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SPECIAL FEATURE: BRIEF COMMUNICATION

Structure and biological activity of Metarhizin C, a stereoisomer of BR-050 from *Tolypocladium album* RK17-F0007

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Abstract

Metarhizin C, a stereoisomer of BR-050 was isolated from a fungus *Tolypocladium album* RK17-F0007 through a screening program to search for new antitumor compounds. A structure of the isomer was determined by spectroscopic methods including detailed analysis of NOESY correlation and mass spectrometry, and found to be identical to that of 3-desacylmetarhizin A with the absolute structure. Previously, it had been isolated by Kikuchi et al and proposed as BR-050 including the stereo-structure. However, detailed analysis for the newly isolated isomer confirmed that 3-desacylmetarhizin A was not identical to BR-050. Therefore, we assigned it metarhizin C as a new BR-050 isomer. Metarhizin C showed selective cytotoxicity against osteosarcoma MG-63 cells in a glucose independent condition with IC_{50} value of 0.79 μg/ml, while > 30 μg/ml of IC_{50} value in a normal condition, and inhibited a mitochondrial respiration.

Microbes, such as actinomycetes and fungi, have been an important source in pharmaceutical, medicinal, and agrochemical fields for their tremendous capacity to produce structurally wide and interesting secondary metabolites with various biological activities [1, 2]. They have been used as drugs, drug leads/seeds, pesticides, and bioprobes, which are chemical tools to investigate a biological function in chemical biology studies [3–5]. Nucleoside antibiotics are one of the major class of bioactive secondary metabolites from microbes. Isono's group in RIKEN has isolated many nucleoside antibiotics based on their UV spectral data, and we succeeded the method using diode-array-detector attached HPLC and database, such as Dictionary of Natural Products. Also, we have been working on isolation by focusing new bioactive compounds based on the combination of unique bioassay systems from microbial metabolite libraries including crude broths and semi-purified broths. To this end, we have prepared a microbial broth library by isolating microbes from soils collected in Japan. In addition, we have prepared a microbial fraction library from selected microbes to search minor metabolites efficiently [6, 7]. A fraction is a semi-purified compound/compounds prepared by a basic chromatographic separation using HPLC and MPLC and minor metabolites should be concentrated and nuisance materials could be excluded. Microbial broths and fraction libraries were used by the combination with our original spectral database NPPlot (Natural Product Plot) to search and identify structurally interesting metabolites [6, 7]. The libraries are also screened by our unique screening systems. We have so far constructed the in-house phenotypic evaluation (iHOPE) system, and exhaustively evaluated the biological activities, such as cytotoxicity, differentiation, and morphological changes, of test samples against several cancer cell lines, bacteria, pathogenic fungi, and a malaria parasite. Based on this strategy, we have found and isolated several new metabolites with interesting

This article is dedicated to Dr Kiyoshi Isono with respect and admiration for his achievements in antibiotics research.

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biological activities, such as verticilactam [8], spirotoamides [9], pyrrolizilactone [10, 11], and trachyspic acid 19-butyl ester [12]. Recently, antimycin related metabolites, unantimycin A [13] and opantimycin A [14] were isolated and unantimycin A was identified as an inhibitor of mitochondrial respiration by targeting the mitochondrial complex III [15].

In the present study, about 1200 microbial broths were tested their cytotoxicity against several cancer cell lines including the human cervical epidermoid carcinoma cell line, HeLa, human promyelocytic leukemia cell line, HL-60, rat kidney cells infected with ts25, a T-class mutant of *Rous sarcoma* virus Prague strain, *src*^{ts}-NRK, and MG-63 cells, which were cultured in both normal and glucose independent conditions, antimicrobial activities against *Staphylococcus aureus* 209, *Escherichia coli* HO141, *Aspergillus fumigatus* Af293, *Pyricularia oryzae* kita-1, and *Candida albicans* JCM1542, and antimalarial activity against malaria parasite *Plasmodium falciparum* 3D7. A culture broth of a fungal strain *Tolypocladium album* RK17-F0007, which had been isolated from a soil collected in Hanyu, Saitama, Japan at 2017, showed a selective cytotoxicity for the glucose independent condition of MG-63 cells without remarkable activities against the other cancer cell lines and microbes. We selected the strain for further isolation and identification of the active metabolite.

