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The Conformation of *de Novo* Designed Amphiphilic Peptides with Six or Nine L-2-(2,2,2-Trifluoroethyl)glycines as the Hydrophobic Amino Acid

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Amphiphilic 21-peptides with six and nine L-2-(2,2,2-trifluoroethyl)glycines as the hydrophobic amino acids and lysine and glutamic acid as the hydrophilic amino acids were synthesized. The CD spectra showed that these peptides with L-2-(2,2,2-trifluoroethyl)glycines took a random conformation in H_2O. On the contrary, similar amphiphilic 21-peptides with leucine as the hydrophobic amino acids took a helical conformation in H_2O. The peptides with L-2-(2,2,2-trifluoroethyl)glycines took a helical conformation in H_2O containing a $>$ 20% volume of 2,2,2-trifluoroethanol. These facts suggested the hydrophobic nature of the L-trifluoroethylglycines. The peptide with six L-2-(2,2,2-trifluoroethyl)glycines took a helical structure in methanol, however it slowly changed into the β-structure within 24 h. Interestingly, the peptide with nine L-2-(2,2,2-trifluoroethyl)glycines formed a stable helix under the same conditions. The peptide with nine L-2-(2,2,2-trifluoroethyl)glycines preferred a helical structure, probably because the assembling of the Tfeg side chains was more effective in forming its helix rather than the β-structure.

Recent progress on the *de novo* design and synthesis of polypeptides has opened a new area in biomimetic chemistry.[1] Polypeptides with super secondary structures, such as the assembled α-helices,[2] artificial β-sheets,[3] and their three-dimensional complexes, have been successfully synthesized.[1—7] Not only the synthesis of these complex molecules, but recently, a protein-like function of the artificial peptides have been shown. For instance, some peptides have been found to change their conformation from α-helices to the β-structure.[4] The enzyme-like catalysis of the peptides with the defined three-dimensional structure would be another recent example of the protein-like function.[5] Thus far, several concepts have been employed for the polypeptide architecture; the hydrogen bonding between the amide groups,[3e] the hydrophobic interaction between the amphiphilic peptides,[2d] Coulomb interaction of the amino acid side chains,[2b,2c,2e] the metal chelation,[6] the template-assistance to define the polypeptide super-structure,[7] and so on.

If some new artificial factor can be introduced during the *de novo* synthesis with the brand-new interaction between the amino acids, the possibility of an "artificial super-structure", as described above, will be further expanded. With that in mind, multi-fluorinated amino acids should be highly hydrophobic,[8—13] and therefore, the amphiphilic peptide with such multi-fluorinated amino acids is very interesting. Moreover, fluorinated compounds sometimes gather with each other, suggesting a negative affinity of the fluorocarbons with the hydrocarbon groups.[9d,14] The fluoroalkyl groups in the fluorinated polypeptides have the possibility of an attractive interaction with each other. Interestingly, a novel hydrogen-bond-like interaction with O–H/FC has recently been reported.[15] Though the fluorine atoms have often shown their unique effects in artificial biological molecules,[8,9] few have studied the character of polypeptides incorporating many multi-fluorinated amino acids.[9d,13] Herein, we wish to report on the syntheses and their solution conformation of the 21-residue peptides with six and nine L-2-(2,2,2-trifluoroethyl)glycine (Tfeg, (*S*)-$H_2NCH(CH_2CF_3)CO_2H$)[10] residues (**21Tfeg6** and **21Tfeg9**, Fig. 1). In their α-helix structure, hydrophobic Tfeg would occupy one face of the polypeptide helix surface and the hydrophilic lysine (Lys) and glutamic acid (Glu) residues occupy the other face.[16] We wish to show the effect of the fluoroalkyl groups on the conformation of **21Tfeg6** and **21Tfeg9**, by a comparison with the amphiphilic peptides with leucine (Leu) residues instead of Tfeg (**21Leu6** and **21Leu9**, Fig. 1).[17]

Results and Discussion

Design and Synthesis of Tfeg-Containing Peptide. For the helical peptide with a multi-fluorinated amino acid, L-2-(2,2,2-trifluoroethyl)glycine (Tfeg) was chosen for several reasons. (1) Tfeg can be easily synthesized from 2,2,2-trifluoroethanol (TFE),[10] in contrast to the lengthy synthesis of, for instance, 5,5,5,5′,5′,5′-hexafluoroleucine.[11a] (2) Tfeg can be easily obtained in the L-form by a simple enzymatic resolution.[10] (3) The helical structure can be expected for

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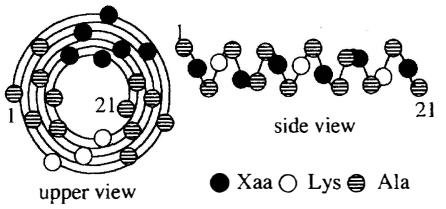
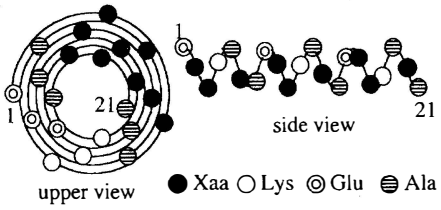
21Tfeg6 (Xaa=Tfeg) and **21Leu6** (Xaa=Leu)



