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ORIGINAL ARTICLE

Paraphaeosphaeride D and berkleasmin F, new circumventors of arbekacin resistance in MRSA, produced by *Paraphaeosphaeria* sp. TR-022

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Two new compounds, designated paraphaeosphaeride D (1) and berkleasmin F (2) together with a previously known compound, berkleasmin A (3), isolated from a culture broth of the fungus *Paraphaeosphaeria* sp. TR-022, proved to be new circumventors of arbekacin (ABK) resistance in methicillin-resistant *Staphylococcus aureus* (MRSA). The structures of 1 and 2 were elucidated by spectroscopic analyses, including various NMR experiments. All compounds showed 10–100 times ABK circumvention activities using the paper disc method and reduced the MIC values of ABK against MRSA from 16 μg ml^{-1} to 4 μg ml^{-1} (fourfold) using the agar dilution method. These new compounds might be promising lead compounds for developing circumventors of ABK resistance in MRSA.
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INTRODUCTION

The World Health Organization has recently classified antibiotic resistance as one of the three greatest threats to human health. In particular, methicillin-resistant *Staphylococcus aureus* (MRSA) has an important role in causing serious nosocomial infection.[1] Launched in Japan at the end of 1990, arbekacin (ABK) is a useful chemotherapeutic agent for the treatment of infections caused by MRSA.[2,3] Soon after it was launched, it was reported that a few percent of MRSA strains were resistant to ABK. Therefore, to maintain the usefulness for the drug, it is important to develop a new agent that helps overcome ABK resistance in MRSA.[4,5] The main mechanism of resistance to aminoglycosides, including ABK, is thought to be via inactivation by enzymatic modification. In particular, the bifunctional enzyme AAC(6′)-Ie/APH(2″)-Ia is thought to play a crucial role in ABK resistance in MRSA, which can catalyze both phosphorylation at 2″-hydroxyl and acetylation at 6′-amino group of aminoglycosides.[6] Consequently, specific inhibitors against this enzyme should help maintain ABK's effectiveness.

During our screening for new circumventors of ABK resistance, we have already found biverlactones, aranorosin and aogacillins among fungal metabolites.[7–9] Our continuing search has now led to the discovery of two new compounds, named paraphaeosphaeride D (**1**) and berkleasmin F (**2**), from a culture broth of the fungus, *Paraphaeosphaeria* sp. TR-022, together with a known compound, berkleasmin A (**3**) (Figure 1).[10] In this paper, the taxonomy of the producing strain, isolation, physicochemical properties and structure elucidation of **1** and **2**, as well as circumvention activities of ABK resistance of the isolated compounds, are described.

RESULTS

Taxonomy of the producing strain TR-022

The fungal strain TR-022 was isolated from a sediment collected in an artificial pond at Machida city, Tokyo. As TR-022 did not produce spores on agar media, molecular identification was carried out. A BLAST search of partial sequences of large subunit and internal transcribed spacer region (ITS) revealed that TR-022 possessed the highest sequence similarities with *Paraconiothyrium brasiliense* CBS 122851 (99.7%) and *Paraphaeosphaeria sporulosa* CBS 824.68 (96.8%), respectively. The phylogenetic tree based on ITS sequences clearly indicated that TR-022 was nested in a *Paraphaeosphaeria-Paraconiothyrium* clade[11] and did not cluster with any species within a clade (Figure 2). Thus, TR-022 was designated as a *Paraphaeosphaeria* sp.

Isolation

The 80% isopropanol extract (4.5 l) was evaporated under reduced pressure to remove isopropanol. The aqueous solution (1 l) was extracted twice with 1 l of ethyl acetate. The organic layer was concentrated to dryness *in vacuo* to afford a crude material (11.8 g) and applied to a silica gel column (60 i.d. × 100 mm), which was

