Synthesis and NMR Studies of RGDSPASS-Containing Cystine Peptides

Yasuo YAMAMOTO and Shosuke SOFUKU*
Department of Chemistry, College of Science, Rikkyo University, Nishi-Ikebukuro, Toshima-ku, Tokyo 171
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Three types of Arg–Gly–Asp–Ser–Pro–Ala–Ser–Ser (RGDSPASS)-containing cystine peptides, FR-1, FR-2, and FR-3, were designed and synthesized. These cyclic peptides exhibited high activities as a platelet aggregation inhibitor. Using rabbit platelet-rich plasma, the IC_{50} values of FR-1, FR-2, and FR-3 were found to be 67, 280, and 135 μM, respectively. In each peptide, the position of the Pro residue was either i+1 or i+2 in the turn, as observed by NMR (ROESY, NOESY, and H–D exchange of amide proton).

The Arg–Gly–Asp–Ser (RGDS) sequence is an active site in the cell binding domain of fibronectin (FN), a cell adhesion protein.[1] Tripeptide RGD is essential as a cell adhesion minimum peptide. The RGD sequence also exists in some bioactive proteins and is predicted to be an important sequence for biological activities.[2] RGD-containing peptides have been shown to inhibit tumor progression in experimental animals[3] and platelet aggregation.[4] We have previously reported that the Pro–Ala–Ser–Ser (PASS) sequence, located adjacent to the RGDS sequence near the C-terminal of FN, also participates in cell binding and cell migration.[5]

Recently, some research groups have synthesized the following RGD-containing peptides: Arg–Gly–Asp–Phe,[6] *cyclo*-(–Arg–Gly–Asp–Ser–Lys–),[7] and Ac–Cys–Arg–Gly–Asp–Cys.[8] These peptides have been assayed in terms of their biological activities, but the preparation of PASS-containing oligopeptides has not been reported. The circumference of the FN-binding site has been expected to form a turn by the method of Chou and Fassman.[1] A conformational study of a RGD-containing protein was carried out by A. L. Main et al.[9] Their NMR (NOESY) and calculation (DIANA) studies showed that the RGD sequence was located in the F-G loop of the tenth type III module of FN. However, they could not identify the exact secondary structure of the circumference of the RGD sequence due to the flexibility of the segment.

We were interested in the secondary structure of the RGDSPASS sequence of FN. We designed 3 types of cyclic oligopeptides: FR-1, FR-2, and FR-3 (Fig. 1). These cyclic peptides seem to have different turn locations for the RGDS and the PASS sequences. The β-turn of a peptide is composed of 4 amino acid residues (i, i+1, i+2, and i+3). In our designed cyclic peptides, FR-1, FR-2, and FR-3, the position of the Pro residue was expected to be i+2, i+1, and i+3, respectively. Chou and Fassman have shown that the Pro residue is located with high probability at the i+1 position in the turn of a protein.[10] If our cyclic peptides, FR-1, FR-2, and FR-3, did not have a disulfide bond, the Pro residue would be located at the i+1 position in the turn. We placed the Cys residues at the end of the C- and N-terminals in the peptide in order to obtain the desired secondary structure (Fig. 1).

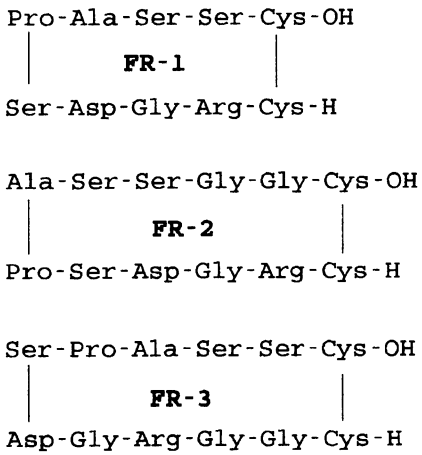


Fig. 1. Design of 3 types of RGDSPASS containing cystine peptides.

In the present study we describe the synthesis, biological activity, and secondary structure of FR-1, FR-2, and FR-3 and discuss the relationships between their secondary structures and biological activities.

H–Cys–Arg–Gly–Asp–Ser–Pro–
Ala–Ser–Ser–Cys–OH : FR – 1

H–Cys–Arg–Gly–Asp–Ser–Pro–
Ala–Ser–Ser–Gly–Gly–Cys–OH : FR – 2

H–Cys–Gly–Gly–Arg–Gly–Asp–
Ser–Pro–Ala–Ser–Ser–Cys–OH : FR – 3

Synthesis

All of the protected peptides were synthesized by the solution method with DCC–HOBt[11] except **4**, **6**, and **9** (Figs. 2, 3, and 4). The Boc group, Tce ester, and Bzl ester were deprotected by HCl/dioxane, Zn/AcOH, and H_2/Pd-C, respectively. The preparations of **4**, **6**, and **9** were carried out by fragment condensation with EDC–HOBt[11]

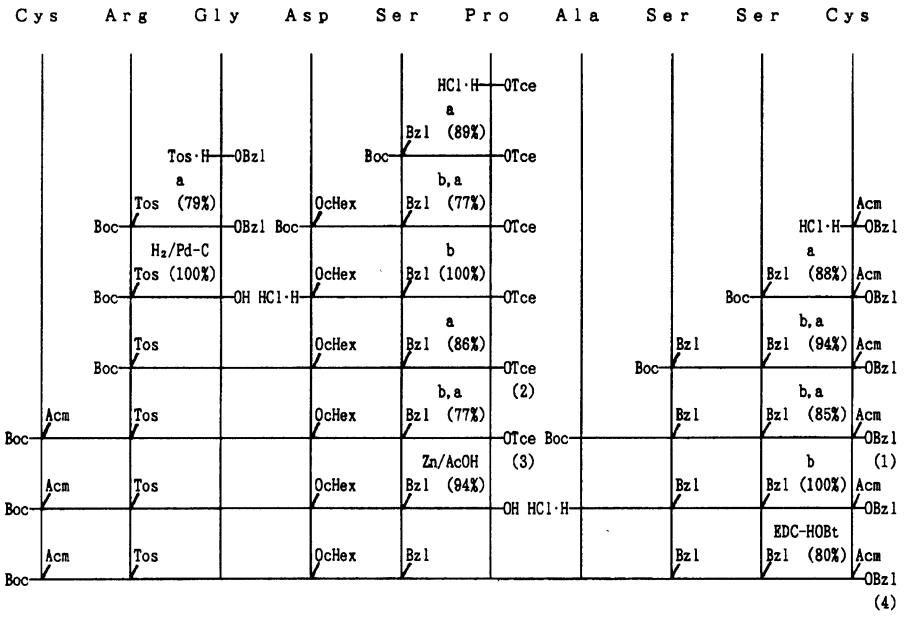


Fig. 2. Synthetic scheme of **4**. a DCC–HOBt; b 4 M HCl/dioxane.

