YM-47141 and YM-47142, New Elastase Inhibitors Produced by *Flexibacter* sp. Q17897

II. Structure Elucidation

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(Received for publication March 20, 1995)

YM-47141 and YM-47142 are new elastase inhibitor produced by *Flexibacter* sp. Q17897. These structures were elucidated by MS and NMR spectral analysis. YM-47141 and YM-47142 were the cyclic peptides containing tricarbonyl moiety hydrated on the center carbonyl carbon in DMSO-d_6.

In the screening program for new elastase inhibitors, we discovered novel substances named YM-47141 (**A**) and YM-47142 (**B**) from the culture broth of *Flexibacter* sp. Q17897. In the preceding paper[1], we have described the taxonomy, fermentation, isolation, physico-chemical properties and biological activities of new elastase inhibitors, **A** and **B**. This article describes the structural elucidations of **A** and **B** by spectroscopic studies including various two-dimensional NMR experiments.

Results

Structure of YM-47141 and YM-47142

The molecular formula of **A** was determined to be $C_{46}H_{62}N_8O_{13}$ based on positive-ion high resolution FAB-MS ($(MH+H_2O)^+$, $C_{46}H_{65}N_8O_{14}$, m/z calcd: 953.4621, found: 953.4646) and negative-ion FAB-MS (m/z 934 $(M)^-$).

In the room temperature, the NMR spectra of **A** showed many peaks due to the conformational changes. Since it was found that **A** formed a single conformation at 17°C in DMSO-d_6 by the variable temperature experiment, all NMR experiments were carried out under this condition. The ^1H NMR spectrum of A is shown in Fig. 2. The structure of A was elucidated by ^1H NMR, ^{13}C NMR, DEPT, doouble-quantum-filtered COSY (DQF-COSY)[2], homonuclear Hartmann-Hahn spectroscopy (HOHAHA)[3], NOESY, ^1H-^{13}C COSY, heteronuclear multiple-bond correlation (HMBC)[4] and ^{15}N-^1H heteronuclear multiple-quantum coherence (^{15}N-^1H HMQC)[5] experiments.

The ^{13}C NMR spectrum (Fig. 3) revealed the presence of 46 carbons, which were assigned to 7 methyls, 5 methylenes, 21 methines and 13 quaternary carbons by DEPT experiments. The ^1H-^{13}C COSY spectrum established all one-bond ^1H-^{13}C correlation.

Fig. 1. Structure of YM-47141 and YM-47142.

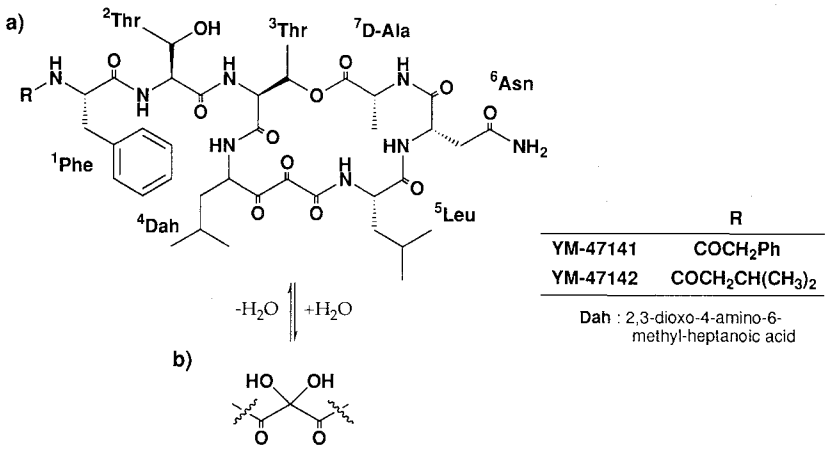


Fig. 2. ^{1}H NMR spectrum of YM-47141.