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This article is dedicated to the fond memory of the late Professor Lester Mitscher, a great scholar, teacher and Emeritus Editor of this Journal.
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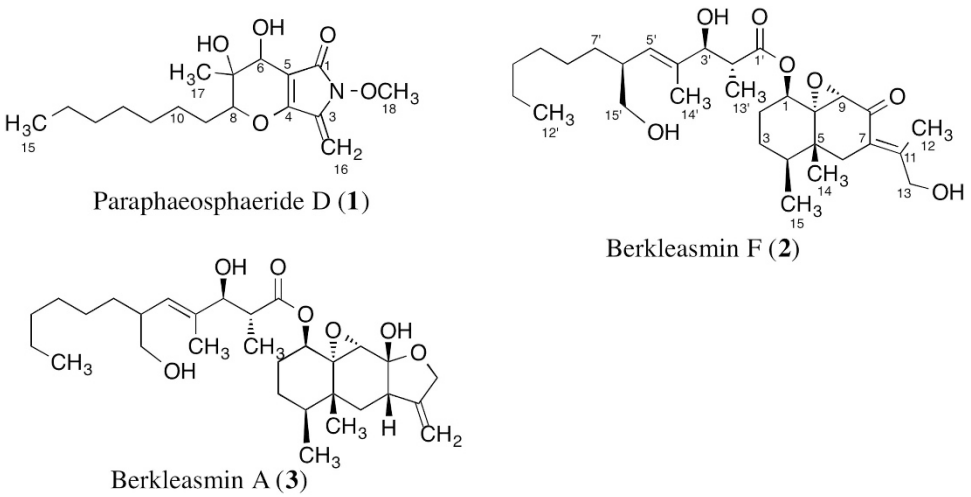

Figure 1 Structures of paraphaeosphaeride D (**1**), berkleasmin F (**2**) and berkleasmin A (**3**).

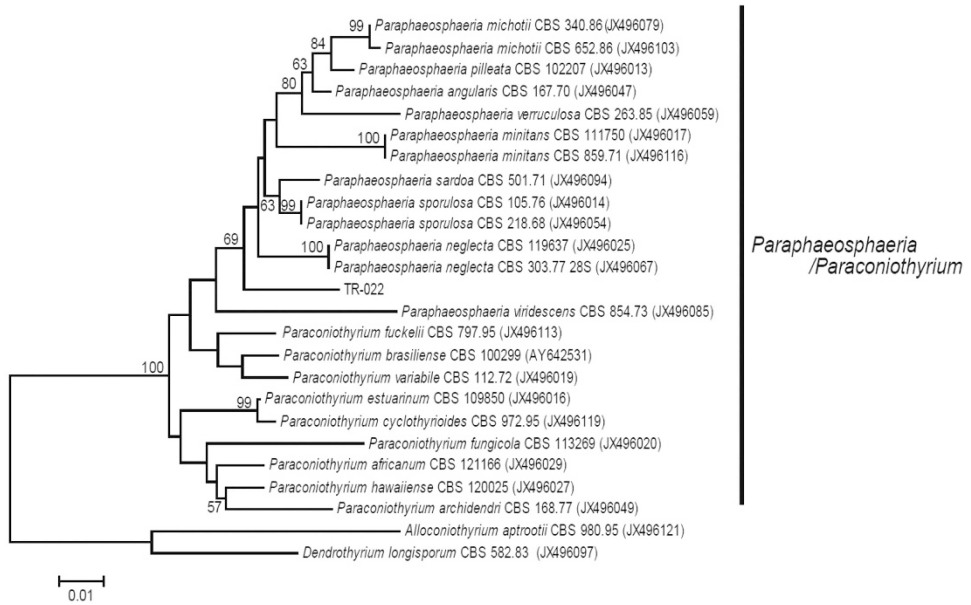

Figure 2 The neighbor-joining tree based on internal transcribed spacer region sequences showing the relationships between TR-022 and *Paraphaeosphaerial Paraconiothyrium* species. The numbers at branch are bootstrap values as a percentage of 1000 replications (only values above 50% are shown). Scale bar = 0.01 substitutions per site.

eluted stepwise with 1 l each of a mixture of $CHCl_3$-MeOH (100:0, 100:2, 100:5, 100:7, 10:1, 1:1 and 0:100) in this order. The $CHCl_3$-MeOH (100:5) fraction (4.1 g) was applied on an octadecylsilyl column (60 i.d. × 50 mm), which was eluted stepwise with 20, 50, 60, 70, 80, 90 and 100% of MeOH/H_2O solvents (each 500 ml). One gram of the active fraction (80% MeOH aq. fraction, 1.1 g) was dissolved in a small amount of MeOH and was applied in 10 aliquots to a preparative HPLC (Capcell pak C18 UG-120, 20 i.d. × 250 mm, Shiseido Co., Tokyo, Japan) with 80% of MeOH/H_2O solvent (flow rate, 7.0 ml min^{-1}; detection, UV 210 nm). Each peak, with retention times of 14, 28 and 36 min, was collected and concentrated *in vacuo* to dryness to afford paraphaeosphaeride D (**1**, 34.4 mg), berkleasmin F (**2**, 62.9 mg) and a known compound, berkleasmin A (**3**, 581 mg), respectively.

