A pair of pyrene groups as a conformational probe for designed four-α-helix bundle polypeptides

Hisakazu Mihara,*† Yuji Tanaka, Tsutomu Fujimoto and Norikazu Nishino *
Department of Applied Chemistry, Faculty of Engineering, Kyushu Institute of Technology, Tobata, Kitakyushu 804, Japan

A 53-residue polypeptide designed to adopt a four-α-helix bundle structure has been synthesized. To probe the bundle structure, a pair of L-pyren-1-ylalanine (Pya) residues were introduced into two α-helix segments (the first and third α-helix segments or the second and third segments, respectively) in the 53-peptide. The peptides showed induced CD in the pyrene absorption region associated with the α-helical CD corresponding to the polypeptide main chain. The probe showed strong excimer emission in the fluorescence spectra, indicating the proximal orientation of the pyrene groups consistent with the formation of the bundle structure. Furthermore, both polypeptides showed similar CD spectra which were split at 350 nm (a positive peak at longer wavelength and a negative peak at shorter wavelength), suggesting that both pairs of pyrene rings on helical rods in parallel and antiparallel orientations are arranged in a right-handed sense in the bundle structure. Moreover, the pyrene excimer energy was efficiently transferred to dodecyl acridine orange, the efficiency of the transfer being due to the trapping of the dodecyl group within the polypeptide bundle.

Artificial proteins with α-helix bundle structures have been designed *de novo* by assembling secondary structures with single-chain polypeptides and with short peptides using templates or metal ions.[1-10] Attempts were made to characterize some of the structures by two-dimensional NMR spectroscopy,[1d,e] crystallographic analysis[1f] and fluorescence measurement.[1e,g,6,8b] We have developed a new method for probing the assembly and orientation of α-helices employing pyrene rings of L-pyren-1-ylalanine (Pya)[11-14] incorporated into designed peptides.[8a,9a,b,10] The method is based on the split CD and excimer fluorescence emission generated from a pair of pyrene rings in close proximity. The probe is able to characterize α-helix–α-helix interactions and the sense of orientation of α-helices in a 2α-helix peptide[9a,b] and a 4α-helix bundle peptide.[8a] Furthermore, β⇄α structural transition[10] can be determined by means of simple measurement of CD and fluorescence spectra.

Although the pyrene group is rather bulky (pyrene is *ca.* 0.9 nm long; for comparison, indole of Trp is *ca.* 0.6 nm long), it has the great advantages of strong fluorescence, excimer formation capability and an extended π-system with which to detect interchromophore interaction. Such pyrene functions have been utilized as a probe for the evaluation of membrane fluidity and polymer conformation.[15-17] Detection of the pyrene excimer has enabled the elucidation of the phase-transition of membranes and the thermal transition of polymer conformations. In particular, Sisido *et al.* have utilized Pya as a chromophoric amino acid in poly(amino acids) to design molecular electronic devices with a chromophoric array along an α-helix.[12] They applied the techniques of regular and fluorescence-detected CD to the evaluation of pyrene geometry on the α-helical structure of poly(Pya). Goedeweeck *et al.* also employed Pya as a tool for a molecular dynamics study of dipeptides by intramolecular pyrene excimer formation.[13] Measurements of pyrene fluorescence and conformational calculation of diastereoisomers of Ac-DL-Pya-DL-Pya-OMe dipeptide enabled the population of the extended and folded conformations of the dipeptides to be determined. Furthermore,

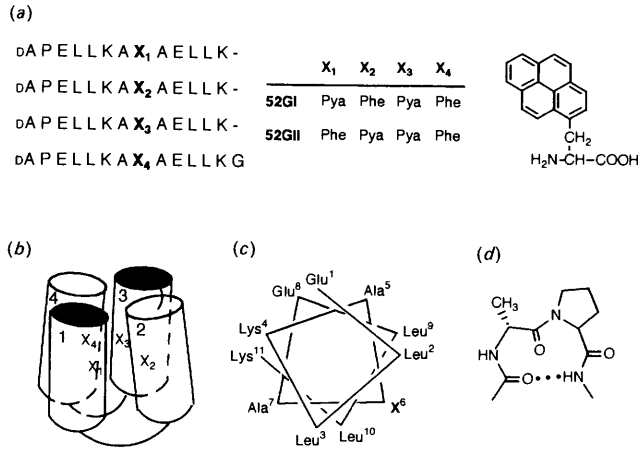

Fig. 1 Structure of the four-α-helix bundle peptides, 52GI and 52GII: (*a*) amino acid sequences of the 53-peptides and structure of pyren-1-ylalanine; (*b*) illustration of the four-α-helix bundle structure; (*c*) α-helix wheel drawing of the 11-peptide segment; (*d*) illustration of the β-turn structure of D-Ala-Pro

Pya residues in hormonal,[14a] antimicrobial[14b] and amphiphilic α-helical[14c] peptides have been utilized as probes for the detection of interactions between peptides and phospholipid membranes. Thus, pyrene is a valuable tool for use in the study of peptide–macromolecular interactions.

Therefore, we have utilized the pyrene probe as an artificial amino acid Pya for the detection of interaction between α-helix segments and conformational changes of polypeptides consisting of four α-helices (Fig. 1). That is, when two pyrene groups are in close proximity in the conformation, the pyrene groups of Pya residues will show an exciton interaction in CD and an excimer emission in fluorescence which may provide information about the arrangement of the α-helix segments in the 3D structure. In a previous study, the conformation of a 4α-helix peptide containing four Pya residues in each α-helix segment was characterized by this principle.[8a] However, the pyrene–pyrene interaction could not define the helix–helix interaction, because the four segments were equivalent. Therefore, in the present study, we introduced a pair of Pya residues into the

† *Present address:* Department of Bioengineering, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta, Yokohama 226, Japan. *E-mail:* hmihara@bio.titech.ac.jp.

same 53-peptide at the two α-helices in parallel or antiparallel orientations to determine the helix–helix interaction in more detail (Fig. 1). Furthermore, we found that the fluorescent energy of the pyrene excimer formed by the bundle structure was transferred to a dye, dodecylacridine orange, through the binding of the alkyl chain to the hydrophobic interior of the bundle.

