Amino Acids and Peptides. XXVII.[1] Solid Phase Synthesis of Fibrinogen-Related Peptides with Disulfide Bond Formed on Solid Support

Masa KAKIUCHI,[a] Yasuhiro IZUNO,[a] Mitsuko MAEDA,[a] Kaori UEDA,[a] Kazuko FUJIWARA,[b] Satoshi KUNITADA,[b] and Koichi KAWASAKI*,[a]

Faculty of Pharmaceutical Sciences, Kobe Gakuin University,[a] Ikawadani-cho, Nishi-ku, Kobe 651-21, Japan and Exploratory Research Laboratories II, Daiichi Pharmaceutical Co., Ltd.,[b] Kita-Kasai 1-16-13, Edogawa-ku, Tokyo 134, Japan. Received December 1, 1995; accepted January 8, 1996

Fibrinogen-related peptides, monomeric cyclic peptides through a disulfide bond [cyclo(H–Cys–Arg–Gly–Asp–Phe–Cys–NH_2), cyclo(H–Cys–Arg–Gly–Asp–Phe–Cys–Gly–NH_2), cyclo(H–Cys–Arg–Gly–Asp–Cys–NH_2) and cyclo(H–Cys–Arg–Gly–Asp–Cys–Gly–NH_2)], were prepared by the solid phase method with disulfide bond formation on the solid support. The acetamidomethyl group was used for protection of the thiol group of Cys and synthetic peptide-resins were treated with iodine to give the disulfide bond. Monomeric cyclic peptides were obtained as main products. Purified *S*-acetamidomethylated peptides were also oxidized with iodine, but the desired materials could not be isolated by HPLC. The disulfide formation from *S*-acetamidomethylcysteine-containing peptide resin by iodine treatment on the solid support was more effective than that from *S*-acetamidomethylcysteine-containing peptide. The inhibitory effects of the cyclic peptides on platelet aggregation were much more potent than that of H–Arg–Gly–Asp–NH_2.

Key words disulfide formation; platelet aggregation inhibitor; fibrinogen; acetamidomethylcysteine; iodine oxidation

Many cyclic peptides with an intramolecular disulfide bond have been prepared to study the structure–activity relationships of the original linear peptides. In solid phase peptide synthesis, the intramolecular disulfide bond is usually formed after separation of the completed peptide chain from the polymeric support, since formation of the intramolecular disulfide bond should be done in a diluted solution of peptide to avoid intermolecular disulfide bond formation. The acetamidomethyl(Acm) group[2] is a valuable protecting group for the thiol group of cysteine. *S*-Acetamidomethylcysteine [Cys(Acm)] is converted to cystine by iodine treatment[3a] and by the silyl chloride–sulfoxide system,[3b] and so it is used for preparation of peptides containing a disulfide bond. However, after the disulfide bond formation reaction by these methods, removal of excess reagent from the reaction mixture is not easy when the reaction is done by the solution method. In the case of oxidation of a small peptide, removal of the used reagents is particularly difficult, since purification by molecular sieve separation may not work. Albericio *et al.*[4a] reported that iodine oxidation of Ac–Cys(Acm)–Pro–D-Val–Cys(Acm)–MBHAResin (Ac, acetyl; MBHAResin, methylbenzhydrylamine resin), followed by HF treatment, gave a crude product (yield, 94%) which contained 52% of monomeric cyclic(Ac–Cys–Pro–D-Val–Cys–NH_2). They determined the yield of the monomeric cyclic peptide by comparing the HPLC peak area of crude peptide to that of a pure peptide standard. Quite recently, Munson and Barany[4b] reported the preparation of α-conotoxin and Camarero *et al.*[4c] reported the preparation of a mimic peptide of the G-H loop of foot-and-mouth disease virus by the solid phase method using Cys(Acm). Both groups succeeded in disulfide formation on the solid support.

