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Glomosporin, a Novel Antifungal Cyclic Depsipeptide from *Glomospora* sp.

II. Structure Elucidation

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The structure of glomosporin, an antifungal antibiotic, was elucidated by NMR and MS spectroscopic studies. Glomosporin is a novel cyclic depsipeptide with an amino acid sequence Ser-Ala-Asp-Asn-Asn-Ser-Thr, and a 3,4-dihydroxy-4-methylhexadecanoic acid side chain.

During the course of our screening for antifungal antibiotics, a novel cyclic depsipeptide was isolated from the culture of *Glomospora* sp. BAUA2825. This strain is a rare fungus, taxonomy of the producing strain, fermentation, isolation and biological properties have been described in the preceding paper[1,2]. Here we report on the structure determination of glomosporin (**1**) on the basis of chemical and spectroscopic studies.

Results

Structure Determination

The physico-chemical properties of **1** were already described[1]. The amino acid component analysis of **1** indicated the presence of 1×Ala, 1×Thr, 2×Ser and 3×Asx.

The ^{1}H-NMR spectrum of **1** in CD_3OD afforded sharp signals, but amide signals disappeared because of deuteration in CD_3OD. The spectrum in DMSO-d_6 afforded amide and hydroxy proton signals, however, other signals was broader than those obtained in CD_3OD. Thus, analysis of the NMR spectra of **1** was carried out mainly with spectra recorded in CD_3OH.

The ^{1}H and ^{13}C-NMR spectra of **1** indicated eleven amide protons (δ 6.8~8.6) and eleven carbonyl carbons (δ 169~176). The presence of an alkyl chain moiety was suggested by a triplet methyl proton signal (δ 0.88) and overlapping methylene proton signals (δ 1.2~1.4) and methylene carbon signals (δ 30.63~30.66) (Table 1).

Eleven partial structures were revealed by the analysis of the DQF-COSY spectrum of **1** (Fig. 2).

The amino acid components of **1** was confirmed by an HMBC spectrum as follows. The correlation from two methine protons (δ 4.36 and 4.11) of the partial structure I to a carbonyl carbon (δ 169.80) indicated the presence of Thr moiety. Ala was indicated by the correlation from methyl and methine protons (δ 1.39 and 4.16) of the partial structure VII to a carbonyl carbon (δ 175.34). Two Ser (Ser-1 and Ser-2) were suggested by the cross peaks from methylene and methine protons (δ 3.80, 3.70 and 4.49) of the partial structure II to a carbonyl carbon (δ 173.02) and from those of the partial structure III (δ 3.99, 3.89 and 4.38) to a carbonyl carbon (δ 172.34). Two Asn (Asn-1 and Asn-2) were indicated by the correlation from a methine proton (δ 4.71) of the partial structure IV to two carbonyl carbons (δ 173.25 and 175.02), from two amide protons (δ 7.54, 6.84) to the carbonyl carbon (δ 175.02), from a methine proton (δ 4.62) of the partial structure V to two carbonyl carbons (δ 173.35 and 174.89) and from two amide protons (δ 7.61, 6.92) to the carbonyl carbon (δ 174.89). Asp was supported by the correlation from methylene and methine protons (δ 2.7~2.9 and 4.62) of the partial structure VI to two carbonyl carbons (δ 172.88 and 174.34).

The amino acid sequence of **1** was determined from the

Table 1. ^{1}H- and ^{13}C-NMR of **1** (in CD_3OH).

