NOTE

Isolation and structural determination of a new macrolide, makinolide, from the newly isolated *Streptomyces* sp. MK-30

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Macrolides are a group of organic compounds that possess a macrolide ring (usually 14-, 15- or 16-membered) as the carbon skeleton. Most of the isolated macrolides are structurally diverse antibiotics such as erythromycin^{1,2} and are produced by microorganisms such as the soil-dwelling bacterium *Streptomyces* via polyketide biosynthesis.³ They exhibit various biological activities, including antitumor, antifungal, antiparasitic and immunosuppressant activities. Among the macrolides, bafilomycins^{4,5} and setamycin^{6,7} are 16-membered macrolides that are reported to exhibit specific membrane ATPase inhibitory activity.⁸ In the course of screening for anti-yeast compounds, we isolated a new bafilomycin analog named makinolide (**1** in Figure 1a), along with the known macrolide JBIR-100 (**2** in Figure 1a)^{9,10} from newly isolated strain, *Streptomyces* sp. MK-30. Herein, we describe the isolation and structural determination of makinolide (**1**).

The new bacterial strain MK-30 was isolated from the soil of a tea field (Makinohara, Shizuoka, Japan) along with 32 other bacterial strains using ISP2 agar medium. With the aim of screening for anti-yeast compounds, these strains were cultured with ISP2 agar media for 7 days. The spent culture media were extracted with acetone. Then, each acetone extract was subjected to anti-yeast activity screening against *Saccharomyces cerevisiae* (NBRC2376), and the strain MK-30 was found to produce a significant amount of anti-yeast substances.

To identify the genetic position of the MK-30 strain, sequencing analysis was performed on the 16S ribosomal RNA (rRNA) coding gene. Using a typical polymerase chain reaction (PCR) method with universal primers for the bacterial 16S rRNA gene, the nearly complete length of the 16S rRNA gene was amplified. The sequence analysis was accomplished using an automated DNA sequencer with eight universal primers. From the obtained sequence, a phylogenetic tree was constructed using the multiple-alignment program ClustalX. It was determined that the genetic position of MK-30 is located in the genus *Streptomyces* (Supplementary Figure S1). The strain MK-30 is closely

related to *Streptomyces sahachiroi*, with a high similarity of 99%. The 16S rDNA sequence of *Streptomyces* sp. MK-30 was deposited in the DDBJ database under the accession number AB691771.

To isolate the anti-yeast compounds, the cultivation of the strain MK-30 was performed with 11 of ISP2 agar media. After 7 days of cultivation, the spent agar culture media were extracted with an equal volume of acetone. After concentration, the acetone extract was subjected to open column chromatography using the synthetic hydrophobic resin CHP-20P and eluted with 20% MeOH, 60% MeOH and then MeOH. As the MeOH fraction showed potent activity, the MeOH fraction was repeatedly subjected to preparatory HPLC to yield the new macrolide makinolide (**1**, 3.3 mg) and the known macrolide JBIR-100 (**2**, 2.0 mg).

Makinolide was isolated as a white amorphous powder. HR-ESI-MS analysis of **1** gave an $[M + Na]^+$ ion at m/z 822.44272, consistent with the molecular formula $C_{44}H_{65}NO_{12}$ (calculated $[M + Na]^+$ ion at m/z 822.44044). To obtain further information on the chemical structure, NMR experiments including ¹H, HMQC, HMBC, DQF-COSY and TOCSY analyses of **1** were performed in acetone-*d*₆ (Supplementary Figures S2–S7).