We also examined disulfide bond formation of a Cys(Acm)-containing peptide on a solid support by iodine treatment. Arg–Gly–Asp–Phe is a partial sequence of fibrinogen[5] and Arg–Gly–Asp-containing peptides were reported to be inhibitors of platelet aggregation.[6] We prepared cyclo(H–Cys–Arg–Gly–Asp–Phe–Cys–NH_2) (I) and cyclo(H–Cys–Arg–Gly–Asp–Phe–Cys–Gly–NH_2) (II), of which the intramolecular disulfide bonds were formed on the solid support. Since the resin may influence the disulfide-forming reaction, *e.g.*, through steric hindrance, the Gly[7] in II was put as a spacer between the resin and Cys(Acm). Furthermore, we prepared cyclo(H–Cys–Arg–Gly–Asp–Cys–NH_2) (III) and cyclo(H–Cys–Arg–Gly–Asp–Cys–Gly–NH_2) (IV) to compare the biological activities and reactivities of disulfide formation of I and II with those of III and IV. Since I and II have 4 amino acid residues between the Cys residues, and III

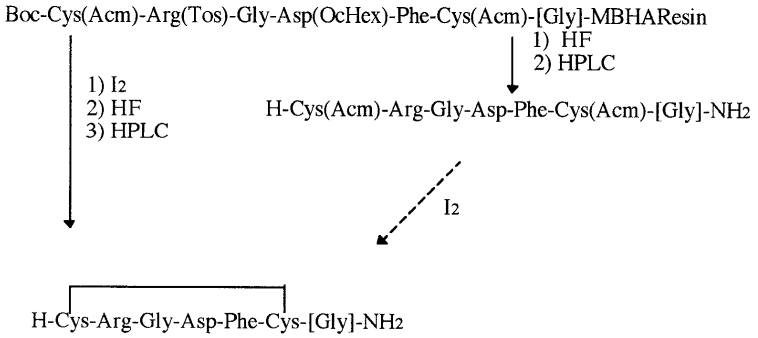

Fig. 1. Synthetic Scheme for Cyclo[CRGDFC]-NH_2(I) and Cyclo[CRGDFCG]-NH_2(II)

* To whom correspondence should be addressed.

© 1996 Pharmaceutical Society of Japan

NII-Electronic Library Service

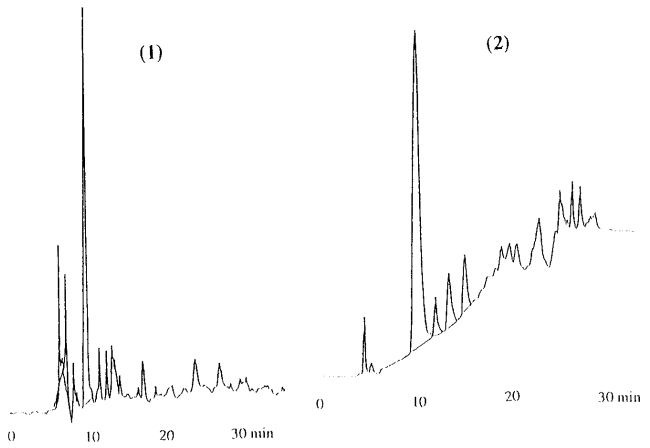

Fig. 2. Preparative HPLC Profiles of Cyclo[CRGDFC]-NH_2(I) (**1**) and Cyclo[CRGDFCG]-NH_2(II) (**2**)

1: Column, YMC J'sphere ODS-M-80 (20×250 mm). Flow rate, 12 ml/min. Solvent, A) 0.05% TFA : water; B) 0.05% TFA : acetonitrile. Gradient, A : B = 90 : 10→50 : 50 (20 min). **2**: Column, Cosmosil 5C18-AR (20×250 mm). Flow rate, 10 ml/min. Solvent, A) 0.05% TFA : water; B) 0.05% TFA - acetonitrile. Gradient, A : B = 85 : 15→70 : 30 (30 min). Compounds I and II were obtained in the main peaks of **1** and **2**.