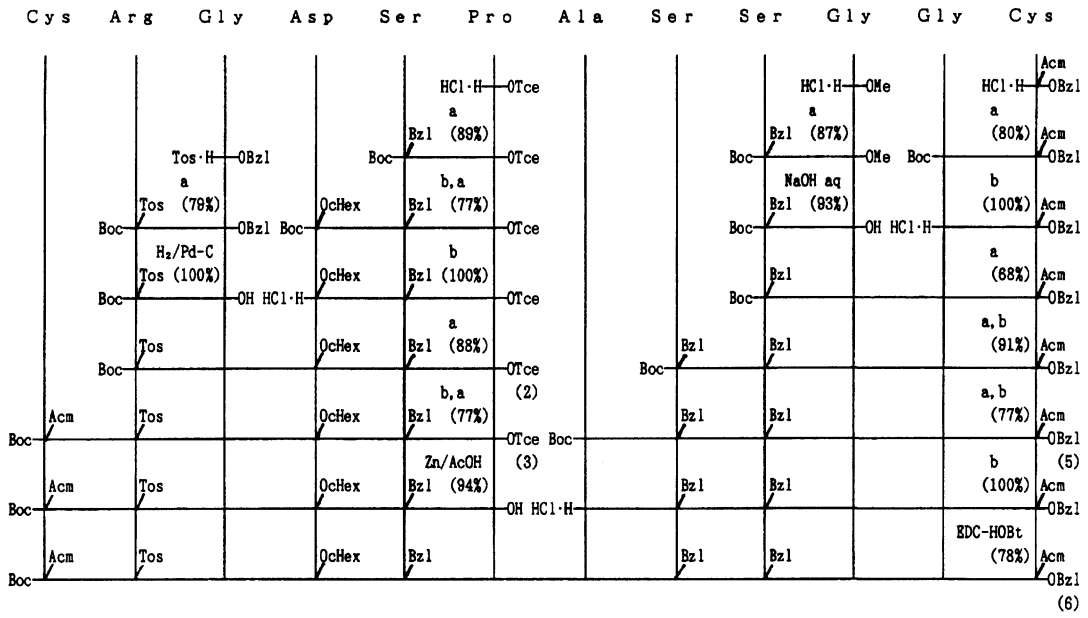


Fig. 3. Synthetic scheme of **6**. a DCC–HOBt; b 4 M HCl/dioxane.

(Figs. 2, 3, and 4). The protected peptides were purified by silica gel column chromatography, and confirmed by NMR and FABMS at each step. The protected deca- and dodecapeptedes, **4**, **6**, and **9**, were treated with liq. HF at 0 °C for 1 h in the presence of anisole. All of the protective groups except the Acm groups[11] were removed (Scheme 1). The formation of the intramolecular disulfide bond of these Acm peptides was not attained by thallium(III) trifluoroacetate (TTFA) oxidation[12] or by iodine oxidation. This bond formation was successfully completed by the silyl chloride-sulfoxide method[13] (Scheme 1). The cyclic peptides (FR-1, FR-2, and FR-3) were prepared in this way, but the yields were very low.[14] We sought a synthetic route for a higher yield of cystine peptides and tried to make the intramolecular disulfide bond from the protected peptide **9** by iodine oxidation. The protected peptides, **4** and **6**, were also oxidized to form an intramolecular disulfide bond using iodine and were confirmed by FABMS. However, the two protected cystine peptides (obtained from **4** and **6**) were not available in the quantity required for the following step; the protected cystine peptide obtained from **9** was treated with liq. HF at 0 °C for 1 h in the presence of anisole (Scheme 1). The resulting product had a yield of 42% from **9** and was confirmed by HPLC and NMR to be the same as authentic FR-3.

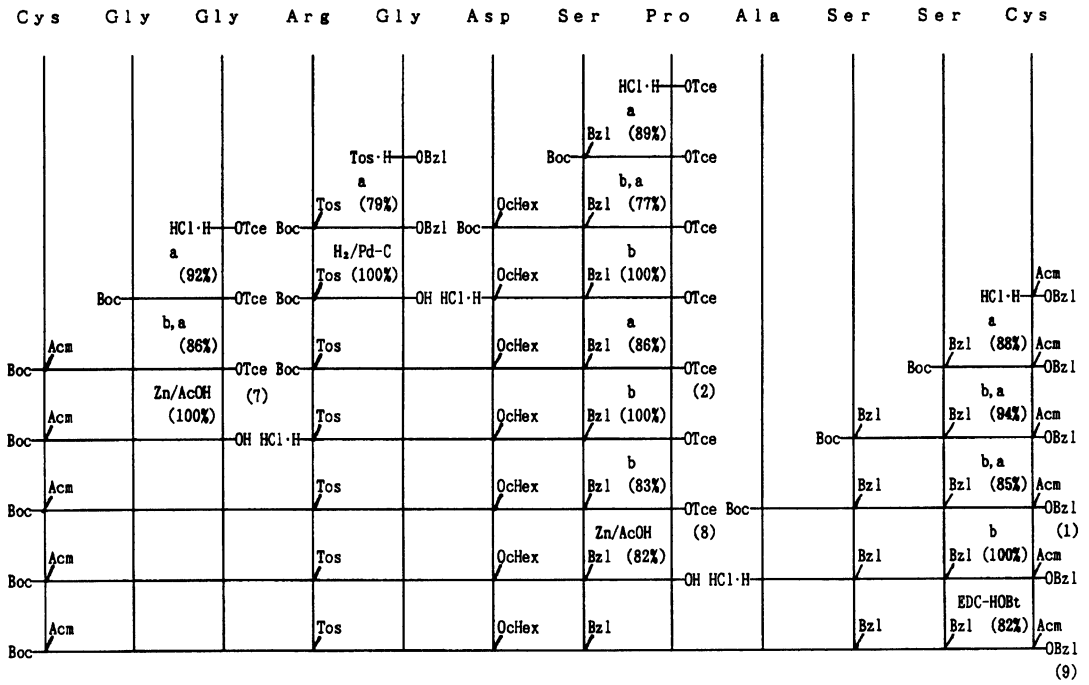


Fig. 4. Synthetic scheme of **9**. a DCC–HOBt; b 4 M HCl/dioxane.