21Tfeg9 (Xaa=Tfeg) and **21Leu9** (Xaa=Leu)



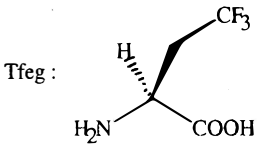


Fig. 1. Structure of H–(Ala–Tfeg–Ala–Lys–Ala–Tfeg–Ala–)$_3$–OH (**21Tfeg6**), H–(Ala–Leu–Ala–Lys–Ala–Leu–Ala–)$_3$–OH (**21Leu6**), H– (Glu– Tfeg– Tfeg– Lys–Ala–Tfeg–Ala–)$_3$–OH (**21Tfeg9**), and H–(Glu–Leu–Leu–Lys–Ala–Leu–Ala–)$_3$–OH (**21Leu9**), in their α-helical conformation.

the Tfeg-containing peptide as ethylglycine (2-aminobutyric acid) was reported to form an α-helix peptide.[18] 4) Though the molecular weight (157 for Tfeg free amino acid) somewhat exceeds that of Leu (131), the covalent bond radius of H (0.30 Å), F (0.58 Å), and C (0.77 Å)[19] indicate that the molecular size may be between Ala and Leu.

We had already synthesized the amphiphilic peptides **21Leu6** (H–(–Ala–Leu–Ala–Lys–Ala–Leu–Ala–)$_3$–OH)[17a] and **21Leu9** (H–(–Glu–Leu–Leu–Lys–Ala–Leu–Ala–)$_3$–OH),[17b,17c,17d] in which the hydrophobic Leu occupies one face of the polypeptide helix surface and the hydrophilic Lys and Glu residues occupy the other in their α-helix structure. Such an amphiphilic nature of the helices can stabilize their α-helical structure. The former peptide **21Leu6** possesses three Lys residues as the charged amino acid residue (side chain –NH_3^+) in its hydrophilic surface. The latter peptide **21Leu9** possesses three Glu–Leu–Leu–Lys units, and the Coulomb interaction between the side chains of Glu and Lys, such as $COO^- \cdots NH_3^+$ (salt-bridging effect),[2b,2c,2e] might exist. Thus, we investigated the effect of fluorine atoms by substituting the hydrophobic Leu of **21Leu6** and **21Leu9** with Tfeg. The structures of these novel peptides, **21Tfeg6** (H– (–Ala– Tfeg– Ala– Lys– Ala– Tfeg– Ala–)$_3$–OH) and **21Tfeg9** (H–(–Glu–Tfeg–Tfeg–Lys–Ala–Tfeg–Ala–)$_3$–OH), in their helical form are depicted in Fig. 1. In the helix structure, Tfeg covers 2/7 of the surface of **21Tfeg6** and 3/7 of the surface of **21Tfeg9**.

For syntheses of Tfeg-containing peptides, a solution-phase synthetic strategy was employed because an excess amount of Tfeg was necessary for the solid-phase synthesis.[9d] In spite of the low basicity of the amino group of Tfeg (pK_{NH2} is 8.169 for Tfeg and 9.747 for Leu, pK_{COOH} is 1.600 for Tfeg and 2.329 for Leu),[20] the reaction including Tfeg smoothly occurred throughout the syntheses. By adopting the usual Boc strategy, the protected 7-peptide Boc–Ala–Tfeg–Ala–Lys(ClZ)–Ala–Tfeg–Ala–OTce (**3**, –OTce is 2,2,2-trichloroethoxy) was synthesized via the coupling of Boc–Ala–Tfeg–Ala–OH and H–Lys-(ClZ)–Ala–Tfeg–Ala–OTce by the EDC–HOBt method (Fig. 2, see Experimental Section for detail).[21] Because some racemization or some side reaction occurred during coupling of the C-terminal multi-fluorinated amino acid residue,[9a,9e,11e] we chose the fragment coupling reactions at the positions of Ala and Lys(ClZ) as the C-termini. Actually, no racemization of the Tfeg moiety was observed and the purity of the protected 7-peptide fragments was confirmed by HPLC and FAB-MS. Unfortunately, the ^{1}H NMR (500 MHz, 1D and 2D-COSY) spectra were too complex for these peptide fragments and also for the 21-peptides, because of the repeated sequential character of our peptides.

