multidimensional NMR spectroscopy and crystallographic analysis. However, these methods are limited in studying designed peptides and proteins, because they have a molten-globule-like nature, *i.e.* stable secondary structures with a less ordered 3D structure.[1] We have therefore developed a new probing method for the detection of assembly and orientation of α-helices by employing a pair of L-1-pyrenylalanine (Pya) residues[19–22] incorporated into designed peptides.[8a,b,9a,b,10a] The method is based on split CD and excimer fluorescence emission generated from a pair of pyrene rings in close proximity. The probe could characterize the α-helix–α-helix interaction and the sense of orientation of α-helices in 2α-helix peptides[8a,b] and 4α-helix bundle peptides,[9a,b] and furthermore, the β-strand interaction in β⇆α structure transitional peptides[10a] simply by means of this CD and fluorescence spectra.

In the present study, we attempted to expand the pyrene probing method to antiparallel β-structures. Gramicidin S (GS), a cyclic decapeptide antibiotic, consists of two antiparallel β-strands connected with two type II′ β-turns.[23] The characteristic feature of the molecule is its amphiphilic deployment of hydrophilic (Orn) and hydrophobic (Leu and Val) side chains in the cyclic framework. This small cyclic peptide offers an ideal model for the investigation of the arrangement of functional side chains on the organized β-strands.[24] A pair of Pya residues were introduced at various positions in the cyclic decapeptide GS and another designed cyclic decapeptide with different amphiphilic nature (Fig. 1). In CD and fluorescence measurements, the pyrene probe sensitively responded to a left-handed orientation of two β-strands and a right-handed twist of a single β-strand. This study enhanced the usefulness of the Pya probe method utilizing the significant nature of pyrene;[25] strong fluorescence, excimer formation and strong exciton interactions.

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Results and discussion

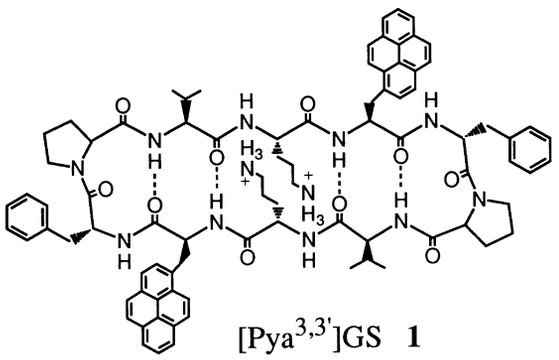
Design and synthesis

Three cyclic decapeptides containing a pair of pyrene groups were designed (Fig. 1). Two of them are *analogues* of GS, in which hydrophobic Val and Leu residues are replaced by Pya. One *analogue* contains two Pya residues on different β-strands ([Pya$^{3,3'}$]GS, **1**), whereas the other includes two Pya on the same strand ([Pya^{1,3}]GS, **2**). The third peptide **3** is designed *de novo* to position two Pya at face-to-face positions in the cyclic framework, *i.e.* hydrophilic Orn residues are placed at 1, 1′, 3 and 3′ positions instead of Val and Leu in GS, and hydrophobic Pya residues are deployed at the 2 and 2′ positions in place of Orn in GS. The β-turn moieties, D-Phe-Pro, in GS are replaced by Phe-D-Pro, which is expected to take a type II β-turn instead of a type II′ in GS. As a result, all peptides were designed to retain their amphiphilic nature when they form an antiparallel β-sheet structure connected with β-turns, which is the characteristic structure of GS. The third peptide has the opposite hydrophilic and hydrophobic character to that of GS.