Structure elucidation of paraphaeosphaeride D (1)

Compound **1** was obtained as a yellow oil ($[\alpha]_D^{27}$ -6; $c = 0.1$, MeOH), readily soluble in $CHCl_3$ and MeOH. It showed UV absorption maxima at 265 nm (sh, ε 2470) and 226 nm (ε 7900) in MeOH. The IR absorption at 1666 and 3355 cm^{-1} indicated the presence of ketone and hydroxy groups. The molecular formula of **1** was elucidated by HR-ESI-MS to be $C_{17}H_{27}NO_5$ (found *m/z* 348.1792 $[M+Na]^+$, calcd. *m/z* 348.1787), requiring five degrees of unsaturation. The ^1H and ^{13}C NMR spectra data of **1** is listed in Table 1. The ^{13}C NMR and HSQC spectra indicated 17 carbons, which were classified into four sp^2 fully substituted carbons, including one carbonyl carbon, two oxygenated sp^3 methines, one oxygenated sp^3 fully substituted carbon, one sp^2 exomethylene, six methylenes and three methyls, including one methoxy group. The ^1H-^1H COSY of **1** indicated the assignments

Table 1 ^{1}H and ^{13}C NMR spectral data of paraphaeosphaeride D (1) in $CDCl_3$

	*Paraphaeosphaeride D (**1**)*			
No.	*δ_C mult.*		*δ_H mult. (J in Hz)*	
1	166.5	C		
3	136.3	C		
4	156.1	C		
5	104.3	C		
6	68.1	CH	4.46	s
7	71.7	C		
8	86.2	CH	4.04	t (6.8)
9	27.5	CH_2	1.79	m
10	26.5	CH_2	1.27–1.34[a]	m
			1.57	m
11	29.2[b]	CH_2	1.27–1.34[a]	m
12	29.3[b]	CH_2	1.27–1.34[a]	m
13	31.8	CH_2	1.27–1.34[a]	m
14	22.6	CH_2	1.27–1.34[a]	m
15	14.1	CH_3	0.88	t (7.2)
16	92.6	CH_2	5.03	d (1.6)
			5.08	d (1.6)
17	16.8	CH_3	1.27[a]	s
18	64.5	CH_3	3.90	s

Abbreviation: mult., multiplicity.
NMR spectra were recorded at 400 MHz for ^{1}H and 125 MHz for ^{13}C.
[a]Overlapped.
[b]Exchangable.

from H-8 to H_2-10 and from H_3-15 to H_2-14 (Figure 3a). The HMBC correlations from H_3-17 (δ_H 1.27) to C-6 (δ_C 68.1), C-7 (δ_C 71.7) and C-8 (δ_C 86.2), from H-6 (δ_H 4.46) to C-7, C-8 and C-17 (δ_C 16.8), from H-8 (δ_H 4.04) to C-6, C-7 and C-17 established the connectivity between C-6 and C-8 and attachment of C-17 to C-7. The presence of 3,4-dihyroxy-3-methyl-3,4-dihydro-2*H*-pyran ring was suggested by the HMBC correlations from H-6 to C-5 (δ_C 104.3) and C-4 and from H-8 to C-4, and the ^{13}C chemical shifts of C-4 (δ_C 156.1). HMBC and COSY correlations suggested that this pyran ring was connected to a *n*-heptyl side chain at C-8 position, as shown in Figure 3a. The exomethylene protons (δ_H 5.03 and 5.08, H_2-16) showed HMBC correlations to carbons at C-4 and C-3 (δ_C 136.3), which indicated C-3 was located between C-16 and C-4. One singlet methyl, one carbonyl group and one atom each of nitrogen and oxygen remain from the molecular formula. The remaining methyl group is very highly deshielded, with ^{13}C chemical shift of δ_C 64.5 and ^{1}H chemical shift of δ_H 3.90 (H_3-18), and hence it must be attached to oxygen. The HMBC correlation from H-6 to C-1 (δ_H 166.5) and remaining one unsaturation degree indicated connectivity between C-1 and C-3 via a nitrogen atom, substituted with a methoxy group, to form a *N*-methoxy-γ-methylidene-α,β-unsaturated-γ-lactam ring. This unique moiety was confirmed by the comparison of ^{1}H and ^{13}C chemical shifts between **1** and phaeosphaeride A,[16] and we consequently designated **1** as paraphaeosphaeride D.