The fungal strain *T. album* RK17-F0007 was cultured in 21 of YMGS culture medium (0.5% yeast extract, 0.5% malt extract, 1% glucose, and 1% soluble starch) for 4 days at 28 °C. The whole culture broth was extracted with the same volume of acetone and filtered to remove mycelia. The acetone extract was evaporated under reduced pressure to obtain H_2O suspension. It was partitioned thrice with the same volume of EtOAc and the resulting organic extract was evaporated to afford a 1.3 g of brown gum. The gum was subjected to SiO_2-MPLC with stepwise elution of $CHCl_3$/MeOH to give eight fractions. The aimed activity was observed at the 3rd fraction and it was separated by C18-MPLC with acetonitrile/H_2O linear gradient to afford crude **1**. It was further purified by C18-HPLC with acetonitrile/H_2O (7:3) to obtain 1.4 mg of purified **1** as colorless amorphous; $[\alpha]_{589}^{22}$ -44.3 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 214 (4.30), 290 (3.78) nm; IR (neat) ν_{max}; 3322, 2925, 2875, 1668, 1565, 1496, 1455, 1436, 1378, 1238, 1114, 1079, 1027, 995, 885 cm^{-1}; ESIMS *m/z* 429 $[M + H]^+$ (Fig. 1).

Compound **1** had a molecular formula of $C_{27}H_{40}O_4$ determined by HRESITOFMS (found *m/z*: 429.3007 $[M + H]^+$ calcd for $C_{27}H_{41}O_4$, 429.2999) (Fig. S1). The IR spectrum implied the presence of a hydroxyl group and pyrone system by showing the absorptions at 3322, 1668, and 1565 cm^{-1} (Fig. S2). The ^{1}H NMR spectrum in methanol-d_4 showed six methyl signals including two methyl signals attached on *sp2* carbons at 2.17 (*s*, 3 H) and 1.90 (*s*, 3 H) ppm, an olefin signal at 5.14 (*t*, $J = 6.8$) ppm, and a signal suggesting the presence of a hydroxyl group at 3.54 (dd, $J = 11.5$, 4.6) ppm (Table 1 and Fig. S3). In addition, two characteristic signals were observed at 4.22 (brs) and 4.47 (t, $J = 2.3$) ppm. The ^{13}C NMR spectrum showed 27 signals containing six methyls, eight methylenes including an unusually low-fielded signal observed at 110.1 ppm, four methines, and nine quaternary carbons including a carbonyl signal at 169.0 ppm, which were confirmed by ^{13}C DEPT experiment (Figs. S4 and S5). The characteristic signals in the ^{1}H NMR spectrum (4.22 (brs) and 4.47 (*t*, $J =$ 2.3) ppm) and the unusual methylene signal at δ_C 110.1 ppm suggested that **1** had an exo-methylene group in the molecule. The detailed structure was investigated using 2D NMR spectra, such as HSQC, DQF-COSY, and HMBC spectra (Fig. 2a, Figs. S6–S8). The connections between proton and carbon were confirmed by the correlations in the HSQC spectrum and established the presence of the exo-methylene group. The DQF-COSY revealed the connectivities from C-1 to C-3, from C-5 to C-7, from C-11 to C-13, and between C-9 and C-19 (Fig. 2a). A decalin skeleton was confirmed by HMBC correlations from H-5 to C-4 and C-10, from Me-17 to C-3, C-4, and C-5, from Me-20 to C-1, C-5, C-9, and C-10, and from H-18 to C-7 and C-9, which also assigned the attachment of the exo-methylene at C-8 supported by an HMBC correlation from H-7 to C-8. A prenyl group was constructed by HMBC correlations from Me-15 and Me-16 to C-14 and C-13, and attached at C-4 position supported by HMBC correlations from H-5 and Me-17 to C-11. A 4-hydroxy-5,6-dimethyl-α-pyrone was constructed by HMBC correlations from Me-7′ and Me-8′ to C-4′, C-5′, C-6′, and C-5′, C-6′, respectively, and from H-19 to C-2′, C-3′, and C-4′, which also confirmed the connection of the α-pyrone to C-19, with the help of ^{13}C NMR chemical shift values of C-2′ and C-6′ observed at 169.0 and 156.4 ppm, respectively. The above 2D NMR correlations established the planar structure of **1** and it was found to be identical to BR-050 (**2**) [16] (Fig. 1). Compound **2** had been isolated by Kagamizono et al. from a fungus, *Penicillifer superimpositus* TF-0402, as a bone resorption inhibitor in 1995, and the only planar structure was reported at that time. Then, Kikuchi et al isolated 3-desacylmetarhizin A (**3**) along with metarhizins A and B from an entomopathogenic fungus, *Metarhizium flavoviride* in 2009, and proposed that **3** was identical to **2** including the absolute stereo-structure [17] (Fig. 1). However, the ^{1}H NMR spectrum of **1** was not identical to that of **2** with slight differences in signals around 3 ppm by the same deuterated solvent, chloroform-d_1/methanol-d_4 (9:1) [16] (Fig. S9), while it was identical to that of **3** compared by the same deuterated solvent, pyridine-d_5 (Figs. S10, S11). Therefore, we investigated the relative stereo-structure of **1** by the

