Synthesis of [Hexafluorovalyl$^{1,1'}$]gramicidin S

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[Hexafluorovalyl$^{1,1'}$]gramicidin S (**8**), in which two valine residues in the natural gramicidin S were replaced by L-hexafluorovaline (Hfv) residues, was synthesized by the solid-phase-synthesis and cyclization-cleavage method on benzophenone oxime resin. Though the racemic hexafluorovaline derivative was employed for the peptide synthesis, the desired product was isolated in moderate yield, probably reflecting the stable cyclic structure of **8**. CD and ^{1}H NMR spectra indicated that the conformation of **8** is similar to that of gramicidin S. The two β-turn structure and antiparallel β-sheet structure with four intramolecular hydrogen bondings were maintained in spite of introducing the hexafluorovaline residues. The dye-release experiment from lecithin vesicle and antimacrobial activity assay also indicated that **8** showed membrane-disturbing activity like gramicidin S, although the activity of **8** was somewhat weaker than gramicidin S.

The introduction of fluorinated amino acids into peptides has been attracted much attention in the biochemical and pharmaceutical fields because of various interesting effects.[1] For instance, the natural system often takes up the fluorinated amino acids because of the small atomic radius of the fluorine atom. Therefore, various fluorinated amino acids are known to be irreversible inhibitors of natural enzymes[2] and have been attracted extensive medicinal studies.[3] Peptide drugs containing fluorinated amino acids might resist to the proteolytic digestion, hence some natural product analogues have been synthesized.[4] Since the strongly hydrophobic character of the fluorine atom is considered to enhance the binding of peptide to the receptor protein, several fluorine-containing amino acids were applied for the drug design.[5] The electronegative nature of the fluorine atom influences the acidity of the carboxyl group. This nature of fluorinated amino acids was utilized for the modification of some antitumor agents.[6] The fluorinated amino acid residues have also been a potential probe of ^{19}F NMR.[7]

In the *de novo* design of an artificial polypeptide, various natural and non-natural amino acids are arranged to create stable secondary- and super-structures.[8] The strongly hydrophobic (in some cases "solvatophobic") nature of the fluorinated amino acids are fascinating for the construction of an artificial polypeptide, though the interaction between fluorocarbon chain and hydrocarbon chain as the amino acid side chains has not been clearly elucidated. In this work, we synthesized [hexafluorovalyl$^{1,1'}$]gramicidin S (**8**), in which the Val residue of gramicidin S (GS) is replaced by L-hexafluorovaline (Hfv).[9] Gramicidin S is a cyclic decapeptide with two β-turn units (D-Phe–Pro) and one set of antiparallel β-sheets. The Val and Leu residues exist on one side of the compact framework, and the Orn residues lie on the other face, therefore the whole molecule shows an amphiphilic feature (Fig. 1).[10] Utilizing the tight framework of GS, we attempted to study the effect of incorporating the multi-fluorinated amino acids on the conformation and hydrophobicity of the molecule. Especially, the antimicrobial activity of the fluorinated GS analog may shed some light on the perturbation mechanism of lipid bilayer membranes. The result may further benefit the *de novo* design of the artificial proteins as an additional structure-constructing tool.

Results and Discussion

Synthesis of [Hfv$^{1,1'}$]GS (8). Boc–DL-Hfv (**4**) was synthesized through the reported procedure with some modification.[4] The DL-Hfv derivatives were reported to resist the enzymatic resolution,[4,6] therefore we used the racemic mixture as a starting material for the peptide synthesis. By using the solid-phase-synthesis and cyclization-cleavage method with benzophenone oxime resin, we attempted to synthesize a mixture of cyclic decapeptides (Scheme 1). The resin was first substituted by Boc–Leu, then linear decapeptides were assembled by the stepwise solid-phase condensations of the Boc–amino acids. The Kaiser test[11] for each coupling step indicated that, except for the condensation of Boc–Pro to the N-terminus DL-Hfv, the coupling completed with the usual condition (see Experimental section). This weak nucleophilicity of the amino group of Hfv might be responsible for the incomplete coupling of Boc–Pro.[12] In these cases, Boc–Pro was recoupled and the unreacted N-terminus Hfv was capped by the acetyl group, if it existed.

Thus, the linear decapeptide-resin should be obtained as a mixture of four diastereomers. The Boc- groups of N-termini were removed by TFA, then AcOH (2 mol amount) and DIEA (2 mol amount) were added to perform the cyclization-cleavage reaction.[13] The cyclic peptides should be obtained as a diastereomeric mixture of *cyclo*(–L-Hfv–Orn(Z)–Leu–D-

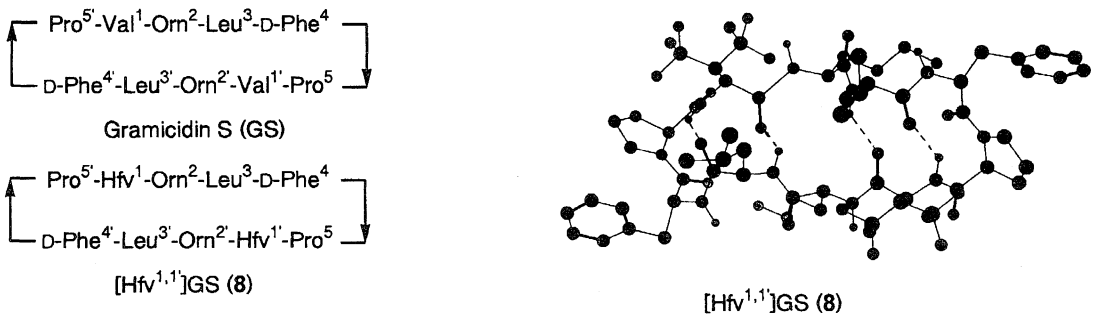
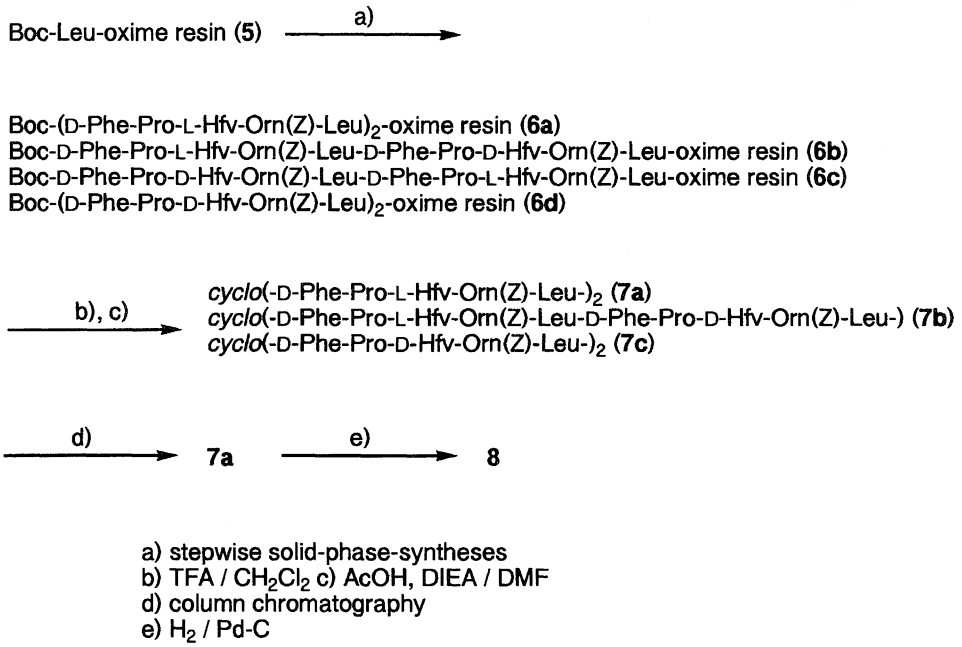