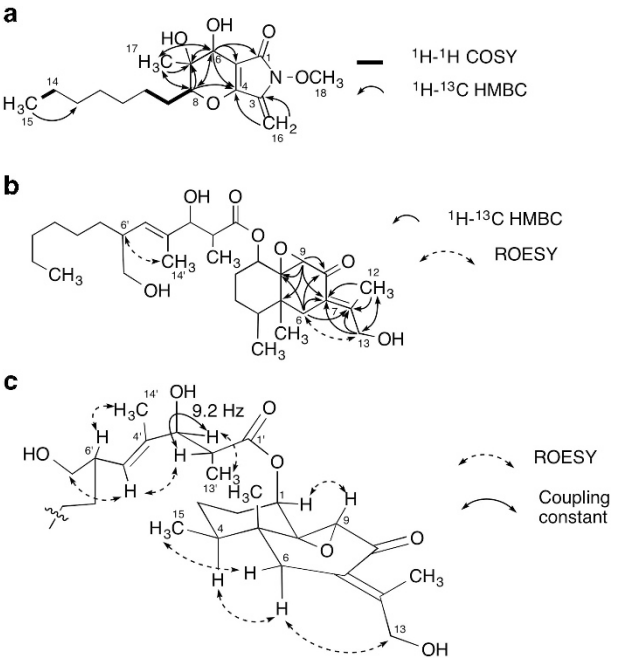

Figure 3 (**a**) COSY and key HMBC correlations of paraphaeosphaeride D (**1**). (**b**) Key HMBC and key ROESY correlations of berkleasmin F (**2**). (**c**) ROESY correlations and coupling constants of berkleasmin F (**2**).

Structure elucidation of berkleasmin F (2)

Compound **2** was obtained as a yellow oil ($[\alpha]_D^{27}$ 0.4; $c = 0.1$, MeOH), soluble in $CHCl_3$ and MeOH. It showed UV absorption maxima at 203 nm (ε 5800) and 273 nm (ε 3300) in MeOH. The IR absorption at 1720 cm^{-1} and 3401 cm^{-1} of **2** suggested the presence of ketone and hydroxy groups. The similarity in physicochemical properties between **2** and the known **3** strongly suggested that **2** is a new analog of **3**[10]. The molecular formula of **2** was elucidated by HR-ESI-MS to be $C_{30}H_{48}O_7$ (found *m/z* 543.3285 $[M+Na]^+$, calcd. *m/z* 543.3298), indicating that **2** had the same molecular formula as that of **3**. The ^{1}H and ^{13}C NMR spectra data of **2** is listed in Table 2. NMR spectra of **2** lacked a tetrahydrofuran ring and the exomethylene of **3** and had new signals of a ketone (δ_C 197.3), an oxymethylene (δ_H 4.10 and 4.29, δ_C 63.6), two fully substituted olefinic carbons (δ_C 127.6 and 150.6) and an olefinic methyl group (δ_H 2.16, δ_C 31.0). The HMBC experiments (Figure 3b) gave the following results; the proposed sesquiterpene core structure was confirmed by the key HMBC correlations: from H-9 (δ_H 3.44) to C-5 (δ_C 36.9), C-7 (δ_C 127.6), C-8 (δ_C 197.3) and C-10 (δ_C 68.0), from H_2-6 (δ_H 2.33 and 2.37) to C-7, C-8, C-10 and C-11 (δ_C 150.6), from H_3-12 (δ_H 2.16) to C-7, C-11 and C-13 (δ_C 63.6) and from H_2-13 (δ_H 4.10) to C-7, C-11 and C-12 to form 1-hydroxy-2-propylidene group at C-7 position. The chemical shifts of the side chain (from C-1′ to C-15′) is almost the same as those of **3**. The ROESY correlation (Figure 3b), observed between H-6 and H-13 and between H_3-14′ and H-6′, were elucidated to be 7*E* and 4′*E*. The ^{1}H and ^{13}C chemical shifts of an allylic methyl group (δ_H 2.16, δ_C 31.0) were shifted downfield by the anisotropic effect of the carbonyl group at C-8. Therefore, the planar structure of **2** was elucidated as shown in Figure 3b and we designated **2** to be berkleasmin F.