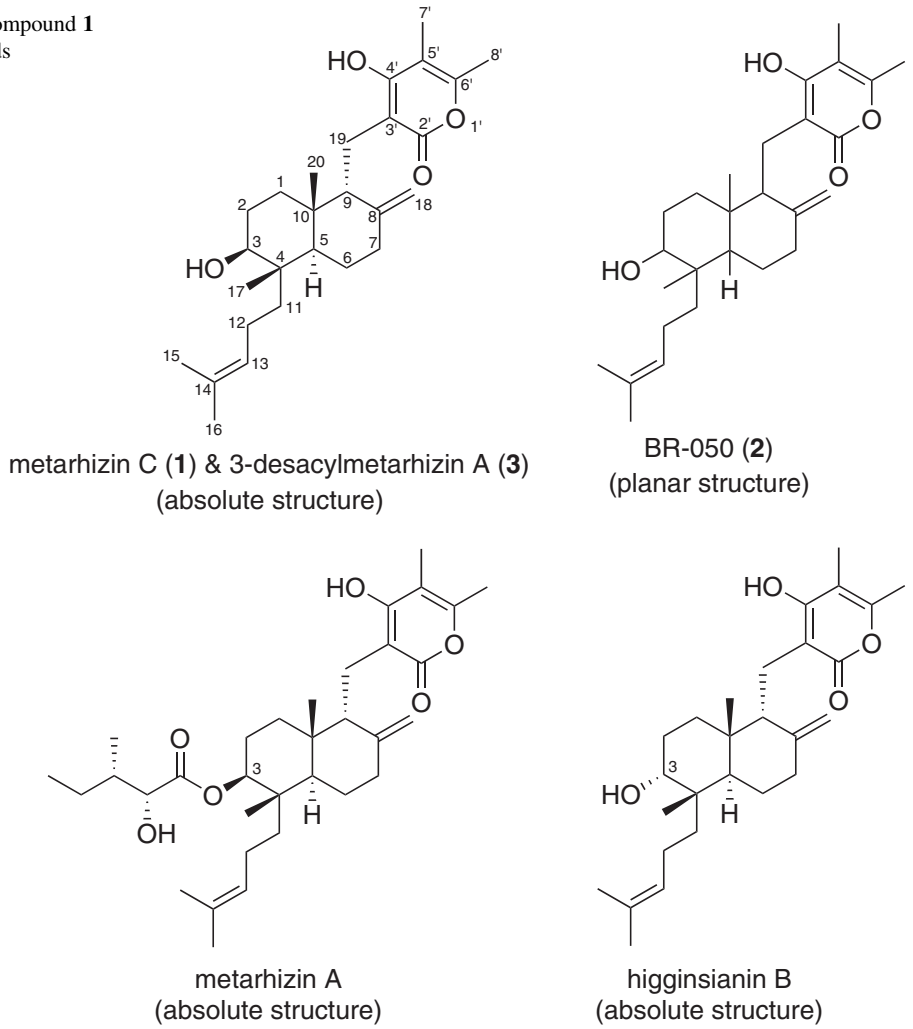

Fig. 1 Structures of compound **1** and related compounds

Table 1 ^{1}H and ^{13}C NMR chemical shift of compound **1** in methanol-d_4

Position	δ_C	δ_H	Multiplicity (*J* in Hz)	Position	δ_C	δ_H	Multiplicity (*J* in Hz)
1	35.7	1.30	m	13	126.5	5.14	t (6.8)
		1.86	ddd (13.8, 13.8, 3.4)	14	131.9	–	
2	28.7	1.68	m	15	18.0	1.66	s, 3 H
		1.76	m	16	26.2	1.69	s, 3 H
3	74.6	3.54	dd (11.5, 4.6)	17	18.2	0.78	s, 3 H
4	42.4	–		18	110.1	4.22	brs