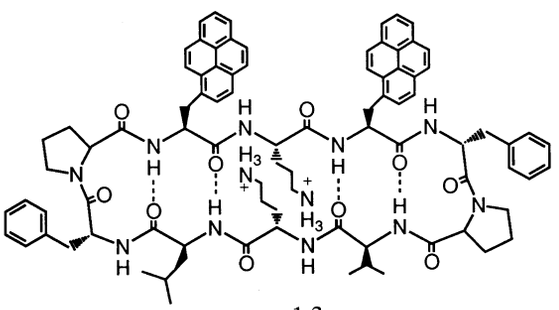
Synthesis of the cyclic peptides was carried out in the solid-phase and the subsequent cyclization–cleavage (SPS–CC) method on *p*-nitrobenzophenone oxime resin[26] as reported previously by Taylor and co-workers[27] and our groups (Fig. 2).[28] The solid-phase method allows the rapid and efficient elongation of Boc-amino acid to linear peptides. The cyclization–cleavage method gives monomer-dominant cyclic peptides in solution in high yield. The SPS-CC method has been demonstrated to be highly efficient in the synthesis of cyclic peptides. In the present studies, as shown in Fig. 2, various protected decapeptides containing Pya residues were assembled on the oxime resin by the single coupling of each Boc-amino acid using BOP−HOBt [BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole].[29] The cleavage of cyclic peptides was accomplished by the treatment of Boc deprotected linear decapeptides with dipropan-2-ylethylamine (DIEA) and AcOH in dimethylformamide (DMF). Fig. 2 also showed the time course of the cyclization reaction for **1**. The cyclization–cleavage reaction was completed within 60 min. The reaction time was much shorter than those reported for other cyclic peptides (*ca.* 10 h).[28] This is probably due to the pre-cyclized conformation of the linear decapeptide precursor forming the rigid β-turn structure. The

cyclo(-Val$^{1,1'}$-Orn$^{2,2'}$-Leu$^{3,3'}$-D-Phe$^{4,4'}$-Pro$^{5,5'}$-)$_2$

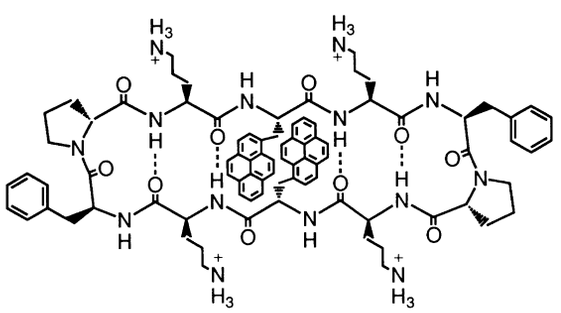
gramicidin S (GS)



[Pya$^{3,3'}$]GS **1**



[Pya^{1,3}]GS **2**



cyclo(-Orn-Pya-Orn-Phe-D-Pro-)$_2$ **3**

Fig. 1 Structure of gramicidin S (GS) and its analogues containing Pya residues **1**–**3**

yields of the crude peptides were high and the monomer : dimer ratios of the products were >90%. The monomeric cyclic decapeptides were purified by silica gel chromatography and the protection groups (Z at Orn side chains) were removed by hydrogenation. The peptides obtained gave a single peak on reversed-phase HPLC analysis, the expected molecular mass in FABMS, and satisfactory results in ^{1}H NMR measurements. The peptides showed characteristic UV–VIS, CD and fluorescence spectra corresponding to the conformation of a pair of pyrene rings.

Backbone conformations

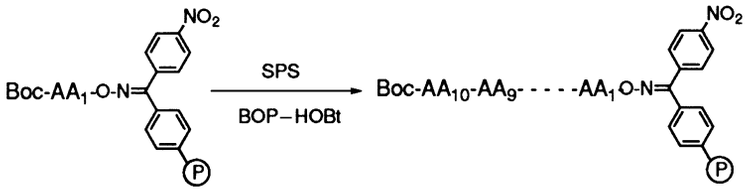
CD spectra of the cyclic decapeptides **1**–**3** in MeOH are shown in Fig. 3. In the amide region, a minimum at 210 nm and a shoulder at 215 nm observed indicated that the backbone conformations of **1**–**3** were similar to that of natural GS with an antiparallel β-sheet structure connected with a β-turn.[23] The peaks of the peptide **3** were shallower than peptides **1** and **2**, probably due to the fact that peptide **3** has a different type of β-turn and β-sheet conformation. Table 1 shows the temperature dependences of NH protons in NMR spectra of **1** and **3** in $(CD_3)_2SO$. Since the NMR spectrum of **2** was not C_2 symmetrical, the temperature coefficients could not be determined. As comparable to GS,[30] the NH protons at the 1,1′ and 3,3′ positions (Val and Leu for GS, Val and Pya for **1** and two kinds of Orn for **3**) showed smaller temperature coefficients than other NH protons. These results indicate that the four NH protons at the 1,1′ and 3,3′ positions participate in hydrogen bonds to form an antiparallel β-sheet structure similar to GS. The coefficients of hydrogen-bonded NH protons of the peptides were very much smaller than those of GS. This may be attributed to the fact that the Pya-introduced peptides have a more rigid conformation than GS.