The relative configuration of **2** was elucidated by the analysis of ROESY and the coupling constants. The β-oriented configuration of H_3-15, and H-9 and the α-oriented configuration of H-1 were

Table 2 ^{1}H and ^{13}C NMR spectral data of berkleasmin F (2) in $CDCl_3$

	*Berkleasmin F (**2**)*			
No.	*δ_C mult.*		*δ_H mult. (J in Hz)*	
1	74.4	CH	4.56	t (2.8)
2	29.2	CH_2	1.90	m
3	25.4	CH_2	1.47	m
			1.70	m
4	39.5	CH	1.71	m
5	36.9	C		
6	36.8	CH_2	2.33	d (15.4)
			2.37	d (15.4)
7	127.6	C		
8	197.3	C		
9	62.6	CH	3.44	s
10	68.0	C		
11	150.6	C		
12	31.0	CH_3	2.16	s
13	63.6	CH_2	4.10	d (14.2)
			4.29	d (14.2)
14	17.7	CH_3	1.04	s
15	16.5	CH_3	0.96	s
1′	174.4	C		
2′	43.3	CH	2.63	dq (9.2, 6.9)
3′	80.4	CH	4.09	d (9.2)
4′	137.1	C		
5′	131.8	CH	5.15	d (10.0)
6′	40.8	CH	2.54	m
7′	31.4	CH_2	1.21–1.29[a]	m
8′	27.2	CH_2	1.21–1.29[a]	m
9′	29.2	CH_2	1.21–1.29[a]	m
10′	31.8	CH_2	1.21–1.29[a]	m
11′	22.6	CH_3	1.21–1.29[a]	m
12′	14.0	CH_3	0.86	t (7.2)
13′	14.2	CH_3	1.03	d (6.9)
14′	11.2	CH_3	1.62	s
15′	66.5	CH_2	3.36	m
			3.57	m

Abbreviation: mult., multiplicity.
NMR spectra were recorded at 400 MHz for ^{1}H and 125 MHz for ^{13}C.
[a]Overlapped.

deduced by ROESY correlations between H_3-15 and H-6β (δ_H 2.33), between H-4 and H-6α (δ_H 2.37), between H-6α and H_2-13 (δ_H 4.29) and between H-1 and H-9 (Figure 3c). The same relative configuration of C-2′, C-3′ and C-6′ on the acyl side chain of **2** was elucidated to be the same as those of berkleasmins A-E, evidenced by a large coupling constant (9.2 Hz) between H-2′ and H-3′ and ROESY correlations between H_3-13′ and H-3′, between H-2′ and H-5′, between H_3-14′ and H-6′ and between H-5′ and H_2-15′. Thus the relative configuration of **2** was elucidated to be 1*R**,4*S**,5*R**,9*S**,10*R**,2′*R**,3′*S**,6′*S**.

Activity of 1, 2 and 3 against ABK resistance in MRSA

The activity of **1, 2** and **3** with respect to overcoming ABK resistance in MRSA was evaluated against the ABK-resistant TH-1466 strain using the paper disc method (Table 3). All compounds enhanced anti-MRSA activity, with the effectiveness of ABK being improved 10-, 30- and 100-fold for **1**, **2** and **3**, respectively.

The compounds were also tested against the TH-1466 strain using the agar dilution assay. The MIC values of **1**, **2** and **3** were 256, 16 and 16 μg ml^{-1}, respectively.