Results and discussion

Design and synthesis

To design the single-chain 53-peptide which was to form a 4α bundle structure, a short 11-peptide was designed containing an amphiphilic α-helical structure[18] with three α-helical turns (Fig. 1). Four Leu and two pairs of Glu and Lys were incorporated such that side-chain salt bridges[19] could be adopted on the helix or between helices. These hydrophobic and hydrophilic groups are separately deployed on the helix rod [Fig. 1(*c*)]. The probe Pya is placed at the centre of the unit segments, *i.e.*, the first and third segments for parallel α-helices (52GI) and the second and third for antiparallel α-helices (52GII) [Figs. 1(*a*) and (*b*)]. D-Ala-Pro was attached to the 11-peptide to make a 13-peptide. This dipeptide sequence between two α-helix segments should terminate the helix by adopting a type II′ β-turn conformation [Fig. 1(*d*)].[20] The 13-peptide was then repeatedly connected starting with a *C*-terminal Gly to give 52G, which was expected to fold into a 4α-helix bundle conformation[8] as illustrated in Fig. 1(*b*).

The synthesis of the peptides was carried out by the convergent method with solid-phase synthesis (SPS) of protected peptide segments on Kaiser's oxime resin[21] and segment condensations of the protected segments in solution.[8,9b] The hexapeptide Boc-D-Ala-Pro-Glu(OcHex)-Leu-Leu-Lys(ClZ)-OH and the heptapeptide Boc-Ala-X-Ala-Glu(OcHex)-Leu-Leu-Lys(ClZ)-OPip (X, Pya or Phe) [cHex, cyclohexyl; ClZ, 2-chlorobenzyloxycarbonyl; Pip, piperidine] were synthesized by the stepwise SPS on the oxime resin. The Boc-6-peptide-OH was coupled with the Boc-removed H-7-peptide-OPip in dimethylformamide (DMF). The Boc-13-peptide-OPip obtained was subsequently reduced with sodium dithionite to give Boc-13-peptide-OH as the protected intermediates for the first, second and third segments. Boc-13G-OBzl (Bzl, benzyl) was similarly synthesized with Boc-6-peptide-OH and H-7G-OBzl. The Boc-13-peptide-OH was condensed consecutively three times with H-13G-OBzl to give the Boc-52G-OBzl. The intermediates, protected 27-, 40- and 53-peptides, were purified with Sephadex LH-60 column (DMF) and could be separated according to the expected molecular weights. The protecting groups were removed with anhydrous HF. The crude 53-peptides were purified with reversed-phase HPLC to give the pure 53-peptides (>95% purity). The 53-peptides showed satisfactory results on amino acid analysis based on Gly. Gel-permeation chromatography of the 53-peptides with Sephadex G-50 (2.0×10^{-2} mol dm^{-3} TrisHCl, pH 7.4) gave an apparent molecular weight of 6000 (calc. 6022), indicating that the peptides assume a monomeric form in solution.

CD measurements

The 53-peptides 52GI and 52GII showed a typical α-helical CD pattern in buffer with ellipticities $[\theta] = -19\,000$ and $-20\,000$ deg cm^2 dmol^{-1} at 222 nm, respectively (60 and 63% α-helcity[22]) (Fig. 2). Since the β-turn requires four amino acids and the remaining nine residues are allowed to take the α-helix conformation, this α-helix content indicates that approximately 90% of the four 9-peptide segments assume the α-helix structure as expected in the design. This calculation suggests that the conformation of 52G consists of almost perfect α-helices and bridging β-turn moieties. The hydrophobic interaction between

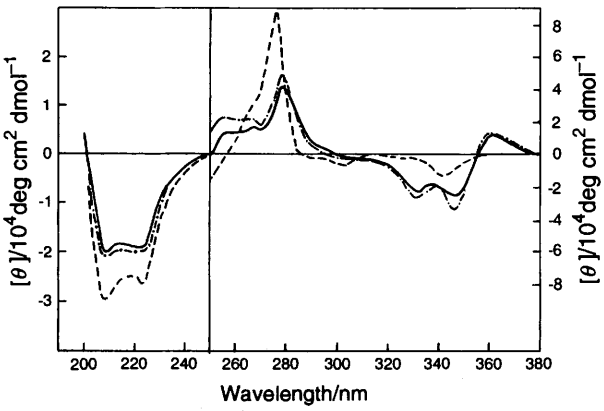

Fig. 2 CD spectra of 52GI and 52GII in 2.0×10^{-2} mol dm^{-3} TrisHCl buffer, pH 7.4 and in MeOH. 52GI in buffer (——), 52GI in MeOH (– – – –) and 52GII in buffer (– · – · – · –); $[\theta]$ in the amide region is the mean residual weight ellipticity, and $[\theta]$ in the pyrene absorption region is the molar ellipticity for the peptide; [peptide] = 3.0×10^{-5} mol dm^{-3}, 25 °C

the amphiphilic α-helix segments stabilized the whole conformation probably by folding into the 4α-helix bundle structure.[1] The 53-peptides did not show significant changes in α-helicity with the peptide concentration ranging from 0.5 to 5.0×10^{-5} mol dm^{-3}. On the other hand, the monomeric 14-peptide (13G) containing a Pya residue showed poor α-helicites, 25% at 1.4×10^{-5} mol dm^{-3} and 39% at 3.0×10^{-5} mol dm^{-3}, respectively, in aqueous solution. The observed concentration dependence of the α-helicity is probably due to the aggregation of the peptide. Pya did not affect the CD spectra in the amide region, though it showed weak Cotton effects around 250 nm.[9a,b,12]

The bundle structure of 52G was further confirmed by CD in the pyrene absorption regions (250 nm at ^1L_a band and 280 nm at ^1B_b band) (Fig. 2). Significant CD observed in the pyrene region indicates that pyrene rings on the α-helix segments are arranged in close proximity in the bundle structure. It is important to note that the split CD spectra at 350 nm with a positive peak at longer wavelength and a negative peak at shorter wavelength were observed both for 52GI and 52GII. This fact indicates that two pyrene groups on different α-helical rods are arranged in a right-handed sense with each other according to the exciton chirality method of Harada and Nakanishi.[23] Moreover, the sense could not be distinguished by the pyrene rings on α-helical rods in parallel and antiparallel orientations. From these results, it could be assumed that the four α-helix segments are similarly arranged in a right-handed sense relative to each other or that the segments on average are oriented in the same sense within mixed conformations like a molten globule.[1a,e] As compared with the previous reports with 2α-helix peptides containing Pya residues,[9a,b] the intensity of the split CD at 350 nm region was much smaller (1/10) in the 4α-helix peptides than that in the 2α-helix peptides ($\Delta[\theta] = 4.0 \times 10^5$ deg cm^2 dmol^{-1}). This fact suggests that the side chains in the 4α-bundle peptides are more mobile than those in the 2α-helix peptides, that is, the 4α-helix peptides are more likely to be in a molten globule, probably because the 4α-helix bundle structure can be considered to have clockwise or counterclockwise topology.[1a] As a comparison, the 53-peptide containing four Pya residues showed a similar split CD in aqueous solution with intensity $\Delta[\theta] = 9.6 \times 10^4$ deg cm^2 dmol^{-1},[8a] which was a reasonable value for four pyrene groups.

