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NOTE

Euvesperins A and B, new circumventors of arbekacin resistance in MRSA, produced by *Metarhizium* sp. FKI-7236

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Methicillin-resistant *Staphylococcus aureus* (MRSA) infection is associated with a high risk of complications in patients with underlying disease, and can cause acute and severe conditions such as pneumonia. Moreover, more than half of MRSA-infected patients are co-infected with other pathogens, such as *Pseudomonas aeruginosa*, and a superinfection poses therapeutic problems in many such cases. Though arbekacin (ABK), vancomycin, teicoplanin, linezolid and daptomycin are used for MRSA infection, resistant strains to each of these drugs have been reported.[1–3]

ABK, an aminoglycoside (AG) antibiotic, was launched in Japan in 1990 as a chemotherapeutic agent for MRSA infection.[4,5] Bacterial resistance to AG antibiotics, caused by the effect of aminoglycoside acetyltransferases (AACs), aminoglycoside nucleotidyltransferases (ANTs) and aminoglycoside phosphotransferases (APHs), is widely recognized as a serious public health threat. ABK is a synthetic derivative of kanamycin and not usually affected by such enzymes.[2] However, a few MRSA strains are resistant to ABK. The resistance is mainly due to the bifunctional enzyme AAC(6′)-Ie/APH(2′′)-Ia, which catalyzes both acetylation of the 6′ of the amino group and phosphorylation of the 2′′ of the hydroxyl group of ABK.[2,6,7] Therefore, inhibitors of this enzyme could be promising drug candidates to use in combination with ABK to help maintain its effectiveness.

Our screening program for microbial metabolites in the search for compounds that circumvent ABK resistance of MRSA strains, led us to discover biverlactones,[8] aranorosin[9] and aogacillins.[10] Our ongoing search has recently discovered two new compounds, designated euvesperins A and B (**2** and **3**, respectively, Figure 1), together with a known compound, PI-091 (**1**), in the culture broth of a fungal strain FKI-7236. All three proved to be circumventors of ABK resistance. In this paper, we describe the producing strain, fermentation, isolation, structural elucidation and biological activity of **1**, **2** and **3**.

The fungal strain FKI-7236 was isolated from a soil sample collected in Niijima, the Izu Islands, Tokyo, Japan. Morphologically, this strain resembles *Paecilomyces carneus*. This species was recently reclassified into the genus *Metarhizium*.[11] The ITS sequence of FKI-7236 was compared with sequences in the GenBank database by BLASTN 2.2.32 analysis.[12] The sequence of FKI-7236 was 90% similar to that of *Paecilomyces carneus* (current name: *Metarhizium carneum*) CBS 239.32 (GenBank accession number AB103379). The producing strain FKI-7236 was thus identified with the genus *Metarhizium*, based on its morphology and the ITS sequence. A 500-ml Erlenmeyer flask containing 100 ml of a seed culture medium (2% glucose, 0.2% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% Polypepton (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 0.1% KH_2PO_4 and 0.1% agar, pH 6.0) was inoculated with one loopful of the strain, *Metarhizium* sp. FKI-7236, grown on an LcA slant (0.1% glycerol, 0.08% KH_2PO_4, 0.02% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% KCl, 0.2% $NaNO_3$ and 1.5% agar, pH 6.0) and incubated on a rotary shaker (210 r.p.m.) at 27 °C for 3 days. Eighty-five 500-ml Erlenmeyer flasks each containing 100 ml of culture medium (0.5% glycerol, 0.5% $NaNO_3$, 0.02% sodium valproate, 1.0% sucrose (Wako Pure Chemical Industries, Ltd.), 0.2% ammonium acetate, 0.002% $FeSO_4$, 0.002% $ZnSO_4$ (Kanto Chemical Co., Inc., Tokyo, Japan) and 1.0% yeast extract (Oriental Yeast Co., Ltd., Tokyo, Japan), pH 6.0) was inoculated with the seed culture (1 ml) and incubated on a rotary shaker (210 r.p.m.) at 27 °C for 6 days. The culture broth (8.5 l) was added to an equal volume of EtOH and filtered. Obtained filtrate was concentrated *in vacuo*. The aqueous solution was applied to an HP20 chromatography column (110 i.d. × 80 mm, Mitsubishi Chemical Co., Tokyo, Japan), which was eluted sequentially with a MeOH-H_2O solvent system (30% MeOH, 50% MeOH and 100% MeOH; 1.5 l each). The 100% MeOH fraction was applied to an ODS chromatography column (45 i.d. × 90 mm, YMC Co., Ltd., Kyoto, Japan), which was eluted stepwise with 50% MeOH (300 ml), 60% MeOH (300 ml), 70% MeOH (300 ml), 80% MeOH (300 ml), 90% MeOH (300 ml) and 100% MeOH (300 ml). Finally, 115 mg of the 70% MeOH fraction (667 mg) was purified by HPLC (Develosil C30, 20 i.d. × 250 mm, Nomura Chemical Co., Aichi, Japan) with an isocratic mobile phase of 65% MeOH-H_2O at a flow rate of 7.0 ml per min to