The direct bonds between each proton and carbon were established in the HMQC spectrum (Table 1). As shown by the bold line in Figure 1b, the analysis of the TOCSY and DQF-COSY spectra revealed proton–proton spin connections in five partial structures: carbon skeletons connecting C5 to C9, C11 to C18, C20 to C25, C2' to C3' and C7' to C8'. The HMBC correlations between the protons of the 2-Me group and C1, C2 and C3, and between the protons of the 4-Me group and C3, C4 and C5, indicated a 2, 4-dimethyl dienone structure. The HMBC correlations between the protons of the 10-Me group and C9, C10 and C11 indicated a connection between the (C5 to C9) and (C11 to C18) partial structures. The HMBC correlations between the protons of the 18-Me group, H18, H20 and C19 indicated a hemiacetal structure, which was also confirmed by the characteristic

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Figure 1 (a) Chemical structure of makinolide (1) and JBIR-100 (2). (b) Selected NMR correlations of 1.

chemical shift value for C19 (δ c 99.4). The HMBC correlation between H23 and C19 was not observed; however, a comparison of the chemical shift values with the related compound JBIR-100 confirmed the cyclic hemiacetal moiety. The presence of the *N*-(3-hydroxy-2-cyclopentenone-2-yl)-fumaric acid monoamide moiety (C1'–C7') was confirmed by the chemical shifts observed in the HMQC spectrum and the molecular formula determined by the HR-ESI-MS analysis. Although no HMBC correlations were observed between H7' and H8', the similarity of the chemical shift values for the 7' and 8' positions to the values for these positions in the related compound R176502¹¹ support the structural assignment. Previously, the acid hydrolysis of bafilomycin B1 was reported to form *N*-(3-hydroxy-2-cyclopentenone-2-yl)-fumaric acid monoamide.¹² To confirm the partial structure containing the *N*-(3-hydroxy-2-cyclopentenone-2-yl)-fumaric acid monoamide moiety (C1'–C7'), mild acid hydrolysis of 1 was accomplished following the procedure described in the previous report.¹² The chemical shifts in the ¹H NMR spectrum (in MeOH-*d*₄) of the obtained compound were identical to those observed in the spectrum of *N*-(3-hydroxy-2-cyclopentenone-2-yl)-fumaric acid monoamide derived from an authentic sample of bafilomycin B1, which confirmed the partial structure of the C1'–C7' skeleton (Supplementary Figures S8 and S9).

In the disc diffusion assay, the sensitivity of various microorganisms was tested against macrolides 1 and 2 following previously reported procedures (Table 2).⁴ Among the bacteria, compounds 1 and 2 showed inhibitory activities only against *Micrococcus luteus* at the doses of 15 and 50 μ g per disc. Bafilomycins have been reported to be specific inhibitors of the membrane ATPase of eucaryotic cells,⁸ and recently, 2 was confirmed to exhibit inhibitory activity against vacuolar-type ATPase.⁹ Considering the inhibitory mechanism of bafilomycins, the related compounds 1 and 2 were thought to be active against yeast and fungi; however, among the six strains of yeast and fungi that were tested, macrolides 1 and 2 exhibited inhibitory activities against only *S. cerevisiae* and *Mucor hiemalis* (Table 2).

EXPERIMENTAL PROCEDURE

General Methods

NMR spectra were obtained using a JEOL ECA-600 in acetone-*d*₆ or MeOH-*d*₄ (JEOL Ltd, Tokyo, Japan) at 27.0 °C. The ESI-MS spectrum was recorded using a JEOL JMS-T100LP mass spectrometer.

