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Aogacillins A and B Produced by *Simplicillium* sp. FKI-5985: New Circumventors of Arbekacin Resistance in MRSA

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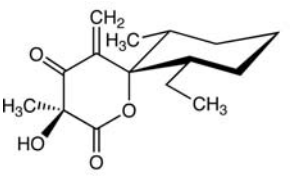
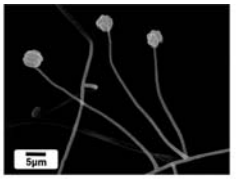
ABSTRACT



Aogacillin A



Simplicillium sp. FKI-5985

Aogacillins A and B, capable of overcoming arbekacin resistance in methicillin-resistant *Staphylococcus aureus* (MRSA), were isolated from a culture broth of *Simplicillium* sp. FKI-5985. Their structures were elucidated by NMR spectroscopic studies and ECD analyses. The aogacillins possessed a novel carbon skeleton, including a β-keto-γ-methyliden-δ-lactone ring connected to a 2-ethyl-6-methylcyclohexane ring by spiro conjugation.

Methicillin-resistant *Staphylococcus aureus* (MRSA) causes severe opportunistic infections, particularly in patients with compromised immune systems. MRSA has also been increasing significantly as a cause of serious community-acquired infection among healthy people.[1] Although four antibiotics, arbekacin (ABK), vancomycin, teicoplanin, and linezolid, are approved as therapeutic agents for MRSA in Japan, microorganisms resistant to these compounds have increasingly been reported.

ABK has more potent bactericidal activity than other anti-MRSA agents, and it inhibits the growth of both Gram-positive and Gram-negative bacteria. ABK resistance is believed to be caused by bacterial aminoglycoside-modifying enzymes, which phosphorylate and acetylate ABK to inactivate it.[2,3] Consequently, any inhibitor of these enzymes can be used in combination with ABK to help maintain its effectiveness.

During our screening program to discover new anti-MRSA agents, we recently found four lactone compounds, biverlactones A–D,[4] from the culture broth of *Penicillium* sp. FKI-4429, plus aranorosin from the culture broth of *Gymnascella aurantiaca*. All are effective ABK-resistance

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circumventors.[5] Further research led to the discovery of two new compounds which could also overcome ABK resistance in MRSA, designated as aogacillins A (**1**) and B (**2**), obtained from culture broth of *Simplicillium* sp. FKI-5985 (Figure 1). In this paper, the isolation, structure elucidation, and biological activity of the two aogacillins are described.

A sample of the fungus *Simplicillium* sp. FKI-5985 was collected in Aogashima, Tokyo, Japan. Ethanol (3 L) was added to the culture broth at the end of fermentation. After filtration and removal of the ethanol *in vacuo*, the remaining aqueous solution was applied to an ODS silica gel column, and then eluted fractions containing **1** and **2** were further purified by reversed-phase HPLC to afford **1** (15.0 mg) and **2** (15.5 mg).

Aogacillin A (**1**) was obtained as yellow syrups. The molecular formula of **1** was elucidated by HR-FAB-MS to be $C_{15}H_{22}O_4$ (*m*/*z* 267.1589 $[M+H]^+$ calcd 267.1597), requiring five degrees of unsaturation. The IR absorption at 1705, 1745, and 3420 cm^{-1} suggested the presence of ketone carobonyl, ester carbonyl, and hydroxyl groups,

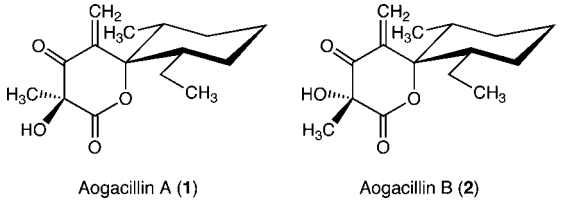

Figure 1. Structures of aogacillins A (**1**) and B (**2**).