On the other hand, the CD particularly in the ^1L_a band region (350 nm) was drastically diminished by the addition of MeOH (Figs. 2 and 3). MeOH presumably causes the 4α bundle

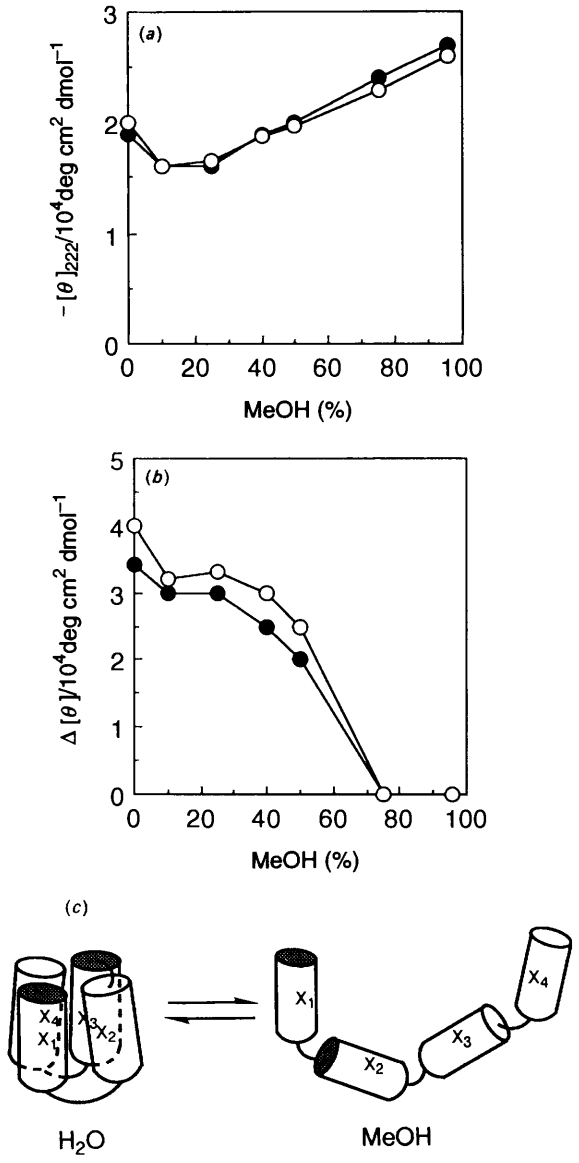

Fig. 3 Dependences of CD spectra of 52GI (●) and 52GII (○) on MeOH content: (*a*) changes of the ellipticity at 222 nm; (*b*) changes in the CD split at 350 nm; $\Delta[\theta]$ denotes the difference of ellipticities between the positive peak (362 nm) and negative peak (346 nm); [peptide] = 3.0×10^{-5} mol dm^{-3}, 25 °C; (*c*) illustrations of peptide conformations in buffer and MeOH

peptide to melt the amphiphilic folding. Up to 30% MeOH, the split CD was not decreased so much. Further addition of MeOH diminished the intensity and finally the CD disappeared at 75% MeOH [Fig. 3(*b*)]. Conversely, the α-helicity was slightly decreased around 20% MeOH, and then it was increased to 85% in MeOH ($[\theta] = -26\,000$ deg cm^2 dmol^{-1}) [Fig. 3(*a*)]. These results indicate that the α-helix structure is temporarily decreased by the weakened hydrophobic interaction around 20% MeOH, but each α-helix segment is stabilized by the effect of further added MeOH. In MeOH there could not be any defined 3D structure [Fig. 3(*c*)], because the hydrophobic interaction is cancelled in this solvent. This assumption was supported by the disappearance of the exciton interaction in the pyrene region. The profiles of the CD changes observed for peptides 52GI and 52GII by the addition of MeOH were not significantly different from each other. This also suggests that the bundle structure is loose enough to exist as a molten globule.

Fluorescent properties

In the fluorescence spectra of 53-peptides in buffer, highly intense excimer emission was observed [Fig. 4(*a*)], the ratio of the intensity of the excimer emission at 470 nm (I_E) to that of the monomer at 400 nm (I_M) being 1.3 and 1.7, respectively, for 52GI and 52GII at 3.0×10^{-5} mol dm^{-3} [Fig. 4(*b*)]. For the 53-peptides there was no observed dependence of the ratio I_E/I_M on concentration ($3.0–30 \times 10^{-6}$ mol dm^{-3}). The monomeric 14-peptide (13G) in aqueous solution, however, showed monomer emission only at 3.0×10^{-6} mol dm^{-3} ($I_E/I_M = 0$), but significant excimer emission at 3.0×10^{-5} mol dm^{-3} ($I_E/I_M = 1.3$). The excimer formation directly demonstrates that the 53-peptides fold into the compact bundle conformation which allows the pyrene rings to be in close proximity in aqueous solution. The 14-peptide assumes a similar bundle structure by intermolecular assembly at higher concentration, but is in monomeric form at lower concentration. Two pyrenes in 52GII, in which Pya-containing α-helices are in an antiparallel orientation, appeared to be better stacked than those in 52GI, in which the orientation is parallel, resulting in slightly higher chance of excimer formation in 52GII. This might correlate to the slightly higher α-helicity of 52GII and the larger split CD ($\Delta[\theta]$) in aqueous solution. The addition of MeOH significantly decreased the excimer intensity as shown in Fig. 4(*b*). With higher MeOH content (75%), the bundle conformation was completely destroyed, and the resulting undefined conformation gave no excimer emission. The results obtained by the fluorescence measurements were well correlated with those from CD studies.