4 Boc-Cys(Acm)-Arg(Tos)-Gly-Asp(OcHex)-Ser(Bzl)-Pro-Ala-Ser(Bzl)-Ser(Bzl)-Cys(Acm)-OBzl

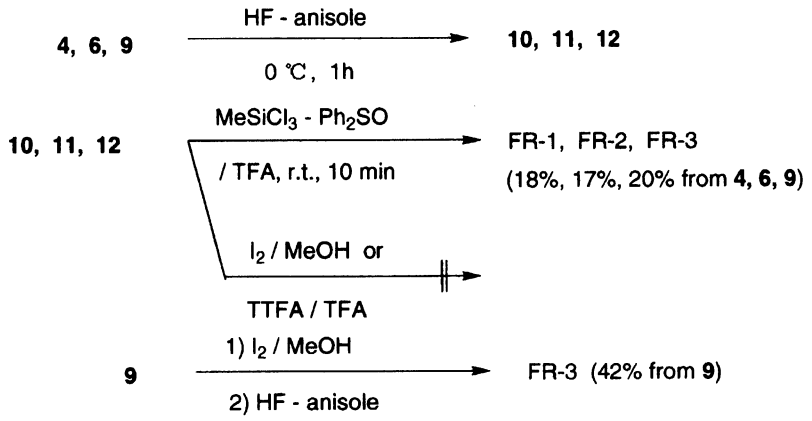
6 Boc-Cys(Acm)-Arg(Tos)-Gly-Asp(OcHex)-Ser(Bzl)-Pro-Ala-Ser(Bzl)-Ser(Bzl)-Gly-Gly-Cys(Acm)-OBzl

9 Boc-Cys(Acm)-Gly-Gly-Arg(Tos)-Gly-Asp(OcHex)-Ser(Bzl)-Pro-Ala-Ser(Bzl)-Ser(Bzl)-Cys(Acm)-OBzl

10 H-Cys(Acm)-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Cys(Acm)-OH

11 H-Cys(Acm)-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Gly-Gly-Cys(Acm)-OH

12 H-Cys(Acm)-Gly-Gly-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Cys(Acm)-OH



Scheme 1.

Platelet Aggregation Assay[15]

Platelet aggregation was measured by an aggregation analyzer T-500 (Erma Inc.). Reaction mixtures consisting of rabbit platelet-rich plasma (200 μl) and various concentrations of sample peptides (10 μl) were prepared. The aggregation was initiated by the addition of 10 $\mu g\,ml^{-1}$ of collagen (10 μl) to the above mixtures. The turbidity was measured by the analyzer at room temperature.

NMR Measurements

NMR measurements were performed with a FT-NMR spectrometer, JEOL-JNM GSX 400 (^1H, 400 MHz). The samples (7—8 $mmol\,dm^{-3}$) contained ca. 6—8 mg of peptides in [$^2H_6$]DMSO (dimethyl sulfoxide) (0.6 ml). Proton chemical shifts were referenced internally to residual [$^2H_5$]DMSO at 2.50 ppm. Assignments were

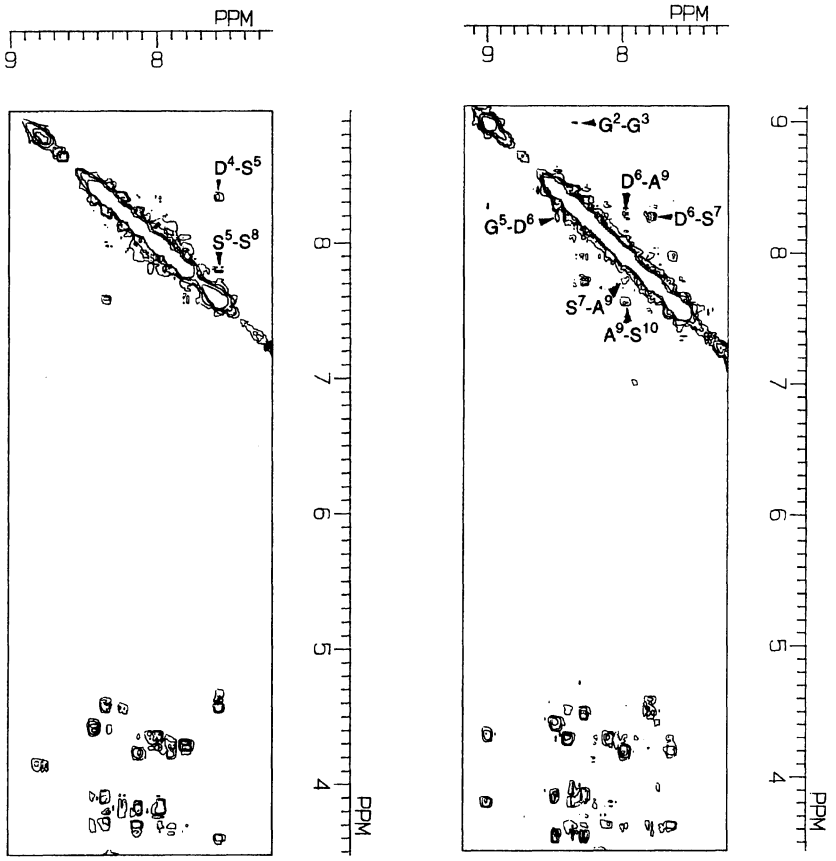


Fig. 5. NOESY spectra of (a) FR-2 and (b) FR-3 in DMSO-d_6 at 27 °C, T_{mix}=400 ms.

made using COSY, ROESY[16] with mixing times of 150 and 250 ms, and NOESY[17] with mixing times of 250 and 400 ms at 27 °C. Conformational studies were made using NOESY with mixing times of 250 and 400 ms at 27 °C and the H–D exchange of amide protons with CD_3OD (0.1 ml) at 27 °C was determined. Two conformers of each cyclic peptide were confirmed by NMR and HPLC; in the present study we discuss the major conformer.

Results and Discussion

Table 1 shows the biological activities of **10**, **11**, **12**, FR-1, FR-2, and FR-3. RGD-containing peptides are known to inhibit FN binding to platelets[18] and FN also inhibits platelet aggregation.[19] Under the same conditions as the compounds in Table 1, RGDS (1 mM, M=mol dm^{-3}) and PASS (1 mM) showed an inhibitory activity of less than 10%. On the other hand, the cyclic peptides, FR-1 (IC_{50}=67 μM), FR-2 (IC_{50}=280 μM), and FR-3 (IC_{50}=135 μM), exhibited higher activity as platelet aggregation inhibitors than their linear peptides. The difference in IC_{50} values indicates the difference in the secondary structures of RGDSPASS in each peptide. These results indicate that FR-1 has a high affinity for fibrinogen receptor on the platelet surface.

Table 1. Inhibition of Platelet Aggregation in Rabbit Platelet-Rich Plasma[a]

Compound	IC_{50} (μM)
10	740
11	760
12	510
FR-1	67
FR-2	280
FR-3	135

a) Platelet aggregation was induced by collagen (10 μg cm^{-3}).