respectively. The ^1H and ^{13}C NMR spectral data of **1** are listed in Table 1. The ^{13}C NMR, HSQC, and HMBC spectra indicated 15 carbons, which were classified into two carbonyl carbons, one sp^2 methylene carbon, one sp^2 quaternary carbon, two oxygenated sp^3 quaternary carbons, two sp^3 methine carbons, four sp^3 methylene carbons, and three methyl carbons, thus accounting for three degrees of unsaturation. Therefore, the remaining two degrees of unsaturation should be due to two ring structures. As shown by the bold lines for **1** in Figure 2, two partial structures were revealed by proton spin networks from H-7 (δ_H 1.42) to H_3-15 (δ_H 0.82) and from H_2-8 (δ_H 1.38, 1.81) to H_3-16 (δ_H 0.95) in the $^1H-^1H$ COSY. On the basis of $^1H-^{13}C$ HMBC experiments, the correlations from H_2-8 to C-14 (δ_C 23.9) and from H_2-14 (δ_H 0.92, 1.24) to C-8 (δ_C 27.3) indicated the C-8 is linked to C-7. The correlations from H_3-16 (δ_H 0.95), H_2-10 (δ_H 1.50, 1.62), and H_2-14 to C-6 (δ_C 91.6) in HMBC spectra revealed the presence of a 1-ethyl-3-methylcyclohexane moiety by the connection of C-7 and C-11 via C-6. The correlations from H_2-13 (δ_H 5.77, 6.44) to C-4 (δ_C 197.4), C-5 (δ_C 145.5), and C-6 and from H_3-12 (δ_H 1.54) to C-2 (δ_C 174.9), C-3 (δ_C 77.8), and C-4 established the alignment from C-2 to C-6 and attachment of C-13 to C-5. A δ-lactone ring formed by the connection between C-2 and C-6 via an oxygen atom was suggested by the characteristic ester carbonyl absorption at 1745 cm^{-1} in the IR spectrum. The broad signal (δ_H 3.52 in $CDCl_3$) in ^1H NMR indicated the presence of a hydroxyl group. This was confirmed by the presence of a hydroxyl group at C-3 by high-field shifts of C-3 from δ_c 77.818 (in CD_3OD) to δ_c 77.782 (in CD_3OH) in the H/D exchange experiment. Thus, the structure of **1** was elucidated to be a novel compound

Table 1. NMR Data of Aogacillins A (**1**) and B (**2**) in CD_3OD

	Aogacillin A (**1**)			Aogacillin B (**2**)		
no.	δ_H (mult, *J* in Hz)	δ_C	gHMBC	δ_H (mult, *J* in Hz)	δ_C	gHMBC
2	–	174.9		–	175.0	
3	–	77.8		–	77.9	
4	–	197.4		–	197.6	
5	–	145.5		–	145.6	
6	–	91.6		–	91.9	
7	1.42 (m)	54.6	8, 15	1.84 (m)	48.7	
8a	1.38 (m)	27.3	9, 14	1.36 (m)	26.4	9
8b	1.81 (m)			1.91 (m)		
9a	1.45 (m)	26.2	8	1.46 (m)	26.2	
9b	1.83 (m)			1.84 (m)		8
10a	1.50 (dddd, 14.0, 8.8, 8.8, 6.2)	30.2		1.52 (2H, m)	31.5	6, 11
10b	1.62 (dddd, 14.0, 6.4, 2.2, 2.2)		6, 9, 11			
11	2.12 (dqd, 8.8, 7.2, 5.4)	42.4	10, 16	1.68 (m)	48.1	6, 10, 16
12	1.54 (s)	27.2	2, 3, 4	1.55 (s)	27.3	2, 3, 4
13a	5.77 (s)	125.5	3, 4, 5, 6	5.73 (s)	125.1	4, 5, 6
13b	6.44 (s)		4, 5, 6	6.42 (s)		4, 5, 6
14a	0.92 (m)	23.9	7, 8, 15	1.14 (m)	24.7	7, 8, 15
14b	1.24 (m)		6, 7, 8, 15	1.66 (m)		6, 7, 8, 15
15	0.82 (t, 7.2)	12.3	7, 14	0.94 (t, 7.2)	12.3	7, 14
16	0.95 (d, 7.2)	17.2	6, 10, 11	0.71 (d, 7.2)	16.1	6, 10, 11

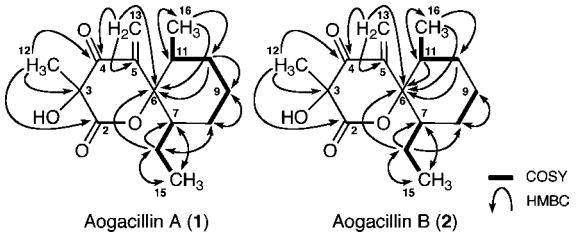

Figure 2. COSY and HMBC correlations of aogacillins A (**1**) and B (**2**).

including a β-keto-γ-methyliden-δ-lactone ring connected to a 2-ethyl-6-methylcyclohexane ring by spiro conjugation. Only a few natural product compounds having δ-lactone with a spiro-conjugated cyclohexane have been reported, but all of their cyclohexanes are fused with the other ring, such as vitexifolin E from the fruit of *Vitex rotundifolia*.[6] The relative configuration of **1** was elucidated to be 3*S**,6*R**,7*S**,11*R** by ROESY and differential NOE correlations of H-13a/H-7, H-13a/H-11, H-7/H-11, and H_3-16/H_3-12 (Figure 3a).