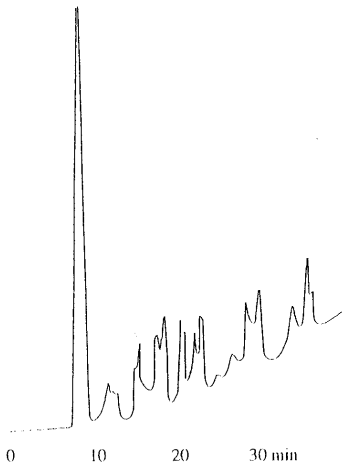

Fig. 3. HPLC Profile of the Product of Iodine Oxidation of IIA

Column: Cosmosil 5C18AR (4.6×250 mm). Solvent, A) 0.05% TFA : water; B) 0.05% TFA : acetonitrile. Flow rate, 1.5 ml/min. Gradient, A : B = 95 : 5→70 : 30 (40 min).

Table 1. Inhibition of Platelet Aggregation with Synthetic Peptides

	Inhibition (%)	
	10 μM	100 μM
RGD	−11.0	15.9
Cyclo[CRGDFC] (I)	34.1	90.2
Cyclo[CRGDFCG] (II)	45.1	87.8
Cyclo[CRGDC] (III)	57.3	98.8
Cyclo[CRGDCG] (IV)	52.4	98.8
C(Acm)RGDFC(Acm) (IA)	42.7	89.0
C(Acm)RGDFC(Acm)G (IIA)	1.2	52.4
C(Acm)RGDC(Acm) (IIIA)	22.0	80.5
C(Acm)RGDC(Acm)G (IVA)	1.2	62.2

All peptides are the hydrochlorides.

and IV have 3 amino acid residues between the Cys residues, the reaction rates of disulfide formation of I and III (or II and IV) may be different. Compounds I and II were prepared as shown in Fig. 1.

Boc–Cys(Acm)–Arg(Tos)–Gly–Asp(OcHx)–Phe–Cys(Acm)–MBHA-resin (IR) (Boc, *tert*-butoxycarbonyl; Tos, tosyl; cHx, cyclohexyl) and Boc–Cys(Acm)–Arg(Tos)–Gly–Asp(OcHx)–Phe–Cys(Acm)–Gly–MBHA-resin (IIR) were prepared by the N^{α}-Boc strategy. Compounds IR and IIR were each treated with iodine (3 eq to the resin) in a mixture of DMF and methanol for 3 h at room temperature. The peptide resin was washed with DMF, and a mixture of methanol and dichloromethane (DCM) successively, and dried. It was then treated with HF and the product was purified by RP-HPLC. The desired products, I and II, were obtained as the main peak on HPLC as shown in Fig. 2.

Amino acid ratios and mass spectra of I and II indicated that they were the desired monomeric disulfide compounds. Yields of I and II based on the amino group content of the starting resin were 11% and 10% respectively. The yields may indicate that the Gly spacer in II is not particularly suitable for the disulfide formation reaction on the resin. Acm derivatives of I and II, H–Cys(Acm)–Arg–Gly–Asp–Phe–Cys(Acm)–NH_2 (IA) and H–Cys(Acm)–Arg–Gly–Asp–Phe–Cys(Acm)–Gly–NH_2 (IIA), were also prepared by HF treatment of the corresponding synthetic protected peptide resins[Boc–Cys(Acm)–Arg(Tos)–Gly–Asp(OcHx)–Phe–Cys(Acm)-resin and Boc–Cys(Acm)–Arg(Tos)–Gly–Asp(OcHx)–Phe–Cys(Acm)–Gly-resin] and were purified by HPLC. Compound IA and IIA were treated with iodine in 80% acetic acid to form the disulfide bond, and excess iodine was reduced with ascorbic acid. Each reaction mixture was passed through a Sephadex G-25 column, but the peptide and the other reagents could not be separated. Pure I and II could not be obtained by HPLC. The HPLC profile of the reaction mixture of IIA after Sephadex column treatment is shown in Fig. 3. The main peak contained colored impurities and the desired product (II) was not found in other peaks.

Compounds III and IV were prepared by disulfide bond formation on the resins in the same way as described for I and II. Yields of III and IV were 16% and 17% calculated from the amino group content of the starting resin respectively. The result means that the Gly spacer did not lead to any improvement on the disulfide formation reaction. A slight difference of the yields between (I, II) and (III, IV) was observed. Compounds I and II contain Arg–Gly–Asp–Phe, and III and IV contain Arg–Gly–Asp between the cysteine residues. The presence of Phe may have influenced the reaction rate of disulfide formation.