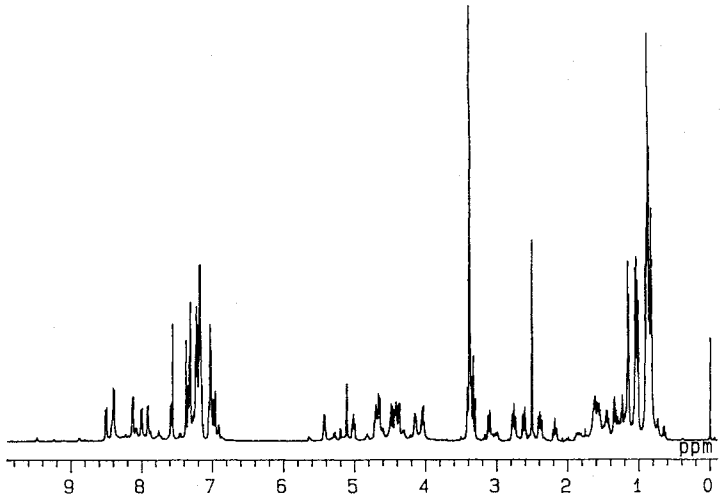
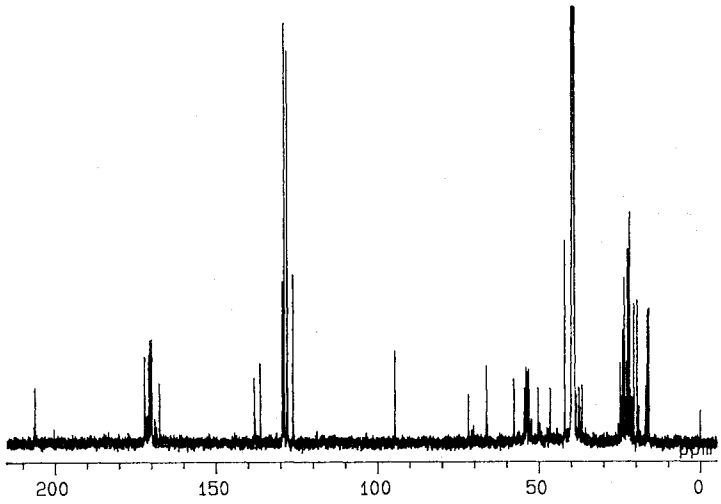


Fig. 3. ^{13}C NMR spectrum of YM-47142.



The proton spin networks were obtained from phase-sensitive HOHAHA and DQF-COSY experiments and it was clarified that **A** contained seven amino acids (Ala, Asn, Phe, Leu, two Thr and one unknown amino acid). Although the pattern of the proton spin network cannot distinguish between Asn and Asp, it was confirmed to be Asn from the ^{13}C-^{1}H long-range couplings between two exchangeable protons (δ 6.95 and δ 7.38) of the $CONH_2$ group and γ carbon (δ 170.5) of Asn by HMBC experiment, and from the geminal ^{15}N-^{1}H couplings by ^{15}N-^{1}H HMQC experiment. The amino acid sequence of **A** was determined by the sequential NOEs between amide protons and α protons, and the ^{13}C-^{1}H long-range couplings between carbonyl carbons and, amide and α protons. These analyses established the partial structures of **1** and **2** (Fig. 4-a).

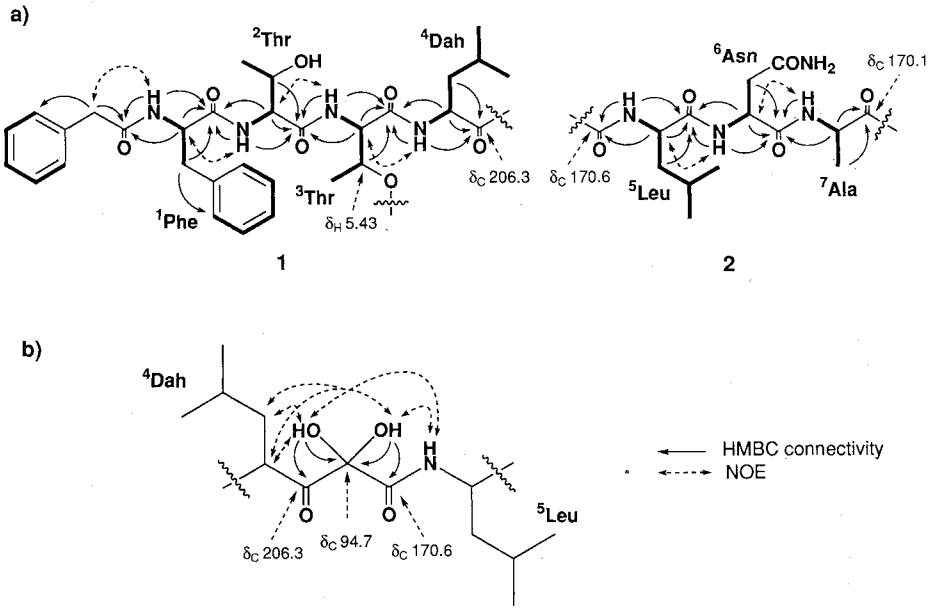
A ^{13}C-^{1}H long-range coupling was observed between the low field shifted β proton (δ 5.43) of ^{3}Thr of partial structure **1** and the carbonyl group (δ 170.1) of ^{7}Ala of partial structure of **2**, suggesting the linkage of ^{3}Thr and ^{7}Ala through the ester bond. Two exchangeable protons (δ 7.38 and δ 7.56) showed the ^{13}C-^{1}H long-range couplings to both a quaternary carbon (δ 94.7) and two carbonyl groups of δ 206.3 (the carbonyl carbon adjacent to the amino-methine of 2,3-dioxo-4-amino-6-methyl-heptanoic acid; ^{4}Dah) and δ 170.6 (the carbonyl carbon directly binding to the N-terminal of ^{5}Leu) (Fig. 4-b). The NOEs shown in Fig. 4-b (dot line) were observed in the NOESY spectrum. These results established the exchangeable protons were hydroxy protons and attached to the quaternary carbon (δ 94.7), and its quaternary carbon was connected to both the carbonyl