Fig. 1. Structure of Gramicidin S and [Hfv$^{1,1'}$]GS (**8**). Only the amide protons of Hfv–NH and Leu–NH were depicted for clarity.



Scheme 1. Synthesis of **8**.

Phe–Pro–)$_2$ (**7a**), *cyclo*(–D-Hfv–Orn(Z)–Leu–D-Phe–Pro–L-Hfv–Orn(Z)–Leu–D-Phe–Pro–) (**7b**), and *cyclo*(–D-Hfv–Orn-(Z)–Leu–D-Phe–Pro–)$_2$ (**7c**). Three peaks were indeed detected by the HPLC analysis of the supernatant phase of the reaction mixture. Small signals were observed at the retention times of 23.4 (fraction a) and 24.5 min (fraction b) (see Experimental section for the HPLC conditions), and a large signal at 33.4 min (fraction c). The large signal at 33.4 min was, fortunately, the desired peptide containing two L-Hfv residues as described below. Figure 2 shows the time course of this cyclization-cleavage reaction of the diastereomeric peptide-resin mixture. The signal at the retention time of 33.4 min (fraction c) was formed from the initial stage of the reaction. The other two products were formed slowly. This result suggested that fraction c is of preferred conformation for the cyclization. Such a relationship of the peptide assembly on the oxime resin and the yield of the cyclic peptide has been also observed for various peptides.[13b]

The reaction was terminated after 9 h and the solution phase was collected; it contained 67% amount of fraction c after evaporation. The three protected cyclic peptides (fractions a, b, and c) were separated by silica-gel column chromatography and at first their CD spectra were examined in MeOH (Fig. 3). Fraction c showed a negative Cotton effect with minima at 207 and 217 nm, which resembles closely that of the protected natural gramicidin S, [Orn(Z)$^{2,2'}$]GS. But, fractions a and b showed only weak Cotton effects around 220 nm. These facts indicated that fraction c was of similar conformation to [Orn(Z)$^{2,2'}$]GS, and suggested that this fraction was **7a**.

In the ^{1}H NMR spectrum of fraction c in DMSO-d_6 (Fig. 4c), four kinds of amide protons were observed. For the α-protons and the side chain-protons, only one set of signals were observed in the ^{1}H NMR spectra of fraction c. Unambiguous assignments of the signals were accomplished by the 2D-COSY spectra (δ = 9.15, Orn–NH; 9.09, D-Phe–NH; 8.08, Leu–NH; 7.61, Hfv–NH). These facts strongly suggest that fraction c is of C_2 symmetry like GS. But, ^{1}H NMR spectra of fractions a and b (Figs. 4a and 4b) are very complicated, and suggest that these cyclic peptides are mixtures of the conformers. Introduction of one or two D-Hfv residues would destroy the stable cyclic structure of GS.

Thus, fraction c showed similar conformation to natural GS; it therefore was considered to be [L-Hfv$^{1,1'}$, Orn(Z)$^{2,2'}$]-

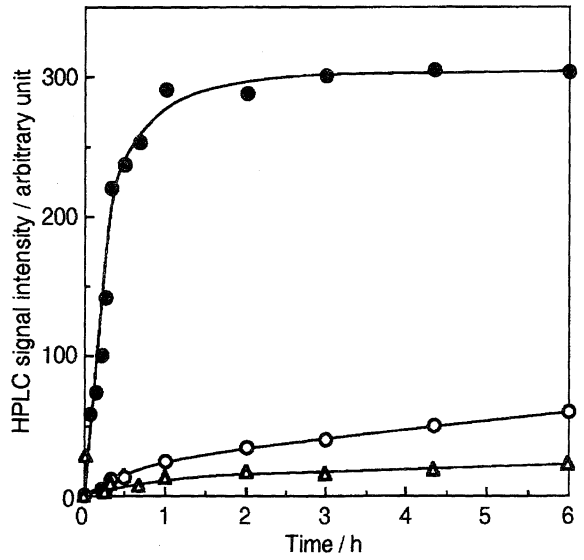


Fig. 2. Time course of the formation of cyclic peptides; (○) fraction a, retention time 23.4 min, (△) fraction b, retention time 24.5 min, (●) fraction c, retention time 33.4 min.