The chemical shift assignments were obtained by the combined use of COSY, ROESY, and NOESY experiments; the data concerning the amide protons are shown in Table 2. By increasing the mixing time in the ROESY measurements, the amide proton signals collapsed. The H–D exchange rate of amide protons exhibited an obvious difference with each amino acid as follows — FR-1: 2-Arg (NH), 10-Cys (NH) (fast)>5-Ser (NH), 8-Ser (NH), 9-Ser (NH) (medium)>3-Gly (NH), 4-Asp (NH), 7-Ala (NH) (slow); FR-2: 2-Arg (NH), 11-Gly (NH), 12-Cys (NH)>4-Asp (NH), 5-Ser (NH), 7-Ala (NH), 10-Gly (NH)>3-Gly (NH), 8-Ser (NH), 9-Ser (NH); FR-3: 2-Gly (NH), 3-Gly (NH), 7-Ser (NH), 12-Cys (NH)>10-Ser (NH), 11-Ser (NH)>4-Arg (NH),

Table 2. Chemical Shift and H–D Exchange Rate of Amide Proton, and Coupling Constant[a)]

	FR-1			FR-2			FR-3		
	NH	D/H	J_{NH-CH}	NH	D/H	J_{NH-CH}	NH	D/H	J_{NH-CH}
Cys	—	—	—	—	—	—	—	—	—
Gly							9.04	f	B
Gly							8.36	f	B
Arg	8.74	f	8.0	8.82	f	B	7.75	s	B
Gly	8.35	s	A	8.44	s	B	8.52	s	B
Asp	8.33	s	A	8.35	m	8.1	8.30	s	7.3
Ser	7.58	m	7.3	7.57	m	7.3	7.83	f	7.7
Pro	—	—	—	—	—	—	—	—	—
Ala	7.88	s	7.0	8.01	m	A	8.02	s	7.0
Ser	7.63	m	7.0	7.80	s	7.3	7.65	m	7.3
Ser	7.75	m	7.7	7.91	s	7.0	8.06	m	A
Gly				8.13	m	B			
Gly				7.95	f	A			
Cys	8.16	f	8.1	8.22	f	7.2	8.41	f	7.4

a) Measurements were carried out by ^1H 400MHz NMR spectrometer in [$^2H_6$] DMSO at 300 K; NH chemical shift (δ); H–D exchange rate (D/H): f=fast, m=medium, s=slow; J_{NH-CH} coupling constant (Hz), A: overlapped with other peak, B: broad.

5-Gly (NH), 6-Asp (NH), 9-Ala (NH) (Table 2). A slow H–D exchange rate indicates that the amide proton is shielded from the solvent. In FR-2, 8-Ser (NH) was shielded from the solvent and might be involved in intramolecular hydrogen bonding. In FR-3, 6-Asp (NH) and 9-Ala (NH) were shielded from the solvent and might be involved with intramolecular hydrogen bonds.

No nonsequential (spatial) ROEs were observed in FR-1, FR-2, or FR-3. Spatial NOEs were observed between 5-Ser (NH) and 8-Ser (NH) of FR-2 (Fig. 5a), 6-Asp (NH) and 9-Ala (NH), and 7-Ser (NH) and 9-Ala (NH) of FR-3 (Fig. 5b).[20)] None of the peptides showed a NOE between the α proton (i+1) and the amide proton (i+3) of the typical type-I and type-II β-turns in DMSO. However, it was predicted that in the former, 5-Ser (NH) would be close to 8-Ser (NH), and in the latter, 6-Asp (NH) and 7-Ser (NH) would be close to 9-Ala (NH) (Fig. 6). These results indicate that these cyclic peptides do not form the typical β-turn in DMSO but that the Ser–Pro–Ala–Ser in FR-2 and the Asp–Ser–Pro–Ala in FR-3 each form a turn (Fig. 6).[20)] FR-1 did not show any spatial NOEs. However, the sequential ROEs and NOEs between αH(i) and NH-(i+1) were observed in all of the residues except for the Pro residue. A NOE between 5-Ser (αH) and 6-Pro (δH) was also observed. This indicates that FR-1 has an antiparallel pleated sheet structure and that this cyclic peptide bends at the Ser–Pro position. In addition, the H–D exchange rates of the amide protons of FR-1 were similar to those of FR-3 (Table 2). From the NMR data we concluded that the turn structure of FR-1 is similar to that of FR-3. The H–D exchange rates of the amide protons in the vicinity of the C- and N-terminal amino acid residues in FR-2 and FR-3 were fast. FR-1 forms a turn at the Asp–Ser–Pro–Ala and a fast H–D exchange of amide protons occurs in the C-terminal and N-terminal amino acid residues in FR-1 (Table 2). This presupposition also suggests that the turn structure of FR-1 is similar to that of FR-3. These observations suggest that the backbone in the circumference of the C- and N-terminals is flexible and expanded and that the center of the turn is rigid and slim.

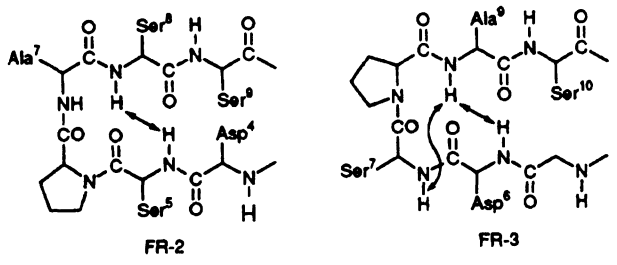


Fig. 6. Turn structures and NOEs of FR-2 and FR-3. Arrows indicate the protons giving NOEs by NOESY.

The turn structures of FR-1 and FR-2 were retained as designed (the position of the Pro residue in the turn was at i+2 and i+1, respectively) but the turn structure of FR-3 was not retained as designed (Figs. 1 and 6). The few possibility of the Pro residue being located at the i or i+3 position in the turn of the FN cell adhesion site was suggested by these results. It should be noted that both RGDS and PASS have an antiparallel location in the cyclic peptide, FR-1, which exhibited high activity as a platelet aggregation inhibitor. It is well-known that the RGD(S) plays an important role in the binding to receptors. Previously, we reported that the binding of exogenous FN to the primary mesenchyme cells (PMCs) of sea urchins was inhibited by synthetic tetrapeptide, PASS, but not by RGDS.[5)] However, FN-promoted PMC migration was inhibited by both PASS and RGDS. These results suggest that the PASS se-

quence is a novel PMC surface interaction site in FN.[5] We expect that the Pro residue in the PASS plays an important role in the fixing or tightening of the turn structure for binding to fibrinogen receptor on the cell surface.

The fibrinogen receptor on the platelet surface is able to recognize the unique structure of RGDSPASS. These results suggest that the structure of FR-1 is appropriate for binding to the fibrinogen receptor and that the structure corresponds to that of the cell binding active site of FN. NMR measurements of the aqueous solution of these cyclic peptides are currently in progress.

Experimental

All the melting points were measured on a Mel-Temp apparatus and are uncorrected. ^{1}H NMR spectra were measured on a JEOL-JNM GSX 400 (^{1}H, 400 MHz) spectrometer using tetramethylsilane as an internal standard. Mass spectra were measured on a JEOL JMS-SX mass spectrometer operating under FAB conditions. Optical rotations were measured on a JASCO DIP-370. Amino acid analyses were obtained on a Hitachi L-8500 amino acid analyzer, after hydrolysis of samples in 6 M HCl at 110 °C for 24 h. TLC was performed on Merck silica gel F254 plates using UV light and/or 5% molybdophosphoric acid in ethanol for detection. Merck silica gel (silica gel 60) was used for column chromatography. Platelet aggregation was measured on a T-500 (Erma Inc.).