PCR amplification, sequencing and phylogenetic analysis of 16S rRNA genes

The extraction of all DNA from the cells of the strain MK-30 was performed according to the literature procedure.¹³ The 16S rRNA-encoding sequence was amplified from the total DNA by the PCR method with two sets of universal primer pairs: 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 926R (5'-CCG TCAATTCCTTTGAGTTT-3'); and 686F (5'-TAGCGGTGAAATGCGTAGA-3') and 1510R (5'-GGCTACCTTGTTACGA-3'). For PCR, the reaction mixture was prepared by adding 1.0 μ l of the total DNA of the MK-30 strain (100 ng), 1.0 μ l of Pfu DNA polymerase (Bioneer, Daejeon, South Korea), 5.0 μ l of X10 reaction buffer (Bioneer), 4 μ l of 2.5 mM dNTP mix solution (Bioneer) and 31 μ l of distilled water into the PCR reaction tube. PCR amplification was carried out using a thermal cycler using the following program: initial denaturation for 10 min at 94 °C, followed by 34 cycles consisting of denaturation for 40 s at 94 °C, annealing for 60 s at 55 °C and DNA synthesis for 1 min at 72 °C. A final extension of 5 min at 72 °C was included at the end of the 34 cycles. The PCR product was purified with a Qiagen PCR PURE kit (Qiagen, Venlo, Netherlands) following the manufacturer's instructions. The reactions for sequencing were performed using a Beckman DTCS-Quick Start Kit following the manufacturer's instructions. Four primers were used for the reaction: 339F (5'-CTCC TACGGGTGAGTAACAC-3'), 536R (5'-GTATTACCGCGG CTGCTG-3'), 686F (5'-TAGCGGTGAAATGCGTAGA-3') and 1099F (5'-GCAACGAGCGC AACCC-3'). The sequencing was performed with the capillary DNA sequencer CEQ8000XL (Beckman Coulter, Brea, CA, USA).

Isolation of the bacterial strains

The soil samples were collected from a tea-field ground in Makinohara, Shizuoka, Japan. The soil samples were spread onto ISP2 agar medium. After 4–5 days of incubation at 30 °C, the colonies that developed were isolated and stored in a refrigerator at –80 °C. In total, 33 strains were collected and cultured for anti-yeast compound screening. Briefly, the ISP agar culture (25 ml) of each strain was extracted with an equal volume of acetone. Each acetone extract was evaporated and dissolved in dimethyl sulfoxide to adjust the concentration to 10 mg ml^{–1}. The anti-yeast activity was evaluated by determining the inhibitory zone that was caused by a 10- μ g inoculation of the acetone extract sample on a culture of the yeast *S. cerevisiae* using ISP2 agar medium.

Isolation of makinolide

ISP2 agar medium (1 l) was used for culturing *Streptomyces* sp. MK-30. After 7 days at 30 °C, an equal volume of acetone was added to the agar culture for extraction. The acetone extract was concentrated to an aqueous suspension,

Table 1 NMR chemical shift values of makinolide (1) in acetone- d_6

Position	δ_C	δ_H (J in Hz)
1	174.0	
2	121.3	
2-CH3	13.5	2.02 (s)
3	147.9	7.25 (s)
4	134.0	
4-CH3	15.4	1.96 (s)
5	148.4	6.04 (d, 9.0)
6	38.0	2.56 (m)
6-CH3	17.5	1.03 (d, 6.9)
7	81.0	3.29 (m)
8	39.0	1.89 (s)
8-CH3	22.4	0.90 (d, 6.9)
9	43.0	1.73 (m)
10	145.5	
10-CH3	20.2	1.90 (s)
11	125.0	5.77 (d, 11.0)
12	134.0	6.67 (d, 15.1, 10.3)
13	127.0	5.12 (dd, 15.1, 9.6)
14	84.0	4.07 (m)
14-OCH3	56.0	3.20 (s)
15	77.0	4.91 (m)
16	38.0	2.08 (m)
16-CH3	12.5	0.83 (d, 6.8)
17	72.0	4.10 (m)
18	42.8	1.80 (m)
18-CH3	24.0	0.96 (d, 7.6)
19	99.4	
20	40.5	1.26 (m), 2.31 (dd, 11.5, 4.8)
21	76.0	5.01 (m)
22	38.2	1.60 (m)
22-CH3	22.6	0.84 (d, 6.9)
23	75.2	3.59 (dd, 10.3, 2.1)
24	28.5	1.91 (m)
25	21.7	0.92 (d, 6.9)
26	14.8	0.77 (d, 6.9)
1'	164.9	
2'	130.2	7.52 (d, 15.1)
3'	135.0	6.80 (d, 15.1)
4'	166.9	
5'	—	
6'	—	
7'	36.3	2.76 (br)
8'	26.4	2.44 (br)
9'	—	