The inhibitory effect of the synthetic cyclic peptides on platelet aggregation was examined *in vitro*. As shown in Table 1, these peptides inhibited ADP-induced platelet aggregation. The inhibition of platelet aggregation with these cyclic peptides was more potent than that with cognate non-cyclic peptides. In particular, though the peptides which possess a Gly residue at the carboxyl terminus were less potent antagonists of platelet aggregation, the disulfide formation of these peptides increased the antagonistic activity, as with the Gly-deleted cyclic peptides.

NII-Electronic Library Service

Experimental

p-Methylbenzhydrylamine resin(amino content, 0.68 meq/g) was purchased from Peptide Institute, Inc. For amino acid analysis, synthetic peptides were hydrolyzed in 6 N HCl at 110 °C for 24 h. Amino acid compositions of acid hydrolysates were determined with a Kyowa K-202SN amino acid analyzer. Determination of Cys(Acm) by analysis of an acid hydrolysate was not reliable since an acid hydrolysate of commercial Boc–Cys(Acm)–OH gave a heterogeneous hydrolysate. Thus, analytical data of Cys(Acm) in acid hydrolysates of IA, IIA, IIIA and IVA were omitted. RP-HPLC was conducted with a Waters 600 on a YMC Pack ODS-M80 column or a Cosmosil C_{18}AR-300 column using gradient systems of CH_3CN/H_2O containing 0.05% TFA. Optical density of the eluates was measured at 220 nm. FAB-MS were measured on a VG Analytical ZAV-SE spectrometer.

General Procedure for Peptide Synthesis by the Solid Phase Method A) Peptides were prepared by an Applied Biosystem peptide synthesizer 430A on *p*-methylbenzhydrylamine resin using the following amino acid derivatives; Boc–Cys(Acm)–OH, Boc–Gly–OH, Boc–Arg(Tos)–OH, Boc–Asp(OcHx)–OH, Boc–Phe–OH. Coupling reactions were performed by means of a double coupling system with dicyclohexylcarbodiimide.

B) Manual method. The synthetic protocol for manual solid phase peptide synthesis is shown below. Reactions were checked by means of the ninhydrin test.[7]

step	reagents	reaction time	
1	NMM/DCM	10 min	×2
2	DCM	3 min	×3
3	Boc-amino acid (2 eq) in DMF (or DCM) 1 M DCC/DCM (2 eq)	120 min	
4	50% MeOH/DCM	5 min	×3
5	DCM	2 min	1
6	50% TFA/DCM, anisole	2 min	1
		45 min	1
7	DCM	3 min	×4

For the activation of Boc–Arg(Tos)–OH, 1 M HOBt/DMF (2 eq) was added.

Disulfide Formation on the Solid Support Peptide resin (peptide content, 0.5 mmol) was swollen with DCM (10 ml) and suspended in DMF (10 ml). A solution of iodine (381 mg, 1.5 mmol) in MeOH (10 ml) was combined with the suspension and the mixture was stirred for 3 h at room temperature. The resin was washed successively with DMF and a mixture of DCM and MeOH (1 : 1) repeatedly. The resin was dried for the next HF treatment.

HF Treatment and Purification A synthetic peptide resin was treated with 5% anisole/HF at 0 °C for 1 h. After removal of the HF, the residue was washed repeatedly with ether and extracted with 5% AcOH, followed by lyophilization. The product was purified by RP-HPLC. The peptide purified by HPLC was converted to its hydrochloride by repeated lyophilization from HCl-containing water. Yields of the desired peptides were calculated from the amino content of the starting resin.

Cyclo(H–Cys–Arg–Gly–Asp–Phe–Cys–NH_2) (I) The peptide was prepared by the manual method. Yield 83 mg (11%), $[\alpha]_D^{28}$ −89.4° (c = 1.0, water), MS m/z: 697 $(M+1)^+$. Amino acid ratios in an acid hydrolysate: Cys 1.89, Arg 0.89, Gly 1.00, Asp 1.07, Phe 1.06 (average recovery 93%).

