Synthesis of Cyclic Oligopeptides Containing an Arg–Gly–Asp (RGD) Sequence[1)]

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The synthesis of cyclic RGD peptides with 8—10 residues cyclized by an amide bond and the relationship between their structure and activity (*i.e.* circular dichroism spectrum and inhibition of platelet aggregation) are reported. The linear peptides were synthsized by the solution method and their cyclization was performed in high dilution using DPPA.

Key words Arg–Gly–Asp (RGD) sequence; platelet aggregation inhibitor; cyclic peptide; circular dichroism spectrum

We have studied the differentiation of sea urchin embryo using synthetic peptides related to the partial structure of the cell adhesion domain of fibronectin.[2)] In order to obtain a specific conformation of the RGDSPASS peptide, we designed and synthesized a cyclic peptide, CRGDSPASSC, having a disulfide bond (FR-1).[3)] FR-1 exhibited activity as a platelet aggregation inhibitor and some FR-1 analogs were found to be more potent than FR-1 itself.[4)]

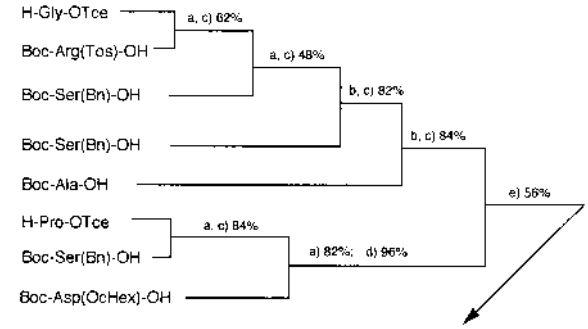
In the present investigation, we synthesized RGD peptides (**1**—**5**) cyclized by an amide bond, and studied the relationship between their structure and activity, in particular, their size and the side-chain effect of an amino acid residue located adjacent to the RGD sequence. We evaluated the circular dichroism (CD) spectra and the inhibition of platelet aggregation in human platelet-rich plasma.

cyclo(–Arg–Gly–Asp–Ser–Pro–Ala–Ser–Ser–)	**1**
cyclo(–Gly–Arg–Gly–Asp–Ser–Pro–Ala–Ser–Ser–)	**2**
cyclo(–Arg–Gly–Asp–Ser–Pro–Ala–Ser–Ser–Lys–Pro–)	**3**
cyclo(–Arg–Gly–Asp–Phe–Pro–Ala–Ser–Ser–)	**4**
cyclo(–Arg–Gly–Asp–Cha–Pro–Ala–Ser–Ser–)	**5**

Protected peptides **6**—**10** were synthesized by the solution method in the same manner as for FR-1.[5)] The synthetic route for **6** is shown in Chart 1 and the routes for **9** and **10** are the same as that for **6**. Chart 2 shows the route for **8**. Peptide **7** was synthesized in a similar manner to that of **8** using Gly–OTce in place of Lys(Z)–Pro–OTce.

Cyclization of **6**—**10** was performed in high dilution using DPPA after removal of the protective groups on the N- and C-termini. Cyclic protected peptides **11**—**15** were treated with liquid HF and then subjected to HPLC purification to obtain the final cyclic peptides **1**—**5** (Chart 3).

Peptides **4** and **5** were the most potent inhibitors of human platelet aggregation among the five cyclic peptides (**1**—**5**) as shown in Table 1. CD spectra of these cyclic peptides, the linear peptide RGDSPASSKP and FR-1 are shown in Fig. 1. The CD spectrum of peptide **3** was characterized by double-minima at 200 and 220 nm (π–π^* and n–π^* transition bands, respectively). This spectrum is similar in shape to that of gramicidin S,[6)] which is a cyclic decapeptide containing two prolyl residues and a fixed conformation. Peptides **1** and **2** also have similar CD spectra. On the other hand, peptides **4** and **5** were characterized by the increased ratio of the π–π^*/n–π^* bands in their CD spectra. These spectra are not consistent with the preferred β-turn structure,[7)] and show that these cyclic peptides have unordered structures caused by the steric effect of Phe or Cha, compared with peptide **1**. Needless to say, their structures need to be confirmed by another method such as NMR. The results of biological assay suggest that peptides **4** and **5** have a relatively flexible cyclic struc-



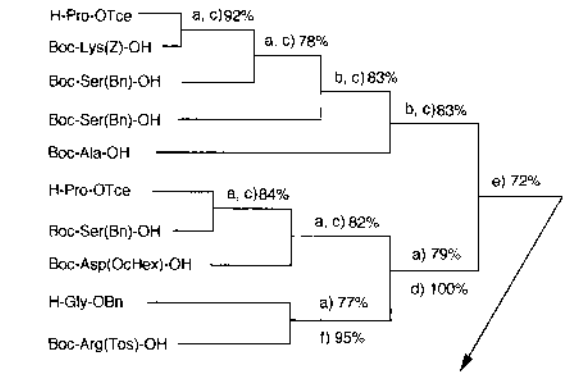
Boc-Asp(OcHex)-Ser(Bn)-Pro-Ala-Ser(Bn)-Ser(Bn)-Arg(Tos)-Gly-OTce (6)