Table 3 Circumvention of ABK resistance in MRSA TH-1466 using the paper disc method

	Inhibition zone (mm)/6 mm disc					
	1		*2*		*3*	
μg per disc	*ABK(+)*	*ABK(–)*	*ABK(+)*	*ABK(–)*	*ABK(+)*	*ABK(–)*
100.0	20	12	14	8	NT	NT
30.0	16	7	12	—	20	11
10.0	12	—	10	—	17	8
3.0	8	—	8	—	13	—
1.0	—	—	7	—	14	—
0.3	—	—	—	—	11	—
0.1	NT	NT	—	—	—	—

Abbreviations: ABK, arbekacin; MRSA, methicillin-resistant *Staphylococcus aureus*; NT, not tested.

A population analysis was subsequently undertaken of the impact of the compounds on anti-MRSA activity against 26 clinical isolated strains harboring the gene of aminoglycoside-modifying enzyme *aac (6′)-Ie/aph(2″)-Ia* (Table 4). Compounds **1**, **2** and **3** showed anti-MRSA activity, with MIC values ranging from 128 to >256 μg ml^{-1}, 16 μg/ml and from 8 to 16 μg ml^{-1}, respectively. The circumvention impact of **1**, **2** and **3** against 26 clinical isolated strains was 1- to 16-fold, and it was similar to the impact against TH-1466 strain (4-fold).

The inhibitory activity against the bifunctional enzyme AAC(6′)-Ie/APH(2″)-Ia was also evaluated using a cloned recombinant enzyme from the MRSA TH-1466 strain.[8] Surprisingly, all three compounds failed to inhibit phosphorylation and acetylation, even at 1 mg ml^{-1} (data not shown).

DISCUSSION

We found **1** and **2**, isolated from the culture broth of *Paraphaeosphaeria* sp. TR-022, to be circumventors of ABK resistance in MRSA, similar to the known compoyund **3**. It is interesting that the microorganism simultaneously produces metabolites with a variety of different skeletal structures. Especially, **1** has an unusual skeleton containing a 3-pyrrolin-2-one moiety, which has been reported previously in seven compounds.[12–14] Very recently, biosynthetic pathways of pyranonigrins, which have skeleton similar to **1**, have been reported,[15] in which the polyketide synthase-non-ribosomal peptide synthetase (PKS-NRPS) hybrid enzyme was proposed to form a precursor of pyraonigrin E using one acetyl-CoA, six malonyl-CoAs and one L-serine as substrates. Phaeosohaerides were reported to inhibit the signal transducer and activate the transcription 3 (STAT3) pathway, acting as anti-tumor reagents, but the action mechanisms are poorly understood. However, other biological activities of the phaeosphaerides have not been reported. Compound **2** has an eremophilane sesquiterpenoid skeleton similar to that of **3**. Many eremophilane-type compounds possess unique chemical structures and a variety of bioactivities.[16,17] Compounds **2** and **3** showed ABK circumvention activity against MRSA.

Both **1** and **2** reduced the MIC values of ABK against MRSA, from 16 μg ml^{-1} to 4 μg ml^{-1} (fourfold), but they did not inhibit the bifunctional enzyme AAC(6′)-Ie/APH(2″)-Ia, even at 1 mg ml^{-1}. These results indicate that they might have another mechanism for lowering drug resistance. Such inhibition mechanisms remain poorly defined or understood. ABK uptake may be modified by changing membrane permeability and energy metabolism in MRSA, due to

Table 4 Circumvention of ABK resistance in MRSA TH-1466 and 26 clinical isolate strains using the agar dilution method

	MIC (μg ml^{-1})			*Cirucumvention activity by combination assay[a] (times)*		
		26 clinical isolates			*26 clinical isolates*	
	TH-1466	*Average*	*Range[b]*	*TH-1466*	*Average*	*Range[b]*
ABK	16	15.7	8–32			
1	256	187	128–>256	4	6.2	2–16
2	16	16.0	16	4	3.0	1–8
3	16	15.7	8–16	4	2.8	1–4

Abbreviations: ABK, arbekacin; MRSA, methicillin-resistant *Staphylococcus aureus*.
[a]Concentrations of the compounds (1–3) were used 1/4 MIC values.
[b]Compounds were evaluated against 26 clinical isolated strains.

effects such as the proton motive force.[18,19] We identified **1** and **2** as new chemicals that might prove to be promising lead compounds for developing circumventors of ABK resistance in MRSA.