Cyclo(H–Cys–Arg–Gly–Asp–Phe–Cys–Gly–NH_2) (II) The peptide was prepared by the manual method. Yield 79 mg (10%), $[\alpha]_D^{26}$ −88.8° (c = 1.0, water), MS m/z: 754 $(M+1)^+$. Amino acid ratios in an acid hydrolysate: Cys 1.99, Arg 0.99, Gly 2.00, Asp 0.99, Phe 1.06 (average recovery 86%).

Cyclo(H–Cys–Arg–Gly–Asp–Cys–NH_2) (III) The peptide was prepared by the synthesizer. Yield 51 mg (16%), $[\alpha]_D^{28}$ −68.8° (c = 1.0, water), MS m/z: 550 $(M+1)^+$. Amino acid ratios in an acid hydrolysate: Cys 1.45, Arg 1.09, Gly 1.00, Asp 1.01 (average recovery 78%).

Cyclo(H–Cys–Arg–Gly–Asp–Cys–Gly–NH_2) (IV) The peptide was prepared by the synthesizer. Yield 55 mg (17%), $[\alpha]_D^{28}$ −59.7° (c = 1.0, water), MS m/z: 607 $(M+1)^+$. Amino acid ratios in an acid hydrolysate: Cys 1.70, Arg 1.08, Gly 2.00, Asp 0.95 (average recovery 80%).

H–Cys(Acm)–Arg–Gly–Asp–Phe–Cys(Acm)–NH_2 (IA) The peptide was prepared by the manual method. The main peak of HPLC, containing IA, had a shoulder and the HPLC purification was repeated 3 times. Yield 53 mg (6%), hygroscopic powder, $[\alpha]_D^{28}$ −30.1° (c = 1.0, water), MS m/z: 841 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Arg 0.85, Gly 1.00, Asp 1.00, Phe 1.00 (average recovery except Cys 86%).

H–Cys(Acm)–Arg–Gly–Asp–Phe–Cys(Acm)–Gly–NH_2 (IIA) The peptide was prepared by the manual method. Yield 140 mg (16%), hygroscopic powder, $[\alpha]_D^{29}$ −27.4° (c = 1.0, water), MS m/z: 898 $(M+1)^+$. Amino acid ratios in an acid hydrolysate: Arg 0.93, Gly 2.00, Asp 1.14, Phe 1.04 (average recovery except Cys 85%).

H-Cys(Acm)–Arg–Gly–Asp–Cys(Acm)–NH_2 (IIIA) The peptide was prepared by the synthesizer. Yield 55 mg (16%), hygroscopic powder, $[\alpha]_D^{28}$ −22.0° (c = 1.0, water), MS m/z: 694 $(M+1)^+$. Amino acid ratios in an acid hydrolysate: Arg 1.09, Gly 2.00, Asp 0.99 (average recovery except Cys 72%).

H–Cys(Acm)–Arg–Gly–Asp–Cys(Acm)–Gly–NH_2 (IVA) The peptide was prepared by the synthesizer. Yield 80 mg (17%), $[\alpha]_D^{28}$ −22.7° (c = 1.0, water), MS m/z: 752 $(M+1)^+$. Amino acid ratios in an acid hydrolysate: Arg 1.09, Gly 2.00, Asp 0.99 (average recovery except Cys 77%).

Iodine Oxidation of IIA Iodine oxidation was performed according to the procedure reported by Kumagaye *et al.*[8] An iodine solution [115 mg (0.9 mmol)/20 ml 80% AcOH] was added to a solution of H–Cys(Acm)–Arg–Gly–Asp–Phe–Cys(Acm)–Gly–NH_2 (IIA) (100 mg, 0.09 mmol) in 80% AcOH (869 ml) and the mixture was stirred for 2 h at room temperature. Ascorbic acid was added to decolorize the mixture and the solvent was removed *in vacuo*. The residue was passed through a Sephadex G-25 column (3 × 140 cm) using water as an eluent. Each fraction (15 g each) was checked by use of the ninhydrin test. The ninhydrin-positive fractions (fractions 55—65) were lyophilized and gave 490 mg of crude product. The peptide could not be isolated from the contaminating reagents. The HPLC profile is shown in Fig. 3. The desired product could not be isolated on an ODS column under the conditions shown in Fig. 3.