Position		δ ^{1}H ppm (multiplicity, J)[a]	δ ^{13}C ppm[b]
Ser-1	NH	8.10 (d, 8.2)	
	C-1		173.02
	C-2	4.49 (ddd, 8.2, 6.4, 5.5)	56.30
	C-3	3.80 (dd, 11.3, 6.4), 3.70 (dd, 11.3, 5.5)	62.75
Ala	NH	8.55 (d, 5.2)	
	C-1		175.34
	C-2	4.16 (dq, 5.2, 7.3)	52.34
	C-3	1.39 (d, 7.3)	16.78
Asp	NH	8.07 (d, 7.6)	
	C-1		172.88
	C-2	4.62 (m)	51.43
	C-3	2.7-2.9 (m)	35.89
	COOH		174.34
Asn-1	NH	8.10 (d, 8.2)	
	C-1		173.25
	C-2	4.71 (m)	51.95
	C-3	2.7-2.9 (m)	38.02
	$CONH_2$	7.54 (s), 6.84 (s)	175.02
Asn-2	NH	8.37 (d, 6.7)	
	C-1		173.35
	C-2	4.62 (m)	52.76
	C-3	2.7-2.9 (m)	37.38
	$CONH_2$	7.61 (s), 6.92 (s)	174.89
Ser-2	NH	8.22 (d, 7.6)	
	C-1		172.34
	C-2	4.38 (ddd, 7.6, 4.0, 4.6)	56.96
	C-3	3.99 (dd, 11.3, 4.0), 3.89 (dd, 11.3, 4.6)	63.04
Thr	NH	8.30 (d, 7.6)	
	C-1		169.80
	C-2	4.36 (dd, 7.6, 4.6)	60.66
	C-3	4.11 (dq, 4.6, 6.7)	68.93
	C-4	1.28 (d, 6.7)	20.27
Acyl	1		173.60
	2	2.66 (dd, 14.3, 10.1), 2.58 (dd, 14.3, 2.1)	38.47
	3	5.23 (dd, 10.0, 2.1)	78.76
	4		74.68
	5	1.4-1.6 (m)	39.18
	6-13	1.2-1.4*	31.20, 30.66*, 30.63*30.33, 24.18
	14	1.2-1.4*	32.90
	15	1.2-1.4*	23.60
	16	0.88 (t, 6.7)	22.67
	17	1.17 (s)	14.30

[a] Chemical shifts are shown with reference to CD_3OH as 3.30 ppm

[b] Chemical shifts are shown with reference to CD_3OH as 49.80 ppm

* Overlapped signals in column.

Fig. 1. Structure of glomosporin (**1**).

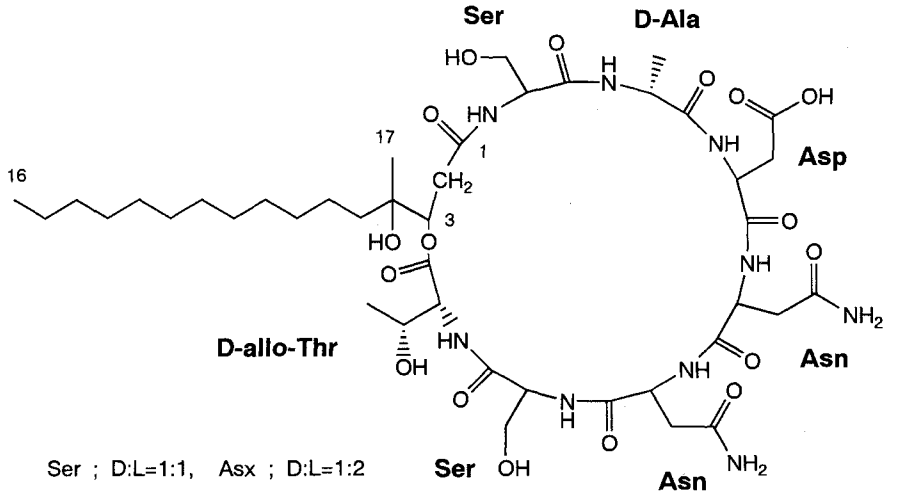
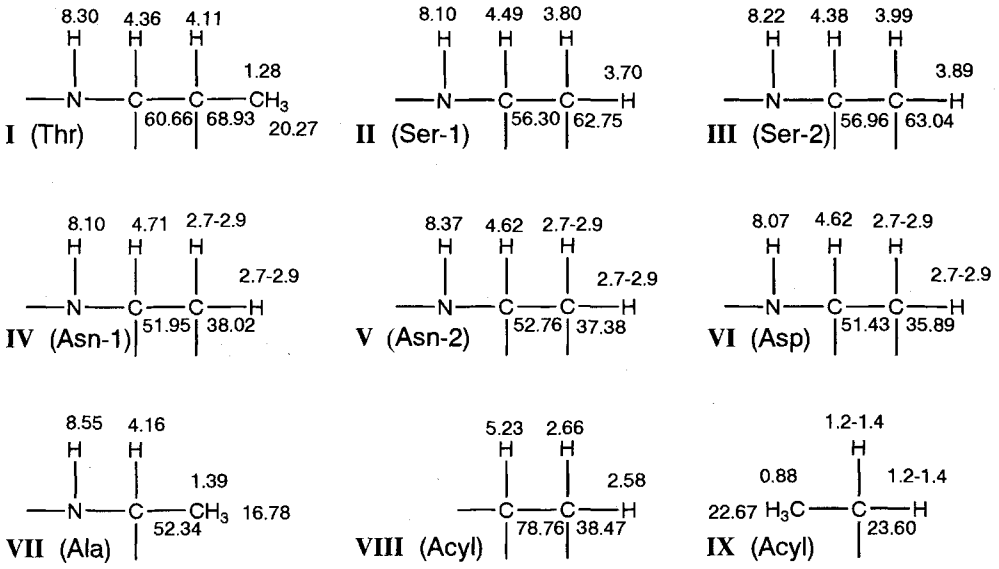