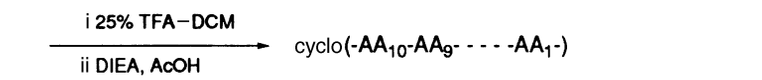
CD properties of Pya

The cyclic peptides **1**–**3** in MeOH showed CD in the pyrene absorption regions [245 (^1B_a), 280 (^1B_b), 340 (^1L_a) and 365 nm (^1L_b) regions] (Figs. 3 and 4). These results indicate that two pyrene rings on the β-strands interact with each other. [Pya$^{3,3'}$]GS **1** especially, showed a split CD at 342 nm with a negative peak at 344 nm and a positive peak at 340 nm (−/+ from longer wavelength). On the other hand, [Pya^{1,3}]GS **2** also showed the split CD at 344 nm with a different sign [346 positive and 342 nm negative (+/−)]. These findings suggest that the two pyrene rings on the different β-strands in **1** are arranged with a left-handed twist, while the two pyrenes on the same β-strand in **2** are right-handed according to the exciton chirality principle.[31] Peptide **3**, which has opposite amphiphilicity to GS and two Pya facing each other on different β-strands, showed split CD at 245, 280 and 365 nm with −/+ sign from longer wavelength, indicating that the two pyrenes are orientated in a left-handed sense. The peptides **1**, **2** and **3** showed only featureless (not split) CD in H_2O (data not shown).

In protein structures,[32] β-strands in β-sheet structures are generally arranged with a left-handed twist. Each β-strand itself tends to twist in a right-handed direction from the *N*-terminus to *C*-terminus. The orientation of pyrenes in three different peptides gave a left-handed twist between different strands (**1** and **3**), and a right-handed sense in the same strand (**2**) (Fig. 5). These facts coincide with the arrangement of β-strands in a β-sheet and the twisting conformation of a single β-strand in a protein structure. The sense of pyrene orientation reflects the arrangement and conformation of the β-strand backbone. We reported a similar result that a pair of Pya probes on a two-β-strand peptide connected in parallel on a bipyridine template showed a left-handed orientation.[10a] On the other hand, we also reported that in two- and four-α-helical polypeptides, two pyrenes orientated in a right-handed sense.[8b,9b] These were consistent with a right-handed arrangement of α-helices in proteins.

In peptides **1** and **2**, the split CD was observed in the 340 nm region (^1L_a), whereas no split but a positive CD at 280 nm (^1B_b) was seen. Neither peptides showed any significant CD at 245 (^1B_a) or 365 nm (^1L_b). Furthermore, only peptide **3** showed a split CD at 245 and 365 nm as well as a weak signal at 280 nm. The difference in the CD pattern depending on pyrene absorption bands should indicate the difference in pyrene orientation between the peptides. The induced CD of pyrene was characterized in a liquid crystal system.[33] The ^1B_b and ^1L_b transitions at 280 and 365 nm were known to be transversely polarized (short axis in-plane), while the ^1B_a and ^1L_a transitions at 240 and 365 nm are longitudinally polarized (long axis in-plane). The split CD derived from the exciton coupling may suggest a more exact orientation of pyrenes. That is, the two pyrenes in peptides **1** and **2** interacted mainly *via* the long axis direction; in peptide **3** both long and short axis interactions were observed. The exciton interaction would be influenced not only by the distance between the two pyrenes but also by the difference in the micro-orientation derived from the micro-conformation of the peptides.