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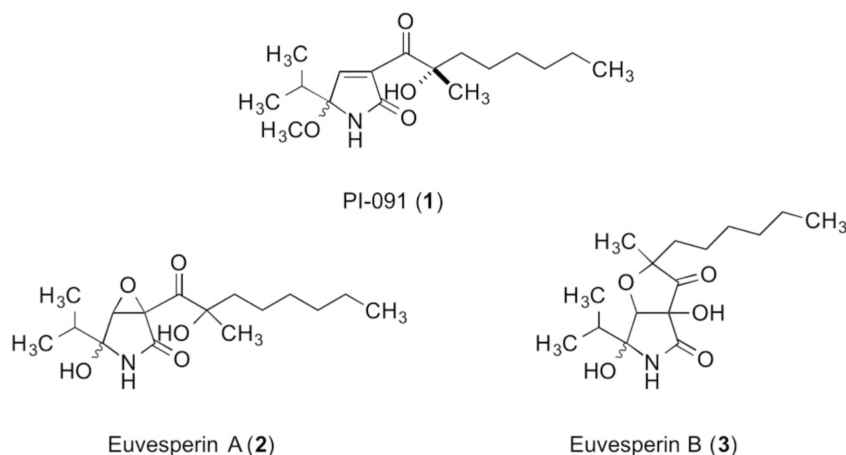

Figure 1 Structures of PI-091 (**1**), euvesperins A (**2**) and B (**3**).

Table 1 NMR spectroscopic data for each epimer of euvesperins A and B in pyridine-d_5

	Euvesperin A				*Euvesperin B*			
Position	δ_H *(J in Hz)*	δ_C	δ_H *(J in Hz)*	δ_C	δ_H *(J in Hz)*	δ_C	δ_H *(J in Hz)*	δ_C
1		171.1		170.7		172.1		171.9
2		64.7		63.7		88.2		88.1
3	4.71 d (1.8)	65.1	4.75 d (2.2)	65.4	5.07 s	77.6	5.09 s	77.8
4		88.4		88.1		91.4		91.6
5-NH	10.14 d (1.8)		10.17 d (2.2)		10.31 s		10.38 s	
6		206.7		206.2		209.3		209.0
7		80.2		80.0		84.1		84.8
8	1.83, 2.06 br s	40.7	2.08, 2.36 br s	39.3	2.06, 2.45 br s	39.6	1.76, 2.00 br s	42.3
9	1.23, 1.28 m	30.2	1.23, 1.28 m	30.2	1.18, 1.28 m	30.0	0.93, 0.99 m	29.8
10	1.48, 1.80 m	23.7	1.67, 1.72 m	23.9	1.40, 1.68 m	24.0	1.48, 1.80 m	23.7
11	1.16, 1.24 m	32.1	1.02, 1.14 m	32.1	1.20, 1.24 m	32.2	1.02, 1.14 m	32.0
12	1.16, 1.22 m	23.0	1.16, 1.22 m	23.0	1.16, 1.22 m	23.0	1.05, 1.09 m	22.9
13	0.78 t (6.0)	14.3	0.76 t (6.0)	14.3	0.79 t (8.0)	14.3	0.71 t (8.0)	14.2
14	2.35 dq (7.3, 7.3)	34.6	2.37 dq (7.3, 7.3)	34.6	2.63 dq (7.6, 7.6)	32.7	2.67 dq (7.4, 7.4)	32.4
15	1.31 d (7.3)	18.1	1.35 d (7.3)	16.9	1.24 t (7.6)	17.8	1.19 d (7.4)	17.8
16	1.34 d (7.3)	16.9	1.32 d (7.3)	18.1	1.38 t (7.6)	17.3	1.43 d (7.4)	17.3
17	1.83 s	24.9	1.59 s	28.0	1.61 s	28.2	1.85 s	24.2