Table 1. ^{1}H and ^{13}C chemical shifts of YM-47141 and YM-47142 in DMSO-d_6 (17°C).

	YM-47141		YM-47142	
	δ_H	δ_C	δ_H	δ_C
BzCO				
1		169.9		
2	3.32 (d, 13.4 Hz), 3.39 (d, 13.4 Hz)	40.1		
3		136.4		
4, 8	7.03 (m)	129.0		
5, 7	7.18 (m)	128.1		
6	7.17 (m)	126.2		
iVal				
1				171.5
2			1.86 (m)	44.5
3			1.81 (m)	25.5
4			0.63 (d, 6.1 Hz)	22.1
			0.73 (d, 6.7 Hz)	22.2
[1]Phe				
CO		172.7		172.4
NH	8.40 (d, 8.6 Hz)		8.08 (d, 8.6 Hz)	
α	4.70 (m)	54.1	4.70 (m)	53.9
β	2.76 (dd, 13.4, 11.6 Hz), 3.11 (dd, 13.4, 2.5 Hz)	37.8	2.72 (dd, 13.4, 11.6 Hz) 3.08 (dd, 13.4, 2.5 Hz)	37.5
γ		138.1		138.3
δ	7.32 (m)	129.5	7.32 (m)	129.0
ε	7.23 (m)	127.9	7.22 (m)	128.1
ζ	7.17 (m)	126.2	7.15 (m)	126.1
[2]Thr				
CO		170.4		170.4
NH	8.50 (d, 8.6 Hz)		8.37 (d, 8.6 Hz)	
α	4.37 (dd, 8.6, 2.5 Hz)	57.9	4.37 (dd, 8.6, 2.5 Hz)	57.7
β	4.15 (m)	66.3	4.14 (m)	66.3
γ	1.04 (q, 6.1 Hz)	19.7	1.06 (q, 6.1 Hz)	19.6
[3]Thr				
CO		167.6		167.7
NH	7.58 (d, 8.6 Hz)		7.58 (d, 9.2 Hz)	
α	4.66 (dd, 8.6, 2.4 Hz)	54.6	4.64 (d, 9.2, 2.4 Hz)	54.6
β	5.43 (m)	71.9	5.42 (m)	71.9
γ	1.15 (d, 6.1 Hz)	16.1	1.15 (d, 6.1 Hz)	16.0
[4]2,3-dioxo-4-amino-6-methyl-heptanoic acid				
CO		170.6		170.5
NH	8.00 (d, 8.6 Hz)		8.00 (d, 8.0 Hz)	
α	7.38 (s), 7.56 (s)	94.7	7.38 (s), 7.55 (s)	94.6
β CO		206.3		206.2
γ	5.02 (m)	53.7	5.01 (m)	53.7
δ	1.31 (m), 2.18 (m)	38.6	1.33 (m), 2.17 (m)	38.6
ε	1.61 (m)	24.1	1.62 (m)	24.1
ζ	0.87 (d, 6.1 Hz) 0.90 (d, 6.7 Hz)	20.7 22.6	0.88 (d, 6.1 Hz) 0.90 (d, 6.7 Hz)	20.6 22.6
[5]Leu				
CO		172.2		172.2
NH	8.13 (d, 7.9 Hz)		8.13 (d, 7.9 Hz)	
α	4.04 (m)	53.2	4.04 (m)	53.2
β	1.45 (m), 1.56 (m)	39.7	1.45 (m), 1.56 (m)	39.9
γ	1.60 (m)	24.9	1.60 (m)	24.9
δ	0.83 (d, 6.7 Hz) 0.88 (d, 6.1 Hz)	22.0 23.6	0.83 (d, 6.1 Hz) 0.87 (d, 6.1 Hz)	21.9 23.6
[6]Asn				
CO		170.5		170.5
NH	7.91 (d, 6.7 Hz)		7.91 (d, 6.7 Hz)	
α	4.42 (m)	50.4	4.42 (m)	50.4
β	2.38 (dd, 15.9, 11.0 Hz) 2.62 (dd, 15.9, 3.1 Hz)	36.7	2.38 (dd, 15.9, 11.0 Hz) 2.62 (dd, 15.9, 2.5 Hz)	36.7
γ		170.9		170.9
δ NH_2	6.96 (s), 7.34 (s)		6.95 (s), 7.38 (s)	
[7]D-Ala				
CO		170.01		170.1
NH	6.99 (d, 9.2 Hz)		6.99 (d, 9.2 Hz)	
α	4.48 (m)	46.6	4.48 (m)	46.6
β	1.02 (d, 7.3 Hz)	16.5	1.02 (d, 7.3 Hz)	16.5

