Ro 09-1679, A NOVEL THROMBIN INHIBITOR

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Among the serine proteases involved in thrombosis and hemostasis, thrombin plays a key role in blood coagulation; for example, it converts fibrinogen into fibrin and activates the cofactor V and VIII by a speciffc cleavage of certain peptide bonds next to arginine in physiological substrates. It also acts as a potent agonist for platelet aggregation. Therefore, thrombin is an effective target for an anticoagulant agent. During our screening program for thrombin inhibitors, we isolated a novel inhibitor, Ro 09-1679 (**1**), from the culture broth of strain NR6773 collected at Minamishitara, Aichi Prefecture, Japan. The strain was identified as *Mortierella alpina* Peyronel[1,2] on the basis of its morphological properties. In this paper, the fermentation, isolation, structural elucidation and biological properties of **1** are reported.

The stock culture of the producing organism, *M. alpina* NR6773, (stored at −80°C) was thawed and 200 μl was inoculated into five, 500-ml Erlenmeyer flasks each containing 100 ml of seed medium consisting of glycerol 3%, glucose 2%, Polypepton 0.5%, yeast extract 0.2%, NaCl 0.3% and $CaCO_3$ 1%. All were agitated on a rotary shaker for 3 days at 27°C and 190 rpm. Then, the cultures were combined and transferred to a 50-liter jar fermenter containing

Fig. 1. Isolation procedure of Ro 09-1679.

Broth filtrate (85 liters)
↓ carbon adsorption, eluted with 50% aqueous acetone
Eluate
↓ SP-Toyopearl column chromatography, eluted with 50 mM NaCl
Concentrated active fractions
↓ prep HPLC Capcell pak C18, eluted with MeOH-phosphate buffer (pH 9.0) (5:95)
Concentrated active fractions
↓ SP-Toyopearl column chromatography, eluted with 50 mM NaCl
Concentrated active fractions
↓ Sephadex LH-20 column chromatography, eluted with MeOH
Ro 09-1679 (70 mg)

Table 1. Physico-chemical properties of Ro 09-1679.

Appearance	Colorless powder
HRFAB-MS (*m/z*)	Calcd: 526.3103
$(M+H)^+$	Found: 526.3121
Molecular formula	$C_{22}H_{39}N_9O_6$
UV $\lambda_{max}^{H_2O}$	End absorption
$[\alpha]_D^{24}$	−38.7° (*c* 1.06, H_2O)
IR (KBr) cm^{-1}	3400~3200, 1660, 1560, 1395
Color reaction:	
Positive	Lydon-Smith, I_2 vapor, Sakaguchi, triphenyl, tetrazolium chloride
Negative	$FeCl_3$, ninhydrin
Amino acid composition	L-Leu (1), L-Arg (1)

Table 2. NMR chemical shifts of **2** in D_2O.

	^{13}C	^1H
Fumaric acid		
1	176.1	
2[c]	133.0	6.69 (d, J=15.6)[a]
3[c]	140.4	6.73 (d, J=15.6)
4	170.5	
Arginine 1		
1′	176.3	
2′	56.5	4.40 (dd, J=7.8, 6.3)
3′	31.0	1.89, 1.78[b]
4′	27.2	1.65[b]
5′	43.4	3.20 (t, J=7.1)
6′	159.6	
Leucine		
1″	176.5	
2″	55.5	4.35 (dd, J=10.0, 4.5)
3″	42.3	1.69, 1.63[b]
4″	27.2	1.64[b]
5″	25.1	0.92 (d, J=5.9)
6″	23.5	0.87 (d, J=5.9)
Arginine 2		
1‴	180.9	
2‴	57.4	4.16 (dd, J=7.8, 4.9)
3‴	31.7	1.85, 1.70[b]
4‴	27.2	1.57[b]
5‴	43.5	3.18 (t, J=7.1)
6‴	159.6	