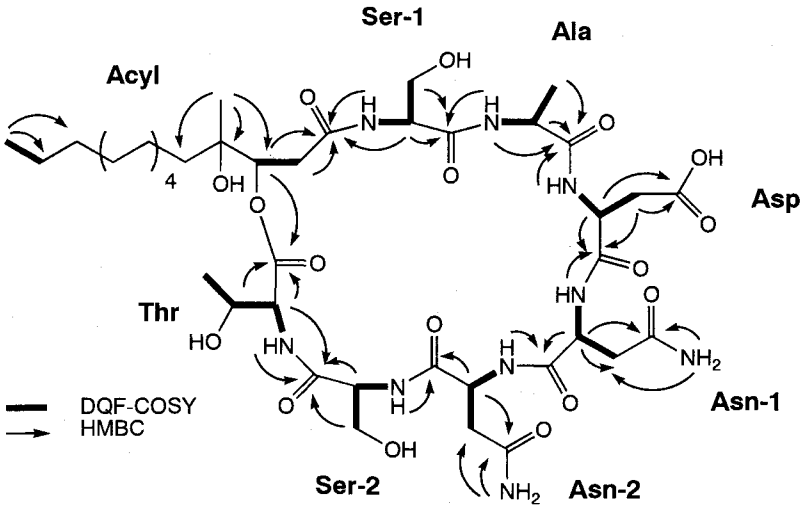
Fig. 2. Partial structures of **1**.



HMBC spectrum as follows. The correlation from an amide proton (δ 8.30) of Thr to the carbonyl carbon of Ser-2, from an amide proton (δ 8.22) of Ser-2 to carbonyl carbon (δ 173.34) of Asn-2, from an amide proton (δ 8.37) of Asn-2 to the carbonyl carbon (δ 173.25) of Asn-1, from an amide proton (δ 8.10) of Asn-1 to the carbonyl carbon (δ 172.88) of Asp, from an amide proton (δ 8.07) of Asp to the carbonyl carbon of Ala, and from an amide proton (δ 8.55) of Ala to the carbonyl carbon of Ser-1 indicated that the amino acid sequence of **1** is Ser-Ala-Asp-Asn-Asn-Ser-Thr.