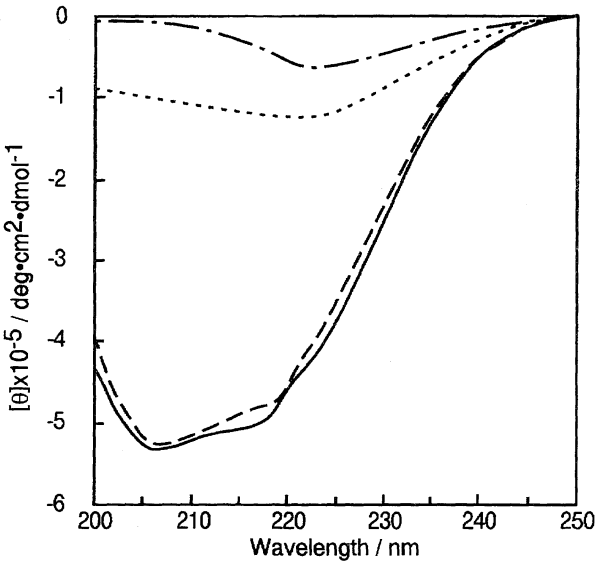


Fig. 3. CD spectra of the protected cyclic peptides in MeOH; (–•–) fraction a, (••••) fraction b, (——) fraction c, (- - -) [Orn(Z)$^{2,2'}$]GS.

GS (**7a**). On cyclization-cleavage, **7a** was generated in high yield from the corresponding linear peptide resin **6a**. **7b** and **7c** were obtained in lesser yield from **6b**, **6c**, and **6d**, although the molar ratio of **6a/6b/6c/6d** on the oxime resin can not be evaluated. Unfortunately, compounds **7b** and **7c** were contaminated with some impurities and the exact yields of these minor cyclic peptides were not determined. Anyway, the linear diastereomeric peptide-resins might be of different reactivities because of their conformations. The β-turn and β-strand parts of **7a** afford a less strained structure. However, **7b** and **7c** include one or two D-Hfv instead of L-Hfv, therefore these cyclic peptides are unfavorable structures for cyclization. The HPLC retention time of **7a** was prolonged compared with the other diastereomers, which might be reflected by the amphiphilic nature of **7a** like natural GS.

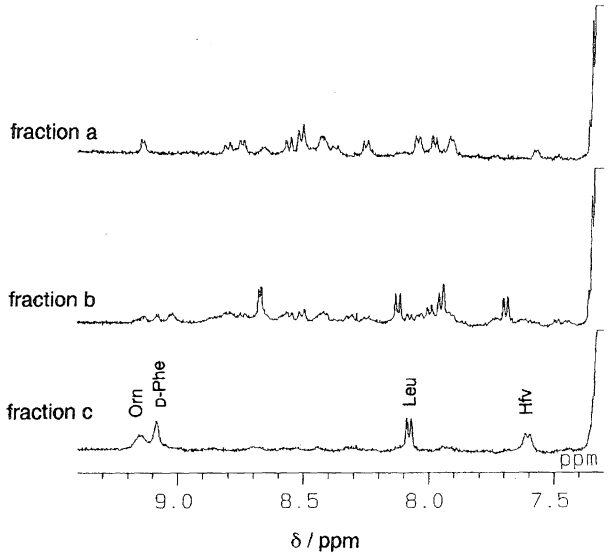


Fig. 4. ^{1}H NMR spectra (amide region) of the protected cyclic peptides in DMSO-d_6.

The deprotected peptide, [Hfv$^{1,1'}$]GS (**8**) was obtained as a HCl salt after hydrogenation of **7a**. The yield of **8** was 2.0% based on **4** used in the solid-phase synthesis. This yield is moderately high, because 3 molar amounts of **4** (per peptide-resin) is used for the solid-phase peptide condensation, and the desired peptide containing two L-Hfv is 1/4 of all the decapeptide, therefore the theoretical yield is $1/12 = 8.3\%$. The solid-phase peptide synthesis with ten residues and the cyclization reaction, both in general proceed with less efficiency. Therefore, Hfv was revealed to be utilized in the solid-phase-synthesis regardless of the weak nucleophilicity of the amino group.

Spectroscopic Study for the Conformation of [Hfv$^{1,1'}$]-GS (8). In the CD spectra of **8** in MeOH (Fig. 5), the negative Cotton effect was observed at minima 207 and 217 nm and was very similar to natural GS. These CD patterns were of typical type II′ β-turn and β-sheet structure of GS framework. ^{1}H NMR spectra of **8** (in DMSO-d_6) showed four amide NH proton signals, five α-proton signals, and a set of side chain proton signals. This fact indicates that **8** is of C_2 symmetry in the time scale of ^{1}H NMR. The ^{19}F NMR showed two multiplet signals at $\delta = 99.60$ and 98.91, indicating the two CF_3– groups of a Hfv residue are in diastereomeric circumstances. The β-proton of Hfv was sixtet (or double quartet), owing to the H_α–H_β coupling and H_β–F_γ couplings. Unambiguous assignments of the ^{1}H NMR spectra were performed by 2D-COSY spectra (see Experimental section). 2D-ROESY spectra showed cross peaks at Hfv–αH with Orn–NH, Orn–αH with Leu–NH, and Leu–αH with D-Phe–NH, suggesting the extended structure of Hfv–Orn–Leu–D-Phe unit.

The chemical shifts, their temperature coefficients, and the J values of the amide protons are summarized in Table 1. Temperature coefficients of amide proton chemical shifts were calculated in the range of 303—333 K. If an amide

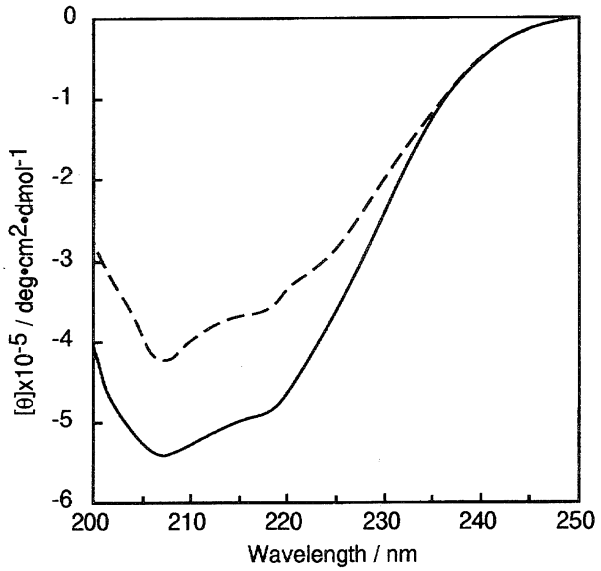


Fig. 5. CD spectra of the cyclic peptides in MeOH; (——) [Hfv$^{1,1'}$]GS (**8**), (- - -) GS.