METHODS

Taxonomic study

For molecular identification of TR-022, a nuclear ribosomal large subunit and ITS were analyzed. Primers of ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) were used for PCR amplification. The PCR product was sequenced using primers of ITS5, ITS4 (5′-T CCTCCGCTTATTGATATGC-3′), NL1 (5′-GCATATCAATAAGCGGAGGA AAAG-3′) and NL4. The determined DNA sequences were deposited to the DNA Data Bank of Japan as accession number LC115035. BLAST searches were performed to compare large subunit and ITS sequences of TR-022 with DNA sequences in public databases. The ITS sequence of TR-022 was aligned with those of 24 fungal strains selected based on the BLAST result as well as on taxonomical references.[11,20] The phylogenetic tree was constructed by MEGA ver. 6.0.[21]

Fermentation

A piece of TR-022 grown on agar was inoculated into 10 ml seed medium [mashed potato (Megmilk Snow Brand Co., Ltd., Tokyo, Japan) 3%, glucose 2%, yeast extract 0.5%, (pH 6.0) in a test tube and shaken for 5 days (280 r.p.m. at 25 °C). Five ml of the seed culture was inoculated into 45 ml of the same medium in each of 11 Erlenmeyer flasks and cultured for 2 days (200 r.p.m. at 25 °C). The mixed culture (24 ml) was seeded onto fermentation medium (oat meal (Tomizawa Shouten Inc., Tokyo, Japan) 70 g, soybean meal (J-Oil Mills, Inc., Tokyo, Japan) 7 g, 140 ml distilled water) in 20 polypropylene containers and left standing for 12 days at 25 °C.

General experiments

NMR spectra were measured by a Varian XL-400 spectrometer (Agilent Technologies, CA, USA), with ^{1}H NMR at 400 MHz and ^{13}C NMR at 100 MHz in $CDCl_3$. The chemical shifts are expressed in p.p.m. and are referred to $CHCl_3$ (7.26 p.p.m.) in the ^{1}H NMR spectra and to $CDCl_3$ (77.0 p.p.m.) in the ^{13}C NMR spectra. ESI-MS spectra were measured with a JMS AX-505 HA mass spectrometer (JEOL Ltd., Tokyo, Japan). IR spectra (ATR) were observed using a FT-210 Fourier transform IR spectrometer (Horiba Ltd., Kyoto, Japan). UV spectra were measured with a Hitachi U-2801 spectrophotometer (Hitachi Ltd., Tokyo, Japan). Optical rotation was measured with a JASCO P-2200 polarimeter (JASCO Corporation, Tokyo, Japan).

Assay for circumvention of ABK resistance in MRSA

Using an MRSA TH-1466 strain, a clinical ABK-resistant isolate harboring the gene for the aminoglycoside-modifying enzyme *aac(6′)-Ie/aph(2″)-Ia*, circumvention of ABK resistance in MRSA was evaluated by the paper disc method and the agar dilution method. The paper disc method was carried out according to the following protocol; the MRSA was cultured in 4 ml of Difco Mueller Hinton broth (MHB; Becton Dickinson, NJ, USA) at 37 °C for 20 hours and adjusted to 1×10^{8} CFU per ml. Seven hundred and fifty microliters of the culture broth was transferred to a square plate (10 × 14 cm, Eiken Chemical Co., Ltd., Tokyo, Japan) containing 20 ml of Difco Mueller Hinton agar (MHA; Becton Dickinson), with or without ABK (8 μg ml^{-1}, Meiji Seika Pharma Co., Ltd., Tokyo, Japan) whose concentration has no effect on the growth of MRSA. Paper disks (6 mm, Advantec Toyo Kaisha, Ltd., Tokyo, Japan) containing various amounts of a sample (or 7 μg per disc of vancomycin as a positive control) were placed on the MHA plate and incubated at 37 °C overnight. Anti-MRSA activity was expressed as the diameter (mm) of the inhibition zone.

The agar dilution assay was carried out according to the method recommended by NCCLS.[22] Population analysis of the MIC value of ABK together with **1–3** was studied against 26 clinical isolated MRSA.[23] Concentrations of the 1/4 MIC values of **1–3** were used for the combination assay.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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