

Note

Isolation and Structural Determination of Makinolide B from *Streptomyces* sp. MK-19

Michitaka HIDAOKA,¹ Mitsuru YOSHIDA,^{2,*} Naoto OGAWA,¹ and Shinya KODANI^{1,3,†}

¹Graduate School of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

²National Food Research Institute, National Agriculture and Food Research Organization, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

³Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

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A new 16-membered macrolide named makinolide B (1) was isolated from *Streptomyces* sp. MK-19. The structure of makinolide B (1) was determined on the basis of 2D NMR experiments involving DQF-COSY, TOCSY, HSQC, and HMBC methods. Application of the paper disk diffusion method to makinolide B (1) showed weak antibacterial activity against *Staphylococcus aureus* at the dose of 100 µg/disk.

Key words: *Streptomyces*; macrolide; bafilomycin analogue; NMR spectrum

Macrolides are structurally diverse antibiotics like erythromycin^{1,2)} and are produced by such a micro-organism as the soil-dwelling bacterium *Streptomyces* via polyketide biosynthesis.³⁾ They exhibit various biological activities, including antitumor, antifungal, antiparasitic, and immunosuppressant effects. Among the macrolides, bafilomycins^{4,5)} and setamycin^{6,7)} are 16-membered macrolides that have been reported to exhibit specific membrane ATPase inhibitory activity.⁸⁾ We have recently isolated a bafilomycin analogue named makinolide A (2)⁹⁾ together with JBIR-100¹⁰⁾ as an anti-yeast compound from *Streptomyces* sp. MK-30. In the course of a further chemical investigation of the related strains, we isolated the new bafilomycin analogue, makinolide B, from *Streptomyces* sp. MK-19. We describe here the isolation and structural determination of makinolide B (1).

The new MK-19 bacterial strain was isolated from soil in a tea field (Makinohara, Shizuoka, Japan) as described in a previous paper.⁹⁾ A sequencing analysis was performed on the 16S rRNA coding gene to identify the genetic position of the MK-19 strain. The PCR method was used with universal primers for the bacterial 16S rRNA gene to amplify the nearly complete length of this gene. A sequence analysis was conducted by an automated DNA sequencer with universal primers. A phylogenetic tree was constructed from the obtained sequence by using the ClustalX multiple-alignment program. The genetic position of MK-19 was determined to be located in the *Streptomyces* genus. Strain MK-19 was closely related to previously isolated *Streptomyces* sp. MK-30 with close 99% similarity.

The 16S rRNA gene sequence of *Streptomyces* sp. MK-19 has been deposited in the DDBJ database under accession no. AB770481. Although strains MK-30 and MK-19 were thought to be closely related, a comparative HPLC analysis of the MeOH extracts between MK-30 and MK-19 only indicated the presence of the new bafilomycin analogue in the MK-19 extract. We therefore isolated and structurally determined the new bafilomycin analogue named makinolide B from *Streptomyces* sp. MK-19.

Strain MK-19 was cultivated on 1 L of an ISP2 agar medium. The culture medium and mycelia were extracted with an equal volume of acetone after 7 d of cultivation. The acetone extract was concentrated and then subjected to open column chromatography, using the CHP-20P synthetic hydrophobic resin and eluting with 20% MeOH, 60% MeOH, and then MeOH. The MeOH fraction was repeatedly subjected to preparatory HPLC to yield the new macrolide, makinolide B (1 in Fig. 1a).

Compound 1 was isolated as a white amorphous powder. A high-resolution electrospray ionization-mass spectrometry (HR-ESI-MS) analysis of 1 gave an [M + Na]⁺ ion at *m/z* 611.3906, consistent with the molecular formula C₃₅H₅₆O₇Na (calculated [M + Na]⁺ ion at *m/z* 611.3923). To obtain further information on the chemical structure, NMR experiments involving ¹H, ¹³C, DEPT-135, DQF-COSY, TOCSY, HSQC, and HMBC were performed on 1 in acetone-*d*₆ (Table 1). The direct bonds between each proton and carbon were established in the HSQC spectrum. As shown by the bold line in Fig. 1b, an analysis of the DQF-COSY and TOCSY spectra revealed proton–proton spin connections in three partial structures: carbon skeletons connecting C5 to C9, C11 to C18, and C20 to C26. The HMBC correlations (2-Me/C1, 2-Me/C2, 2-Me/C3, 4-Me/C3, 4-Me/C4, 4-Me/C5, shown by open-ended arrows in Fig. 1b) indicated a 2, 4-dimethyl dienone structure. The HMBC correlations, 10-Me/C9, 10-Me/C10, 10-Me/C11, indicated a connection between the C5 to C9 and C11 to C18 partial structures. The lactone ring flanked with C1 and C15 indicated the HMBC correlation between H15/C1. The HMBC correlation between the oxymethyl (δ 3.22) and C14