Boc-Asp(OcHex)-Phe-Pro-Ala-Ser(Bn)-Ser(Bn)-Arg(Tos)-Gly-OTce (9)

Boc-Asp(OcHex)-Cha-Pro-Ala-Ser(Bn)-Ser(Bn)-Arg(Tos)-Gly-OTce (10)

a) DCC, HOBt, DIEA / DMF b) DPPA, DIEA / DMF c) 4M HCl / dioxane
d) Zn / AcOH e) EDC, HOBt, DIEA / DMF

Chart 1



Boc-Arg(Tos)-Gly-Asp(OcHex)-Ser(Bn)-Pro-Ala-Ser(Bn)-Ser(Bn)-Lys(Z)-Pro-OTce (8)

Boc-Arg(Tos)-Gly-Asp(OcHez)-Ser(Bn)-Pro-Ala-Ser(Bn)-Ser(Bn)-Gly-OTce (7)

a) DCC, HOBt, DIEA / DMF b) DPPA, DIEA / DMF c) 4M HCl / dioxane d) Zn / AcOH
e) EDC, HOBt, DIEA / DMF f) H_2 / Pd-C / MeOH

Chart 2

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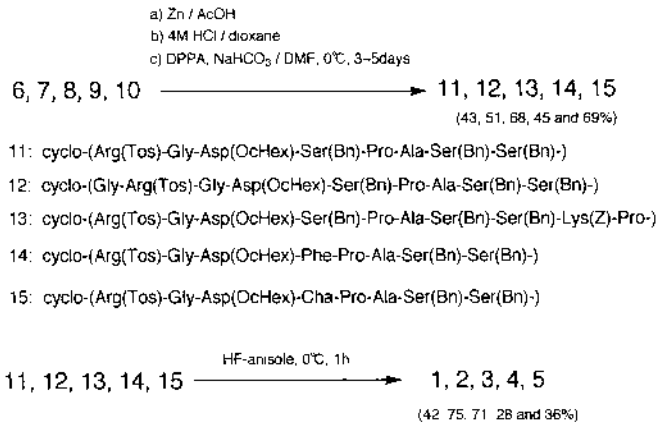

Chart 3

Table 1. Inhibition of Human Platelet Aggregation[a)]

Peptide	IC_{50} (μM)
1	12.0
2	14.0
3	37.0
4	3.0
5	2.0
RGDSPASSKP	22.0
FR-1	11.0

a) Platelet aggregation in human platelet-rich plasma was induced by ADP (2.3 μM).

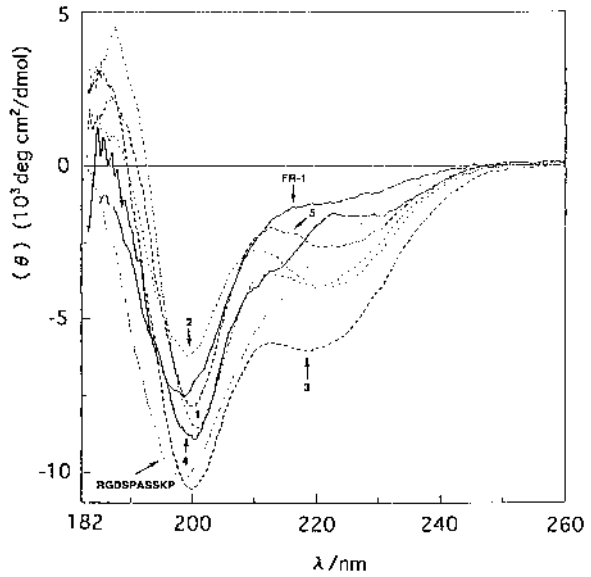

Fig. 1. CD Spectra of Peptides **1**, **2**, **3**, **4**, **5**, RGDSPASSKP, and FR-1 in Water

ture and can change to a conformation favorable for the receptors on human platelets.

Experimental

Column chromatography was performed using Silica-gel 60 from Merck. Mass spectra were measured on a JEOL JMS-SX mass spectrometer operating under FAB conditions. HF treatment was performed in an HF-reaction apparatus type II from Peptide Institute, Inc. The final products were isolated by HPLC on a Jasco Gulliver system equipped with a PU-980/UV-970 detector (210 nm) using a Megapak SIL C18 column, 10×250 mm. CD spectra of 0.015% peptide solution (*ca*. 1 mM) in water were recorded at room temperature on a Jasco J-725 spectropolarimeter using a cuvette with a 0.99 mm path length. CD extrema are expressed as (θ): the mean residue molecular ellipticity (deg cm^2/dmol).