5	40.3	1.78	m			4.47	brt (2.3)
6	24.4	1.35	m	19	22.8	2.57	dd (13.2, 4.6)
		1.57	m			2.75	dd (13.2, 13.2)
7	32.6	2.05	m	20	23.9	0.96	s, 3 H
		2.52	ddd (13.7, 13.7, 5.7)	2′	169.0	–	
8	150.8	–		3′	103.4	–	
9	56.7	2.16	m	4′	171.5	–	
10	38.9	–		5′	110.5	–	
11	38.8	1.24	m	6′	156.4	–	
		1.57	m	7′	10.7	1.90	s, 3 H
12	23.0	2.02	m, 2 H	8′	17.5	2.17	s, 3 H

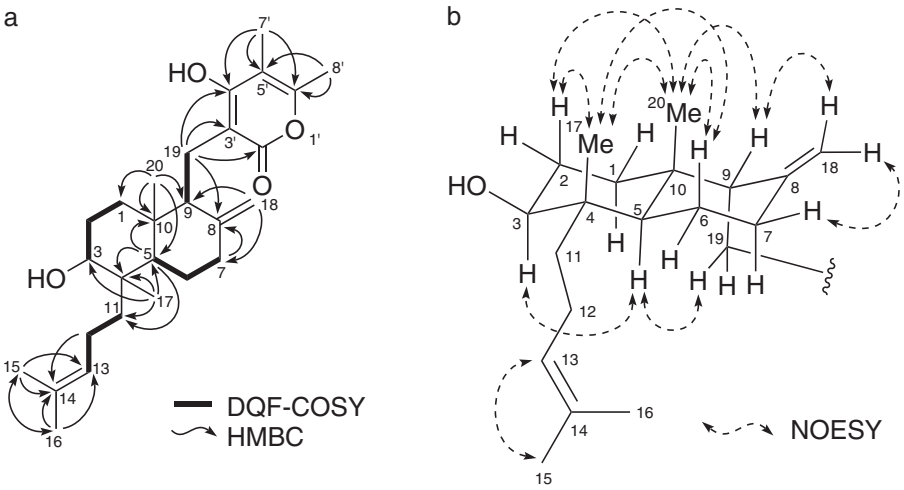

Fig. 2 Key HMBC correlations (**a**) and key NOESY correlations (**b**) for compound **1**