proton participates in the intramolecular hydrogen bonding, its chemical shift little depends on the temperature, and the temperature coefficient is smaller than 3 ppb K^{-1}. If the amide proton is free from intramolecular hydrogen bondings and exposed to the solvent DMSO, the temperature coefficient would be large.[14] Table 1 indicates that amide protons of Hfv and Leu in **8** take part in the intramolecular hydrogen bonding, but those of Orn and D-Phe do not. Like natural GS, the strong intramolecular hydrogen bondings of NH(Hfv)–CO(Leu) and NH(Leu)–CO(Hfv) exist in **8**, as depicted in Fig. 1. Coupling constants between amide proton and α proton ($^3J_{HN\alpha}$) are also summarized in Table 1. As has been reported, the $^3J_{HN\alpha}$ value reflects the secondary structure of the peptides.[15] For the amino acid residue in the β-sheet structure, the $^3J_{HN\alpha}$ value of 8.9 to 9.2 Hz is reported. For the amino acid residue in the helix or tight turn structure, the $^3J_{HN\alpha}$ value is about 4 Hz. The $^3J_{HN\alpha}$ value of **8** is almost similar to that of GS. The Hfv, Orn, and Leu residues are of the extended structure and probably consist of a β-sheet structure. The D-Phe residue would take part in the type II′ β-turn D-Phe–Pro, suggested by the $^3J_{HN\alpha}$ value of 2.1 Hz. The $^3J_{HN\alpha}$ value of Orn (6.4) in **8** might imply that the β-sheet moiety of **8** is somewhat more distorted than that of GS, which might be owing to the effect of the Hfv. In this meaning, it is interesting that the $^3J_{HN\alpha}$ value of Orn was 9.0 Hz at 333 K, which was a typical value for the β-sheet residue.

Interaction of 8 with Phospholipid Bilayer. The antimicrobial activity of natural GS against Gram-positive bacteria has been considered to originate from the perturbation of the phospholipid bilayer. The amphiphilic nature of GS molecule has been recognized to be essential for the interaction with the membrane. The interaction of **8** with phospholipid bilayer was studied to elucidate whether the amphiphilic nature of GS is changed or not by introducing Hfv residues instead of Val.[16] The unilamellar vesicles of egg yolk L-α-phosphatidylcholine entrapping 5(6)-carboxylfluorescein (CF) were prepared. When **8** or GS was added to the vesicle solution in buffer, CF immediately released from the vesicle, which was quantified by the fluorescence intensity. Figure 6 showed the concentration-dependence of the CF leakage by the addition of cyclic peptides. The addition of GS solution to the vesicle solution up to 3 μmol dm^{-3} concentration resulted in the complete release of the dye. However, **8** showed somewhat weaker membrane-disturbing ability. This is probably because the difference in the interactions of GS and **8** with the phospholipids. GS may aggregate with each other in the near-surface of the hydrophobic phase of the membrane, in which the Val and Leu residues are penetrating to the lipid. The interaction of trifluoromethyl groups of **8** with the hydrophobic phase of the membrane might be rather weaker than the interaction of aliphatic side chains of GS with the lipid.

The minimum inhibition concentration of **8** against the growth of *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli* was evaluated by the liquid dilution method.[17] The antimicrobial activity of **8** was 25, 25, and 100 μg ml^{-1} for each bacteria, while GS showed the activity of 3.13, 1.56, and 12.5 μg ml^{-1}, respectively. Thus, **8** exhibited antimicrobial activity like GS, but the activity was relatively weak. As described above, the interaction of **8** with lipid bilayer is weaker than that of GS, and this might be the reason for the weaker antimicrobial activity of **8**.

Table 1. ^{1}H NMR Parameters of Amide Protons of **8** and GS[a]

	[Hfv$^{1,1'}$]GS (**8**)			GS		
Amide proton	Chemical shift δ/ppm[b]	Temperature coefficients $\Delta\delta$/ppb K^{-1} [c]	Coupling constant $^3J_{HN\alpha}$/Hz[d]	Chemical shift δ/ppm[b]	Temperature coefficients $\Delta\delta$/ppb K^{-1} [c]	Coupling constant $^3J_{HN\alpha}$/Hz[d]
Hfv	7.63	−1.74	9.8 (9.5)	—	—	—
Val	—	—	—	7.20	−1.83	9.5 (9.8)
Orn	9.31	−4.61	6.4 (9.0)	8.65	−4.83	9.5 (8.9)
Leu	8.00	−2.45	9.2 (9.2)	8.31	−2.72	9.2 (9.5)
D-Phe	9.21	−6.65	2.1 (3.4)	9.04	−7.03	3.7 (4.0)

a) Measured in DMSO-d_6. b) At 303 K. c) Measured at 303, 313, 323, and 333 K. d) At 303 K. Value in parentheses, at 333 K.

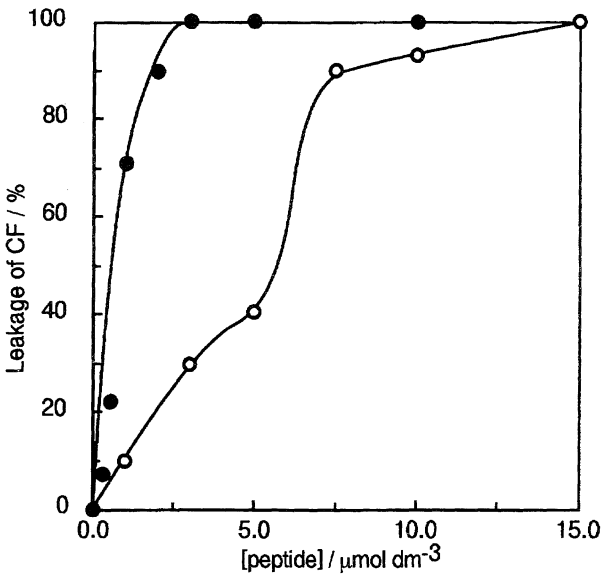


Fig. 6. Concentration dependence of (○) [Hfv$^{1,1'}$]GS (**8**) and (●) GS on the CF-leakage from egg yolk lecithin vesicles at 298 K.