which was subjected to an open column chromatography (Mitsubishi Chemical, Tokyo, Japan, CHP20P, 3 × 5 cm), eluting with 50 ml each of 20% MeOH, 60% MeOH and MeOH. The MeOH fraction was subjected to reverse-phase HPLC using an ODS column (Nacalai Tesque, Kyoto, Japan, Cosmosil C18-MSII 4.6 × 250 mm) with an isocratic elution of 85% MeCN containing 0.05% trifluoroacetic acid (TFA) at an absorbance of 220 nm to yield makinolide (1, 3.3 mg) and JBIR-100 (2, 2.0 mg).

Mild acid hydrolysis

To obtain *N*-(3-hydroxy-2-cyclopentenone-2-yl)-fumaric acid monoamide, mild acid hydrolysis was performed. Makinolide (1.0 mg) or authentic bafilomycin B1 (1.0 mg, purchased from Wako, Japan) was dissolved in concentrated TFA and incubated at 80 °C for 16 h. After evaporation of TFA, 1 ml of distilled water was added. The hydrolysate solution was subjected to reverse-phase HPLC using an ODS column (Nacalai Tesque, Cosmosil MSII

Table 2 Antimicrobial activities of 1 and 2

Microorganisms	Diameter of inhibition zone (mm)			
	1		2	
	15 µg	50 µg	15 µg	50 µg
<Bacteria>	—	—	—	—
<i>Escherichia coli</i> (NBRC 1002203)	—	—	—	—
<i>Pseudomonas aeruginosa</i> (NBRC 12689)	—	—	—	—
<i>Serratia marcescens</i> (NBRC 1002204)	—	—	—	—
<i>Vibrio harveyi</i> (NBRC 15634)	—	—	—	—
<i>Bacillus subtilis</i> (NBRC13719)	—	—	—	—
<i>Staphylococcus aureus</i> (NBRC 1009110)	—	—	—	—
<i>Micrococcus luteus</i> (NBRC33333)	15	18	13	20
<i>Streptomyces antibioticus</i> (NBRC 3117)	—	—	—	—
<Yeast>	—	—	—	—
<i>Saccharomyces cerevisiae</i> (NBRC2376)	12	13	11	9
<i>Schizosaccharomyces pombe</i> (NBRC 0340)	—	—	—	—
<i>Kloeckera apiculata</i> (NBRC 0154)	—	—	—	—
<Fungi>	—	—	—	—
<i>Aspergillus niger</i> (NBRC 33023)	—	—	—	—
<i>Aspergillus oryzae</i> (NBRC 4290)	—	—	—	—
<i>Mucor hiemalis</i> (NBRC 9405)	15	18	11	12

Paper discs (i.d. 6 mm for 15 µg per disc and i.d. 8 mm for 50 µg per disc) were used. The minus (—) means no inhibition.

4.6 × 250 mm) using isocratic elution of 13% MeCN containing 0.05% TFA and a UV detector set at an absorbance of 220 nm to yield *N*-(3-hydroxy-2-cyclopentenone-2-yl)-fumaric acid monoamide: HR ESI-MS showed an ion peak at *m/z* 234.0383 [*M* + Na]⁺ calculated for C₉H₉O₅Na (Δ 0.55 mmu). The ¹H-NMR signals (MeOH-*d*₄) were detected at δ_H H2': 7.18 (d, 15.1 Hz, 1 H), H3': 6.81 (d, 15.1 Hz, 1 H), and H8' and H9': 2.54 (s, 4 H).

Bacterial strains for antimicrobial assay

All microorganisms shown in Table 1 were obtained from the NBRC culture collection (NITE Biological Resource Center, Kisarazu, Japan). Cultivation was performed using ISP2 liquid medium at 37 °C for 24 h. The disc diffusion assay using a paper disc (i.d. 6 mm, thin type for 15 µg; and i.d. 8 mm, thick type for 50 µg) was performed to determine the antimicrobial activity of 1 and 2.

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