Denaturation with guanidine hydrochloride

The unfolding experiment with guanidine hydrochloride (GuHCl) was carried out to investigate the stability of 52GI (Fig. 5). The process was followed by CD and fluorescence spectroscopy. The peptide 52GI retained its conformation up to 3.0 mol dm^{-3} of GuHCl, then gradually unfolded on further addition of GuHCl, and finally was almost in random structure at 7.5 mol dm^{-3}. The concentration of GuHCl at the midpoint of the transition was 5.0 mol dm^{-3}, indicating that the α-helical structure was rather stable as reported for other reported designed proteins.[1,8] With increasing GuHCl concentration, the I_E/I_M value in the fluorescence spectrum gradually decreased up to 2 mol dm^{-3}, but decreased more steeply at higher concentration. At higher GuHCl concentration (> 3 mol dm^{-3}), the decrease in the I_E/I_M value was almost parallel with the decrease in ellipticity at 222 nm. The former fact indicates that environments around side chains are more easily disrupted by the denaturant than is the secondary structure, that is, the 3D structure starts to be denatured faster than the secondary structure. The latter demonstrates that pyrene excimer formation strictly requires the α-helix conformation. The random structure does not allow the pyrene groups to aggregate in excimer proximity.

Temperature dependence

Since the conformation of proteins depends on temperature, the thermal stability of the α-helix conformation was examined by CD measurements. Judging from Fig. 6, the main-chain α-helix ($[\theta]_{222}$) started to unfold from 30 °C. The CD due to the side chain of pyrene ($\Delta[\theta]$) was diminished almost parallel to the main chain denaturation over 30 °C, but decreased gradually under 30 °C. These results demonstrate that a pair of Pya residues can probe the unfolding of the 4α-helix bundle structure and support the finding obtained by fluorescent measurements with the denaturant that the environments around side chains are more easily disrupted than is the secondary structure. The fluorescence experiments could not be used for the evaluation of the thermal stability, because the fluorescent intensities are strongly influenced by temperature.

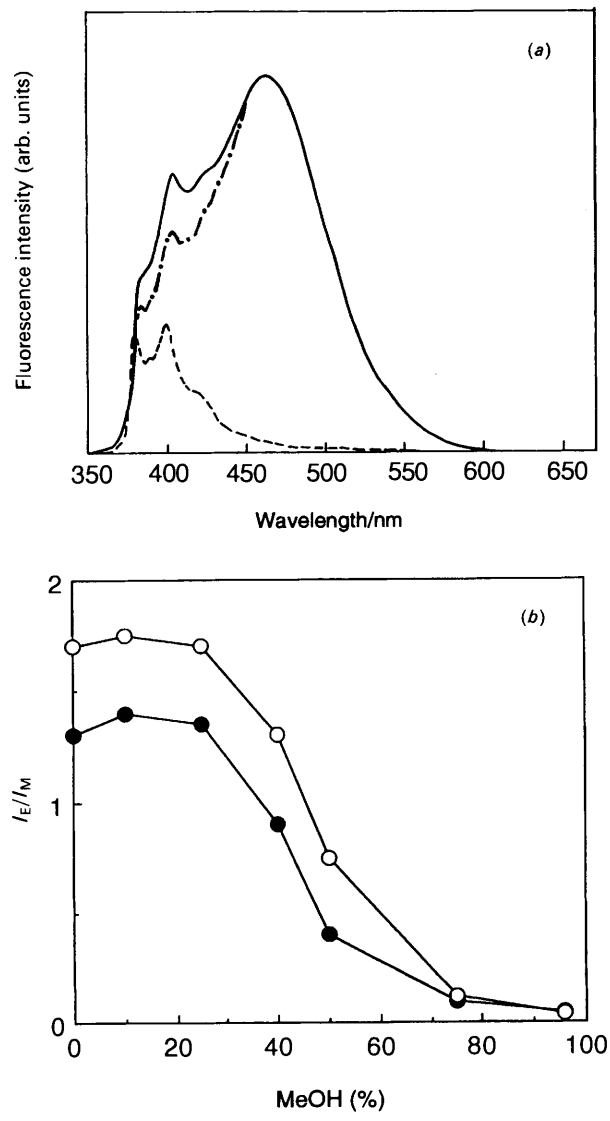

Fig. 4 Fluorescence spectra of 52GI and 52GII in 2.0×10^{-2} mol dm^{-3} TrisHCl, pH 7.4 and in MeOH: (*a*) 52GI in buffer (——), 52GI in MeOH (– – – –) and 52GII in buffer (– · – · –); [peptide] = 3.0×10^{-5} mol dm^{-3}; excited at 345 nm at 25 °C; (*b*) changes on the ratio I_E/I_M of 52GI (●) and 52GII (○) by the addition of MeOH; I_E/I_M denotes the ratio of fluorescence intensities at 470 nm to 400 nm

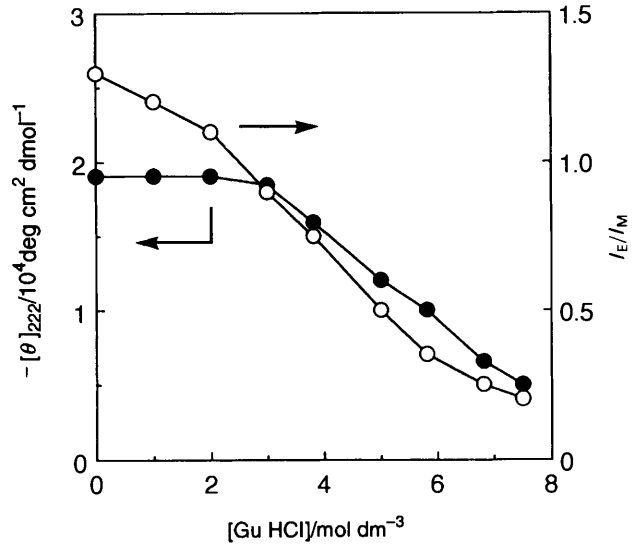

Fig. 5 Dependences of CD and fluorescence spectra of 52GI on the addition of guanidine hydrochloride; ellipticity at 222 nm (●) and the ratio I_E/I_M (○); [peptide] = 3.0×10^{-5} mol dm^{-3}, 25 °C