A 3,4-dihydroxy-4-methylhexadecanoic acid portion was proposed by the correlations from methylene and methine protons (δ 2.66, 2.58 and 5.23) of the partial structure VIII to a carbonyl carbon (δ 173.60) and from a methyl proton (δ 1.17) to the methine carbon (δ 78.76), a quaternary carbon (δ 74.68) and a methylene carbon (δ 39.18). The bond between the remaining alkyl chain moiety containing the partial structure IX and the 3,4-dihydroxy-4-methylhexadecanoic acid portion was deduced by the

Fig. 3. HMBC and DQF-COSY correlation of **1** in CD_3OH.



degree of unsaturation and calculated from the molecular formula obtained by HRFAB-MS. Therefore, the acyl chain moiety was deduced to be a 3,4-dihydroxy-4-methylhexadecanoic acid.

The cyclic structure of **1** was evident from the correlation from H-3 of acyl chain to carbonyl carbon of Thr and from amide and methine protons of Ser-1 to carbonyl carbon of the acyl chain.

In the ^{1}H-NMR spectrum of **1** in DMSO-d_6, five additional protons exchangeable protons with D_2O, were observed at δ 12.29 (br s), 5.07 (d), 5.06 (br s), 4.87 (br s) and 4.36 (s). The proton at δ 12.29 was assigned to the γ-COOH of Asp from its chemical shift. From the DQF-COSY spectrum in DMSO-d_6, two protons at δ 5.07 and 4.87 were assigned to the OH of Thr and the OH of Ser-1, respectively. In the HMBC spectrum, the signal at δ 4.36 showed a correlation to C-4 of the acyl chain moiety. The remaining exchangeable proton was assigned to the OH of Ser-2. Thus, the structure of **1** was suggested as shown in Fig. 1.

To confirm the structure, **1** was applied to electrospray ionization MS/MS (ESI-MS/MS), but **1** did not give fragments. To obtain fragments, **2** and **3** were prepared by treatment with 50% dimethylamine followed with 1% $SOCl_2$/MeOH.

In the ESI-MS/MS spectra of **2** and **3**, b-type ($(M+H-X)^+$) and y-type ($(M+2H-X)^+$) fragments were obtained as shown in Fig. 4. Due to methylation at the γ-COOH of Asp and the terminal COOH of Thr, the Asp and Thr portions of **3** were 14 mass units larger than that of **2**. These results indicated that the amino acid sequence of **1** is Ser-Ala-Asp-Asn-Asn-Ser-Thr and the molecular weight of the acyl chain moiety was 285 ($C_{17}H_{33}O_3$). These results were in accord with NMR results, thus the structure of **1** was determined as shown in Fig. 1.

The absolute configuration of the amino acids were determined by MARFEY's method[3]. Usually this method was detected by UV absorption at 340 nm for FDAA (1-fluoro-2,4-dinitrophenyl-5-L-alanyl-amide) derivative, however we used ESI-MS for detection as described by HARADA *et al.*[4]. As shown in Fig. 5, the amino acids of **1** include D-Ala and D-*allo*-Thr. Moreover it was indicated that the configuration of Ser was D : L = 1 : 1, and that of Asx was D : L = 1 : 2.

Discussion

The structure of **1** is a cyclic depsipeptide containing a β-hydroxy fatty acid and a heptapeptide. Among the compounds of this class, surfactins[5,6], halobacillins[7,8], pumilacidins[9], iturins[10,11] were isolated from bacteria *Bacillus* sp. and CDPC 3510s[12,13] was isolated from *Fusarium* sp. The amino acid components of these compounds produced from bacteria are rich in hydrophobic amino acids, such as Leu, Ile and Val. In contrast, **1** is formed from hydrophilic amino acids, such as Ser, Thr and Asx. Although CDPC 3510s contains D-*allo*-Thr common to **1**, its component amino acids and the sequence, D-*allo*-

Fig. 4. MS/MS analysis of **1** derivatives.