Boc–Ala–Ser(Bzl)–Ser(Bzl)–Cys(Acm)–OBzl (1). Boc–Ser(Bzl)–Ser(Bzl)–Cys(Acm)–OBzl (9.00 g, 12.1 mmol) was treated with 4 M HCl/dioxane (60.5 ml) for 1 h at room temperature. The reaction mixture was concentrated and the residue was dried over NaOH pellets in vacuo. This residue, Boc–Ala–OH (2.51 g, 13.3 mmol), and HOBt (1.96 g, 14.5 mmol) were dissolved in DMF (35 ml) and this mixture was cooled in an ice bath. After the addition of NMM (1.33 ml, 11.2 mmol) and DCC (2.99 g, 14.5 mmol) at 0 °C, the reaction mixture was stirred for 2 h at 0 °C and overnight at room temperature. The reaction mixture was filtered to remove dicyclohexylurea. Ethyl acetate was added to the filtrate, and the organic layer was washed with 0.5 M HCl, 5% $NaHCO_3$ aq, and saturated NaCl aq. The organic layer was dried over anhydrous sodium sulfate and then evaporated. This coupling procedure is defined as method A. The crude peptide was purified by silica-gel column chromatography ($CHCl_3$–MeOH=25 : 1) to give **1** (8.32 g, 10.3 mmol) as crystals: Yield of **1**, 85%; mp 123—124 °C; $[\alpha]_D^{23}$ −29.7 (*c* 1.16, MeOH); ^{1}H NMR (CD_3OD) δ=1.33 (3H, d, *J*=7.3 Hz), 1.44 (9H, s), 1.95 (3H, s), 2.89 (1H, dd, *J*=14.5, 8.1 Hz), 3.01 (1H, dd, *J*=14.1, 4.8 Hz), 3.73—3.78 (2H, complex), 3.79—3.85 (2H, complex), 4.13 (1H, m), 4.23—4.29 (2H, complex), 4.47—4.58 (5H, complex), 4.69—4.77 (2H, complex), 5.18 (2H, s), 7.24—7.38 (15H, complex); FABMS *m/z* 808 (MH^+). Found: C, 60.72; H, 6.74; N, 8.53%. Calcd for $C_{41}H_{53}N_5O_{10}S$: C, 60.95; H, 6.61; N, 8.67%.

Boc–Arg(Tos)–Gly–Asp(OcHex)–Ser(Bzl)–Pro–OTce (2). Boc–Asp(OcHex)–Ser(Bzl)–Pro–OTce (14.4 g, 20.0 mmol) was treated with 4 M HCl/dioxane (100 ml) for 1 h at room temperature. The reaction mixture was concentrated and the residue was dried over NaOH pellets in vacuo. This residue and Boc–Arg(Tos)–Gly–OH (9.73 g, 20.0 mmol) were coupled by method A with HOBt (2.97 g, 22.0 mmol), NMM (2.20 ml, 20.0 mmol), and DCC (4.54 g, 22.0 mmol). The crude peptide was purified by silica-gel column chromatography ($CHCl_3$–MeOH=25 : 1) to give **2** (15.9 g, 14.6 mmol) as foam: Yield of **2**, 73%; $[\alpha]_D^{23}$ −29.5 (*c* 1.17, MeOH); ^{1}H NMR (CD_3OD) δ=1.32—1.52 (4H, complex), 1.48 (9H, s), 1.53—1.67 (4H, complex), 1.71—1.85 (6H, complex), 2.07—2.15 (3H, complex), 2.31 (1H, m), 2.41 (3H, s), 2.74 (1H, dd, *J*=16.7, 7.7 Hz), 2.93 (1H, dd, *J*=15.9, 5.9 Hz), 3.20 (2H, br), 3.68—3.83 (6H, complex), 4.04 (1H, m), 4.50—4.62 (3H, complex), 4.76 (1H, m), 4.80—4.97 (4H, complex), 7.29—7.40 (7H, complex), 7.77 (2H, d, *J*=8.1 Hz); FABMS *m/z* 1089 (MH^++2). Found: C, 49.48; H, 6.28; N, 9.92%. Calcd for $C_{47}H_{65}N_8O_{13}S\cdot 3H_2O$: C, 49.41; H, 6.26; N, 9.81%.

Boc–Cys(Acm)–Arg(Tos)–Gly–Asp(OcHex)–Ser(Bzl)–Pro–OTce (3). Pentapeptide **2** (3.50 g, 3.20 mmol) was treated with 4 M HCl/dioxane (16 ml) for 1 h at room temperature. The reaction mixture was concentrated and the residue was dried over NaOH pellets in vacuo. This residue and Boc–Cys(Acm)–OH (935 mg, 3.20 mmol) were coupled by method A with HOBt (475 mg, 3.52 mmol), NMM (352 µl, 3.20 mmol), and DCC (727 mg, 3.52 mmol). The crude peptide was purified by silica-gel column chromatography ($CHCl_3$–MeOH=25 : 1) to give **3** (3.08 g, 2.45 mmol) as foam: Yield of **3**, 77%; $[\alpha]_D^{24}$ −29.2 (*c* 1.08, MeOH); ^{1}H NMR (CD_3OD) δ=1.31—1.52 (4H, complex), 1.47 (9H, s), 1.52—1.67 (4H, complex), 1.67—1.89 (6H, complex), 2.01 (3H, s), 2.04—2.23 (3H, complex), 2.32 (1H, m), 2.43 (3H, s), 2.75—2.83 (2H, complex), 2.93 (1H, dd, *J*=15.9, 6.1 Hz), 3.04 (1H, dd, *J*=16.0, 5.7 Hz), 3.20 (2H, s), 3.70—3.92 (6H, complex), 4.25—4.33 (2H, complex), 4.42 (1H, m), 4.48—4.66 (5H, complex), 4.76—4.95 (5H, complex), 7.29—7.40 (7H, complex), 7.78 (2H, d, *J*=8.0 Hz); FABMS *m/z* 1263 (MH^++2). Found: C, 47.79; H, 6.01; N, 10.53%. Calcd for $C_{53}H_{75}N_{10}O_{15}S_2Cl_3\cdot 5.5H_2O$: C, 47.87; H, 6.52; N, 10.53%.

Boc–Cys(Acm)–Arg(Tos)–Gly–Asp(OcHex)–Ser(Bzl)–Pro–Ala–Ser(Bzl)–Ser(Bzl)–Cys(Acm)–OBzl (4). To a solution of **3** (2.00 g, 1.59 mmol) in AcOH (25 ml)–H_2O (1.5 ml), zinc powder was added. The mixture was stirred for 1.5 h at room temperature and after removal of the precipitate, the filtrate was evaporated. This residue was dissolved in chloroform and the solution was washed with 0.5 M HCl and saturated NaCl aq, and dried over anhydrous sodium sulfate. After concentration of the solution, the residue was dried over NaOH pellets, concd H_2SO_4, and $CaCl_2$ in vacuo at room temperature. Yield of Boc–Cys(Acm)–Arg(Tos)–Gly–Asp(OcHex)–Ser(Bzl)–Pro–OH (1.70 g, 1.50 mmol) was 94%.