As a temporary protection of the C-terminus, –OTce

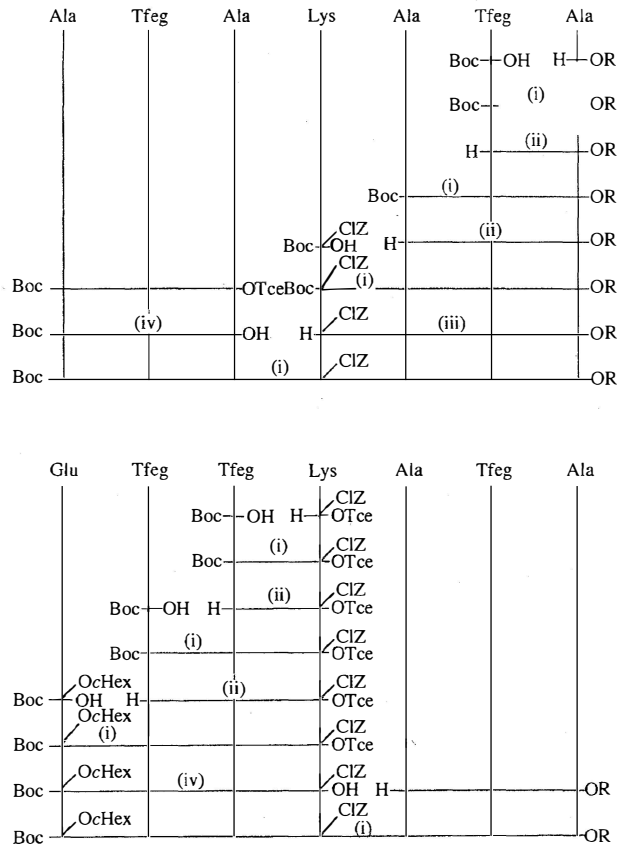


Fig. 2. Syntheses of the protected heptapeptides (above) Boc–Ala–Tfeg–Ala–Lys(ClZ)–Ala–Tfeg–Ala–OR (OR = OTce (**3**), OBzl (**4**)) and (below) Boc–Glu(O*c*Hex)–Tfeg–Tfeg–Lys(ClZ)–Ala–Tfeg–Ala–OR (OR = OTce (**7**), OBzl (**8**)). (i) EDC–HOBt; (ii) HCl/dioxane; (iii) TFA; (iv) Zn–AcOH.

was chosen, which is selectively removable with Zn–AcOH.[22] Solution-phase fragment coupling between Boc–Ala–Tfeg–Ala–Lys(ClZ)–Ala–Tfeg–Ala–OH and H–Ala–Tfeg–Ala–Lys(ClZ)–Ala–Tfeg–Ala–OBzl was performed in CH_2Cl_2–TFE (10 : 3, v/v), which is known to dissolve various protected peptides (Fig. 3).[23] Because HPLC and FAB-MS showed that the obtained 14-peptide was sufficiently pure, this peptide was not thoroughly purified, but again coupled at its N-terminal. A fully protected 21-peptide (**6**) with six Tfeg residues was thus obtained. The protected 21-peptide with nine Tfeg residues (**9**) was similarly synthesized. These protected 21-peptides (**6** and **9**) were then treated with anhydrous hydrogen fluoride-10% anisole (v/v) to deprotect the Boc–, –OBzl, –ClZ, and –O*c*Hex groups.[24] The crude 21-peptides **21Tfeg6** and **21Tfeg9** were purified by size-exclusion chromatography (Sephadex® G-25 eluting with 40% AcOH), in which these peptides were eluted as a single band. The elution volumes of **21Tfeg6** and **21Tfeg9** as well as those of **21Leu6** and **21Leu9** were similar. The FAB-MS (both low MS and high resolution MS, see Experimental Section) clearly indicated the molecular ion signals for both peptides, **21Tfeg6** (2091, $[M+H]^+$) and **21Tfeg9** (2469, $[M+H]^+$). HPLC analyses for these peptide showed that these peptide were obtained in high purity (see Experimental Section).

Solution Conformation of Tfeg-Containing Peptides. In H_2O (containing 3% HFIP), CD spectra indicated that the Tfeg-containing peptides **21Tfeg6** and **21Tfeg9** took the random coil structure with $[\theta]_{min}$ at 197 nm (Fig. 4, curves a and b); peptide concentration was 30 μM (1 M = 1 mol dm^{-3}).[25] These facts were in contrast to the facts that the Leu-containing amphiphilic peptides, **21Leu6** and **21Leu9**, showed typical CD spectra for the α-helices in the same solvents (Fig. 4, curves c and d) with $[\theta]_{min}$ at 222 and 208 nm and $[\theta]_{max}$ at 192 nm.[25,26] The values of $[\theta]_{min}$ at 222 nm were −35000 deg cm^2 $dmol^{-1}$ for **21Leu6** and −29000 deg cm^2 $dmol^{-1}$ for **21Leu9**. The reasons why the Tfeg-containing peptides were of a distorted conformation in H_2O are: (1) compared to the side chain of Leu (isobutyl group), the side chain of Tfeg ($-CH_2CF_3$) might be too hydrophobic; therefore, the side chain of Tfeg preferred to aggregate randomly in H_2O than to arrange to form a helical structure; (2) compared to the side chain of Leu, the side chain of Tfeg might be sterically too small to isolate the amide groups from the solvent H_2O; therefore, the solvent H_2O could cleave the intramolecular hydrogen bonds.

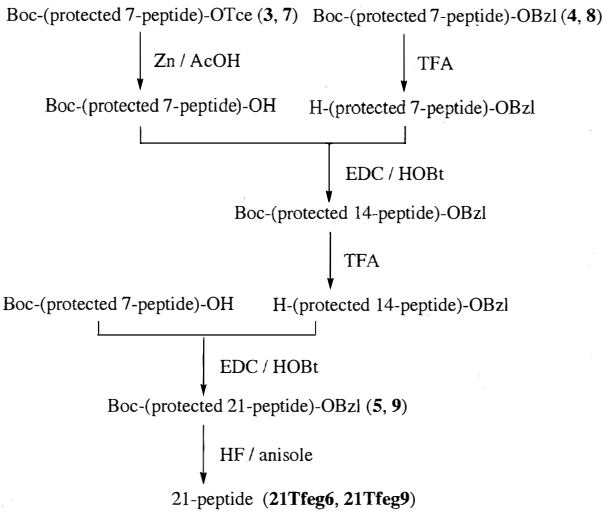


Fig. 3. Syntheses of the 21-peptides (**21Tfeg6** and **21Tfeg9**) via the stepwise fragment condensations.