[†] To whom correspondence should be addressed. Tel/Fax: +81-54-238-5008; E-mail: askodan@ipc.shizuoka.ac.jp

* Present address: Faculty of Applied Bioscience, Nippon Veterinary and Life Science University, 1-7-1 Kyonan-cho, Musashino, Tokyo 180-8602, Japan

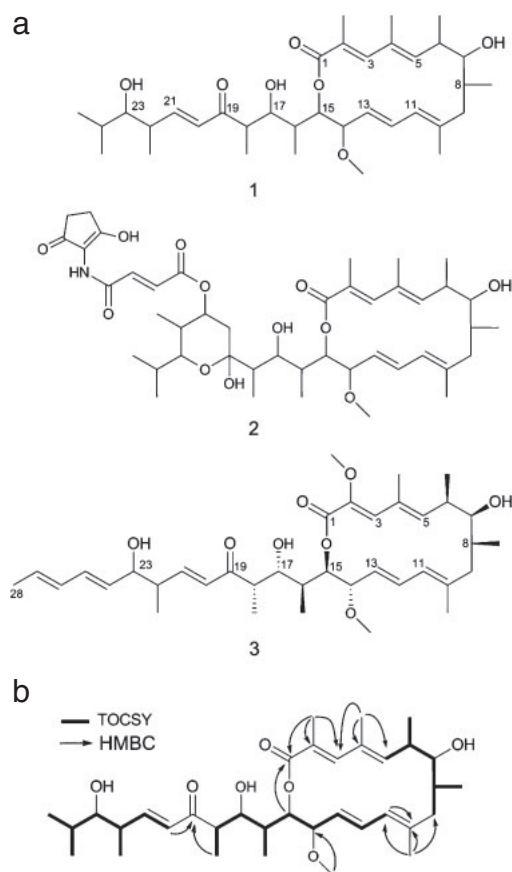


Fig. 1. a) Chemical Structures of Makinolide B (**1**), Makinolide A (**2**), and Micromonospolide C (**3**); b) Key NMR Correlations of **1** in Acetone- d_6 .

indicated a methoxy residue attached to C14. The characteristic ^{13}C -NMR chemical shift (δ 202.2), which was assigned to be a carbon without a C–H bond by HSQC and DEPT-135 experiments, indicated the presence of a ketone residue. The HMBC correlations of 18Me/C19, H18/C19, H20/C19, and H21/C19 elucidated the connection between the C11 to C18 and C20 to C26 partial structures flanking the ketone. The olefins at positions 12 and 13 and positions 20 and 21 were demonstrated to be of *E*-configuration by the respective coupling constants (14.9 and 15.9 Hz).

The relative stereochemistry of bafilomycin analogues micromonospolides A–C had previously been determined by comparing the coupling constants with those of bafilomycin A₁.¹¹⁾ We compared the ^1H - and ^{13}C -NMR chemical shifts and the magnitude of the coupling constants of **1** with the relevant data for micromonospolide C (**3**). Figure 1a shows the structural difference in the macrolactone rings of **1** and **3** to be the methyl residue at position 2. The chemical shifts and coupling constants from position 4 to 19 in **1** and **3** were similar (Table 1). The proton-proton coupling constants at positions 5–7, 11–15, 17, and 18 of **1** and **3** had particularly close values. We speculated that the relative stereochemistry of **1** was the same as that of **3** by the similarity of their NMR spectral data (Fig. 1a).

Compound **1** was subjected to an antimicrobial activity test by the paper disk diffusion method against the six microorganisms, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Micrococcus luteus*,

Saccharomyces cerevisiae, and *Mucor hiemalis*, applying the previously reported procedure⁹⁾ at a dose of 100 μg /disk. Compound **1** only showed weak antibacterial activity by the formation of a 9-mm-diameter inhibition zone against *S. aureus*.