Tetrapeptide **1** (727 mg, 900 µmol) was treated with 4 M HCl/dioxane (5 ml) for 20 min at room temperature. The reaction mixture was concentrated and the residue was dried over NaOH pellets in vacuo. This residue, Boc–Cys(Acm)–Arg(Tos)–Gly–Asp(OcHex)–Ser(Bzl)–Pro–OH (850 mg, 750 µmol), and HOBt (122 mg, 900 µmol) were dissolved in DMF (4 ml) and the solution was cooled in an ice bath. After the additions of NMM (99 µl, 900 µmol) and EDC·HCl (172 mg, 900 µmol) at 0 °C, this solution was stirred for 3 h at 0 °C and overnight at room temperature. Chloroform was added to the reaction mixture and the solution was washed in the routine manner, dried over anhydrous

sodium sulfate, and then evaporated. This coupling method is defined as method B. The crude peptide was purified by silica-gel column chromatography ($CHCl_3$–MeOH=10 : 1) to give **4** (1.11 g, 610 μmol): Yield of **4**, 81%; $[\alpha]_D^{25}$ −28.2 (*c* 1.0, DMF); FABMS *m/z* 1820 (MH^+). Found: C, 56.41; H, 6.38; N, 11.19%. Calcd for $C_{87}H_{117}N_{15}O_{22}S_3 \cdot 1.5H_2O$: C, 56.54; H, 6.54; N, 11.37%.

Boc–Ala–Ser(Bzl)–Ser(Bzl)–Gly–Gly–Cys(Acm)–OBzl (5). Boc–Ser(Bzl)–Ser(Bzl)–Gly–Gly–Cys(Acm)–OBzl (4.20 g, 4.94 mmol) was treated with 4 M HCl/dioxane (25.0 ml) for 40 min at room temperature. The reaction mixture was concentrated and the residue was dried over NaOH pellets in vacuo. This residue and Boc–Ala–OH (945 mg, 5.00 mmol) were coupled by method A with HOBt (743 mg, 5.50 mmol), NMM (550 μl, 5.00 mmol), and DCC (1.13 g, 5.50 mmol). The crude peptide was purified by silica-gel column chromatography ($CHCl_3$–MeOH=15 : 1) to give **5** (3.51 g, 3.81 mmol) as crystals: Yield of **5**, 77%; mp 137—140 °C (decomp); $[\alpha]_D^{24}$ −24.6 (*c* 1.00, MeOH); ^{1}H NMR (CD_3OD) δ=1.34 (3H, d, *J*=7.0 Hz), 1.44 (9H, s), 1.95 (3H, s), 2.95 (1H, dd, *J*=13.1, 8.8 Hz), 3.11 (1H, dd, *J*=9.7, 4.8 Hz), 3.61—3.87 (6H, complex), 3.95 (2H, complex), 4.15 (1H, m), 4.31—4.38 (2H, complex), 4.50—4.63 (6H, complex), 4.76 (1H, m), 5.20 (2H, s), 7.28—7.41 (15H, complex); FABMS *m/z* 922 (MH^+). Found: C, 34.82; H, 4.74; N, 9.22%. Calcd for $C_{45}H_{59}N_7O_{12}S \cdot 3H_2O$: C, 34.46; H, 4.60; N, 9.46%.

Boc–Cys(Acm)–Arg(Tos)–Gly–Asp(OcHex)–Ser(Bzl)–Pro–Ala–Ser(Bzl)–Ser(Bzl)–Gly–Gly–Cys(Acm)–OBzl (6). To a solution of **3** (2.00 g, 1.59 mmol) in AcOH (25 ml)–H_2O (1.5 ml), zinc powder was added. The mixture was stirred for 1.5 h at room temperature and, after removal of the precipitate, the filtrate was evaporated. This residue was dissolved in chloroform and the solution was washed with 0.5 M HCl and with saturated NaCl aq, and then dried over anhydrous sodium sulfate. After concentration of the solution, the crude peptide was dried over NaOH pellets, concd H_2SO_4, and $CaCl_2$ in vacuo at room temperature. Yield of Boc–Cys(Acm)–Arg(Tos)–Gly–Asp(OcHex)–Ser(Bzl)–Pro–OH (1.70 g, 1.50 mmol) was 94%.

Hexapeptide **5** (460 mg, 500 μmol) was treated with 4 M HCl/dioxane (3 ml) for 20 min at room temperature. The reaction mixture was concentrated and the residue was dried over NaOH pellets in vacuo. This residue and Boc–Cys(Acm)–Arg(Tos)–Gly–Asp(OcHex)–Ser(Bzl)–Pro–OH (537 mg, 474 μmol) were coupled by method B with HOBt (64 mg, 474 μmol), NMM (55 μl, 474 μmol), and EDC·HCl (96 mg, 500 μmol). The crude peptide was purified by silica-gel column chromatography ($CHCl_3$–MeOH=8 : 1) to give **6** (566 mg, 370 μmol): Yield of **6**, 78%; $[\alpha]_D^{24}$ −26.3 (*c* 0.99, DMF); FABMS *m/z* 1934 (MH^+). Found: C, 54.28; H, 6.79; N, 11.48%. Calcd for $C_{91}H_{123}N_{17}O_{24}S_3 \cdot 4H_2O$: C, 54.45; H, 6.58; N, 11.86%.

Boc–Cys(Acm)–Gly–Gly–OTce (7). Boc–Gly–Gly–OTce (1.82 g, 5.00 mmol) was treated with 4 M HCl/dioxane (25.0 ml) for 40 min at room temperature. The reaction mixture was concentrated and the residue was dried over NaOH pellets in vacuo. This residue and Boc–Cys(Acm)–OH (1.51 g, 5.50 mmol) were coupled by method A with HOBt (1.02 g, 5.50 mmol), NMM (550 μl, 5.00 mmol), and DCC (1.13 g, 5.50 mmol). The crude peptide was purified by silica-gel column chromatography ($CHCl_3$–MeOH=20 : 1) to give **7** (2.25 g, 4.19 mmol) as foam: Yield of **7**, 84%; $[\alpha]_D^{25}$ −12.4 (*c* 1.05, MeOH); ^{1}H NMR ($CDCl_3$) δ=1.41 (9H, s), 2.03 (3H, s), 2.89 (1H, dd, *J*=15.2, 7.3 Hz), 2.98 (1H, dd, *J*=14.3, 5.1 Hz), 3.97—4.09 (2H, complex), 4.18 (2H, d, *J*=5.9 Hz), 4.30 (1H, dd, *J*=13.9, 5.9 Hz), 4.43 (1H, m), 4.52 (1H, dd, *J*=13.9, 6.6 Hz), 4.79 (2H, s), 5.81 (1H, d, *J*=7.3 Hz), 7.08 (1H, bs), 7.39 (1H, bs), 7.59 (1H, d, *J*=5.9 Hz); FABMS *m/z* 539 (MH^++2H). Found: C, 34.83; H, 4.74; N, 9.22%. Calcd for $C_{17}H_{27}N_4O_7SCl_3 \cdot 3H_2O$: C, 34.46; H, 4.60; N, 9.46%.