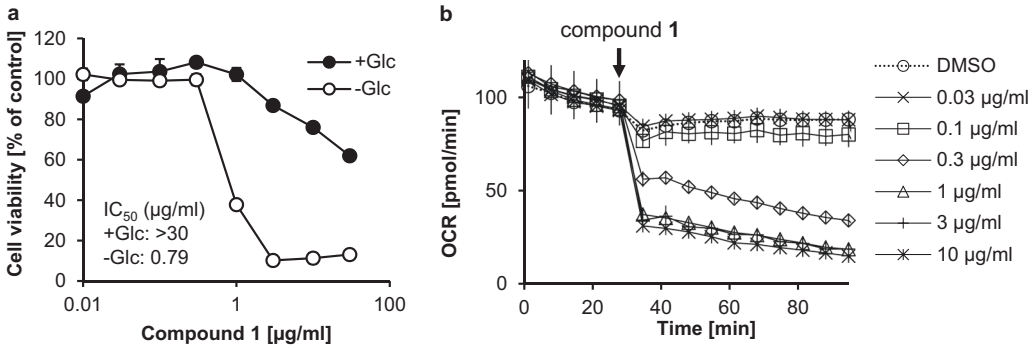

Fig. 3 Effect on glucose (Glc) independent growth of MG-63 cells (**a**) and effect on a mitochondrial respiration using a Flux analyzer (**b**)

detailed interpretation of NOESY spectrum to confirm the stereo-structure.

Compound **1** showed NOESY correlations between Me-17 and H-2ax, H-6ax, and Me-20, which also correlated to H-2ax and H-6ax (Fig. 2b, Fig. S12). H-5 showed NOESY correlations to H-3ax and H-19. These observations suggested that the decalin skeleton had a *trans*-configuration at C-5 and C-10 positions with axial-orientations of H-5 and Me-20, and also confirmed an equatorial-orientation of the hydroxyl group at C-3 and axial-orientations of Me-17 and α-pyrone moiety. It resulted the relative stereo-structure of **1** with the hydroxyl group, Me-17, and Me-20 on the same side and the prenyl group at C-4 and α-pyrone moiety on the other side of the decalin (Fig. 1). These results confirmed that the relative stereo-structure of **1** was the same as that of **3** supported by the identical ^{1}H NMR spectra as mentioned above (Figs. S9, S10). The absolute structure of **1** was supposed to be the same as that of **3** by the identical specific rotation values (**1**: $[\alpha]_{589}$ −44.3 (*c* 0.13, MeOH), **3**: $[\alpha]_{D}$ −32.1 (*c* 0.38, MeOH)) [17]. Therefore, we concluded that the structure of **1** was identified as 3-desacylmetarhizin A (**3**) and designated as metarhizin C.

The in vitro cytotoxicity was evaluated against MG-63 cells in both normal and glucose independent conditions. Compound **1** showed the selective activity in a glucose independent condition with IC_{50} value of 0.79 μg/ml against >30 μg/ml of IC_{50} value by a normal condition (Fig. 3a). It confirmed **1** was the active principle of *T. album* RK17-F0007 strain and might influence on a metabolic pathway exclude a glycolysis. Recently, Katou et al. have reported that metarhizin A inhibits cytochrome C oxidase and suppresses the mitochondrial activity [18]. Therefore, we investigated an effect on a mitochondrial respiration in MG-63 cells using a flux analyzer. It showed the decrease of oxygen consumption rate (OCR) when **1** was added to the culture by dose depending manner, suggesting **1** inhibited a mitochondrial respiration (Fig. 3b).

Until now, stereochemistry of this class of compounds is being controversial. The stereochemistry of BR-050 (**2**) had been considered to be identical to that of 3-desacylmetarhizin A (**3**), however, our present data clearly denied that possibility. Recently, higginsianin B has been reported as a new epimer at C-3 position of **2**, which is based on the stereo-structure of **3**, by Cimmino

et al. (Fig. 1) [19]. As far as we are concerned, the ^{1}H NMR spectrum of higginsianin B is seemed to be identical to that of **2**, even though the NMR spectra of **2** and higginsianin B were measured in different deuterated solvents; **2** in chloroform-*d*/methanol-d_4 (9:1) and higginsianin B in chloroform-*d*, respectively. In addition, specific rotation values also showed the same negative signs [16, 19]. Taken together, we suggest that the stereo-structure of **2** should be investigated by the direct comparison with both of **1** and higginsianin B to confirm the structure. In this report, we confirmed that **2** did not have the same stereo-structure as that of metarhizins on the decalin skeleton and 3-desacylmetarhizin A (**1** and **3**) was the new isomer of **2** as metarhizin C.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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