NMR spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C.

give **1** (8.9 mg, $R_t = 58$ min) as a nearly 1:1 mixture, epimeric at the γ-ketal carbon, and an inseparable mixture of **2** and **3** (7.5 mg, $R_t = 46$ min), each of them are also epimeric mixtures. We conducted structure elucidation and biological evaluation using this inseparable mixture of **2** and **3**.

In comparison with reported NMR and MS data (Supplementary Data), **1** was identified as PI-091 as shown in Figure 1.[13,14] Compound **1** was originally isolated as a new aggregation inhibitor from a fermentation broth of *Paecilomyces* sp. F-3430.[16] Compound **1** has a γ,γ-*C*,*O*-disubstituted α,β-unsaturated γ-lactam skeleton with a 2-hydroxy-2-methyloctanoyl moiety.[13–15] The mixture of **2** and **3** was obtained as a yellow oil. The HR-ESI-MS of **2** and **3** showed one sodium adduct peak of *m/z* 336.1773 [M+Na]$^+$ (calculated for $C_{16}H_{27}NO_5Na$, 336.1787), which suggested the molecular formula of **2** and **3** were the same. The IR spectra showed characteristic absorptions at 3324, 3301, 2954 and 1712 cm^{-1}, suggesting the presence of hydroxyl and carbonyl groups. The structures of **2** and **3** were mainly elucidated by NMR, including 2D NMR. The ^{1}H and ^{13}C NMR spectra and HSQC experiments of the epimers of **2** each indicated 16 carbons, which were classified into four *sp*^{3} methyl carbonyl carbons, five *sp*^{3} methylene carbons, two *sp*^{3} methine carbons, two carbonyl carbons and three fully substituted carbons (Table 1). The ^{1}H NMR data of the epimers of **2** were similar to that of **1**. However, methoxy and olefinic signals of **1** did not exist, whereas the oxygenated methine signals (δ_H 4.71, 4.75) as epimeric pairs appeared in ^{1}H NMR spectra of **2**. The ^{1}H–^{13}C HMBC experiment showed correlations from H-3 (δ_H 4.71, 4.75 (for each epimer)) to C-1 (δ_C 171.1, 170.7) and C-4 (δ_C 88.4, 88.1), from 5-NH (δ_H 10.14, 10.17) to C-2 (δ_C 64.7, 63.7), C-3 (δ_C 65.1, 65.4) and C-4, and from H-14 (δ_H 2.35, 2.37) to C-3 and C-4 (Figure 2a), which indicated that the γ-lactam ring was oxidized at C-2 and C-3 to form an epoxide group. This was confirmed by large $^1J_{C–H}$ coupling constant (194 Hz) at the C-3 position. The COSY correlations observed between H-14 and H-15 (δ_H 1.31, 1.35) and between H-14 and H-16 (δ_H 1.34, 1.32)

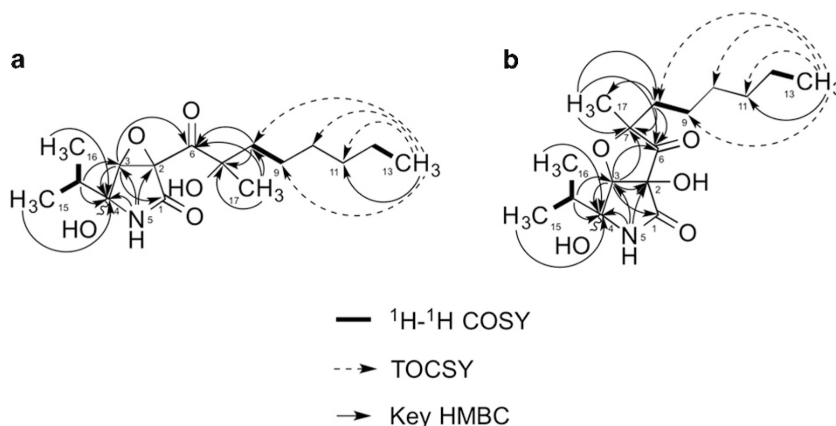

Figure 2 Key correlations of ^{1}H-^{1}H COSY, TOCSY and HMBC in (**a**) euvesperin A (**2**) and (**b**) euvesperin B (**2**). Bold lines show proton spin networks, dotted arrows indicate ^{1}H–^{1}H long range correlations, and arrows show ^{1}H–^{13}C long range correlations.