Boc–Cys(Acm)–Gly–Gly–Arg(Tos)–Gly–Asp(OcHex)–Ser(Bzl)–Pro–OTce (8). To a solution of **7** (1.63 g, 3.00 mmol) in AcOH (54 ml)–H_2O (6.0 ml), zinc powder was added. The mixture was stirred for 3 h at room temperature and after removal of the precipitate, the filtrate was evaporated. This residue was dissolved in chloroform and the solution was washed with 0.5 M HCl and saturated NaCl aq, and then dried over anhydrous sodium sulfate. After concentration of the solution, the crude peptide was dried over NaOH pellets, concd H_2SO_4, and $CaCl_2$ in vacuo at room temperature. Yield of Boc–Cys(Acm)–Gly–Gly–OH was theoretical.

Pentapeptide **2** (1.63 g, 1.50 mmol) was treated with 4 M HCl/dioxane (7.5 ml) for 20 min at room temperature. The reaction mixture was concentrated and the residue was dried over NaOH pellets in vacuo. This residue and Boc–Cys(Acm)–Gly–Gly–OH (771 mg, 1.90 mmol) were coupled by method B with HOBt (259 mg, 1.90 mmol), NMM (165 μl, 1.50 mmol), and EDC·HCl (363 mg, 1.90 mmol). The crude peptide was purified by silica-gel column chromatography ($CHCl_3$–MeOH=8 : 1) to give **8** (1.63 g, 1.17 mmol): Yield of **8**, 78%; $[\alpha]_D^{24}$ −26.8 (*c* 0.63, MeOH); ^{1}H NMR (DMSO-d_6) δ=1.18—1.57 (8H, complex), 1.37 (9H, s), 1.57—1.77 (6H, complex), 1.84 (3H, s), 1.92—1.99 (3H, complex), 2.23 (1H, m), 2.33 (3H, s), 2.50 (DMSO peak, overlapped with 1H), 2.64—2.72 (2H, complex), 2.95 (1H, dd, *J*=13.8, 4.4 Hz), 3.03 (2H, bs), 3.55—3.62 (2H, complex), 3.64—3.79 (8H, complex), 4.14—4.25 (4H, complex), 4.46 (1H, dd, *J*=10.1, 3.6 Hz), 4.50 (2H, s), 4.60—4.78 (3H, complex), 4.86 (2H, s), 6.69 (2H, br), 6.99 (1H, d, *J*=8.4 Hz), 7.08 (1H, bs), 7.20 (1H, br), 7.27 (1H, d, *J*=8.0 Hz), 7.31—7.34 (5H, complex), 7.62 (1H, d, *J*=8.1 Hz), 7.39 (1H, bs), 7.59 (1H, d, *J*=5.9 Hz), 8.10 (2H, br), 8.17 (1H, d, *J*=8.1 Hz), 8.21 (1H, d, *J*=7.7 Hz), 8.29 (2H, br), 8.62 (1H, br); FABMS *m/z* 1399 (MNa^++2H). Found: C, 34.83; H, 4.74; N, 9.22%. Calcd for $C_{17}H_{27}N_4O_7SCl_3 \cdot 3H_2O$: C, 34.46; H, 4.60; N, 9.46%.

Boc–Cys(Acm)–Gly–Gly–Arg(Tos)–Gly–Asp(OcHex)–Ser(Bzl)–Pro–Ala–Ser(Bzl)–Ser(Bzl)–Cys(Acm)–OBzl (9). To a solution of **8** (1.13 g, 822 μmol) in AcOH (15 ml)–H_2O (1.5 ml), zinc powder was added. The mixture was stirred for 3 h at room temperature and, after removal of the precipitate, the filtrate was evaporated. This residue was dissolved in chloroform and the solution was washed with 0.5 M HCl and saturated NaCl aq, and dried over anhydrous sodium sulfate. After concentration of the solution, the residue was dried over NaOH pellets, concd H_2SO_4, and $CaCl_2$ in vacuo at room temperature. Yield of Boc–Cys(Acm)–Gly–Gly–Arg(Tos)–Gly–Asp(OcHex)–Ser(Bzl)–Pro–OH was 82%.

Tetrapeptide **1** (690 mg, 800 μmol) was treated with 4 M HCl/dioxane (3.0 ml) for 50 min at room temperature. The reaction mixture was concentrated and the residue was dried over NaOH pellets in vacuo. This residue

and Boc–Cys(Acm)–Gly–Gly–Arg(Tos)–Gly–Asp(OcHex)–Ser(Bzl)–Pro–OH (840 mg, 675 μmol) were coupled by method B with HOBt (108 mg, 800 μmol), NMM (88 μl, 800 μmol), and EDC·HCl (153 mg, 800 μmol). The crude peptide was purified by silica-gel column chromatography ($CHCl_3$–MeOH=10:1) to give **9** (1.08 g, 559 μmol): Yield of **9**, 83%; $[\alpha]_D^{25}$ −32.6 (*c* 1.06, DMF); FABMS *m/z* 1934 (MH^+). Found: C, 56.19; H, 6.61; N, 11.58%. Calcd for $C_{91}H_{123}N_{17}O_{24}S_3 \cdot 1.5H_2O$: C, 55.70; H, 6.47; N, 12.13%.

H–Cys(Acm)–Arg–Gly–Asp–Ser–Pro–Ala–Ser–Ser–Cys(Acm)–OH (10). Decapeptide **4** (500 mg, 270 μmol) was treated with liq. HF (30 ml) in the presence of anisole (1.0 ml) for 1 h at 0 °C. HF was removed and the residue was dried over NaOH pellets in vacuo at room temperature. The residue was dissolved in 10% AcOH, and the aqueous solution was washed with diethyl ether and then lyophilized. The crude product (343 mg) was obtained. For measurement of mass spectra, elemental analysis, and biological assay, this crude peptide (20 mg) was purified by HPLC (Solvent: 6% MeCN–2% MeOH–0.1% TFA aq; Column: Biofine RPC-SC 18L, 10×250 mm) to give **10** (14.8 mg): FABMS *m/z* 1124 (MH^+). Found: C, 37.57; H, 5.08; N, 14.22%. Calcd for $C_{41}H_{69}N_{15}O_{18}S_2 \cdot 3TFA \cdot 1.5H_2O$: C, 37.80; H, 5.06; N, 14.07%.

H–Cys(Acm)–Arg–Gly–Asp–Ser–Pro–Ala–Ser–Ser–Gly–Gly–Cys(Acm)–OH (11). Dodecapeptide **6** (400 mg, 199 μmol) was treated with liq. HF (30 ml) in the presence of anisole (1.0 ml) for 1 h at 0 °C. HF was removed and the residue was dried over NaOH pellets in vacuo at room temperature. The residue was dissolved in 10% AcOH, and the aqueous solution was washed with diethyl ether and then lyophilized. The crude product (301 mg) was obtained. For measurement of mass spectra, elemental analysis, and biological assay, this crude peptide (20 mg) was purified by HPLC (Solvent: 6% MeCN–2% MeOH–0.1% TFA aq; Column: Biofine RPC-SC 18L, 10×250 mm) to give **11** (15.3 mg): FABMS *m/z* 1238 (MH^+). Found: C, 35.87; H, 4.46; N, 13.13%. Calcd for $C_{45}H_{75}N_{17}O_{20}S_2 \cdot 4TFA \cdot 4H_2O$: C, 36.04; H, 4.96; N, 13.48%.