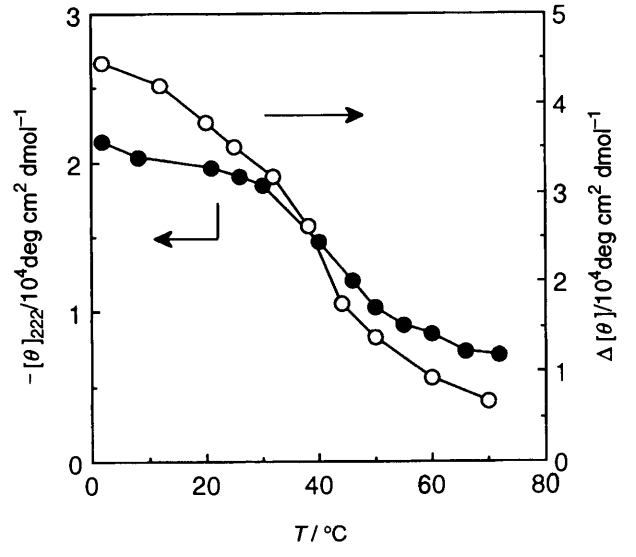

Fig. 6 Effects of temperature on the CD spectra of 52GI; ellipticity at 222 nm (●) and the $\Delta[\theta]$ value (○); [peptide] = 3.0×10^{-5} mol dm^{-3}

Energy transfer of pyrene excimer to dodecyl acridine orange

To characterize further the bundle structure of the 53-peptides, dodecylacridine orange (DAO) was added to the aqueous solution of 52GI.[6,24] When the pyrene was excited at 345 nm, the pyrene fluorescence (monomer and excimer) almost disappeared and strong fluorescence of DAO was observed at 530 nm (Fig. 7). The fluorescence energy of pyrene excimer was almost completely transferred to DAO, because DAO has the broad absorption band around 480 nm. When the fluorescence intensity of DAO was measured at various concentrations of DAO, the intensity increased along with the concentration (data not shown). The apparent binding constant was 1.5×10^5 mol^{-1} dm^3. It is interesting to note that the addition of methylacridine orange (MAO) resulted in less effective energy transfer (Fig. 7). These results suggest that the long alkyl chain in DAO might be trapped within the inner hydrophobic space of the bundle structure and the acridine moiety would be oriented in a close position to the pyrenes, resulting in the highly efficient energy transfer. On the other hand, MAO, with no long alkyl chain, cannot stay around the peptide, therefore, the energy transfer is not significant.

It is further noteworthy that the excimer emission of the monomeric 13G at 3.0×10^{-5} mol dm^{-3} was also quenched by the addition of DAO, whereas emission of DAO fluorescence was not observed (data not shown). Therefore, the compact folding of 52GI in the 4α-helix bundle structure is essential for efficient energy transfer. As expected, the addition of MeOH to the 52GI-DAO mixture resulted in a gradual decrease of energy transfer (Fig. 7). This is attributed to the prevention of excimer formation by the addition of MeOH. The incidence of energy transfer could be regulated by the polypeptide conformation under various circumstances.

Conclusions

The pseudoprotein designed to form a four-α-helix bundle structure was synthesized. The conformation was successfully probed by the pyrene side chains in the incorporated Pya residues. All the CD and fluorescent data were consistent with

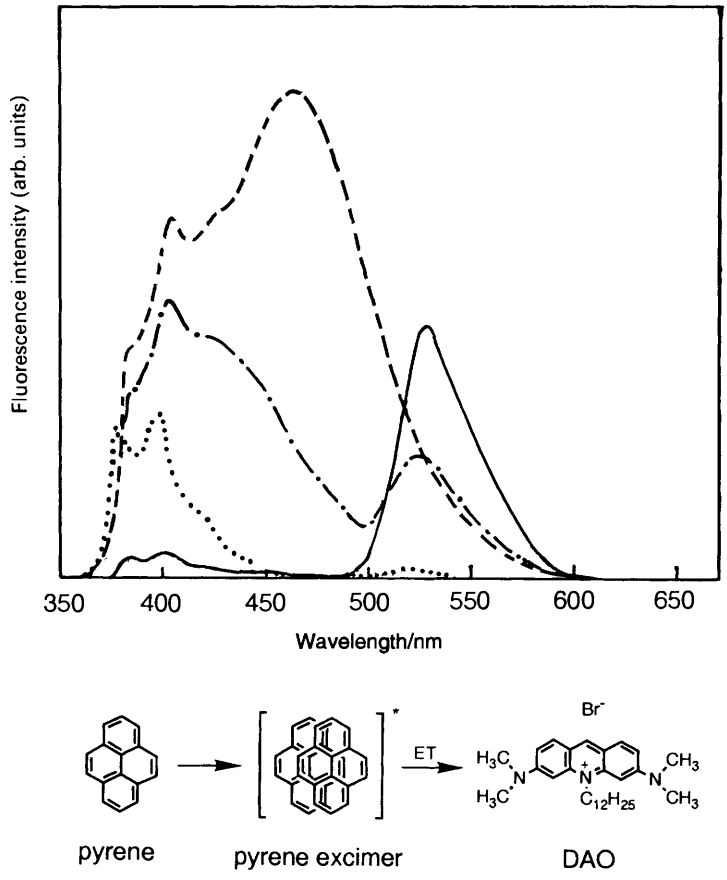

Fig. 7 Fluorescence spectra of 52GI in the presence of dodecylacridine orange (DAO) and methylacridine orange (MAO) in buffer and MeOH: 52GI with DAO in buffer (——); 52GI with DAO in MeOH (· · · ·); 52GI with MAO in buffer (– · – · –); and 52GI without DAO in buffer (– – – –); [peptide] = [dyes] = 3.0×10^{-5} mol dm^{-3}; excited at 345 nm at 25 °C. The illustration explains the fluorescence energy transfer.

four-α-helix bundle formation and the conformational changes of the 53-peptides, though the β-turn structure consisting of D-Ala-Pro is not characterized at present. Furthermore, the efficient energy transfer from pyrene excimer to DAO was observed and the incidence could be regulated by the formation and the deformation of the bundle structure. All the results demonstrated the usefulness of Pya as a probe for the conformation of designed polypeptides. Moreover, the information on the oriented pyrene groups in the 3D structure of polypeptide could be utilized in the design of artificial proteins carrying arranged functional chromophores and catalytic groups.

Experimental

Materials and methods

Amino acid derivatives and reagents for peptide synthesis were purchased from Watanabe Chemical Co. (Hiroshima, Japan). Boc-L-pyren-1-ylalanine was synthesized according to the method reported previously.[9b] *p*-Nitrobenzophenone oxime resin was prepared according to the reported method.[21d,e] Solid-phase peptide synthesis was carried out manually in a glass vessel. Dodecylacridine orange bromide was purchased from Dojindo Laboratories (Kumamoto, Japan) and the methylated dye was prepared from acridine orange in our laboratory. Fast atom bombardment mass spectra (FAB-MS) 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. 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, Tokyo) (4.6 × 150 mm or 10 × 250 mm) employing a Hitachi L-6200 HPLC System.