Experimental

General methods. Makinolide B (1 mg) was dissolved in acetone- d_6 (0.5 mL), and spectral measurements of ^1H -NMR at 800.13 MHz, ^{13}C - and DEPT-135 NMR at 201.22 MHz, ^1H - ^1H correlation 2D NMR (DQF-COSY, NOESY, and TOCSY), and ^1H - ^{13}C correlation 2D NMR (HSQC and HMBC) were taken at 298 K with an AVANCE 800 spectrometer fitted with a CryoProbe (Bruker Biospin, Karlsruhe, Germany). The resonance of residual acetone at δ H 2.04 and δ C 29.9 were used as respective internal references for the ^1H - and ^{13}C -NMR spectra. The ESI-MS data were recorded with a Jeol JMS-T100LP mass spectrometer, and the UV spectrum was recorded with UV-1200 UV/VIS photospectrometer (Hitachi, Japan).

Polymerase chain reaction (PCR) amplification, sequencing, and phylogenetic analysis of the 16S rRNA genes. All DNA was extracted from the cells of strain MK-19 according to the literature procedure.¹²⁾ The 16S rRNA-encoding sequence was amplified from total DNA by the PCR method with the universal primer pairs, 9F (5'-GAGTTT-GATCCTGGCTCAG-3') and 1510R (5'-GGTACCTTGTTACGA-3'). The PCR reaction was performed by using the EmeraldAmp PCR Master mix (Takara Bio, Japan) according to the manufacturer's instructions. PCR amplification was carried out with a thermal cycler using the following program: initial denaturation for 10 min at 94 °C, and then 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 an AxyPrep DNA gel extraction kit (Axygen Bioscience, USA) according to the manufacturer's instructions. The reactions for sequencing were performed by using a BigDye Terminator cycle sequencing kit according to the manufacturer's instructions. The four primers used for the reaction were 339F (5'-CTCCTACGGGTGAGTAACAC-3'), 536R (5'-GTAT-TACCGCGG CTGCTG-3'), 686F (5'-TAGCGGTGAAATGCGT-AGA-3'), and 1099F (5'-GCAACGAGCGCAACCC-3'). Sequencing was performed with an ABI 3130 capillary DNA sequencer (Applied Biosystems, USA).

Isolation of makinolide B. An ISP2 agar¹³⁾ medium (1 L) was used for culturing *Streptomyces* sp. MK-19. After 7 d at 30 °C, an equal volume of acetone was added to the agar culture medium and mycelia for extraction. The resulting acetone extract was concentrated to an aqueous suspension which was subjected to open column chromatography (CHP-20P, 3 \times 5 cm; Mitsubishi Chemicals), eluting with 50 mL each of 20% MeOH, 60% MeOH, and MeOH. The MeOH fractions were subjected to preparatory reverse-phase HPLC in an ODS column (Cosmosil C18-MSII, 4.6 \times 250 mm; Nacalai Tesque) with isocratic elution of 85% MeCN containing 0.05% trifluoroacetic acid (TFA) at an absorbance of 220 nm to yield 2.7 mg of makinolide B (**1**). UV absorption in MeOH at λ_{max} 280 nm (ϵ 700) and λ_{max} 238 nm (ϵ 3350); chemical shift values are shown in Table 1.

Antimicrobial assay. The NBRC culture collection (NITE Biological Resource Center, Japan) afforded the testing microorganisms of *Escherichia coli* (NBRC 1002203), *Pseudomonas aeruginosa* (NBRC 12689), *Staphylococcus aureus* (NBRC 1009110), *Micrococcus luteus* (NBRC 3333), *Saccharomyces cerevisiae* (NBRC 2376) and *Mucor hiemalis* (NBRC 9405). All microorganisms were cultivated in an ISP2 agar¹³⁾ medium with incubation at 30 °C. A paper disk diffusion assay (8 mm i.d., thick type) was performed to determine the antimicrobial activity of **1**. Tetracycline (against *E. coli*, *P. aeruginosa*, *S. aureus*, and *M. luteus*) and cycloheximide (against *S. cerevisiae* and *M. hiemalis*) were used as the positive controls. After incubating for 24 h, tetracycline (10 μg /disk) formed inhibition zones with respective diameters of 20 mm, 16 mm, 21 mm, and 25 mm against *E. coli*, *P. aeruginosa*, *S. aureus*, and *M. luteus*, while cycloheximide (10 μg /disk for *S. cerevisiae* and 50 μg /disk for *M. hiemalis*) formed respective inhibition zones with diameters of 30 mm and 10 mm. An MeOH solution was used as a negative control which did not show any inhibition under the same conditions.