The molecular formula of **2** was elucidated by ESI-MS to be $C_{15}H_{22}O_4$ (*m*/*z* 289.1428 $[M+Na]^+$ calcd 289.1416), the same mass units as **1**. The ^1H and ^{13}C NMR spectral data of **2** are listed in Table 1. The analysis of 1D and 2D NMR revealed **2** has the same planar structure to **1** as shown in Figure 2. Although the ^1H and ^{13}C NMR spectra of **2** were very similar to those of **1** (Table 1), both ^1H and ^{13}C chemical shifts of CH_2-14 and CH_3-15 shifted high-field (CH_2-14: $\Delta\delta_H = -0.22$ and -0.42 ppm, $\Delta\delta_C = -0.8$ ppm/CH_3-15: $\Delta\delta_H = -0.12$ ppm) in **1** (Figure 3c). Conversely, those of CH_3-16 largely shifted low-field ($\Delta\delta_H = +0.24$ ppm, $\Delta\delta_C = +1.1$ ppm) in **1**. This observation is explainable by a magnetic anisotropic effect induced by the ester carbonyl group at C-2, on which the ethyl group (C-14−C-15) in **1** and the methyl group (C-16) in **2** are located. On the other hand, steric compression between H-13a and H-11 in **1** and between H-13a and H-7 in **2** caused opposite shifts of ^1H and ^{13}C chemical shifts at C-11 ($\Delta\delta_H = +0.44$ ppm and $\Delta\delta_C = -6.7$ ppm) and C-7 ($\Delta\delta_H = -0.42$ ppm and $\Delta\delta_C = +5.9$ ppm). This difference of chemical shifts and the correlations of ROESY and differential NOE of H-13a/H-7, H-13a/H-11, H_3-12/H_2-14, and H_3-12/H_3-15 revealed the relative configuration of **2** to be 3*R**,6*R**,7*S**,11*R**, and thus **2** is a C-3 epimer of **1** (Figure 1).

The absolute stereochemistries of **1** and **2** were deduced by electronic circular dichroism (ECD) spectra[7,8] in comparison to their calculated spectra[9] (Figure 4). The

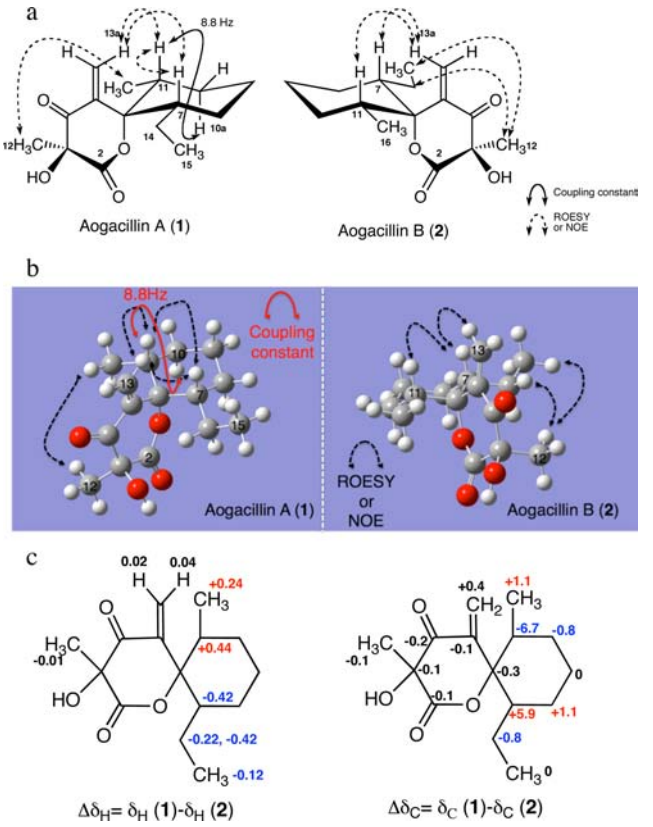

Figure 3. Elucidation of the relative stereochemistry of aogacillins A (**1**) and B (**2**); (a) key ROESY and differential NOE correlations, (b) key ROESY and differential NOE correlations with models of caliculated conformers, (c) difference of chemical shifts between **1** and **2**.

conformational analyses of **1** and **2** started from MM2 force field structures further optimized by semiempirical PM3 calculations and then further refined by density functional theory (DFT) at the B3LYP/6-311G level of theory which yielded additional relevant conformers. Structures of resulting calculated conformers[10] were supported by our NOE experiments (Figure 3b). The ECD spectra of **1** and **2** were simulated using time-dependent DFT (TD-DFT) at the same level of theory on the relevant conformers.