	AA_{10}	AA_9	AA_8	AA_7	AA_6	AA_5	AA_4	AA_3	AA_2	AA_1
1	D-Phe	Pro	Val	Orn(Z)	Pya	D-Phe	Pro	Val	Orn(Z)	Pya
2	D-Phe	Pro	Val	Orn(Z)	Pya	D-Phe	Pro	Pya	Orn(Z)	Leu
3	Phe	D-Pro	Orn(Z)	Pya	Orn(Z)	Phe	D-Pro	Orn(Z)	Pya	Orn(Z)

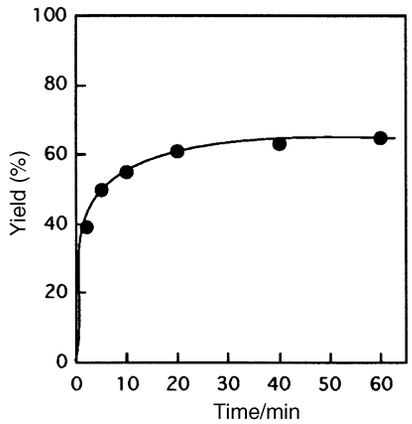


Fig. 2 Synthesis of the cyclic peptides by the solid phase synthesis and cyclization–cleavage (SPS-CC) method. The time course of the cyclization–cleavage of **1** is also shown.

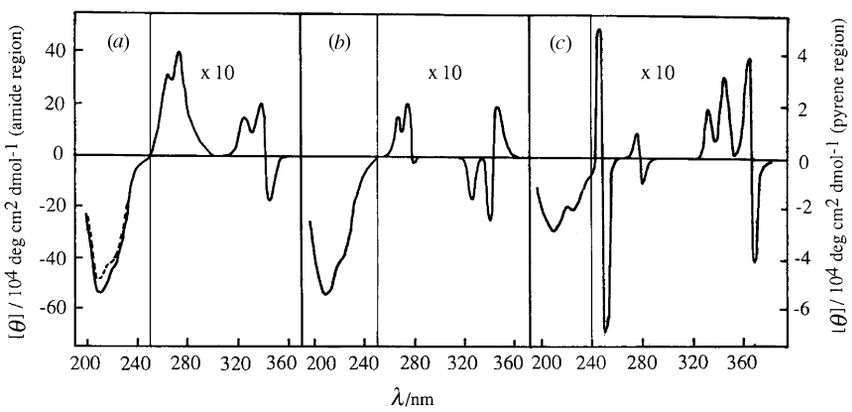


Fig. 3 CD spectra of the cyclic peptides in MeOH; (*a*) **1**, (*b*) **2** and (*c*) **3**. The broken line in (*a*) shows the CD spectrum of GS.

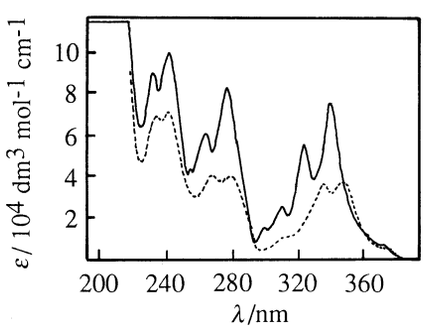


Fig. 4 Absorption spectra of the cyclic peptide **1** in MeOH (——) and H_2O (-----). Other peptides showed similar spectra (see Experimental).

Table 1 Temperature dependence of the chemical shift of NH protons observed in ^{1}H NMR spectra of the cyclic peptides[a]

	Temperature coefficient/10^{-3} ppm K^{-1}		
NH proton	**1**	**3**	**GS**
Val	−0.67		−2.2
Orn	−4.2	−0.25	−5.4
		−1.50	
Leu			−3.3
D-Phe	−3.3		−8.1
L-Phe		−6.25	
Pya	−0.67	−3.50	

[a] The concentration of peptides, 5 mmol dm^{-3}; in $(CD_3)_2SO$; 25 °C.