Boc–Asp(OcHex)–Ser(Bn)–Pro–Ala–Ser(Bn)–Ser(Bn)–Arg(Tos)–Gly–OTce (6) Activated zinc powder was added to a solution of Boc–Asp(OcHex)–Ser(Bn)–Pro–OTce (1.79 g, 2.48 mmol) in 12 ml 90% AcOH aq. and the reaction mixture was stirred at room temperature overnight. After removal of the powder, the filtrate was concentrated *in vacuo*. The residue was dissolved in methylene chloride, which was then washed with 0.5 M HCl and saturated NaCl aq., and dried over anhydrous Na_2SO_4. After concentration of the solution, the residue was dried *in vacuo* to afford Boc–Asp(OcHex)–Ser(Bn)–Pro–OH (1.40 g, 2.37 mmol) in 96% yield; FAM-MS *m/z*: 590 (MH^+). To prepare the amine component, Boc–Ala–Ser(Bn)–Ser(Bn)–Arg(Tos)–Gly–OTce (0.93 g, 0.89 mmol) was treated with 4 M HCl/dioxane (10 ml) at room temperature for 30 min. The reaction mixture was concentrated to afford an amorphous compound which was dried *in vacuo*. The compound (*i.e*. H–Ala–Ser(Bn)–Ser(Bn)–Arg(Tos)–Gly–OTce·HCl, 0.89 mmol), Boc–Asp(OcHex)–Ser(Bn)–Pro–OH (0.80 g, 1.36 mmol), and HOBt·H_2O (0.20 g, 1.31 mmol) were dissolved in 10 ml DMF. DIEA (0.23 ml, 1.35 mmol) and EDC·HCl (0.25 g, 1.30 mmol) were added to the solution chilled in an ice bath. The reaction mixture was stirred at room temperature overnight. Then methylene chloridethe was added to the mixture, which was washed successively with 0.5 M HCl, saturated $NaHCO_3$ aq. and saturated NaCl aq. After filtration of the solution through anhydrous Na_2SO_4, the solvent was evaporated. The crude peptide was purified on a silica-gel column ($CHCl_3$: MeOH = 50 : 1—30 : 1) to afford **6** (0.75 g, 0.50 mmol) in 56% yield; FAB-MS *m/z*: 1512 (MH^+).

Boc–Arg(Tos)–Gly–Asp(OcHex)–Ser(Bn)–Pro–Ala–Ser(Bn)–Ser(Bn)–Lys(Z)–Pro–OTce (8) Boc–Arg(Tos)–Gly–Asp(OcHex)–Ser(Bn)–Pro–OTce (4.14 g, 3.80 mmol) was treated with zinc powder in 90% AcOH aq. at room temperature overnight to give Boc–Arg(Tos)–Gly–Asp(OcHex)–Ser(Bn)–Pro–OH (3.49 g, 3.65 mmol, 96%). To prepare the amine component, Boc–Ala–Ser(Bn)–Ser(Bn)–Lys(Z)–Pro–OTce (2.07 g, 2.0 mmol) was treated with 4 M HCl/dioxane to give H-Ala–Ser(Bn)–Ser(Bn)–Lys(Z)–Pro–OTce·HCl (2.0 mmol). Both the peptides (2.0 mmol each) were coupled with EDC·HCl (0.42 g, 2.2 mmol), HOBt (0.30 g, 2.2 mmol), and NMM (0.24 ml, 2.4 mmol) in DMF (5 ml) at room temperature overnight in a similar manner to that of **6**. The crude peptide was purified on a silica-gel column ($CHCl_3$: MeOH = 50 : 1—20 : 1) to afford **8** (2.68 g. 1.43 mmol) in 72% yield; FAB-MS *m/z*: 1871 (MH^+).

