CHEMISTRY LETTERS, pp. 415-418, 1994. © 1994 The Chemical Society of Japan

Design and Synthesis of Fibronectin-related Peptides. Ala-analogs of RGDSPASS Containing Cystine Peptide (FR-1)

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The Arg-Gly-Asp (RGD) sequence is an active site in the cell-binding domain of fibronectin. Ala-analogs of RGDSPASS containing cystine peptide (FR-1) were synthesized to investigate the relationship between the functional group and the biological activity of FR-1.

The Arg-Gly-Asp (RGD) sequence exists in some bioactive proteins and is predicted to be an important sequence for biological activity.[1] Previously, we had found that Pro-Ala-Ser-Ser (PASS) sequence participated in cell binding and cell migration.[2] We were interested in the secondary structure of the RGDSPASS sequence in fibronectin (FN), the cell adhesion protein, and we have already synthesized RGDSPASS containing cyclic peptides.[3] We reported that both RGDS and PASS had an antiparallel location in cyclic peptide, FR-1, which exhibited high activity as a platelet-aggregation inhibitor.[4] This result suggests that this secondary structure of RGDSPASS in FR-1 is appropriate for binding to the fibrinogen receptor on the platelet surface and the structure corresponds to that of the active site of FN.

H-Cys^{1}-Arg^{2}-Gly^{3}-Asp^{4}-Ser^{5}-Pro^{6}-Ala^{7}-Ser^{8}-Ser^{9}-Cys^{10}-OH FR-1

H-Cys^{1}-Ala^{2}-Gly^{3}-Asp^{4}-Ser^{5}-Pro^{6}-Ala^{7}-Ser^{8}-Ser^{9}-Cys^{10}-OH [Ala^{2}]-FR-1

H-Cys^{1}-Arg^{2}-Gly^{3}-Ala^{4}-Ser^{5}-Pro^{6}-Ala^{7}-Ser^{8}-Ser^{9}-Cys^{10}-OH [Ala^{4}]-FR-1

H-Cys^{1}-Arg^{2}-Gly^{3}-Asp^{4}-Ser^{5}-Pro^{6}-Ala^{7}-Ala^{8}-Ala^{9}-Cys^{10}-OH [Ala^{8,9}]-FR-1

In order to investigate the relationship between the functional group and the biological activity of FR-1, we synthesized three Ala-analogs, [Ala^{2}]-FR-1, [Ala^{4}]-FR-1, and [Ala^{8,9}]-FR-1. The peptides were synthesized by the solution method.

Hexapeptides (Boc-Cys(Acm)-X-Pro-OTce)[5] and tetrapeptides (Boc-Ala-X-Cys(Acm)-OBzl)[5] were synthesized by the DCC-HOBt[5] method. Peptides 1, 2, and 3 were synthesized from the corresponding peptides, Boc-Cys(Acm)-X-Pro-OH and H-Ala-X-Cys(Acm)-OBzl by the fragment condensation with EDC-HOBt.[5] Peptides 1, 2, and 3 were confirmed by FABMS (MH^+ 1581, MNa^+ 1716, and MH^+ 1608, respectively) and ^{1}H-NMR. The formation of intramolecular disulfide bond was achieved by I_2 oxidation (r.t., 10 min.) in high dilution (1×10^{-3}M)(Scheme 1). The yields of the peptides 4, 5, and 6 were 77%, 77%, and 78%, respectively. These cyclic peptideds were confirmed by FABMS (4, MNa^+ 1459; 5, MH^+ 1550; 6, MH^+ 1464) and were treated with liq. HF (0 °C, 1 h) for deprotection (Scheme 1). The final products[6] were purified by HPLC (Biofine RPC-SC18 column, 10 x 250mm, JASCO Co.).

Boc-Cys(Acm)-Ala-Gly-Asp(OcHex)-Ser(Bzl)-Pro-Ala-Ser(Bzl)-Ser(Bzl)-Cys(Acm)-OBzl 1

Boc-Cys(Acm)-Arg(Tos)-Gly-Ala-Ser(Bzl)-Pro-Ala-Ser(Bzl)-Ser(Bzl)-Cys(Acm)-OBzl 2

Boc-Cys(Acm)-Arg(Tos)-Gly-Asp(OcHex)-Ser(Bzl)-Pro-Ala-Ala-Ala-Cys(Acm)-OBzl 3

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

Boc-Cys-Arg(Tos)-Gly-Ala-Ser(Bzl)-Pro-Ala-Ser(Bzl)-Ser(Bzl)-Cys-OBzl 5

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

1,2,3 —(1) I_2/MeOH, (2) HF-anisole→ [Ala^{2}]-FR-1 (54%), [Ala^{4}]-FR-1 (48%), [Ala^{8,9}]-FR-1 (69%)

Scheme 1.