and HMBC correlations from H-15 and H-16 to C-4 indicated an isopropyl group was attached to the γ-position of α,β-epoxy-γ-hydroxyl-γ-lactam. The remaining partial structures were established by COSY, TOCSY and HMBC experiments. The cross peaks between H-8 (δ_H 2.06, 2.36) and H-9 (δ_H 1.28, 1.23), and between H-12 (δ_H 1.16, 1.22) and H-13 (δ_H 0.78, 0.76) were observed in COSY. The cross peaks from H-13 to H-8, H-9, H-10 (δ_H 1.80, 1.67) and H-11 (δ_H 1.24, 1.02) were observed in TOCSY. In addition, HMBC correlations from H-13 to C-11 (δ_C 32.1), from H-8 to C-6 (δ_C 206.7, 206.2) and C-7 (δ_C 80.2, 80.0) and from H-17 (δ_H 1.83, 1.59) to C-6, C-7 and C-8 (δ_C 40.7, 39.3) indicated the partial structure of 2-hydroxyl-2-methyl-1-octanoyl moiety. The connectivity of two partial structures was elucidated by HMBC correlation from H-3 to C-6. Thus, the planar structure of **2** was elucidated as an α,β-epoxy-4-demethoxy-4-hydroxy analog of **1**, and **2** was designated euvesperin A. Compound **1** was isolated as a nearly 1:1 epimeric mixture from a fungal culture broth.[13] As the culture broth of the **2**-producing strain also contained **1**, they may share the same biosynthetic pathway of α,β-epoxy-γ-hydroxyl-γ-lactam and epimerize at the hemiaminal moiety. In fact, several similar compounds were synthesized and obtained as two diastereomers.[16,17] The ^{1}H and ^{13}C NMR spectra of **3** were similar to those of **2**. However, signals at the C-3 position were shifted downfield to 5.07 and 5.09 p.p.m. in ^{1}H NMR and to 77.6 and 77.8 p.p.m. in ^{13}C NMR. We assumed that the hydroxyl group 7-OH of **2** attacked its epoxy group (C-3). Smaller $^1J_{C\text{–}H}$ coupling constant (149 Hz) at the C-3 position of **3** also suggested the presence of a tetrahydrofuran ring instead of an epoxide ring. In addition, the ^{1}H–^{13}C HMBC experiment showed key correlation from H-3 to C-7 (δ_C 84.1, 84.8) (Figure 2b), indicating the presence of an unprecedented carbon skeleton with a 5,5-bicyclic ring system comprising a 2,4-dihydroxy-γ-lactam fused with a 3-furanone moiety. Thus, the planar structure of **3**, comprising a 3a-hydroxydihydro-2*H*-furo[2,3-*c*]pyrrole-3,4(3a*H*,5*H*)-dione ring, was elucidated as shown in Figure 2b and **3** was named euvesperin B.

A literature search identified several fungal metabolites having α,β-epoxy-γ-lactam ring, such as epolactaene and the fusarins, that might have biosynthetic pathways similar to that of **2** and **3**.[18,19] The structural similarity of **2** and **3** indicates that **2** might be a precursor of **3** as a consequence of nucleophilic opening of the epoxy group by the 7-OH of **2**. Though there are several microbial metabolites structurally related to **1**, **2** and **3**, such as epolactaene, fusarins, lactacystin, lucilactaene and L-755,807, their reported biological activities are different from those of **2** and **3**.[18–22]