Conclusion

We successfully synthesized [hexafluorovalyl$^{1,1'}$]gramicidin S (**8**), in which two valine residues of GS were replaced by Hfv residues, by the solid-phase-synthesis and cyclization-cleavage method on the benzophenone oxime resin. Though racemic Boc–DL-Hfv was employed for the peptide synthesis, the desired diastereomeric product was obtained in fair yield, probably because of the stable cyclic structure of **8**. The conformational similarity of **8** and GS, which consists of two β-turn and the antiparallel β-sheet with four intramolecular hydrogen bondings, was elucidated by CD and ^{1}H NMR spectra. Introducing multi-fluorinated Hfv residues, essentially induced no conformational change on the GS framework. The dye-release experiment from egg yolk lecithin vesicle and antimacrobial activity assay also suggested that **8** showed membrane-disturbing activity like GS, although the activities of **8** were weaker than those of GS. Probably the hydrophobic interaction of **8** with lipid membrane (fluorocarbon–hydrocarbon interaction) may be weaker than that of GS (hydrocarbon–hydrocarbon interaction), suggesting that the fluorocarbon–hydrocarbon solvatophobicity is not neligible in peptide–lipid interaction in aqueous circumstances. These results would be helpful for the *de novo* design and synthesis of the artificial proteins, for instance the artificial model of the membrane-proteins.

Experimental

Analytical Methods. TLC analyses were performed on Wako B-5 plates with the following solvent systems (by volume): R_{f1} = $CHCl_3$/MeOH (19 : 1), R_{f2}=$CHCl_3$/MeOH/AcOH (25 : 5 : 1), R_{f3}= BuOH/AcOH/pyridine/H_2O (4 : 1 : 1 : 2). The HPLC analysis was carried out on a Hitachi L-6300 intelligent pump equipped with a Hitachi L-4200 UV-vis detector and a Hitachi D-2500 chromato-integrator. The analysis was performed on a MS-GEL C18 PAC DF-5-120 Å column (4.6×150 mm) eluting with a linear gradient of CH_3CN/H_2O/TFA = 55/45/0.1 to 100/0/0.1 (v/v) over 30 min (eluent 1) or with a linear gradient of CH_3CN/H_2O/TFA=33/67/0.1 to 100/0/0.1 (v/v) over 30 min (eluent 2), flow rate of 1.0 ml min^{-1}, and detection at 220 nm. CD spectra were recorded on a JASCO J-500A spectropolarimeter using a quartz cell of 1 mm pathlength at 298 K, with 50 mmol ml^{-1} of peptide concentration. The Cotton effect was evaluated by the molar ellipticity [θ]. ^{1}H and ^{19}F NMR spectra including COSY and ROESY spectra were measured with a JEOL JNM α-500 spectrometer equipped with a tunable TH5AT probe at 298 K unless otherwise noted. The spectrometer operated at 500.0 MHz (^{1}H) and 470.4 MHz (^{19}F), respectively, and the chemical shifts were determined with respect to internal TMS for ^{1}H NMR and with respect to internal C_6F_6 for ^{19}F NMR. The resolution of the 1D spectrum was 0.31 Hz for ^{1}H and 0.86 Hz for ^{19}F NMR. EI and FAB-MS spectra were obtained with a JEOL DX-300 mass spectrometer. Fluorescence spectra were recorded on a Hitachi 650-10S fluorescence spectrophotometer at 298 K. UV-vis spectra were recorded on a Hitachi 150-20 spectrophotometer using quartz cells of 1 and 10 mm pathlength at 298 K.

Materials. Hexafluoroacetone trihydrate was a generous gift from Nippon Mectron Co., Ltd. The amino acid derivatives and the reagent for the peptide synthesis were purchased from Peptide Institute Inc. and Watanabe Chemical Industries, Ltd. L-α-Phosphatidylcholine (type XVI-E: from fresh egg yolk) was purchased from Sigma. 5(6)-Carboxyfluorescein was purchased from Kodak. Other reagent and solvents were purchased from Wako Pure Chemical Industries, Ltd.

Ethyl 4,4,4-Trifluoro-3-trifluoromethyl-2-butenoate (1).[4a,18] Under N_2 atmosphere, a mixture of hexafluoroacetone trihydrate (32 ml, 210 mmol) and molecular sieves 4A (61 g) was heated at 400 K to generate anhydrous hexafluoroacetone. The gaseous hexafluoroacetone was dried and was introduced to the ethyl ether (25 ml) solution of (ethoxycarbonylmethylene)triphenylphosphorane (10 g, 29 mmol) chilled at 195 K. The reaction mixture was stirred at 195 K for 3 h, then warmed to room temperature overnight. After addition of pentane (20 ml), the white precipitate (Ph_3P=O) was filtered off. Then the low boiling point material (ethyl ether and pentane) was carefully distilled off. The bulb-to-bulb distillation of the residual liquid gave **1** (5.8 g, 24 mmol, 84% based on (ethoxycarbonylmethylene)triphenylphosphorane) as a clear oil. R_{f1} 0.46. ^{1}H NMR ($CDCl_3$) δ = 6.88 (1H, s, CH), 4.32 (2H, q, J = 7.1 Hz, CH_2), 1.32 (3H, q, J = 7.1 Hz, CH_3).

DL-Hfv-OEt (2).[4a] Anhydrous NH_3 (generated by heating 25% NH_3 (50 ml), dried, and distilled from Na) was introduced to a solution of **1** (6.3 g, 27 mmol) in ethyl ether (30 ml) at 195 K. The mixture was stirred at that temperature for 1 h, then warmed to room temperature overnight. The bulb-to-bulb distillation under reduced pressure afforded **2** (5.8 g, 23 mmol, 86%) as a clear oil. R_{f2} 0.81 (ninhydrin test, yellow). ^{1}H NMR ($CDCl_3$) δ = 4.27 (2H, m, CH_2), 4.11 (1H, s, C_αH), 3.78 (1H, heptet, J = 7.8 Hz, C_βH), 1.30 (3H, t, J = 7.2 Hz, CH_3).