Table 1. NMR Chemical Shift Values for Makinolide B (**1**) and Micromonospolide C (**3**)

Makinolide B in acetone- <i>d</i> ₆			Micromonospolide C in CD ₃ OD ⁽¹⁾		
Position	δ _C	δ _H (J in Hz)	Position	δ _C	δ _H (J in Hz)
1	171.7		1	168.0	
2	122.6		2	143.4	
2-CH ₃	14.1	2.02 (s)	2-OCH ₃	61.4	3.62 (s)
3	147.0	7.24 (s)	3	135.1	6.68 (s)
4	134.1		4	133.8	
4-CH ₃	15.3	1.96 (br s)	4-CH ₃	14.8	1.96 (br s)
5	146.8	6.02 (brd, 8.8)	5	145.9	5.88 (brd, 9.0)
6	38.1	2.56 (ddq, 8.8, 7.1, 1.8)	6	39.6	2.51 (ddq, 9.0, 7.1, 1.7)
6-CH ₃	18.3	1.04 (d, 7.1)	6-CH ₃	19.0	1.05 (d, 7.1)
7	80.3	3.29 (dd, 6.9, 1.9)	7	82.0	3.27 (dd, 6.0, 1.7)
8	40.9	1.87 (m)	8	41.9	1.86 (m)
8-CH ₃	22.3	0.91 (d, 6.6)	8-CH ₃	23.7	0.93 (d, 6.9)
9	42.3	2.01 (m)	9	43.4	2.08 (dd, 14.3, 11.1), 2.01 (m)
10	143.9		10	145.2	
10-CH ₃	19.9	1.87 (br s)	10-CH ₃	20.4	1.84 (br s)
11	125.1	5.76 (br d, 10.8)	11	126.5	5.78 (br d, 10.9)
12	133.6	6.60 (dd, 14.9, 10.8)	12	134.9	6.56 (dd, 15.1, 10.9)
13	126.9	5.14 (dd, 14.9, 8.7)	13	127.6	5.16 (dd, 15.1, 8.3)
14	84.3	3.99 (dd, 8.7, 7.5)	14	85.9	3.94 (dd, 8.3, 6.7)
14-OCH ₃	49.6	3.28 (s)	14-OCH ₃	56.9	3.23 (s)
15	76.5	5.08 (dd, 7.5, 1.8)	15	78.2	5.18 (dd, 6.7, 2.0)
16	39.8	2.02 (m)	16	41.3	1.98 (m)
16-CH ₃	11.0	0.95 (d, 7.0)	16-CH ₃	12.2	0.97 (d, 6.9)
17	72.8	3.75 (dd, 9.2, 3.4)	17	74.6	3.80 (dd, 8.9, 3.8)
18	46.7	2.97 (dq, 6.9, 3.4)	18	48.4	3.06 (dq, 6.9, 3.8)
18-CH ₃	9.3	1.07 (d, 6.9)	18-CH ₃	10.6	1.10 (d, 6.9)
19	202.2		19	205.7	
20	129.3	6.25 (d, 15.9)	20	130.7	6.23 (d, 16.0)
21	149.2	6.87 (dd, 15.9, 8.8)	21	151.7	6.87 (dd, 16.0, 8.0)
22	41.0	2.52 (m)	22	45.3	2.43 (ddq, 8.0, 6.9, 6.6)
22-CH ₃	17.8	1.05 (d, 6.9)	22-CH ₃	16.8	1.03 (d, 6.9)
23	80.0	3.11 (dd, 6.9, 4.5)	23	77.5	3.97 (dd, 7.1, 6.6)
24	32.3	1.60 (m)	24	133.0	5.50 (dd, 15.1, 7.1)
25	18.1	0.91 (d, 6.8)	25	134.1	6.17 (dd, 15.1, 10.3)
26	19.7	0.88 (d, 6.8)	26	133.0	6.05 (ddd, 14.9, 10.3, 1.4)
			27	131.5	5.69 (dq, 14.9, 6.9)
			28	19.0	1.73 (dd, 6.9, 1.4)

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