Iodine Oxidation IVA H–Cys(Acm)–Arg–Gly–Asp–Cys(Acm)–Gly–NH_2 (IVA) (60 mg, 0.08 mmol) was dissolved in 80% AcOH (87 ml). A solution of iodine (60.96 mg, 0.24 mmol) in 80% AcOH (20 ml) was added to the peptide solution (0.75 mmol/l), and the mixture was stirred for 2 h at room temperature. Powdered ascorbic acid was added to decolorize the reaction mixture and the solvent was removed *in vacuo*. The residue was taken up in water and the solution was applied to a Sephadex G-25 column (3 × 150 cm) and eluted with water. Ninhydrin-positive fractions (59—67) (15 g each) were collected and the solvent was removed *in vacuo* to give 75 mg of the crude product. The desired pure product could not be isolated by HPLC using the gradient system described in Fig. 3.

Bioassay Blood was collected from the antecubital vein of normal healthy volunteers with sodium citrate (9 vol. blood–1 vol. of 3.13% sodium citrate) as anticoagulant. The platelet-rich plasma was obtained by centrifugation for 7 min at 1100 × *g*. Platelet aggregation was induced by the addition of 2 mM ADP and the turbidity was measured (PAM-8C, Mebanix Co.). The platelet-rich plasma was incubated with 10 or 100 mM synthetic peptide for 3 min before the addition of the inducer. The efficacy of the peptides was expressed in terms of inhibition of maximum aggregation.

Acknowledgment This work was supported in part by a grant from The Science Research Promotion Fund of the Japan Private School Promotion Foundation. The authors are grateful to Ms. Naoko Ohtsuji for her technical assistance.

References and Notes

1) A part of this paper was reported at the 4th International Symposium of Solid Phase Synthesis and Combinational Chemical Libraries held in Edinburgh, UK, Sep. 1995. Standard abbreviations are used for amino acids, protecting groups, and peptides [*Eur. J. Biochem.*, **138**, 9 (1984)]. Other abbreviations include: DMF = dimethylformamide, TFA = trifluoroacetic acid, DCM = dichloromethane, NMM = *N*-methylmorpholine, cHx = cyclohexyl, HOBt = 1-hydroxybenzotriazole.
2) Veber D. F., Milkowski J. D., Varga, S. L., Denkewalter R. G., Hirschman R., *J. Am. Chem. Soc.*, **94**, 5456—5461 (1972).
3) *a*) Kamber B., Rittel W. Q., *Helv. Chim. Acta*, **51**, 2061—2064 (1968); *b*) Akaji K., Tatsumi T., Yoshida M., Kimura T., Fujiwara Y., Kiso Y., *J. Am. Chem. Soc.*, **114**, 4137—4143 (1992).

NII-Electronic Library Service

4) *a*) Albericio F., Hammer R. P., Barcia-Echeverria C., Molins M. A., Chang J. L., Munson M. C., Pons M., Giralt E., Barany G., *Int. J. Peptide Protein Res.*, **37**, 402—413 (1991); *b*) Munson M. C., Barany G., *J. Am. Chem. Soc.*, **115**, 10203—10210 (1993); *c*) Camarero J. A., Giralt E., Andreu D., *Tetrahedron Lett.*, **36**, 1137—1140 (1995).
5) Watt K. W. K., Cottrell B. A., Strong D. D., Doolittle R. F., *Biochem.*, **18**, 5410—5416 (1979).
6) Gartner T. K., Bennet J. S., *J. Biol. Chem.*, **260**, 11891—11894 (1985).
7) Kaiser E., Colescott R. L., Bossinger C. D., Cook P. I., *Anal. Biochem.*, **34**, 595—598 (1970).
8) Kumagaye S., Kuroda H., Nakajima K., Watanabe T., Kimura T., Masaki T., Sakakibara S., *Int. J. Peptide Protein Res.*, **34**, 455—462 (1988).

NII-Electronic Library Service