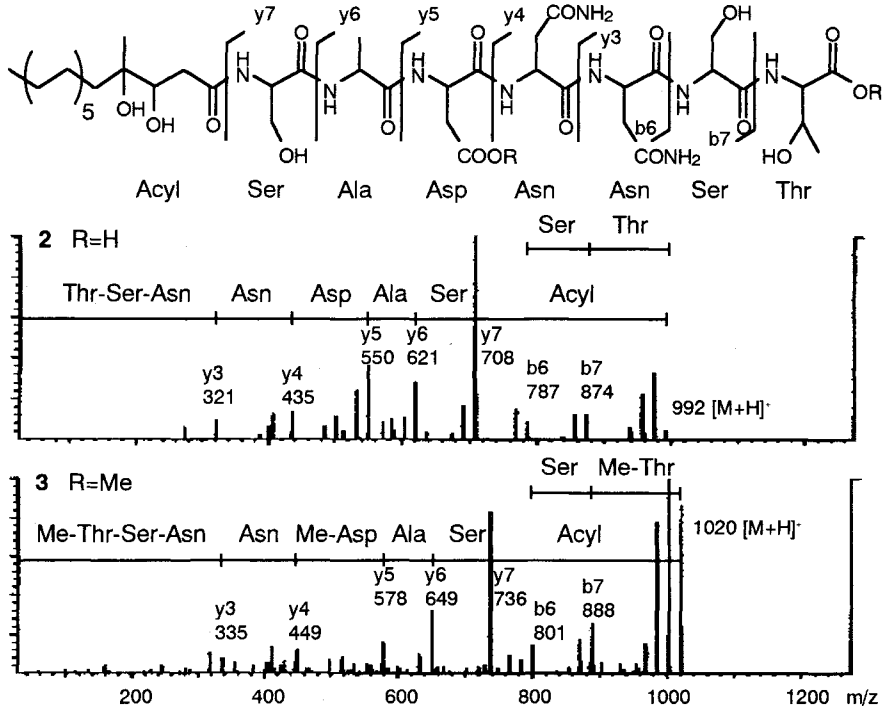
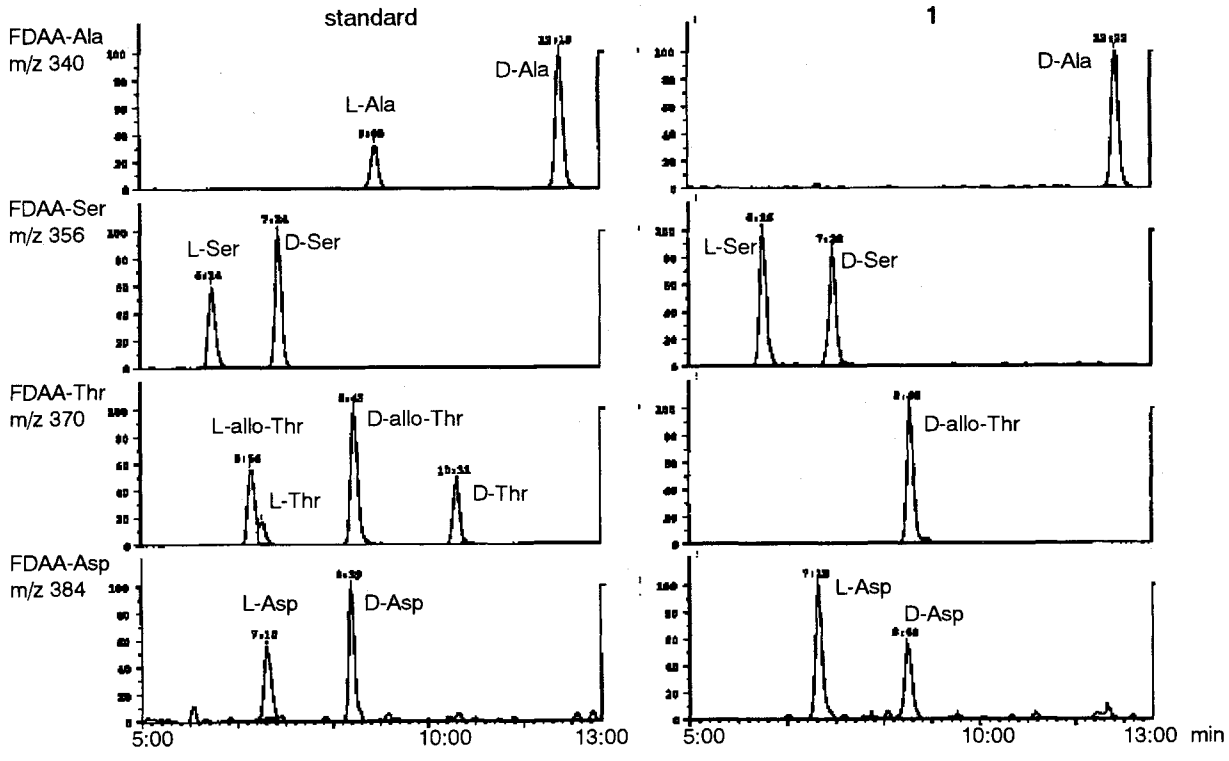