As a result of the comparison between the experimental ECD spectrum and calculated ECD spectrum, the absolute stereochemistry of **1** was elucidated to be 3*S*,6*R*,7*S*,11*R*. In the same manner, that of **2** was elucidated to be 3*R*,6*R*,7*S*,11*R*.

A plausible biosynthetic pathway for **1** and **2** is illustrated in Scheme 1. One acetate and five malonate units might be required to assemble the polyketide skeleton for **1** and **2**. The linear polyketide precursor further undergoes a series of reactions, including methylation by *S*-methyladenosylmethionine (SAM), aldol cyclization to form a spiro skeleton,

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(10) Calculated conformers for compounds **1** and **2** are shown in the SI, together with their atomic coordinates and energies.

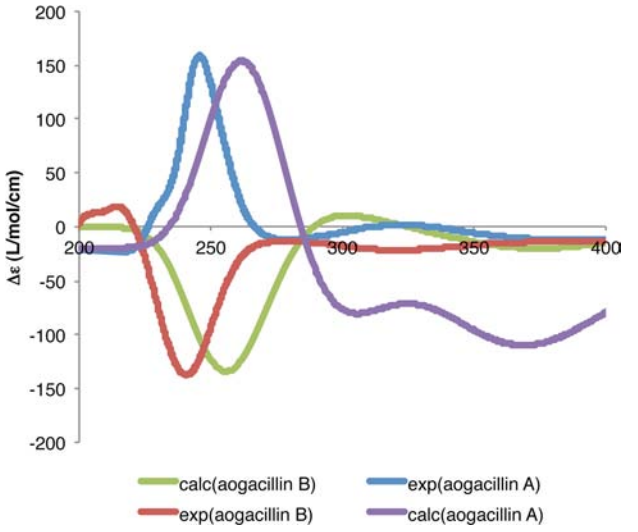


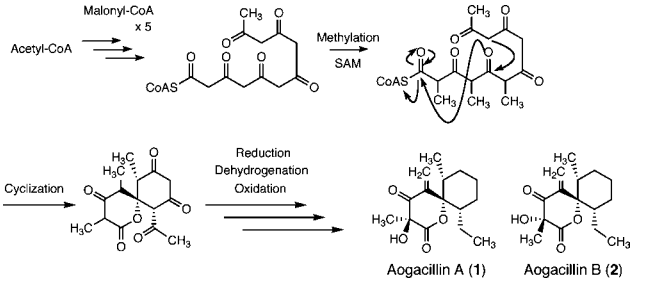
Figure 4. Experimental CD spectra of **1** and **2** overlaid with calculated spectra.

reduction, and then dehydrogenation. As a last step, oxidation at C-3 might produce two epimers at C-3 of the aogacillins.

We investigated the effect of aogacillins on the activity of ABK against ABK-resistant MRSA by the liquid microdilution method. The effect of aogacillins A (**1**) and B (**2**) were tested at first on the growth of MRSA, resulting in equivalent MIC values of 2.0 μg/mL. Therefore, the concentration of aogacillins for combination with ABK was set at 0.5 μg/mL (one-fourth of each MIC value, with no effect on growth of the MRSA) allowing us to investigate any effect on the activity of ABK against ABK-resistant MRSA.

Aogacillins A (**1**) and B (**2**) markedly reduced the MIC value of ABK against ABK-resistant MRSA, from 256 to 8 μg/mL, which was similar to the MIC value of ABK against ABK-sensitive MRSA (1 μg/mL). Moreover, we investigated the circumvention effect of aogacillins against ABK-resistant MRSA using various antibiotics by the liquid microdilution method (see Supporting Information (SI)). The concentrations of aogacillins used were 0.5 μg/mL for each antibiotic. The aogacillins showed no effect, or only slight potentiation, with respect to the MIC values of aminoglycosides tested (kanamycin, dibekacin, and streptomycin) and other antibiotics (ampicillin, ciprofloxacin, and vancomycin) against the MRSA (1−4-fold). These data suggested that the aogacillins could be a specific circumventor of ABK activity for ABK-resistant MRSA. Investigation of the mode of action of the aogacillins is now underway.

Scheme 1. Plausible Biosynthetic Pathways for Aogacillins A (**1**) and B (**2**)



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Supporting Information Available. Experimental procedures; UV, IR, ^{1}H NMR, ^{13}C NMR, gCOSY, gHSQC, gHMBC, and ROESY spectra of aogacillins A (**1**) and B (**2**). This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.