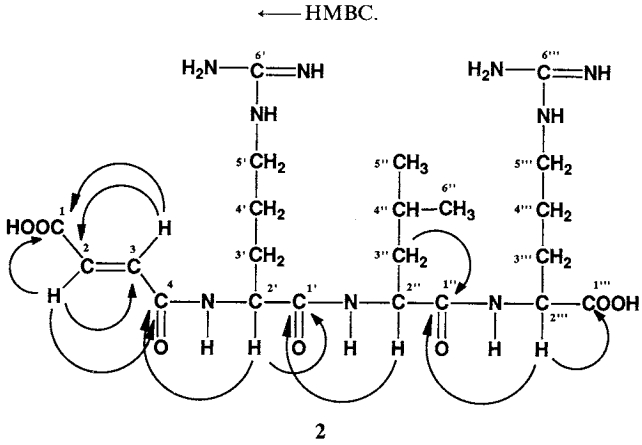
[a] Multiplicity and coupling constant (Hz) are in parentheses.
[b] J value could not be determined.
[c] These assignments are referred to ^{13}C NMR chemical shift values of synthetic fumaramic acid[8].

30 liters of production medium which was the same as the seed medium above. The fermentation was carried out at 27°C for 3 days with agitation at 300 rpm. The thrombin inhibitory activity of **1** was measured by using the Boc-Val-Pro-Arg-methyl-coumarylamide (MCA) as a substrate, and the product was measured at the excitation wavelength of 380 nm and emission wavelength of 460 nm[3].

The purification procedure is shown in Fig. 1. We isolated 70 mg of **1** as an amorphous powder from 85 liters of culture broth. The physico-chemical properties of **1** are summarized in Table 1. The molecular formula of **1** was determined to be $C_{22}H_{39}N_9O_6$ on the basis of HRFAB-MS. The ^1H and ^{13}C NMR spectra of **1** in D_2O indicated that **1** existed as an equilibrium mixture similar to that of leupeptin[4,5] and suggested the presence of an aldehyde group at the *C*-terminal since both spectra were complex. This assumption was supported by 1) the reducing properties of **1** which gave a positive reaction to the triphenyl tetrazolium chloride reagent (Table 1), and 2) the presence of weak signal at δ 9.48, assignable to an aldehyde proton in ^1H NMR spectrum of **1**.

In order to exclude a state of equilibrium, **1** was treated with PtO_2-O_2 at pH 7.5 to give oxidation products, **2** and **3**. Since **2** and **3** showed quite similar ^1H and ^{13}C NMR spectra and almost identical

Fig. 2. Structure and HMBC correlation of **2**.



2

Fig. 3. Structure and mass fragmentation pattern of Ro 09-1679 (**1**).

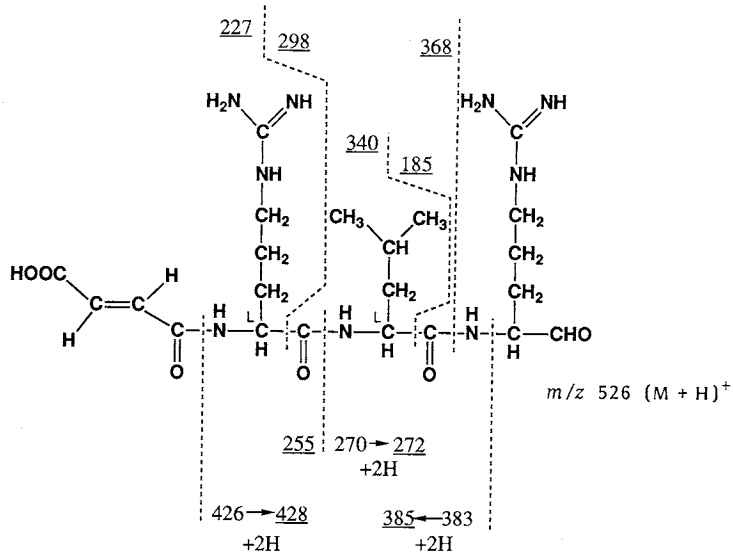


Table 3. Enzyme inhibitory activity of Ro 09-1679.