Peptide synthesis

Protected 6-, 7- and 8-peptides. Boc-D-Ala-Pro-Glu(OcHex)-Leu-Leu-Lys(ClZ)-resin and Boc-Ala-X-Ala-Glu(OcHex)-Leu-Leu-Lys(ClZ)-resin (X: Phe or Pya) were synthesized manually by stepwise elongation of Boc-amino acids on a *p*-nitrobenzophenone oxime resin[21] according to the reported procedure[9b] using benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP)[25] and 1-hydroxybenzotriazole hydrate (HOBt).

To obtain Boc-D-Ala-Pro-Glu(OcHex)-Leu-Leu-Lys(ClZ)-OH, the peptide resin was shaken with HOPip (4.0 equiv.) in DMF for 24 h. The peptide solution was filtered and washed twice with DMF. The solvent was evaporated off and the residues (Boc-peptide-OPip) were dissolved in 95% aqueous acetic acid. To the solution was added sodium dithionite ($Na_2S_2O_4$) (5.0 equiv.) and the solution was stirred for 1.0 h. The solvent was evaporated off and the residues were solidified with 10% citric acid to give protected 6-peptide-OH. Boc-Ala-X-Ala-Glu(OcHex)-Leu-Leu-Lys(ClZ)-OPip was obtained without reductive removal of the Pip ester. To obtain Boc-Ala-X-Ala-Glu(OcHex)-Leu-Leu-Lys(ClZ)-Gly-OBzl, the peptide resin was shaken with Gly-OBzl toluene-*p*-sulfonate (4.0 equiv.) in the presence of AcOH (4.0 equiv.) and dipropan-2-ylethylamine (4.0 equiv.) in DMF for 24 h. The peptide solution was filtered and washed twice with DMF. The solvent was evaporated off and the residues were solidified with 10% citric acid, collected on filter and washed with water.

All protected peptides were re-precipitated with MeOH–diethyl ether to over 92% purity (yield 80–90%). The purity was checked by HPLC (MS-GEL C4 column, 4.6 × 150 mm, with a linear gradient of 30–100% acetonitrile–0.1% TFA over 30 min). Peptides were identified by the molecular ion peak $(M + H)^+$ or $(M + Na)^+$ on FAB-MS; Boc-D-Ala-Pro-Glu(OcHex)-Leu-Leu-Lys(ClZ)-OH, *m/z* 1043 [$(M + Na)^+$]; Boc-Ala-Phe-Ala-Glu(OcHex)-Leu-Leu-Lys(ClZ)-OPip, *m/z* 1247 [$(M + Na)^+$]; Boc-Ala-Pya-Ala-Glu(OcHex)-Leu-Leu-Lys(ClZ)-OPip, *m/z* 1349 [$(M + H)^+$]; Boc-Ala-Phe-Ala-Glu(OcHex)-Leu-Leu-Lys(ClZ)-Gly-OBzl, *m/z* 1289 [$(M + H)^+$]; Boc-Ala-Pya-Ala-Glu(OcHex)-Leu-Leu-Lys(ClZ)-Gly-OBzl, *m/z* 1413 [$(M + H)^+$].

Protected 13-peptides and 13-peptide-Gly-OBzl: Boc-D-Ala-Pro-Glu(OcHex)-Leu-Leu-Lys(ClZ)-Ala-X-Ala-Glu(OcHex)-Leu-Leu-Lys(ClZ)-Gly-OBzl (Boc-13G-OBzl). Boc-Ala-X-Ala-Glu(OcHex)-Leu-Leu-Lys(ClZ)-Gly-OBzl [1.29 g (Phe), 1.41 g (Pya), 1.0 mmol] was treated with TFA (5 cm^3) at 0 °C for 30 min. After evaporation, the residues were solidified with diethyl ether. The obtained H-8-peptide-OBzl·TFA (100%) and Boc-D-Ala-Pro-Glu(OcHex)-Leu-Leu-Lys(ClZ)-OH (1.02 g, 1.0 mmol) were condensed with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (190 mg, 1.0 mmol) in the presence of HOBt (150 mg, 1.0 mmol) and Et_3N (0.14 cm^3, 1.0 mmol) in DMF (20 cm^3) at 0 °C for 24 h. The solvent was evaporated off, after which the residues were solidified with water and washed with 4% $NaHCO_3$, 10% citric acid and water to give the protected Boc-13-peptide-Gly-OBzl. The crude peptide was purified by re-precipitation with MeOH–diethyl ether. The crude peptides were purified by gel filtration chromatography with Sephadex LH-60 (DMF, 2.0 × 90 cm): X = Phe 1.64 g (75%); X = Pya 1.71 g (74%).

Boc-D-Ala-Pro-Glu(OcHex)-Leu-Leu-Lys(Clz)-Ala-X-Ala-Glu(OcHex)-Leu-Leu-Lys(ClZ)-OH (Boc-13-OH). Boc-D-Ala-Pro-Glu(OcHex)-Leu-Leu-Lys(ClZ)-OH (1.02 g, 1.0 mmol) and H-Ala-X-Ala-Glu(OcHex)-Leu-Leu-Lys(ClZ)-OPip·TFA obtained from Boc-7-peptide-OPip [1.23 g (Phe), 1.35 g (Pya), 1.0 mmol] were condensed in the same manner. The coupling solution was diluted with the same volume of AcOH and then $Na_2S_2O_4$ (5.0 equiv.) was added. The solution was stirred at room temperature for 1 h to give Boc-13-peptide-OH. The crude peptides were purified by gel filtration chromatography with Sephadex LH-60 (DMF, 2.0 × 90 cm): X = Phe 1.64 g (80%); X = Pya 1.66 g (76%).

53-Peptides: Boc-[D-Ala-Pro-Glu(OcHex)-Leu-Leu-Lys(ClZ)-Ala-Pya-Ala-Glu(OcHex)-Leu-Leu-Lys(ClZ)]-[D-Ala-Pro-Glu(OcHex)-Leu-Leu-Lys(ClZ)-Ala-Phe-Ala-Glu(OcHex)-Leu-Leu-Lys(ClZ)]-Gly-OBzl (Boc-26G-OBzl). Boc-13-OH (1.08 g, 0.5 mmol) and H-13G-OBzl·TFA obtained from Boc-13G-OBzl (1.09 g, 0.5 mmol) were condensed in the same manner using EDC (1.0 mmol) and HOBt (1.0 mmol) for 2 days to give the Boc-26G-OBzl. The crude peptide was purified by gel filtration chromatography with Sephadex LH-60 (DMF, 2.0 × 90 cm): 1.61 g (76%).