Fluorescence properties of Pya

In the fluorescence spectra of peptides **1**–**3** (Fig. 6), strong excimer emission of pyrene at 465–470 nm as well as weak monomer emission in H_2O was observed, indicating that two pyrene rings are stacked in water to form the excimer. This was supported by the large hypochromism (ε 38 000 dm^3 mol^{-1} cm^{-1} at 348 nm), red-shift (*ca.* 5 nm) and broadening of the pyrene absorption spectra in water as compared with that in MeOH (ε 75 000 dm^3 mol^{-1} cm^{-1} at 342 nm) (Fig. 4). This interaction observed in UV spectra suggests that the two pyrenes can form a dimer in the ground state.[25] The ratios of the excimer (I_E) to the monomer emission (I_M) were 1.6, 2.8 and 3.0 for **1**, **2** and **3**, respectively. The slightly weaker excimer formation of **1** may be due to the fact that the two pyrenes between 3 and 3′ protons are more separated (distances between α-carbons are *ca.* 9, 7 and 6 Å, respectively, for **1**, **2** and **3**). The excimer intensities were proportional to the distances. On the other hand, the excimer emission was decreased linearly by the addition of MeOH. Two pyrene rings on the β-strands tend to move more freely on addition of MeOH and have less chance of stacking. The ratios (I_E/I_M) were 0.7, 0.5 and 0.6 for **1**, **2** and **3**, respectively, in MeOH. The weak excimer intensities were similar to each other. Note that the pyrene excimer was almost completely diminished in MeOH in the case of α-helical peptides[8b,9b] and the two-β-strand peptide[10a] due to the complete separation of segments. However, in the case of cyclic peptides, although the pyrenes are free to move on the cyclic scaffold, the distances are still restricted by the cyclic structure, resulting in weak excimer formation even in MeOH.

Antimicrobial activity

Antimicrobial activity of the peptides against *Bacillus subtilis*,

Staphylococcus aureus and *Escherichia coli* was examined by the standard dilution method in solution media.[34] The Pya-containing peptides did not show antimicrobial activity up to 100 μg ml^{-1}, while GS showed 3.13, 3.13 and 12.5 μg ml^{-1}, respectively, as minimum inhibitory concentrations.

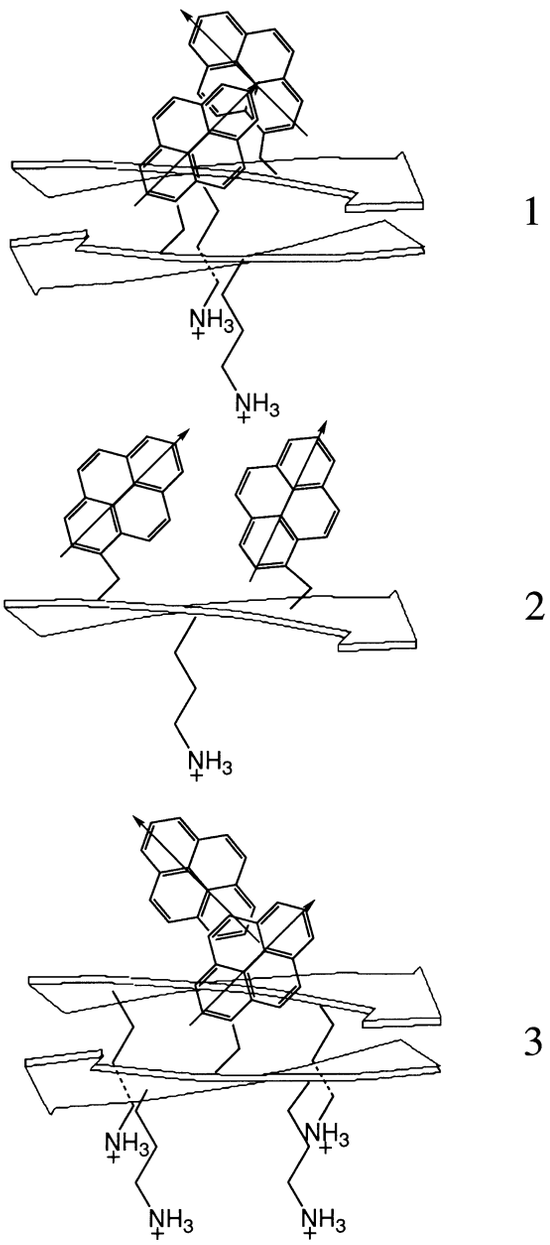


Fig. 5 Illustration of β-sheet conformation and orientation of Pya residues; (top and bottom) a left-handed orientation of β-sheet and Pya residues in **1** and **3**; (middle) a right-handed twist of a single β-strand and the same orientation of Pya residues in **2**