	Enzyme inhibition (IC_{50} μM)			
	Thrombin	Factor Xa	Trypsin	Papain
Ro 09-1679	33.6	3.3	0.04	0.0346
Antipain	12.5	5.1	0.055	0.0458
Leupeptin	15.5	7.9	0.045	0.0124

IC_{50}: The mixture of 195 μl of enzyme solution (final concentration; 0.08 u/ml thrombin, 0.015 u/ml factor Xa, 0.03 u/ml trypsin, 0.076 u/ml papain) and 50 μl inhibitor solution was incubated at 37°C. After 1 minute, 5 μl substrate solution in 100% DMSO (final concentration; 20 μM Boc-Val-Pro-Arg-MCA for thrombin, 120 μM Boc-Ile-Glu-Gly-Arg-MCA for factor Xa, 120 μM Bz-Arg-MCA for trypsin and papain) was added. Fluorescence of 7-amino-4-methylcoumarin (AMC) produced was monitored in 30 seconds intervals up to 180 seconds using spectrophotomeric centrifugal analyzer (Cobas Bio, Roche Diagnostica Inc.) at 380 nm excitation and 460 nm emission wavelength.

HRFAB-MS spectra (**2**: m/z 542.3090 $(M+H)^+$, **3**: m/z 542.3002 $(M+H)^+$; calcd for $C_{22}H_{40}N_9O_7$: 542.3051), it was suggested that the PtO_2 oxidation of **1** afforded two epimers. The amino acid analysis of **2** indicated that **2** possessed 2 mol of arginine and 1 mol of leucine, while **1**, 1 mol of arginine and 1 mol of leucine (Table 1). This result indicated that the *C*-terminal end of **1** was arginal.

The ^1H and ^{13}C NMR data of **2** are summarized in Table 2. The ^1H signals of amino acid residues were mainly assigned by ^1H-^1H correlation spectroscopy (COSY) and selective coherence transfer experiments[6,7]. The presence of a fumaric acid residue in **2** was clarified by the ^1H NMR, ^{13}C NMR and heteronuclear multiple-bond connectivity (HMBC) experiments of **2**. The coupling constant (J = 15.6 Hz) between two olefinic protons (2-H and 3-H) indicated an *E*-geometrical configuration. In the HMBC spectrum of **2**, two olefinic protons (2-H and 3-H) showed long-range CH couplings with carbonyl carbons, C-1 (δ 176.1) and C-4 (δ 170.5) (Fig. 2). These results indicated the presence of a fumaric acid residue in **2**. The sequence of each unit in **2** was assigned by the HMBC experiment of **2** (Fig. 2). All of the α-methine protons of the amino acid residues showed long-range CH coupling with the carbonyl carbon of the adjacent unit. Therefore, the structure of **2** was determined to be fumaryl-arginyl-leucyl-arginine.

From the structure of **2**, we deduced that the structure of Ro 09-1679 is **1**. The structure of **1** was confirmed by FAB-MS fragment analysis (Fig. 3). The configurations of amino acids derived from **1** were determined by HPLC analyses† and were found to be all L-configurations. Therefore, the structure of Ro 09-1679 was determined to be fumaryl-L-arginyl-L-leucyl-arginal.

The inhibitory activity of Ro 09-1679 against thrombin, factor Xa, trypsin and papain is shown in Table 3. Ro 09-1679 inhibited the enzyme activities, with IC_{50} values ranging from 0.03 μM to 34 μM. Ro 09-1679 had no antimicrobial activity.

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† Configurations of the amino acids were determined by the following HPLC conditions. Leucine: Column YMC-pack FL-ODS3, 4.6 i.d. × 30 mm; mobile phase 2 mM *N*,*N*-dipropyl-L-alanine, 1 mM copper acetate; flow rate 1.0 ml/minute; detection UV 230 nm. Arginine: Column TSK-GEL Enantio L-1, 4.6 i.d. × 250 mm; mobile phase 1 mM copper sulfate; flow rate 1.0 ml/minute; detection UV 210 nm.

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