ABK-circumventing activity was measured using the MRSA TH-1466 strain, which is an ABK-resistant clinical isolate that harbors the *aac(6′)-Ie/aph(2″)-Ia* gene coding AG-modifying enzyme. First, the activity was evaluated by the paper disc method using the following protocol. The TH-1466 strain was cultured in 4 ml of Difco Mueller Hinton broth (Becton Dickinson, Franklin Lakes, NJ, USA) at 37 °C for 20 h, and the bacterial concentration was adjusted to 1×10^8 CFU ml^{-1}. A total of 750 μl of the culture broth was transferred to a square plate (10 × 14 cm, Eiken Chemical Co., Ltd., Tokyo, Japan) containing 20 ml Difco Mueller Hinton agar (MHA; Becton Dickinson). Some plates contained 8 μg ml^{-1} of ABK (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), the concentration of which has no effect on the growth of TH-1466. Paper discs (6 mm, Advantec Toyo Co., Ltd., Tokyo, Japan) containing various amounts of a sample or 7 μg of vancomycin as a positive control were placed on the MHA plate and incubated at 37 °C overnight. Diameter in millimeters of the inhibition zone was measured and used as the anti-MRSA activity indicator.

Compound **1** and the mixture of **2** and **3** showed anti-MRSA activity with inhibition zone of 9 mm (at 10 μg per disc) and 8 mm (at 30 μg per disc) without ABK, respectively. Notably, they caused identical inhibitions on the MHA plate containing 8 μg ml^{-1} of ABK at 0.3 and 3 μg per disc, respectively (Supplementary Figure 1). These results suggested that **1** and the mixture of **2** and **3** circumvented ABK resistance at 30- and 10-fold in MRSA, respectively. However, vancomycin did not show any difference in anti-MRSA activity between presence and absence of ABK.

The ABK circumvention activity of **1** and the mixture of **2** and **3** were subsequently measured by the microdilution method.[9] The MIC values of **1** and the mixture of **2** and **3** against MRSA were 48 and 384 μg ml^{-1}, respectively. In order to investigate the ABK circumvention activities, the concentrations of these compounds in combination with ABK were set to one-third of those MIC values, which had no effect on the growth of MRSA. Both **1** and the mixture of **2** and **3** markedly reduced the MIC values of ABK against MRSA from 256 to 7 μg ml^{-1} (Supplementary Table 1). The circumvention ratios were both 36-fold.

We then examined the effect of **1** and the mixture of **2** and **3** on the activity of ABK modifying bifunctional enzyme, AAC(6′)-Ie/APH(2″)-Ia. The enzyme was cloned from the MRSA TH-1466 strain.

Details of the assay procedure were previously reported by Suga *et al.*[9] Briefly, ABK was incubated with the enzyme (112 μg ml^{-1}) containing acetyl-CoA or ATP and the reaction mixture was analyzed by TLC. The TLC plates were developed with 5% KH_2PO_4, and acetylated or phosphorylated ABK were detected by ninhydrin. Acetylated and phosphorylated ABKs were more lipophilic than ABK and showed larger R_f values than ABK on the TLC plate. As a result, **1** and the mixture of **2** and **3** did not inhibit acetylation, and only marginally inhibited phosphorylation at 1 mg ml^{-1}.

In conclusion, **1**, **2** and **3** were isolated from the fungus *Metarhizium* sp. FKI-7236. From the results of morphological characteristics and sequence analysis, strain FKI-7236 was identified as a novel species of *Metarhizium*. Taxonomic details will be reported elsewhere. The structures of **2** and **3** were elucidated as new fungal metabolites containing a γ-lactam ring or γ-lactam fused to furanone unit. Each of them existed as epimers, and thus the structure elucidation was performed in the mixture of four compounds. Compound **2** gradually changed to **3** in solution. Compound **1** and the mixture of **2** and **3** circumvented ABK resistance 36-fold against an MRSA strain in the microdilution assay. However, **1** and the mixture of **2** and **3** only inhibited phosphorylation by AAC(6′)-Ie/APH(2″)-Ia at 1 mg ml^{-1}. No inhibition was seen, even at high concentration, of **1** and the mixture of **2** and **3**, suggesting an alternative action mechanism. We hypothesize that the specific mode of action of **1** and the mixture of **2** and **3** against ABK-resistant MRSA might be their effect on the proton motive force's component, such as transmembrane electric potential ($\Delta\psi$), transmembrane pH gradient (ΔpH) and intracellular ATP levels, thereby increasing the uptake of ABK.[23,24] Owing to the unique structures of **1**, **2** and **3**, they are promising lead candidates for developing therapeutic compounds against ABK-resistant MRSA.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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