Conclusions

The present study demonstrates that pyrene chromophores could be arranged on the antiparallel β-strands of the cyclic peptides and could probe the orientation of the two β-strands and the twisted conformation of the β-strand. In conjunction with previous studies, it was generally demonstrated that arrangement of the fluorogenic chromophores could be regulated by polypeptide tertiary structures such as β-sheet in antiparallel and parallel orientations[10a] as well as α-helix bundle structures.[8b,9b] Porphyrins,[1,3,8c,e,f] flavin,[8f,9d] metal complexes[3,7,8d] have been deployed in 3D structures of α-helix polypeptides by *de novo* design. Construction of the 3D structure of polypeptides by combination of α-helices and β-strands may achieve the arrangement of functional chromophoric side chains to afford artificial proteins such as polypeptide electronic devices—'polypeptide architecture'.

Experimental

Materials and methods

Amino acid derivatives and reagents for peptide synthesis were purchased from Watanabe Chemical Co. (Hiroshima, Japan). Boc-L-1-pyrenylalanine was synthesized according to the method reported previously.[9b] *p*-Nitrobenzophenone oxime resin was prepared according to the reported method.[28] Solid-phase peptide synthesis was carried out manually in a glass vessel. Fast atom bombardment mass spectra (FABMS) were recorded on a JEOL JMS-DX-300 mass spectrometer using *threo*-1,4-dimercaptobutane-2,3-diol as a matrix and xenon for bombardment. NMR spectra were measured on a JEOL GX-400 spectrometer in $(CD_3)_2SO$ at various temperatures. Amino acid analyses were carried out on a JEOL JLC-300 system with ninhydrin detection after hydrolysis in 6 mol dm^{-3} HCl at 110 °C for 24 h in a sealed tube. HPLC was carried out on a MS-GEL C4 column (Asahi Glass, Co. Ltd., Tokyo) (4.6 × 150 nm or 10 × 250 nm) employing a Hitachi L-6200 HPLC System.

Peptide synthesis

General method for protected cyclic peptides. The synthesis of cyclic peptides was conveniently accomplished by a recently developed solid phase synthesis and cyclization–cleavage (SPS-CC) method.[27,28] The linear decapeptides were synthesized by stepwise elongation of Boc-amino acids on a *p*-nitrobenzophenone oxime resin[26] according to the reported procedure[8b] using BOP[29] and HOBt. The protected cyclic decapeptides were cleaved from the oxime resin as follows. The Boc group of Boc-decapeptide-resin was removed with 25% trifluoroacetic acid (TFA) in dichloromethane for 30 min. The resin was washed with CH_2Cl_2 (×2), propan-2-ol (×1) and CH_2Cl_2 (×3). Then the resin was treated with a mixture of AcOH (2.0 equiv.) and DIEA (2.0 equiv.) in DMF at room temp. for 3 h. The mixture was filtered and the resin was washed twice with DMF. The combined solution was evaporated and the crude product was solidified by addition of water. The crude protected peptides

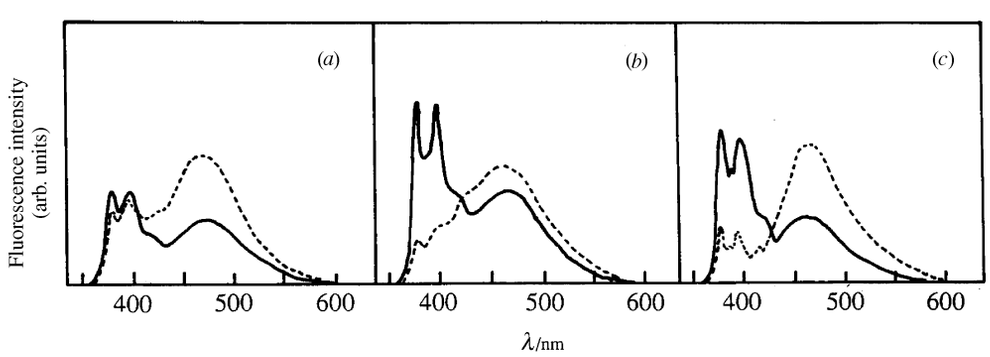


Fig. 6 Fluorescence spectra of the cyclic peptides in MeOH (——) and H_2O (-----); **1** (*a*), **2** (*b*) and **3** (*c*)

were purified by silica gel chromatography ($CHCl_3$–MeOH 97:3 as eluent). The purity was checked as >95% by HPLC (C4 column with a linear gradient of 60–100% CH_3CN–0.1% CF_3CO_2H over 30 min).