DL-Hfv (3).[4a] The solution of **2** (3.3 g, 13 mmol) in 6 M HCl (40 ml) (1 M = 1 mol dm^{-3}) was refluxed for 7 h. The mixture was evaporated to dryness and the residue was recrystallized from MeOH–ethyl ether, yielding 2.6 g (12 mmol, 91%) of **3**. R_{f3} 0.71. ^{1}H NMR (DMSO-d_6) δ = 8.45 (2H, br, NH_2), 4.55 (1H, s, C_αH), 4.23 (1H, t, J = 7.2 Hz, C_βH). ^{19}F NMR (DMSO-d_6) δ = 100.21 (3F, t, J = 9 Hz), 98.72 (1F, m), 98.58 (2F, d, J = 8 Hz). This product was proved to be identical to the commercially available DL-Hfv (PCR).

Boc–DL-Hfv (4).[4b] To a solution of **3** (4.1 g, 18 mmol) and Et_3N (2.8 g, 27 mmol) in 50% dioxane, Boc_2O (0.48 g, 22 mmol)

was added at room temperature and stirred overnight. The solvent was evaporated and 2 M HCl (20 ml) was added to the residue. The mixture was extracted with EtOAc, washed with 2 M HCl, and dried over $MgSO_4$. After evaporation, the residue was twice reprecipitated from EtOAc–hexane to afford **4** as a white solid; 4.3 g (13 mmol, 72%). R_{f2} 0.60. FAB-MS (glycerol) *m/z* 326 ($M+H^+$). ^{1}H NMR (DMSO-d_6) δ = 7.43 (1H, d, J = 9.8 Hz, NH), 4.91 (1H, d, J = 9.8 Hz, C$_\alpha$H), 4.46 (1H, quintet, J = 8.2 Hz, C$_\beta$H), 1.39 (9H, s, *t*-Bu). ^{19}F NMR (DMSO-d_6) δ = 100.61 (3F, m), 96.96 (3F, m).

Boc–Leu–Oxime Resin (5).[13c] To the *p*-nitrobenzophenone oxime resin (4.0 g) pre-swelled with CH_2Cl_2 in a glass reaction vessel, the CH_2Cl_2 (60 ml) solution of Boc–Leu (1.0 g, 4.0 mmol) and DCC (0.83 g, 4.0 mmol) was added. After shaking the mixture for 24 h, the resin was filtered, washed by CH_2Cl_2 and CH_2Cl_2–EtOH (1/1, v/v), then dried to yield Boc–Leu–oxime resin (0.55 mmol/g-resin, evaluated by a picrate assay[19]).

Boc–(D-Phe–Pro–(D or L)-Hfv–Orn(Z)–Leu)$_2$–Oxime Resins (Mixture of 6a, 6b, 6c, and 6d). The elongation of peptide chain was carried out by manual solid-phase-synthesis. The Boc protection group of **5** (2.0 g, 1.1 mmol) was removed by treating with 25% TFA in CH_2Cl_2 (v/v) for 30 min. After washing the resin with CH_2Cl_2 and 2-propanol, a mixture of Boc–Orn(Z) (1.2 g, 3.3 mmol), BOP (1.5 g, 3.3 mmol), HOBt·H_2O (0.51 g, 3.3 mmol), and DIEA (0.71 g, 5.5 mmol) in DMF (30 ml) was added. After shaking for 30 min, the coupling efficiency was checked by the Kaiser test,[11] which indicated the disappearance of the free amino group. The resin was washed by DMF and CH_2Cl_2, then subjected to the next condensation.

The coupling of Boc–Pro to the peptide resin with the D- or L-Hfv residues as N-terminus did not complete in the usual coupling procedure described above. In such case, the resin was recoupled by Boc–Pro and then capped with acetyl group. After the usual coupling of Boc–Pro, 1) repeated coupling reaction of Boc–Pro with the resin and work-up as usual, 2) addition of a DMF solution of Ac_2O (3 molar amount for the total of the peptide-resin) and DIEA (2 mol amount) to cap the free amino group, if existed. After this recoupling and capping process, the Kaiser test indicated the disappearance of the free amino group.

Cyclization-Cleavage of the Peptide Resins, the Mixture of 6a, 6b, 6c, and 6d to Yield the Cyclic Decapeptides. *Cyclo*(–(D or L)-Hfv–Orn(Z)–Leu–D-Phe–Pro–)$_2$ (7a, 7b, and 7c). The Boc groups of the N-termini of the peptide-resin mixture, **6a**, **6b**, **6c**, and **6d** (1.1 mmol) were removed by adding 25% TFA in CH_2Cl_2 (v/v). After 30 min, the resin was filtered, washed, and then a DMF (20 ml) solution of DIEA (0.28 g, 2.2 mmol) and AcOH (0.13 g, 2.2 mmol) was added. A small aliquot (5 µl) was withdrawn at appropriate intervals to analyze the reaction product by HPLC. After 9 h, the resin was filtered off, and the solution was concentrated. Addition of H_2O precipitated the mixture of cyclic peptides as a white solid; 0.19 g (0.12 mmol, 11% based on **5**).

Isolation of 7a. The mixture of peptides obtained by the cyclization-cleavage reaction as described above (0.19 g) was subjected to the silica-gel column chromatography eluted with $CHCl_3$/MeOH (97/3, v/v). Three fractions containing cyclic peptides were collected, the R_{f1} were 0.44, 0.38, and 0.30, respectively. The HPLC analyses indicated that the former two fraction contained **7b** and **7c**, and the last one with R_{f1} of 0.30 contained mainly **7a**. The fraction containing **7a** were evaporated to dryness, and reprecipitated from ethyl ether–petroleum ether to give 58 mg of a white solid. This crude **7a** was rechromatographed over silica-gel eluted with $CHCl_3$/MeOH/AcOH (98/1/1, v/v/v). The appropriate fractions were collected, evaporated to dryness, and reprecipitated from ethyl ether–petroleum ether; 46 mg, 53 µmol; R_{f1} 0.30. FAB-MS (2,2′-dithiodiethanol) *m/z* 1626 ($M+H^+$), 1583 ($M-NCO^+$), 1581 ($M-NH_2CO^+$), 1520 ($M-OBzl^+$), 1518 ($M-H_2OBzl^+$), 1493 ($M-CO_2Bzl+H^+$), HPLC (eluent 1) 33.34 min. ^{1}H NMR (DMSO-d_6) δ = 9.15 (2H, d, J = 6.4 Hz, Orn–NH), 9.09 (2H, d, J = 2.1 Hz, D-Phe–NH), 8.08 (2H, d, J = 9.2 Hz, Leu–NH), 7.61 (2H, d, J = 9.8 Hz, Hfv–NH), 7.3—7.1 (22H, m, aromatic CH of D-Phe, aromatic CH of Z, and Orn ω-NH), 5.35 (2H, t, Hfv–αH), 5.00 (4H, s, CH_2 of Z), 4.68 (2H, q, Orn–αH), 4.58 (2H, q, Leu–αH), 4.34 (4H, m, D-Phe–αH and Pro–αH), 4.16 (2H, q, Hfv–βH), 3.38 (2H, t, Pro–δH), 3.0—2.8 (8H, m, Orn–δH and D-Phe–βH), 2.35 (2H, m, Pro–δH), 1.83 (2H, m, Pro–βH), 1.7—1.3 (20H, m, Orn–βH, Orn–γH, Leu–βH, Leu–γH, Pro–βH, and Pro–γH), 0.81 (12H, t, Leu–δH). ^{19}F NMR (DMSO-d_6) δ = 99.86 (3F, m), 98.90 (3F, m).