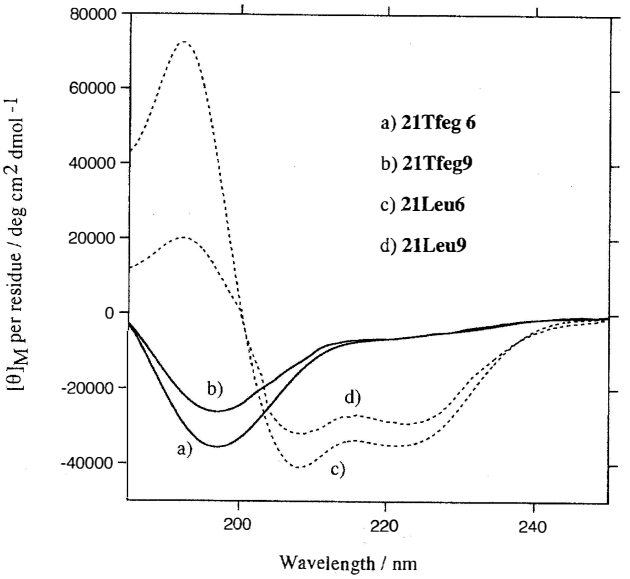


Fig. 4. CD spectra of a) **21Tfeg6**, b) **21Tfeg9**, c) **21Leu6**, and d) **21Leu9** in H_2O (3% HFIP). [Peptide] = 30 μM.

In TFE, not only the Leu-containing peptides, **21Leu6** and **21Leu9**, but also the Tfeg-containing peptides, **21Tfeg6** and **21Tfeg9**, took the helical structure (Fig. 5) with $[\theta]_{min}$ at 218 and 205 nm. TFE is more hydrophobic than H_2O;[27] therefore, the strong aggregation of Tfeg residues (the hydrophobic interaction between $-CH_2CF_3$ groups) may be weakened,

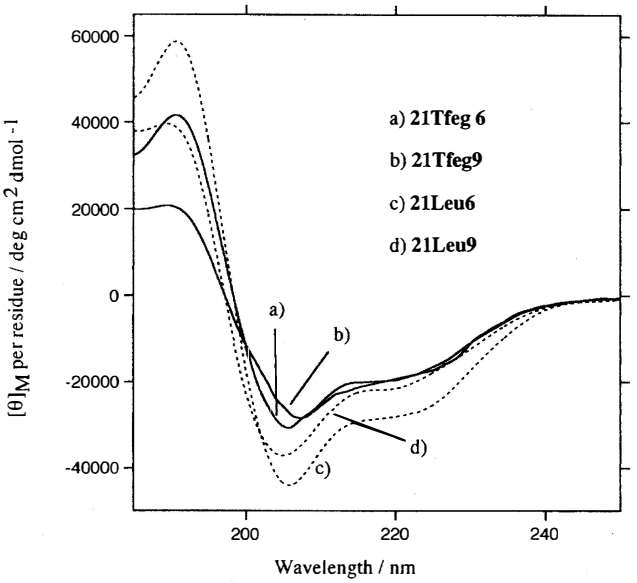


Fig. 5. CD spectra of a) **21Tfeg6**, b) **21Tfeg9**, c) **21Leu6**, and d) **21Leu9** in TFE (3% HFIP). [Peptide] = 30 μM.

resulting in the helix formation of **21Tfeg6** and **21Tfeg9**. The fact that hydrophobic TFE was necessary for the helix formation of **21Tfeg6** and **21Tfeg9** might suggest that Tfeg might be more hydrophobic than Leu.

As we have reported earlier, **21Leu6** and **21Leu9** assembled with each other to form the helix bundle structure, in which the Leu residues gathered inside via the hydrophobic interaction.[17] The ratio of $[\theta]_{ca.\ 220}/[\theta]_{ca.\ 208}$ in the CD spectra, which is a marker of the compactness of the helices,[28] decreased in TFE (**21Leu6**; 0.86 in H_2O and 0.65 in TFE, **21Leu9**; 0.91 in H_2O and 0.64 in TFE). The α-helicities ($[\theta]_{ca.\ 220}$) also decreased in TFE.[26] These facts suggested that the bundle structure of **21Leu6** and **21Leu9** collapsed into monomeric helices in hydrophobic TFE. This fact may imply that the helices of **21Tfeg6** and **21Tfeg9** in TFE also exist as the solvated form, not as the assembled helix form.