cyclo(-Val-Orn-Pya-D-Phe-Pro-)$_2$·2HCl (1). Starting from Boc-Pya-oxime resin (1.0 g, 0.5 mmol), cyclo[-Val-Orn(Z)-Pya-D-Phe-Pro-]$_2$ was obtained as the general procedure described above; 580 mg (66%). The peptide was hydrogenated overnight in the presence of 5% Pd/C in AcOH–EtOH–H_2O (5:1:1, 30 cm^3). After removal of the catalyst, the solution was evaporated and the residues were dissolved in MeOH. An aliquot of 2% HCl was added to the solution and the solution was evaporated to give the cyclic decapeptide hydrochloride. The product was solidified with diethyl ether; 240 mg (32% from the first amino acid); mp 284 °C (decomp.); $[\alpha]_D^{25}$ −95.6 (*c* 0.2, MeOH); FAB-MS *m*/*z* 1458 [(M + H)$^+$]; λ_{max}(H_2O)/nm 348 (ε/dm^3 mol^{-1} cm^{-1} 38 400), 332 (38 000), 279 (39 800), 268 (40 100) and 244 (71 000); λ_{max}(MeOH)/nm 342 (75 200), 326 (53 100), 312 (27 000), 276 (82 000), 264 (59 000), 242 (101 000) and 234 (89 000).

cyclo(-Pya-Orn-Pya-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-)·2HCl (2). Starting from Boc-Leu-oxime resin (1.0 g, 0.5 mmol), *cyclo*[-Pya-Orn(Z)-Pya-D-Phe-Pro-Val-Orn(Z)-Leu-D-Phe-Pro] was obtained as above; 650 mg (75%). The peptide was hydrogenated and worked up by the same procedure as described for **1**; 290 mg (38% from the first amino acid); mp 283 °C (decomp.); $[\alpha]_D^{25}$ −140 (*c* 0.2, MeOH); FABMS *m*/*z* 1470 [(M + H)$^+$]; λ_{max}(H_2O)/nm 347 (ε/dm^3 mol^{-1} cm^{-1} 39 200), 331 (40 700), 278 (45 600), 268 (41 000) and 244 (82 000); λ_{max}(MeOH)/nm 342 (72 600), 326 (56 500), 313 (25 800), 276 (86 300), 265 (57 000), 242 (106 700) and 233 (98 100).

cyclo(-Orn-Pya-Orn-Phe-D-Pro-)$_2$·4HCl (3). Starting from Boc-Orn(Z)-oxime resin (1.0 g, 0.5 mmol), cyclo[-Orn(Z)-Pya-Orn(Z)-Phe-D-Pro-]$_2$ was obtained; 560 mg (55%). The peptide was hydrogenated and worked up by the same procedure as described for **1**; 200 mg (25% from the first amino acid); mp 255 °C (decomp.); $[\alpha]_D^{25}$ +45.7 (*c* 1.0, MeOH); FABMS *m*/*z* 1488 [(M + H)$^+$]; λ_{max}(H_2O)/nm 348 (ε/dm^3 mol^{-1} cm^{-1} 37 900), 334 (38 200), 280 (41 000), 269 (38 900) and 244 (73 000); λ_{max}(MeOH)/nm 342 (71 500), 326 (52 200), 313 (26 500), 276 (81 900), 264 (60 100), 242 (103 000) and 233 (88 900).

CD measurements

CD spectra were recorded on a JASCO 500A spectropolarimeter equipped with Taiyo thermo supplier EZ-100 using a quartz cell with 1 mm path length. Peptides were dissolved in H_2O or MeOH–H_2O in peptide concentration of 3.0–5.0 × 10^{-5} mol dm^{-3}.

Fluorescence measurements

Fluorescence spectra were run on a Hitachi 605-10S fluorescence spectrophotometer at 25 °C. Peptides were dissolved in H_2O or MeOH–H_2O at a peptide concentration of 3.0–5.0 × 10^{-6} mol dm^{-3}.

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