Boc[D-Ala-Pro-Glu(OcHex)-Leu-Leu-Lys(ClZ)-Ala-X-Ala-Glu(OcHex)-Leu-Leu-Lys(ClZ)]$_3$-Gly-OBzl (Boc-39G-OBzl). Boc-13-OH [410 mg (Phe for 39GI), 430 mg (Pya for 39GII), 0.20 mmol] and H-26G-OBzl·TFA obtained from Boc-26G-OBzl (830 mg, 0.20 mmol) were condensed and purified in the same manner: Boc-39GI-OBzl 900 mg (73%); Boc-39GII-OBzl 1.0 g (79%).

Boc-[D-Ala-Pro-Glu(OcHex)-Leu-Leu-Lys(ClZ)-Ala-X-Ala-Glu(OcHex)-Leu-Leu-Lys(ClZ)]$_4$-Gly-OBzl (Boc-52G-OBzl). Boc-13-OH [220 mg (Phe for 52GI), 200 mg (Pya for 52GII), 0.10 mmol] and H-39G-OBzl·TFA obtained from Boc-39G-OBzl [620 mg (39GI), 630 (39GII), 0.10 mmol] were condensed and purified in the same manner: Boc-52GI-OBzl 510 mg (62%); Boc-52GII-OBzl 490 mg (60%).

Gel-filtration chromatography showed that the protected peptides 13G, 26G, 39G and 52G could be separated on a Sephadex LH-60 (DMF) column (2.0 × 90 cm) and the elution volumes of the peptides correlated well to the molecular weights.

H-[D-Ala-Pro-Glu-Leu-Leu-Lys-Ala-X-Ala-Glu-Leu-Leu-Lys]$_4$-Gly-OH (52G). Boc-52G-OBzl (270 mg, 0.033 mmol) was treated with anhydrous HF (10 cm^3) in the presence of anisole (0.5 cm^3) at 0 °C for 60 min. After removal of HF, the peptide was dissolved in 30% AcOH and the solution was washed with diethyl ether. The aqueous layer was lyophilized to give the crude peptide: 52GI 200 mg (100%); 52GII 195 mg (97%). The crude peptides were purified with HPLC on a MS-GEL C4 column (1.0 × 25 cm) using a linear gradient of 70–100% acetonitrile–0.1% TFA (30 min): 52GI 55 mg (27%), 52GII 45 mg (22%); 52GI (3.0×10^{-5} mol dm^{-3}), λ_{max} (2.0×10^{-2} mol dm^{-3} TrisHCl buffer, pH 7.4)/nm 346 (ε/dm^3 mol^{-1} cm^{-1} 52 900), 330 (44 700), 278 (64 800), 268 (47 500) and 244 (88 400); λ_{max}(MeOH)/nm 342 (76 500), 326 (56 500), 313 (25 800), 276 (86 300), 265 (57 000), 242 (106 700) and 234 (98 100); 52GII (3.0×10^{5} mol dm^{-3}), λ_{max}(2.0×10^{-2} mol dm^{-3} TrisHCl buffer, pH 7.4)/nm 346 (ε/dm^3 mol^{-1} cm^{-1} 53 100), 331 (45 100), 278 (65 500), 268 (46 700), 244 (87 100), 237 (80 600); λ_{max}(MeOH)/nm 342 (76 400), 326 (56 300), 313 (25 400), 276 (85 700), 265 (56 200), 242 (106 700) and 234 (96 500). Amino acid analysis: 52GI, $Glu_{8.08}$ (8), $Gly_{1.0}$ (1), $Pro_{4.10}$ (4), $Ala_{12.3}$ (12), $Leu_{15.9}$ (16), $Phe_{2.01}$ (2), $Lys_{7.68}$ (8). 52GII, $Glu_{8.03}$ (8), $Gly_{1.0}$ (1), $Pro_{4.05}$ (4), $Ala_{12.1}$ (12), $Leu_{16.3}$ (16), $Phe_{2.05}$ (2), $Lys_{7.88}$ (8).

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 pathlength. Peptides were dissolved in 2.0×10^{-2} mol dm^{-3} TrisHCl buffer (pH 7.4), MeOH–buffer or in the presence of GuHCl in peptide concentration of 0.5–5.0×10^{-5} mol dm^{-3}.

Fluorescence measurements

Fluorescence spectra were run on a Hitachi 650-10S fluorescence spectrophotometer at 25 °C. Peptides were dissolved in 2.0×10^{-2} mol dm^{-3} TrisHCl buffer (pH 7.4) or MeOH–buffer in a concentration of $(3.0–30.0) \times 10^{-6}$ mol dm^{-3}.

Acknowledgements

This work was funded in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

References

1 (*a*) S. F. Betz, D. P. Raleigh and W. F. DeGrado, *Curr. Opin. Struct. Biol.*, 1993, **3**, 601; (*b*) W. F. DeGrado, Z. F. Wasserman and J. D. Lear, *Science*, 1989, **243**, 622; (*c*) L. Regan and W. F. DeGrado, *Science*, 1988, **241**, 976; (*d*) J. J. Osterhout Jr., T. Handel, G. Na, A. Toumadje, R. C. Long, P. J. Connolly, J. C. Hoch, W. C. Johnson Jr., D. Live and W. F. DeGrado, *J. Am. Chem. Soc.*, 1992, **114**, 331; (*e*) T. M. Handel, S. A. Williams and W. F. DeGrado, *Science*, 1993, **261**, 879; (*f*) C. P. Hill, D. H. Anderson, L. Wesson, W. F. DeGrado and D. Eisenberg, *Science*, 1990, **249**, 543; (*g*) T. M. Handel, S. A. Williams, D. Menyhard and W. F. DeGrado, *J. Am. Chem. Soc.*, 1993, **115**, 4457.

2 M. Mutter and S. Vuilleumier, *Angew. Chem., Int. Ed. Engl.*, 1989, **28**, 535; M. Mutter, G. G. Tuchscherer, C. Miller, K.-H. Altmann, R. I. Carey, D. F. Wyss, A. M. Labhardt and J. E. River, *J. Am. Chem. Soc.*, 1992, **114**, 1463.