A CD investigation of **21Tfeg6** in various contents of TFE in H_2O further demonstrated that TFE stabilized the helical structure (Fig. 6, filled circle). Below a TFE content of 20% (v/v), **21Tfeg6** showed the CD spectrum of the random coil peptide with $[\theta]_{min}$ at 197 nm as described above. Above a 20% content of TFE, **21Tfeg6** showed a helical CD spectrum with $[\theta]_{min}$ at 205 and 218 nm (data not shown, but almost similar to that in TFE (Fig. 5, curve a)). TFE may probably solvate the Tfeg side chains; therefore, the helical structure of **21Tfeg6** may be stabilized. Interestingly, the α-helicity evaluated with $[\theta]_{218}$ was similar for the 20% TFE and 97% TFE. This fact might suggest that the 20% TFE amount was sufficient to solvate **21Tfeg6**. In contrast, the helical structure of **21Tfeg6** was hardly stabilized by MeOH. The $[\theta]_{218}$ values of the CD spectra of **21Tfeg6** in various MeOH contents in H_2O are shown in Fig. 6 (open circle, measured just after preparing the sample). In contrast to the circumstance in TFE–H_2O, the helicity of **21Tfeg6** only gradually increased ($[\theta]_{218}$ decreased) with an increase in MeOH. Interestingly, the $[\theta]_{218}$ in 97% MeOH was similar to that in 20—97% TFE. These facts suggest that the hydrophobic interaction of MeOH with **21Tfeg6** was not sufficient below 97% MeOH to stabilize its helical structure. This might suggest that the interaction of TFE with Tfeg may be effective in forming the helix structure.

Interestingly, the helical structure of **21Tfeg6** in MeOH was not stable with time. In Fig. 7, the CD spectra of **21Tfeg6** in MeOH is shown 5 min, 12 h, and 24 h after dissolving the peptide. Clearly the helical structure of **21Tfeg6** ($[\theta]_{min}$ was 205 and 218 nm) in MeOH gradually changed into the β-structure ($[\theta]_{min}$ was 215 nm).[25] However, the helical structure of not only **21Leu6** and **21Leu9** but also **21Tfeg9** were stable under the same conditions. In the β-structure, the side chains of the neighboring amino acids are located apart. Therefore, if **21Tfeg9** took the β-structure, the neighboring Tfeg residues in its –Glu–Tfeg–Tfeg–Lys– sequence would be separated from each other. One of the reasons why **21Tfeg9** preferred the helical structure and 21Tfeg6 tended to form β-structure in MeOH, may be because the Tfeg residues in the –Glu–Tfeg–Tfeg–Lys– sequence in **21Tfeg9** could be closely located in the helical structure, but separated in its β-structures. On the other hand, the Tfeg residues of **21Tfeg6** were not in the close proximity both in the helical and in the β-structure. The second possible reason why **21Tfeg9** preferred the helical structure in MeOH is the Coulomb interaction between the Glu and Lys side chains of **21Tfeg9**. The salt bridge between these residue may stabilize its helical structure. Thus, the unstable helical structure of **21Tfeg6** in MeOH slowly transformed into the β-structure.[4] The fact that the **21Tfeg9** formed a stable helix in MeOH, while **21Tfeg6** formed an unstable helix, also suggested that the Tfeg side chains tend to attract each other.

As a conclusion, the amphiphilic peptide with Tfeg residues **21Tfeg6** and **21Tfeg9** had a random structure in

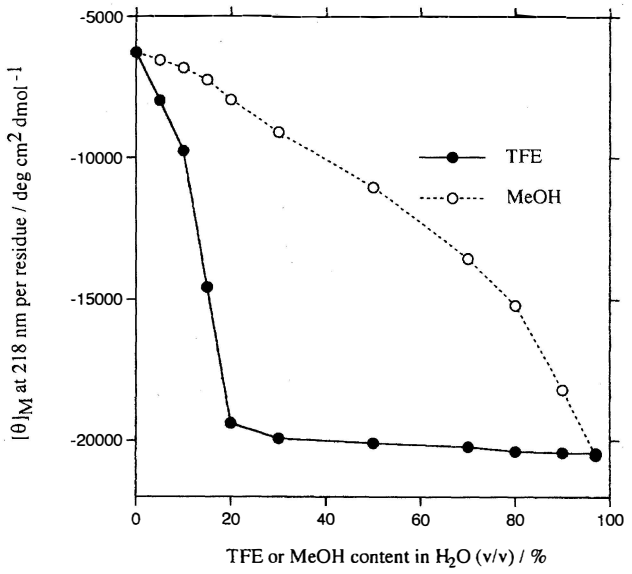


Fig. 6. TFE (●) and MeOH (O)-induced helix structure for **21Tfeg6** evaluated by the $[\theta]_{218}$ value in the CD spectra. [**21Tfeg6**] = 30 μM.

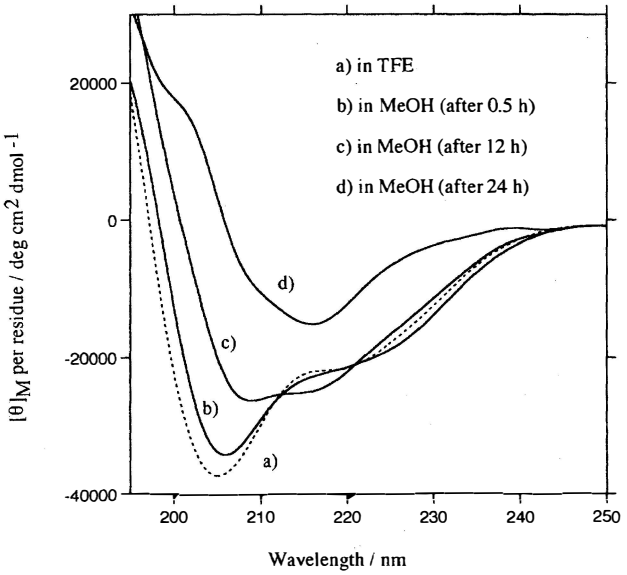


Fig. 7. CD spectra of **21Tfeg6** in a) TFE, b) MeOH (after 5 min), c) MeOH (after 12 h), d) MeOH (after 24 h). [**21Tfeg6**] = 30 μM.