NMR measurements were performed with a FT-NMR spectrometer, JEOL-JNM GSX 400 (^{1}H, 400MHz), in the same manner as FR-1.[4] Chemical shift assignments were obtained by the combined use of COSY and NOESY experiments and the data for amide protons are shown in Table 1. [Ala^{2}]-FR-1, [Ala^{4}]-FR-1, and [Ala^{8,9}]-FR-1 did not show any non-sequential (spatial) NOEs, but each H-D exchange rate of amide protons of [Ala^{8,9}]-FR-1 was similar to that of FR-1. Both [Ala^{2}]-FR-1 and [Ala^{4}]-FR-1 did not show

any difference between the H-D exchange rates of each amide proton. The slow H-D exchange rate indicates that the amide proton is shielded from the solvent. In [Ala^{8,9}]-FR-1, 4-Asp(NH) and 7-Ala(NH) were shielded from the solvent and may be involved in intramolecular hydrogen bonding.

RGD peptides are known to inhibit FN binding to the platelet surface[7] and FN is also known to inhibit platelet aggregation.[8] FR-1 exhibited high activity as a platelet aggregation inhibitor.[3] The IC_{50} of FR-1 was 32 μM. Under the same condition, inhibitory activity of [Ala^{2}]-FR-1(1mM) and [Ala^{4}]-FR-1(1mM) were less than 10% of the platelet aggregation. The IC_{50} of [Ala^{8,9}]-FR-1 was 320 μM which was 10 times lower than that of FR-1.

Table 1. Chemical shift and H-D exchange rate of amide proton, and coupling constant [a]

	FR-1			[Ala^{8,9}]-FR-1			[Ala^{2}]-FR-1			[Ala^{4}]-FR-1		
	NH	D/H	J_{NH-CH}	NH	D/H	J_{NH-CH}	NH	D/H	J_{NH-CH}	NH	D/H	J_{NH-CH}
1												
2	8.74	f	8.0	8.73	f	8.1	8.71	f	7.3	8.81	f	8.1
3	8.35	s	A	8.29	s	B	8.35	f	B	8.31	f	B
4	8.23	s	A	8.36	s	A	8.22	f	8.4	8.11	f	7.7
5	7.58	m	7.3	7.60	s	7.7	7.48	f	7.7	7.71	f	7.3
6												
7	7.88	s	7.0	7.76	s	7.7	7.96	f	6.6	7.77	f	7.7
8	7.63	m	7.0	7.59	m	7.0	7.64	f	7.3	7.52	f	7.7
9	7.75	m	7.7	7.84	f	7.0	7.71	f	7.7	7.88	f	8.1
10	8.16	f	8.1	8.21	f	8.1	8.12	f	8.4	8.24	f	8.1

a) Measurements were carried out by ^{1}H 400 MHz NMR spectrometer in [$^{2}H_6$] 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.

The NMR data suggest that the turn structure of [Ala^{8,9}]- FR-1 is similar to that of FR-1,[4] but [Ala^{2}]-FR-1 and [Ala^{4}]-FR-1 have flexible structures. The biological results indicate that the Arg and the Asp in FR-1 are essential for the activity and that the Ser-Ser in FR-1 is also necessary for the hydroxy groups binding to the receptor. The present results also support that the PASS sequence is a novel interaction site in FN.

The authors are grateful to Dr. Mitsuo Hayashi of Asahi Kasei Kogyo Co., Ltd. for measurement of mass spectra, elemental analysis, and amino acid analysis. We also thank Dr. Koji Yamada and Mr. Ichiro Matsuura of Kyowa Hakko Kogyo Co., Ltd. for the biological assay.

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5) Abbreviations: Acm, acetoamidomethyl; Boc, t-butoxycarbonyl; cHex, cyclohexyl; DCC, dicyclohexylcarbodiimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole; TFA, trifluroacetic acid.
6) [Ala^2]-FR-1: FABMS m/z 895(MH^+); Amino acid analysis(theoretical): Asp 1.01(1), Ser 2.83(3), Gly 1.01(1), Ala 1.00(1), $(Cys)_2$ 0.81(1), Pro 0.96 (1); Found: C,37.12; H,5.46; N,12.73%. Calcd for $C_{32}H_{50}N_{10}O_{16}S_2 \cdot TFA \cdot 5H_2O$: C,37.16; H,5.59; N,12.74%.
[Ala^4]-FR-1: FABMS m/z 936(MH^+); Amino acid analysis(theoretical): Ser 2.79(3), Gly 1.00(1), Ala 2.00(2), $(Cys)_2$ 0.85(1), Arg 0.97(1), Pro 1.00 (1); Found: C,36.86; H,5.59; N,14.57%. Calcd for $C_{34}H_{57}N_{13}O_{14}S_2 \cdot 2TFA \cdot 4.5 H_2O$: C,36.66; H,5.50; N,14.62%.
[$Ala^{8,9}$]-FR-1: FABMS m/z 948(MH^+); Amino acid analysis(theoretical): Asp 1.05(1), Ser 0.99(1), Gly 1.04(1), Ala 3.00(3), $(Cys)_2$ 0.85(1), Arg 0.98 (1), Pro 1.04(1); Found: C,37.26; H,5.36; N, 14.37%. Calcd for $C_{35}H_{57}N_{13} O_{14}S_2 \cdot 2TFA \cdot 4.5H_2O$: C,37.26; H,5.45; N,14.48%.
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(Received October 22, 1993)