Fig. 4. NMR analysis of YM-47141.

a) Partial structure of YM-47141. (Bold lines show proton spin networks obtained by a DQF-COSY).
b) NOE and HMBC connectivity of triketocarbonyl moiety.



carbons of δ 206.3 and δ 170.6. The structure of **A** in DMSO-d_6 was thus elucidated to be (b) in Fig. 1, which was hydrated on 2-ketocarbonyl of 2,3-dioxo-4-amino-6-methyl-heptanoic acid. ^{1}H and ^{13}C assignments of **A** in DMSO-d_6 are shown in Table 1.

The molecular formula of **B** was determined to be $C_{43}H_{64}N_8O_{13}$ based on positive-ion high resolution FAB-MS ($(MH+H_2O)^+$, $C_{43}H_{67}N_8O_{14}$, m/z calcd: 919.4777, found: 919.4825) and negative-ion FAB-MS data, m/z 900 $(M)^-$. All NMR spectra of **B** were carried out by the same experiments as those of **A**.

The ^{1}H and ^{13}C NMR spectra of **B** were very similar to those of **A** except for the presence of the isovarelyl group instead of the phenylacetyl group. The structure of **B** is shown in Fig. 1. ^{1}H and ^{13}C chemical shifts of **B** were shown in Table 1.

YM-47141 and YM-47142 are the first evidence that microorganisms produce peptides containing 2,3-dioxo-4-amino-6-methyl-heptanoic acid.

Stereochemistry of Amino Acids

Acid hydrolysates of YM-47141 and YM-47142 were analyzed using MARFEY's reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA)[6] in the condition as mentioned in the experimental section and each of the peaks was confirmed by co-injection with authentic amino acid derivative. The retention time of the authentic amino acids are listed in Table 2. Unfortunately, the FDAA derivatives of L-Thr and L-allo-Thr were not separated, so amino acid analysis on the chiral column, MCI GEL CRS10W was carried out. On this HPLC analysis, L-Thr and L-allo-Thr were eluted at 10.85 minutes and 19.02 minutes, respectively. As the result, both YM-47141 and YM-47142 contain of one mole of D-Ala, L-Asp, L-Leu, L-Phe and two moles of L-Thr. Stereochemistry of 4-amino-2,3-dioxo-6-methylhepatanoic acid should be determined.

Table 2. HPLC data of FDAA-amino acids derivatives.

FDAA-derivatives	Rt (minutes)
D-Ala	31.78
L-Ala	25.90
D-Asp	24.42
L-Asp	21.25
D-Leu	50.57
L-Leu	43.40
D-Phe	49.10
L-Phe	42.20
D-Thr	27.50
L-Thr	21.73
D-allo-Thr	24.92
L-allo-Thr	21.73

Experimental

Fast atom bombardment spectra (FAB-MS) were measured on JEOL JMS-DX 300 and VG ZAB-VSE spectrometer using 10% 3-nitorobenzyl alcohol-DMSO as a matrix.

NMR spectra were recorded in DMSO-d_6 at 17°C with a JEOL ALPHA-500 spectrometer.

The amino acids in the acid hydrolyzate was derivatized with FDAA[6] and analyzed by reversed-phase HPLC on a TSKgel-ODS-80TS (4.6 i.d. × 150 mm). A linear gradient from 10 to 40% MeCN in 0.05 M triethylamine phosphate buffer at pH 3.0 (flow rate 1.2 ml/minute: UV detection at 340 nm) was used to separate the amino acid derivatives. The absolute stereochemistry of each compound was determined by the comparison of the retention time with those of authentic L or D amino acid derivatives. Separation of L-threonine from L-allo-threonine was carried out on a chiral column. A portion of the acid hydrolyzate was dissolved in 0.1 N HCl and analyzed by HPLC on MCI GEL CRS10W 4.6 i.d. × 50 mm (Mitsubishi Kasei) with 0.2 mM $CuSO_4$ (flow rate 0.5 ml/minute: UV detection at 254 nm).

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