H_2O, though **21Leu6** and **21Leu9** had a helical structure under the same conditions. In a > 20% TFE content in H_2O, **21Tfeg6** and **21Tfeg9** showed a helical structure. These Tfeg-containing peptides were partially helical in MeOH–H_2O, however, the large content of MeOH was necessary to stabilize the helical structure in this case. The helical **21Tfeg6** in MeOH slowly changed into the β-structure, however, the helical **21Tfeg9** in MeOH did not change its structure under the same conditions. The peptide with successive Tfeg residues **21Tfeg9** (–Glu–Tfeg–Tfeg–Lys– sequence) did not tend to transform into the β-structure, which may be because the assembling of the Tfeg side chains may be more effective in forming its helical structure rather than the β-structure. Because the side chains of Tfeg residue may tend to gather with each other, this nature might be beneficial for the design of a sophisticated three-dimensional structure of artificial peptides.

Experimental

Analytical Methods. An HPLC analysis was carried out using a Hitachi L-7100 intelligent pump equipped with a Hitachi L-4200 UV-vis detector and a Hitachi D-7500 chromato-integrator. The analysis was performed on a Wakosil® 5C4 column (4.6×150 mm) eluting with a linear gradient of a) $CH_3CN/H_2O/TFA$ = 37/63/0.1 to 100/0/0.1 (v/v/v) or b) $CH_3CN/H_2O/TFA$ = 0/100/0.1 to 100/0/0.1 (v/v/v) over 30 min at flow rate of 1.0 ml min^{-1} with detection at 220 nm. Size exclusion chromatography was performed with a Sephadex® G-25 column (2.0×76 cm) eluting with 40% AcOH. FAB-MS spectra were obtained with a JEOL DX-300 or SX-102 mass spectrometer. High-resolution MS spectra (HIMS) were calibrated with CsI. The CD spectra were recorded on a JASCO J-500 or J-720 spectropolarimeter using a quartz cell of 1 mm pathlength at 25 °C using a 30 μM peptide concentration. All CD samples contained 3% (v/v) HFIP. All CD samples were measured just after preparing each sample, unless otherwise noted. The Cotton effect was evaluated by the molar ellipticity $[\theta]$ per residue.

Materials. 2-(2,2,2-Trifluoroethyl)glycine (Tfeg) was synthesized and enzymatically resolved into its L-form according to the literature.[10] The amino acid derivatives and the reagents for the peptide synthesis were purchased from Peptide Institute Inc. and Watanabe Chemical Industries, Ltd. Other reagents and solvents were purchased from Wako Pure Chemical Industries, Ltd.

Boc–Ala–Tfeg–Ala–OTce (1). The dicyclohexylamine salt of Boc–Tfeg–OH (1.8 g, 4.0 mmol) and HCl·H–Ala–OTce (1.2 g, 4.5 mmol, synthesized from Boc–Ala–OH and 2,2,2-trichloroethanol with EDC/4-dimethylaminopyridine method,[22] then Boc protection was removed with HCl/dioxane)[29] were suspended in 30 ml of DMF, then EDC·HCl (1.2 g, 6.0 mmol), HOBt·H_2O (0.77 g, 5.0 mmol), and Et_3N (0.63 ml, 4.5 mmol) were added at 0 °C.[21] The mixture was stirred at room temperature overnight and then evaporated. The remained oil was dissolved in ethyl acetate and the organic layer was successively washed with aqueous $NaHCO_3$ and aqueous citric acid. After evaporation, Boc–Tfeg–Ala–OTce was almost quantitatively obtained, which was pure enough for further synthesis. The successive coupling of Boc–Ala–OH with HCl·H–Tfeg–Ala–OTce (generated from Boc–Tfeg–Ala–OTce and HCl/dioxane) was similarly performed. After a work up, **1** was solidified by adding ethyl ether/petroleum ether (yield 1.8 g, 3.4 mmol, 85%). FAB-MS (3-nitrobenzyl alcohol), 530 ($[M+H]^+$), 552 ($[M+Na]^+$).

Boc–Lys(ClZ)–Ala–Tfeg–Ala–OTce (2). The coupling of Boc–Lys(ClZ)–OH (0.33 g, 0.80 mmol) with HCl·H–Ala–Tfeg–Ala–OTce (0.36 g, 0.77 mmol, generated from **1** and HCl/dioxane) was similarly performed and the crude product was chromatographed over silica gel ($CHCl_3$–3% MeOH) giving **2** as an oil (0.50 g, 0.60 mmol, 78% yield). FAB-MS (3-nitrobenzyl alcohol), 828 ($[M+H]^+$), 850 ($[M+Na]^+$).