H–Cys(Acm)–Gly–Gly–Arg–Gly–Asp–Ser–Pro–Ala–Ser–Ser–Cys(Acm)–OH (12). Dodecapeptide **9** (400 mg, 199 μmol) was treated with liq. HF (30 ml) in the presence of anisole (1.0 ml) for 1 h at 0 °C. HF was removed and the residue was dried over NaOH pellets in vacuo at room temperature. The residue was dissolved in 10% AcOH, and the aqueous solution was washed with diethyl ether and then lyophilized. The crude product (423 mg) was obtained. For measurement of mass spectra, elemental analysis, and biological assay, this crude peptide (20 mg) was purified by HPLC (Solvent: 6% MeCN–2% MeOH–0.1% TFA aq; Column: Biofine RPC-SC 18L, 10×250 mm) to give **12** (15.0 mg): FABMS *m/z* 1238 (MH^+). Found: C, 35.60; H, 4.91; N, 13.84%. Calcd for $C_{45}H_{75}N_{17}O_{20}S_2 \cdot 4TFA \cdot 4.5H_2O$: C, 35.85; H, 5.00; N, 13.41%.

FR-1. The crude decapeptide **10** (145 mg) in TFA (100 ml)–anisole (0.5 ml) was treated with CH_3SiCl_3 (2.0 ml, 10.5 mmol) in the presence of Ph_2SO (270 mg, 1.33 mmol) for 10 min at room temperature. After concentration of the reaction mixture to ca. 25 ml, diethyl ether and 10% AcOH were added to the solution. The aqueous layer was concentrated and the residue was purified by HPLC (Solvent: 10% MeCN–0.1% TFA aq and 6% MeCN–2% MeOH–0.1% TFA aq; Column: Biofine RPC-SC 18L, 10×250 mm) to give FR-1 (30.3 mg, 21.1 μmol) as powder: Yield of FR-1, 18% (from **4**); HPLC; t_R=20.16 min (4% MeCN and 0.1% TFA, Biofine RPC-SC 18, 4.6×250 mm); FABMS *m/z* 980 (MH^+); Amino acid analysis (theoretical): Asp 0.92(1), Ser 2.65(3), Gly 1.07(1), Ala 1.00(1), $(Cys)_2$ 0.46(1), Arg 0.88(1), Pro 0.90(1). Found: C, 34.39; H, 5.11; N, 12.31%. Calcd for $C_{35}H_{57}N_{13}O_{16}S_2 \cdot 3TFA \cdot 6.5H_2O$: C, 34.22; H, 5.11; N, 12.65%.

FR-2. The crude dodecapeptide **11** (163 mg) in TFA (100 ml)–anisole (0.5 ml) was treated with CH_3SiCl_3 (2.0 ml, 10.5 mmol) in the presence of Ph_2SO (270 mg, 1.33 mmol) for 10 min at room temperature. After concentration of the reaction mixture to ca. 25 ml, diethyl ether and 10% AcOH were added to the solution. The aqueous layer was concentrated and the residue was purified by HPLC (Solution: 10% MeCN–0.1% TFA aq and 6% MeCN–2% MeOH–0.1% TFA aq; Column: Biofine RPC-SC 18L, 10×250 mm) to give FR-2 (33.7 mg, 22.1 μmol) as powder: Yield of FR-2, 17% (from **6**); HPLC; t_R=18.84 min (4% MeCN and 0.1% TFA, Biofine RPC-SC 18, 4.6×250 mm); FABMS *m/z* 1094 (MH^+); Amino acid analysis (theoretical): Asp 0.92(1), Ser 2.66(3), Gly 2.83(3), Ala 1.00(1), $(Cys)_2$ 0.57(1), Arg 0.94-(1), Pro 0.97(1). Found: C, 34.97; H, 4.93; N, 14.22%. Calcd for $C_{39}H_{63}N_{15}O_{18}S_2 \cdot 3TFA \cdot 5H_2O$: C, 35.41; H, 5.02; N, 13.77%.

FR-3. 1) Silyl Chloride-Sulfoxide Method. The crude dodecapeptide **12** (160 mg) in TFA (100 ml)–anisole (0.5 ml) was treated with CH_3SiCl_3 (2.0 ml, 10.5 mmol) in the presence of Ph_2SO (270 mg, 1.33 mmol) for 10 min at room temperature. After concentration of the reaction mixture to ca. 25 ml, diethyl ether and 10% AcOH were added to the solution. The aqueous layer was concentrated and the residue was purified by HPLC (Solvent: 10% MeCN–0.1% TFA and 6% MeCN–2% MeOH–0.1% TFA aq; Column: Biofine RPC-SC 18L, 10×250 mm), and to give FR-3 (35.7 mg, 22.7 μmol) as powder: Yield of FR-3, 20% (from **9**); HPLC; t_R=27.72 min (4% MeCN and 0.1% TFA, Biofine RPC-SC 18, 4.6×250 mm); FABMS *m/z* 1094 (MH^+); Amino acid analysis (theoretical): Asp 1.03(1), Ser 2.69(3), Gly 2.91(3), Ala 1.00(1), $(Cys)_2$ 0.47(1), Arg 0.99(1), Pro 1.01-(1). Found: C, 34.70; H, 5.36; N, 13.08%. Calcd for $C_{39}H_{63}N_{15}O_{18}S_2 \cdot 3TFA \cdot 7.5H_2O$: C, 34.40; H, 5.20; N, 13.37.

2) Iodine Oxidation Method. To a solution of dodecapeptide **9** (280 mg, 145 μmol) in MeOH (150 ml), I_2 (147 mg, 580 μmol)/MeOH (40 ml) was added. The mixture was stirred for 10 min at room temperature and then a large amount of chloroform was added to the reaction mixture. This solution was washed with saturated $Na_2S_2O_3$ aq and saturated NaCl aq, dried over anhydrous sodium sulfate, and then evaporated. The residue was purified by silica-gel column chromatography ($CHCl_3$: MeOH=10:1) to give cyclic protected peptide (164 mg, 91.2 μmol). This cyclic peptide (130 mg, 72.3 μmol) was treated with liq. HF (30 ml) in the presence of anisole (1.0 ml) for 1 h at 0 °C. HF was removed and the residue was dried over NaOH pellets in vacuo at room temperature. The residue was dissolved in 10% AcOH, and the aqueous solution was washed with diethyl ether and then lyophilized. The crude product (108 mg) was obtained. This sample (19.8 mg) was purified by HPLC (Solvent: 10% MeCN–0.1% TFA aq and 6% MeCN–2% MeOH–0.1% TFA aq; Column: Biofine RPC-SC 18L,

10×250 mm). This compound was identified by HPLC and NMR with FR-3 had been synthesized by the silyl chloride-sulfoxide method. Yield of FR-3 was 42% from **9**.

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11) Abbreviations: Acm, acetamidomethyl; Boc, *t*-butoxycarbonyl; cHex, cyclohexyl; DCC, dicyclohexylcarbodiimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid.
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