Cyclo(–Arg(Tos)–Gly–Asp(OcHex)–Ser(Bn)–Pro–Ala–Ser(Bn)–Ser(Bn)–) (11) Zinc powder (*ca*. 0.6 g) was added to a solution of **6** (0.75 g, 0.50 mmol) in 10 ml 90% AcOH aq. The reaction mixture was stirred at room temperature overnight and filtered. After concentration of the filtrate, a solution of the residue in methylene chloride was washed with 0.5 M HCl and saturated NaCl aq. The solution was filtered through anhydrous Na_2SO_4. Evaporation of the solvent gave Boc–Asp(OcHex)–Ser(Bn)–Pro–Ala–Ser(Bn)–Ser(Bn)–Arg(Tos)–Gly–OH (0.67 g, 0.49 mmol, 98%), which was treated with 4 M HCl/dioxane (13 ml) at room temperature for 1 h. The reaction mixture was concentrated and the residue was dried *in vacuo* to afford a peptide (H–Asp(OcHex)–Ser(Bn)–Pro–Ala–Ser(Bn)–Ser(Bn)–Arg(Tos)–Gly–OH·HCl). DPPA (0.12 ml, 0.56 mmol) and $NaHCO_3$ (0.43 g, 5.0 mmol) were added to a solution of this peptide (0.49 mmol) in 500 ml DMF chilled in a refrigerator. The reaction mixture was stirred at 7—8 °C for 5 d and then the solvent was removed *in vacuo* to afford an amorphous compound. The compound was dissolved in methylene chloride, which was washed with 0.5 M HCl, saturated $NaHCO_3$ aq. and saturated NaCl aq., and dried over anhydrous Na_2SO_4. The residue after solvent evaporation was purified on a silica-gel plate ($60F_{254}$, 2 mm, 20×20 cm from Merck, eluent: $CHCl_3$: MeOH = 10 : 1) to obtain **11**, a colorless foam (0.27 g, 43%); FAB-MS *m/z*: 1264 (MH^+).

Peptide 1 The cyclized peptide **11** (0.27 g, 0.21 mmol) was dissolved in TFA (1 ml) containing anisole (1 ml) and treated with liquid HF (15 ml) at 0 °C for 1 h. After removal of the HF *in vacuo*, the residue was dissolved in 10% AcOH aq., which was washed with diethyl ether and then lyophillized to afford a peptide (0.20 g). This crude peptide was purified by HPLC (solvent: 0.1% TFA/10% CH_3CN aq., 2 ml/min.) to give **1** (42%) with a retention time of 27 min; FAB-MS *m/z:* 758 (MH^+). *Anal*. Calcd for $C_{29}H_{47}N_{11}O_{13}\cdot 2TFA\cdot 3.5H_2O$: C, 37.78; H, 5.38; N, 14.68. Found: C, 37.85; H, 5.12; N, 14.48. The other products (**2**, **3**, **4**, **5** and RGDSPASSKP) were also isolated by HPLC (solvent: 0.1% TFA/7—25% CH_3CN aq.).

Peptide **2**: FAB-MS *m/z*: 815 (MH^+). *Anal.* Calcd for $C_{31}H_{50}N_{12}O_{14}\cdot 2TFA\cdot 3H_2O$: C, 38.32; H, 5.33; N, 15.32. Found: C, 38.70; H, 5.51; N, 15.51.

Peptide **3**: FAB-MS *m/z*: 983 (MH^+). *Anal.* Calcd for $C_{40}H_{66}N_{14}O_{15}\cdot 3TFA\cdot 2H_2O$: C, 40.59; H, 5.41; N, 14.40. Found: C, 40.48; H, 5.41; N, 14.55.

Peptide **4**: FAB-MS *m/z*: 818 (MH^+). *Anal.* Calcd for $C_{35}H_{51}N_{11}O_{12}\cdot 3TFA\cdot 2.5H_2O$: C, 40.86; H, 4.93; N, 12.78. Found: C, 41.40; H, 5.06; N, 13.03.

Peptide **5**: FAB-MS *m/z*: 824 (MH^+). *Anal.* Calcd for $C_{35}H_{57}N_{11}O_{12}\cdot 2TFA\cdot 3H_2O$: C, 42.35; H, 5.92; N, 13.93%). Found: C, 42.63; H, 5.87; N, 13.67.

H–Arg–Gly–Asp–Ser–Pro–Ala–Ser–Ser–Lys–Pro–OH (RGDSPASSKP) Protected peptide **8** (2.63 g, 1.40 mmol) was treated with Zn/90% AcOH aq. at room temperature overnight and then liquid HF at 0 °C for 1 h, as described in reference **5**. The yield of purified RGDSPASSKP was 75%. FAB-MS *m/z*: 1,001 (MH^+). *Anal.* Calcd for $C_{40}H_{68}N_{14}O_{16}\cdot 4TFA\cdot 3H_2O$: C, 38.15; H, 5.20; N, 12.97. Found: C, 37.68; H, 4.89; N, 13.12.

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References and Notes

1) The amino acids used had the L-configuration. Abbreviations used in this paper: Cha=cyclohexylalanine; Boc=*tert*-butoxycarbonyl; Bn=benzyl; cHex=cyclohexyl; Tce=2,2,2-trichloroethyl; Tos=*p*-toluenesulfonyl; Z=benzyloxycarbonyl; ADP=adenosine 5′-diphosphate; DCC=dicyclohexylcarbodiimide; DPPA=diphenylphosphorylazide; DIEA=diisopropylethylamine; DMF=*N*,*N*-dimethylformamide; EDC=1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt=1-hydroxylbenzotriazole; NMM=*N*-methylmorpholine; TFA=trifluoroacetic acid.
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