Boc–Ala–Tfeg–Ala–Lys(ClZ)–Ala–Tfeg–Ala–OTce (3). To the AcOH (12 ml) solution of **1** (0.32 g, 0.60 mmol), 2.3 g of Zn powder was added and the mixture was stirred for 5 h. After filtration, the solvent was evaporated and then washed with aqueous citric acid to give 0.25 g (0.60 mmol) of Boc–Ala–Tfeg–Ala–OH as an oil. This oil was dissolved in 10 ml of DMF; then, TFA·Lys(ClZ)–Ala–Tfeg–Ala–OTce (0.47 g, 0.56 mmol, generated from **2** and TFA), DIEA (0.10 ml, 0.60 mmol), HOBt·H_2O (0.11 g, 0.70 mmol), and EDC·HCl (0.15 g, 0.80 mmol) were added at 0 °C. The mixture was stirred at 0 °C for 24 h and then evaporated. After work-up, **3** was obtained in 93% yield (0.58 g, 0.52 mmol), which was pure enough for further synthesis. HPLC (gradient a), 20.7 min. FAB-MS (2,2′-dithiodiethanol), 1110 ($[M+H]^+$), 1132 ($[M+Na]^+$).

Boc–Ala–Tfeg–Ala–Lys(ClZ)–Ala–Tfeg–Ala–OBzl (4). Starting from HCl·H–Ala–OBzl, **4** was synthesized in a manner similar to **3**. HPLC, 19.9 min. FAB-MS (2,2′-dithiodiethanol), 1067 ($[M+H]^+$), 1089 ($[M+Na]^+$).

Boc–(–Ala–Tfeg–Ala–Lys(ClZ)–Ala–Tfeg–Ala–)$_3$–OBzl (5). The HFIP (0.20 ml) solution of **3** (0.13 g, 0.12 mmol) was diluted with 2.0 ml of AcOH, then Zn powder (0.13 g) was added and the mixture was stirred for 3 h. After filtration, the mixture was concentrated and solidified with the addition of aqueous citric acid. Boc–Ala–Tfeg–Ala–Lys(ClZ)–Ala–Tfeg–Ala–OH was thus obtained as a solid (0.12 g, 0.12 mmol), which was pure enough for further synthesis. This solid was dissolved by adding CH_2Cl_2 (10 ml) and TFE (3 ml),[23] then TFA·H–Ala–Tfeg–Ala–Lys(ClZ)–Ala–Tfeg–Ala–OBzl (0.11 g, 0.10 mmol, generated from **4** and TFA), DIEA (17 μl, 0.10 mmol), HOBt·H_2O (18 mg, 0.12 mmol), and EDC·HCl (29 mg, 0.15 mmol) were added at 0 °C. The mixture was stirred at 0 °C for 36 h. After a work-up, 0.20 g (0.10 mmol) of the protected 14-peptide Boc–(–Ala–Tfeg–Ala–Lys(ClZ)–Ala–Tfeg–Ala–)$_2$–OBzl was obtained, which was pure enough for further synthesis. HPLC (gradient a), 24.8 min. FAB-MS (3-nitrobenzyl alcohol), 1948 ($[M+Na]^+$). Similarly, Boc–Ala–Tfeg–Ala–Lys(ClZ)–Ala–Tfeg–Ala–OH (76 mg, 78 μmol) was again coupled with TFA·H–(–Ala–Tfeg–Ala–Lys(ClZ)–Ala–Tfeg–Ala–)$_2$–OBzl (0.12 g, 60 μmol, generated from the above 14-peptide and TFA) in CH_2Cl_2 (12 ml)–TFE (5 ml) using DIEA (14 μl, 78 μmol), HOBt·H_2O (14 mg, 90 μmol), and EDC·HCl (19 mg, 0.10 mmol). After a work-up, the protected 21-peptide (**5**) was solidified with the addition of aqueous $NaHCO_3$, yielding 0.17 g (60 μmol). This material was also pure enough for further synthesis. FAB-MS (3-nitrobenzyl alcohol), 2807 ($[M+Na]^+$).

H–(–Ala–Tfeg–Ala–Lys–Ala–Tfeg–Ala–)$_3$–OH (21Tfeg6). The protected 21-peptide **5** (88 mg, 32 μmol) was treated with anhydrous HF (9.0 ml)–anisole (1.0 ml) at 0 °C for 1 h.[24] The fully deprotected peptide was washed, lyophilized, and then purified by size exclusion chromatography (Sephadex® G-25) eluting with 40% AcOH. The peptide eluted as a single band. The appropriate fractions were collected, yielding 20 mg (9.6 μmol) of **21Tfeg6**. FAB-MS (2,2′-dithiodiethanol), 2090 ($[M+H]^+$). HIMS, Found: *m/z* 2089.8920. Calcd for $C_{78}H_{123}N_{24}O_{22}F_{18}$: M, 2089.8955. HPLC (gradient b), 16.4 min.