3 T. Sasaki and E. T. Kaiser, *J. Am. Chem. Soc.*, 1989, **111**, 380; T. Sasaki and E. T. Kaiser, *Biopolymers*, 1990, **29**, 79; M. Lieberman

and T. Sasaki, *J. Am. Chem. Soc.*, 1991, **113**, 1470; T. Sasaki and M. Lieberman, *Tetrahedron*, 1993, **49**, 3677.
4 K. W. Harn, W. A. Klis and J. M. Stewart, *Science*, 1990, **248**, 1544.
5 M. Montal, M. S. Montal and J. M. Tomich, *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 6929.
6 H. Morii, K. Ichimura and H. Uedaira, *Chem. Lett.*, 1990, 1987; *Proteins Str. Func. Genet.*, 1991, **11**, 133; H. Morii, S. Honda, K. Ichimura and H. Uedaira, *Bull. Chem. Soc. Jpn.*, 1991, **64**, 396.
7 M. R. Ghadiri, C. Soares and C. Choi, *J. Am. Chem. Soc.*, 1992, **114**, 825; 4000; M. R. Ghadiri and M. A. Case, *Angew. Chem., Int. Ed. Engl.*, 1993, **32**, 1594.
8 (*a*) N. Nishino, H. Mihara, Y. Tanaka and T. Fujimoto, *Tetrahedron Lett.*, 1992, **33**, 5767; (*b*) N. Nishino, H. Mihara, T. Uchida and T. Fujimoto, *Chem. Lett.*, 1993, 53; (*c*) H. Mihara, K. Tomizaki, N. Nishino and T. Fujimoto, *Chem. Lett.*, 1993, 1533.
9 (*a*) H. Mihara, N. Nishino and T. Fujimoto, *Chem. Lett.*, 1992, 1809; (*b*) H. Mihara, Y. Tanaka, T. Fujimoto and N. Nishino, *J. Chem. Soc., Perkin Trans. 2*, 1995, 1133; (*c*) H. Mihara, N. Nishino, R. Hasegawa and T. Fujimoto, *Chem. Lett.*, 1992, 1805; (*d*) H. Mihara, N. Nishino, R. Hasegawa, T. Fujimoto, S. Usui, H. Ishida and K. Ohkubo, *Chem. Lett.*, 1992, 1813.
10 S. Ono, N. Kameda, T. Yoshimura, C. Shimasaki, E. Tsukurimichi, H. Mihara and N. Nishino, *Chem. Lett.*, submitted.
11 H. Lettré, K. Buchholz and M.-E. Fernholz, *Hoppe-Seyler's Z. Physiol. Chem.*, 1941, **267**, 108.
12 S. Egusa, M. Sisido and Y. Imanishi, *Macromolecules*, 1985, **18**, 882; M. Sisido and Y. Imanishi, *Macromolecules*, 1985, **18**, 890.
13 R. Goedeweeck and F. C. De Shryver, *Photochem. Photobiol.*, 1984, **39**, 515; R. Goedeweeck, M. Van der Auweraer and F. C. De Shryver, *J. Am. Chem. Soc.*, 1985, **107**, 2334.
14 (*a*) H. Mihara, S. Lee, Y. Shimohigashi, H. Aoyagi, T. Kato, N. Izumiya and T. Costa, *Int. J. Peptide Protein Res.*, 1987, **30**, 605; (*c*) M. Xu, N. Nishino, H. Mihara, T. Fujimoto and N. Izumiya, *Chem. Lett.*, 1992, 191; (*c*) S. Lee, M. Yoshida, H. Mihara, H. Aoyagi, T. Kato and N. Yamasaki, *Biochim. Biophys. Acta*, 1989, **984**, 174.
15 H. J. Pownall and L. C. Smith, *J. Am. Chem. Soc.*, 1973, **95**, 3136; A. K. Soutar, H. J. Pownall, A. S. Hu and L. C. Smith, *Biochemistry*, 1974, **13**, 2828.
16 S. Tazuke, Y. Iwaya and R. Hayashi, *Photochem. Photobiol.*, 1982, **35**, 621; H. Ringsdorf, J. Venzmer and F. M. Winnik, *Macromolecules*, 1991, **24**, 1678.
17 C. Garcia-Echeverria, *J. Am. Chem. Soc.*, 1994, **116**, 6031.
18 E. T. Kaiser and F. J. Kézdy, *Science*, 1984, **223**, 249; J. P. Segrest, H. De Loof, J. G. Dohlman, C. G. Brouillete and G. M. Anantharamaiah, *Proteins Str. Funct. Genet.*, 1990, **8**, 103.
19 K. R. Shoemaker, P. S. Kim, E. J. York, J. M. Stewart and R. L. Baldwin, *Nature (London)*, 1987, **326**, 563.
20 G. E. Schulz and R. H. Schirmer, *Principles of Protein Structure*, Springer-Verlag, 1979.
21 (*a*) W. F. DeGrado and E. T. Kaiser, *J. Org. Chem.*, 1980, **45**, 1295; (*b*) S. H. Nakagawa and E. T. Kaiser, *J. Org. Chem.*, 1983, **48**, 678; (*c*) E. T. Kaiser, H. Mihara, G. A. Laforet, J. W. Kelly, L. Walters, M. A. Findeis and T. Sasaki, *Science*, 1989, **243**, 187; (*d*) N. Nishino, M. Xu, H. Mihara and T. Fujimoto, *Bull. Chem. Soc. Jpn.*, 1992, **65**, 991; (*e*) T. Sasaki, M. A. Findeis and E. T. Kaiser, *J. Org. Chem.*, 1991, **56**, 3159; (*f*) J. C. Hendrix, K. J. Halverson and P. T. Lansbury Jr., *J. Am. Chem. Soc.*, 1992, **114**, 7930; (*g*) H. Mihara, J. A. Chmielewski and E. T. Kaiser, *J. Org. Chem.*, 1993, **58**, 2207.
22 Y. H. Chen, J. T. Yang and K. H. Chau, *Biochemistry*, 1974, **13**, 3350.
23 N. Harada and K. Nakanishi, *Circular Dichroic Spectroscopy Exciton Coupling in Organic and Bioorganic Chemistry*, University Science Books, Mill Valley, CA, 1983.
24 N. Nishino, Y. Sugita and T. Fujimoto, *Peptide Chemistry 1983*, ed. E. Munekata, Protein Research Foundation, Osaka, 1984, p. 233.
25 P. Rivaille, J. P. Gautron, B. Castro and G. Milhaud, *Tetrahedron*, 1980, **36**, 3413.

Paper 5/00890E
Received 14*th February* 1995
Accepted 16*th May* 1995