***Cyclo*(–L-Hfv–Orn–Leu–D-Phe-Pro-)$_2$·2HCl (Hfv–GS·2HCl, 8).** In AcOH (3.0 ml), **7a** (42 mg, 26 µmol) was hydrogenated by 1 atm H_2 with 10% Pd-charcoal (20 mg) at room temperature for 6 h. After filtration of the catalyst, the solvent was evaporated to give a clear oil. After addition of MeOH (3.0 ml) and 4 M HCl/dioxane (30 µl), the resultant solution was evaporated to dryness. The obtained powder was reprecipitated from ethyl ether–petroleum ether; 37 mg (26 µmol, 100%); R_{f3} 0.80. HPLC (eluent 2) 23.62 min. FAB-MS (2,2′-dithiodiethanol) *m/z* 1358 ($M+H^+$). ^{1}H NMR (DMSO-d_6) δ = 9.31 (2H, d, J = 6.4 Hz, Orn–NH), 9.21 (2H, d, J = 2.1 Hz, D-Phe–NH), 8.00 (2H, d, J = 9.2 Hz, Leu–NH), 7.82 (4H, br, Orn–ω-NH_2), 7.63 (2H, d, J = 9.8 Hz, Hfv–NH), 7.25 (10H, m, aromatic CH of D-Phe), 5.35 (2H, d of d, J = 7.6 and 2.1 Hz, Hfv–αH), 4.66 (2H, d of d, J = 8.5 and 4.6 Hz, Orn–αH), 4.60 (2H, d, J = 7.7 Hz, Leu–αH), 4.36 (4H, m, D-Phe–αH and Pro–αH), 4.27 (2H, sixtet, J = 7.7 Hz, Hfv–βH), 3.45 (2H, m, Pro–δH), 2.99 (2H, d of d, J = 13.0 and 5.8 Hz, D-Phe–βH), 2.85 (4H, m, D-Phe–βH and Orn–δH), 2.79 (2H, m, Orn–δH), 2.42 (2H, m, Pro–δH), 1.89 (2H, m, Pro–βH), 1.78 (2H, q, J = 8.1 Hz, Orn–βH), 1.63 (6H, m, Orn–γH, Orn–βH), 1.43 (10H, m, Leu–γH, Pro–βH, Leu–γH), 1.35 (2H, quintet, J = 6.9 Hz, Leu–βH), 1.27 (2H, quintet, J = 6.9 Hz, Leu–βH), 0.83 (6H, d, J = 6.7 Hz, Leu–δH), 0.81(6H, d, J = 6.7 Hz, Leu–δH). ^{19}F NMR (DMSO-d_6) δ = 99.60 (3F, m), 98.91 (3F, m). Found: C, 46.55; H, 5.86; N, 10.79%. Calcd for $C_{60}H_{80}O_{10}N_{12}F_{12}$·2HCl·6$H_2O$: C, 46.85; H, 6.16; N, 10.93%.

Preparation of CF-Entrapped Vesicles.[16] In a pear-like flask, L-α-phosphatidylcholine (SIGMA type XVI-E: from fresh egg yolk) (24 mg) was dissolved in 2 ml of $CHCl_3$/MeOH (1/1, v/v) and then the solvent was evaporated by a stream of N_2. The dried lipid was hydrated in 3 ml HEPES buffer (10 mmol/l HEPES and 100 mmol/l NaCl, pH = 7.5) containing 0.3 mmol (0.11 g) of CF (5(6)-carboxyfluorescein) (Kodak, used as a sodium salt), using a bath-type sonicator (5 min). The obtained suspension was twice sonicated at 298 K for 10 min under N_2 atmosphere using a Branson Sonifier model 250 at 20 W intensity with 5 min of interval. The vesicles solution was kept standing for 30 min, then the CF-entrapped vesicles were separated from free CF by gel-filtration using Sephadex G-75 (10×200 mm) with the same buffer. The concentration of the vesicles in the eluted buffer was quantified after oxidization with permanganate and transforming to the phosphomolybdate using Phospholipid-test Wako,[20] to be 3.3 mmol dm^{-3} for the L-α-phosphatidylcholine.

CF-Leakage Experiment.[16] The carboxyfluorescein(CF)-release experiments were carried out according to the reported procedure. To 2.0 ml of buffer solution of 10 mM HEPES/100 mM NaCl (pH = 7.5), 20 µl of vesicle solution containing 100 mM CF (eluent of the Sephadex G-75 gel-filtration) was mixed in a 10

mm quartz cuvette. The concentration of the L-α-phosphatidylcholine should be 33 μM. The peptide solution in DMF (1.0 μM) was added to the vesicle solution, then the solution immediately colored. The CF released from the vesicle was quantified by a fluorescence spectrophotometer (λ_{ex} 470 nm and λ_{em} 515 nm). The fluorescence intensity derived from 100% dye-release was determined by adding 10 ml of 20% Triton X-100 (polyoxyethylene(10) octylphenyl ether) to the vesicle-peptide mixture. The percentage of CF-leakage caused by the peptides was calculated by the equation: $100(F-F_0)/(F_t-F_0)$, where F is the fluorescence intensity achieved by adding the peptide, F_0 and F_t are intensities without the peptide and after Triton X-100 treatment, respectively.