Boc–Glu(OcHex)–Tfeg–Tfeg–Lys(ClZ)–OTce (6). The di-

cyclohexylamine salt of Boc–Tfeg–OH (0.87 g, 2.0 mmol) and HCl·H–Lys(ClZ)–OTce (1.1 g, 2.2 mmol) were suspended in 10 ml of DMF, then EDC·HCl (0.58 g, 3.0 mmol), HOBt·H_2O (0.37 g, 2.4 mmol), and Et_3N (0.30 ml, 2.2 mmol) were added at 0 °C. The mixture was stirred at room temperature overnight and then evaporated. After a work-up, almost pure Boc–Tfeg–Lys(ClZ)–OTce was quantitatively obtained. Next, a similar coupling of Boc–Tfeg–OH with HCl·H–Tfeg–Lys(ClZ)–OTce gave Boc–Tfeg–Tfeg–Lys(ClZ)–OTce in 65% yield. The successive coupling of Boc–Glu(O*c*Hex)–OH with HCl·H–Tfeg–Tfeg–Lys(ClZ)–OTce gave **6** in 96% yield. FAB-MS (3-nitrobenzyl alcohol), 1037 ($[M+H]^+$).

Boc–Glu(O*c*Hex)–Tfeg–Tfeg–Lys(ClZ)–Ala–Tfeg–Ala–OTce (7). The treatment of **6** (0.42 g, 0.40 mmol) in 8.0 ml of AcOH with Zn (1.6 g) gave Boc–Glu(O*c*Hex)–Tfeg–Tfeg–Lys(ClZ)–OH quantitatively. The coupling of this tetrapeptide (0.40 mmol) with HCl·H–Ala–Tfeg–Ala–OTce (0.16 g, 0.34 mmol) was performed in 4.0 ml of DMF with DIEA (66 μl, 0.40 mmol), HOBt·H_2O (92 mg, 0.60 mmol), and EDC·HCl (0.11 g, 0.60 mmol) at 0 °C for 24 h. After work-up, **7** was obtained as an oil (0.41 g, 0.31 mmol, 91%). HPLC (gradient a), 25.1 min. FAB-MS (2,2′-dithiodiethanol), 1318 ($[M+H]^+$).

Boc–Glu(O*c*Hex)–Tfeg–Tfeg–Lys(ClZ)–Ala–Tfeg–Ala–OBzl (8). The coupling of Boc–Glu(O*c*Hex)–Tfeg–Tfeg–Lys(ClZ)–OH with HCl·H–Ala–Tfeg–Ala–OBzl was similarly performed to give **8**. HPLC (gradient a), 24.5 min. FAB-MS (2,2′-dithiodiethanol), 1291 ($[M+H]^+$).

Boc–(–Glu(O*c*Hex)–Tfeg–Tfeg–Lys(ClZ)–Ala–Tfeg–Ala–)$_3$–OBzl (9). To the TFE (0.50 ml)–CH_2Cl_2 (1.0 ml) solution of Boc–Glu(O*c*Hex)–Tfeg–Tfeg–Lys(ClZ)–Ala–Tfeg–Ala–OH (generated from **7** (30 mg, 25 μmol) and Zn/AcOH), TFA·H–Glu(O*c*Hex)–Tfeg–Tfeg–Lys(ClZ)–Ala–Tfeg–Ala–OBzl (generated from **8** (32 mg, 25 μmol) and TFA), DIEA (4.0 μl, 20 μmol), HOBt·H_2O (5.0 mg, 30 μmol), and EDC·HCl (7.0 mg, 37 μmol) were added and the mixture was stirred at 0 °C for 48 h. After a work-up, the protected 14-peptide Boc–(–Glu(O*c*Hex)–Tfeg–Tfeg–Lys(ClZ)–Ala–Tfeg–Ala–)$_2$–OBzl (39 mg, 17 μmol) was obtained. This material was pure enough for further synthesis. HPLC, 29.8 min. FAB-MS (3-nitrobenzyl alcohol), 2368 ($[M+Na]^+$). Next, Boc–Ala–Tfeg–Ala–Lys(ClZ)–Ala–Tfeg–Ala–OH (15 mg, 13 μmol) was coupled with TFA·H–(–Glu(O*c*Hex)–Tfeg–Tfeg–Lys(ClZ)–Ala–Tfeg–Ala–)$_2$–OBzl (23 mg, 10 μmol) in TFE–CH_2Cl_2, giving 32 mg (9.4 μmol) of the protected 21-peptide (**9**). This material was pure enough for further synthesis. FAB-MS (3-nitrobenzyl alcohol), 3434 ($[M+Na]^+$).

H–(–Glu–Tfeg–Tfeg–Lys–Ala–Tfeg–Ala–)$_3$–OH (21Tfeg9). The protected 21-peptide **9** (41 mg, 12 μmol) was treated with HF (9.0 ml)–anisole (1.0 ml) at 0 °C for 1 h. The fully deprotected peptide was washed, lyophilized, and then purified by size exclusion chromatography (Sephadex® G-25) eluted with 40% AcOH. The peptide eluted as a single band. The appropriate fractions were collected, yielding 14 mg (5.7 μmol) of **21Tfeg9**. FAB-MS (2,2′-dithiodiethanol), 2469 ($[M+H]^+$), HIMS. Found: *m/z* 2467.8781. Calcd for $C_{87}H_{126}N_{24}O_{28}F_{27}$: M, 2467.8741. HPLC (gradient b), 17.6 min.

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