Fig. 5. LC-ESI-MS (Neg.) analysis for FDAA-derivatives of **1**.



Thr-L-Ala-D-Ala-L-Gln-D-Tyr-L-(Leu,Ile or Val) is quite different from those of **1**.

A quarternary carbon at position 4 of the fatty acid is rare. To our knowledge, 3.4-dihydroxy-4-methylhexadecanoic acid has not been described.

The configurational assignments for Ser, Asp and Asn and the stereochemistry of the β and quartenarys carbon of the fatty acid are yet to be assigned.

Experimental

General Methods

NMR data were collected on a JEOL JNM-A500 spectrometer. FAB-MS data were obtained on a JEOL JMS-SX102 spectrometer. ESI-MS/MS spectra were obtained on a Finnigan MAT TSQ-7000. An amino acid component analysis was performed with a Waters PICO TAG work station and a JEOL JLC-500.

Preparation of **2**

1 (5.0 mg) was dissolved in EtOH (500 μl). 50% dimethylamine (5.0 ml) was added, and the solution was stirred for three hours at room temperature. The reaction mixture was evaporated, and was prepared with C18 solid extraction, affording **2** (4.7 mg). FAB-MS *m/z*: 992 $(M+H)^+$, HR-FAB-MS: 992.5197 (calcd for $C_{42}H_{74}O_{18}N_9$, 992.5152).

Preparation of **3**

2 (100 μg) was dissolved in 1% $SOCl_2$ MeOH solution (100 μl), and was stirred for two hours at room temperature. The reaction mixture was evaporated, and was prepared with C18 solid extraction, affording **3**. FAB-MS *m/z*: 1020 $(M+H)^+$, HR-FAB-MS: 1020.5500 calc. for $C_{44}H_{78}O_{18}N_9$ (1020.5465). 1020.5500 (calcd for $C_{44}H_{78}O_{18}N_9$, 1020.5465).

Amino Acid Component Analysis of **1**

1 (100 μg) was degraded in 1% phenolic 6 N HCl (200 μl) at 110°C for 24 hours using the PICO TAG work station. The product was dissolved in sodium citric acid buffer pH 2.2 (1.0 ml), and applied to JLC-500.

MARFEY's Method of **1**

The acid hydrolysis of **1** was performed as the amino acid component analysis. Standard procedure of MARFEY was used. The hydrolysis product (100 μg) was dissolved in H_2O (20 μl), and 1% FDAA acetone solution(40 μl) and 1 M $NaHCO_3$ (8.5 μl) was added. After reaction at 40°C for 2 hours, the mixture was treated with 2 M HCl (4 μl), and then evaporated. The residue was dissolved in DMSO (200 μl), the solution (5 μl) was applied to LC-MS (negative mode).

HPLC condition; column: SHISEIDO CAPCELL PAK C18 UG120 4.6×100 mm, Temp=40°C, flow rate: 1 ml/minute, solvent: 10 mM $AcONH_4$ pH 3.5-MeCN, linear gradient from 15% MeCN to 45% MeCN till 25 minutes.

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