Antimicrobial Activity Assay. To the *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538P, and *Escherichia coli* NIHJ stubs incubated at 310 K, various concentrations (100 to 0 μg/μl) of cyclic peptide solution were added. In the case of **8**, minimum amount of DMSO was used to dissolve the peptide. The bacteria was further incubated at 310 K for 20 h with continuous shaking, then the growth of the bacteria was evaluated.

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References

1) For recent review, see: V. P. Kukhar', *J. Fluorine Chem.*, **69**, 199 (1994); "Fluorine-containing Amino Acids; Synthesis and Properties," ed by V. P. Kukhar' and V. A. Soloshonok, John Wiley & Sons, Chichester (1995).

2) M. R. C. Gerstenberger and A. Hass, *Angew. Chem., Int. Ed. Engl.*, **20**, 647 (1981); B. Badet, D. Roice, and C. T. Walsh, *Biochemistry*, **23**, 5188 (1984); M. H. Gelb, J. P. Svaren, and R. H. Abeles, *Biochemistry*, **24**, 1813 (1985).

3) T. Watanabe, A. Yamatodani, K. Maeyama, and H. Wada, *Trends Pharmacol. Sci.*, **11**, 363 (1990); R. B. Silverman and S. M. Nanavati, *J. Med. Chem.*, **33**, 931 (1990).

4) a) W. H. Vine, K.-h. Hsieh, and G. R. Marshall, *J. Med. Chem.*, **24**, 1043 (1981); b) K.-h. Hsieh, P. Needleman, and G. R. Marshall, *J. Med. Chem.*, **30**, 1097 (1987).

5) I. Ojima, K. Kato, K. Nakahashi, T. Fuchigami, and M. Fujita, *J. Org. Chem.*, **54**, 4511 (1989); I. Ojima, K. Kato, F. A. Jameison, J. Conway, K. Nakahashi, M. Hagiwara, T. Miyamae, and H. E. Radunz, *Bioorg. Med. Chem. Lett.*, **2**, 219 (1992).

6) T. Tsushima, K. Kawada, S. Ishihara, N. Uchida, O. Shiratori, J. Higaki, and M. Hirata, *Tetrahedron*, **44**, 5375 (1988).

7) H. Tanaka, F. Osakada, S.-i. Ohashi, M. Shiraki, and E. Munekata, *Chem. Lett.*, **1986**, 391; A. P. Campbell, J. Cachia, and B. D. Sykes, *Biochem. Cell Biol.*, **69**, 674 (1991); J. T. Gerig, *Prog. NMR Spectrosc.*, **26**, 293 (1994).

8) N. Nishino and H. Mihara, in "New Functionality Materials, Volume B Synthesis and Function Control of Biofunctionality Materials," ed by T. Tsuruta, M. Doyama, M. Seno, and Y. Imanishi, Elsevier Science Publishers B. V., Amsterdam (1993), pp. 147—152; S. F. Bets, D. P. Raleigh, and W. F. DeGrado, *Curr. Opin. Struct. Biol.*, **3**, 501 (1993); J. P. Schneider and J. W. Kelly, *Chem. Rev.*, **95**, 2169 (1995).

9) Abbreviations used are according to IUPAC-IUB Commissions, *Eur. J. Biochem.*, **138**, 9 (1984). Other abbreviations: GS, gramicidin S; Hfv, 4,4,4,4′, 4′, 4′-hexafluoro-L-valine; Boc, *t*-butoxycarbonyl ; Z, benzyloxycarbonyl ; BOP, benzotriazol-1-yloxytris (diethylamino)phosphonium hexafluorophosphate; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid; DIEA, *N,N*-diisopropylethylamine; CF, 5(6)-carboxyfluorescein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CD, circular dichroism.

10) N. Izumiya, Y. Kato, H. Aoyagi, M. Waki, and M. Kondo, "Synthetic Aspects of Biologically Active Cyclic Peptides-Gramicidin S and Tyrocidins," Kodansha, Tokyo (1979); G. Némethy and H. A. Sheraga, *Biochem. Biophys. Res. Commun.*, **118**, 643 (1984).

11) E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, *Anal. Biochem.*, **34**, 595 (1970).

12) C. Danzin, P. Bey, D. Schirlin, and N. Claveric, *Biochem. Pharmacol.*, **31**, 387 (1982).

13) a) G. Ösapay, A. Profit, and J. W. Taylor, *Tetrahedron Lett.*, **31**, 6121 (1990); b) M. Xu, N. Nishino, H. Mihara, T. Fujimoto, and N. Izumiya, *Chem. Lett.*, **1992**, 191; c) N. Nishino, M. Xu, H. Mihara, and T. Fujimoto, *Bull. Chem. Soc. Jpn.*, **65**, 991 (1992); d) N. Nishino, T. Arai, J. Hayashida, H. I. Ogawa, H. Yamamoto, and S. Yoshikawa, *Chem. Lett.*, **1994**, 2435.

14) T. Higashijima, T. Miyazawa, M. Kawai, and U. Nagai, *Biopolymers*, **25**, 2295 (1986); A. C. Bach, II, C. J. Eyermann, J. D. Gross, M. J. Bower, R. L. Harlow, P. C. Weber, and W. F. DeGrado, *J. Am. Chem. Soc.*, **116**, 3207 (1994).

15) K. Wüthrich, "NMR of Proteins and Nucleic Acids," John Wiley & Sons, N. Y. (1986).

16) H. Mihara, T. Kanmera, M. Toshida, S. Lee, H. Aoyagi, T. Kato, and N. Izumiya, *Bull. Chem. Soc. Jpn.*, **60**, 697 (1987); H. Mihara, N. Nishino, H. I. Ogawa, N. Izumiya, and T. Fujimoto, *Bull. Chem. Soc. Jpn.*, **65**, 228 (1992); S. Ando, H. Nishikawa, H. Takiguchi, S. Lee, and G. Sugihara, *Biochim. Biophys. Acta*, **1147**, 42 (1993).

17) N. Otsuji, T. Higashi, and J. Kawamata, *Biken J.*, **15**, 49 (1972).

18) Jpn. Patent JP59-157045 (1984).

19) B. F. Gisin, *Anal. Chim. Acta*, **58**, 248 (1972).

20) A. Tani, S. Lee, O. Oishi, H. Aoyagi, and M. Ono, *J. Biochem.*, **117**, 560 (1995).