New Macrodiolide Antibiotics, 11-*O*-Monomethyl- and 11,11′-*O*-Dimethylelaiophylins, from *Streptomyces* sp. HKI-0113 and HKI-0114

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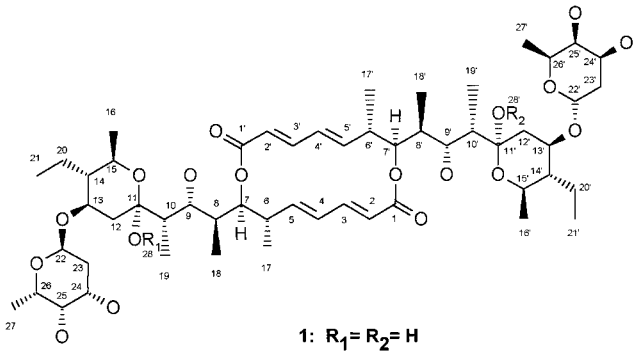
Elaiophylin (**1**) and two new methyl derivatives, 11-*O*-monomethylelaiophylin (**2**) and 11,11′-*O*-dimethylelaiophylin (**3**), were isolated from the mycelium cake of *Streptomyces* strains HKI-0113 and HKI-0114. The structures of **2** and **3** were determined by mass spectrometric and NMR investigations. Compounds **2** and **3** display antimicrobial and moderate cytotoxic activities.

Elaiophylin (**1**, azalomycin B, gopalamycin) is a glycosylated macrodiolide antibiotic occurring in several *Streptomyces* strains.[1−4] Due to its promising antibacterial, antifungal, cytocidal, and anticoccidial activities, this microbial metabolite served as starting material for chemical syntheses of nematicidal and anthelmintic derivatives.[5,6] More recently **1** was shown to enhance rumen efficiency[7] and to possess antiulcer properties.[8] Efomycins[9] were described as homologues of **1** which are discernible either by the presence of methoxyl substituents or an additional methyl instead of an ethyl group at the sugar moieties.[9] In this paper we report the isolation and structural elucidation of two new naturally occurring derivatives of **1**, 11-monomethylelaiophylin (**2**) and 11,11′-dimethylelaiophylin (**3**), from *Streptomyces* strains HKI-0113 and HKI-0114 (Chart 1).

Chart 1



Results and Discussion

Streptomyces strains HKI-0113 and HKI-0014 were both cultivated on a liquid medium using shake flasks. The mycelium was extracted with 10 vol of ethanol, and several subsequent chromatographic steps gave 11-*O*-monomethylelaiophylin (**2**), 11,11′-*O*-dimethylelaiophylin (**3**), and coproduced elaiophylin (**1**) as microcrystalline white powders.

The presence of carbonyl groups and double bonds in **2** and **3** was suggested by their IR spectra which displayed absorptions at λ_{max} 1696 cm^{-1} (CO) and 1633 cm^{-1} (C=C). The pseudomolecular ions of **2** (*m*/*z* 1061 $[M + Na]^+$) and **3** (*m*/*z* 1075 $[M + Na]^+$) in electrospray MS (positive ion mode) distinguished both compounds from the related elaiophylin (**1**; *m*/*z* 1047 $[M + Na]^+$) and indicated that **2** had one additional and **3** had two additional methyl groups. Collision-induced decomposition (CID-MS/MS) of the pseudomolecular ions *m*/*z* 1056 (**2**; $[M + NH_4]^+$) and *m*/*z* 1070 (**3**; $[M + NH_4]^+$) afforded diagnostic daughter ions such as *m*/*z* 729 (**1**, M − 2 × O-sugar, $-CH_3$) and *m*/*z* 744 (**2**, M − 2 × O-sugar, $-CH_3$), respectively. Moreover, *m*/*z* 411 was generated by CID-MS/MS as a common daughter ion of **1**, **2**, and **3**, suggesting its origin from the aglycone part of the three molecules. As with electrospray mass spectrometry, the HR-FABMS also suggested the molecular weight of both compounds. However, the intensity of the FABMS pseudomolecular ions ($[M + Na]^+$) of **1** (*m*/*z* 1061) and **2** (*m*/*z* 1075) was too low for high-resolution experiments.

The chemical structure and relative stereochemistry of **2** and **3** were settled unambiguously by one- and two-dimensional NMR spectroscopic measurements (DEPT, COSY, ROESY, HSQC, HMBC). The ^{1}H and ^{13}C NMR spectra of both **2** and **3** were almost identical to the corresponding spectra of elaiophylin (**1**)[1] (Table 1). In the proton and ^{13}C NMR spectra of zone 3, the only differences from that of **1** concerned the presence of an additional methoxyl signal at $\delta_H = 3.04$ and $\delta_C = 46.9$. However, in the ^{1}H NMR spectrum of **3**, this signal displayed twice the intensity (apparently 3H) as in the spectrum of **2** (1.5H) showing the presence of two additional methyl groups in **3**. Based on their somewhat high upfield chemical shifts, these new signals (H-28) were unambiguously assigned as part of the ketal structure, and the assignments were confirmed by the $^3J_{C,H}$ long-range coupling (HMBC) of these 28-C-methoxyl protons with carbons 11 and 11′, respectively. Due to the structural identity of the two constituting parts of the substituted macrodiolide ring in **3**, their ^{1}H and ^{13}C NMR signals were also equivalent. In the pertinent spectra of **2**, minor shift differences between the upper and the lower parts of the molecule were visible because of the nonsymmetric substitution at C-11 (Table 1).

11-*O*−Monomethylelaiophylin (**2**) and 11,11′-*O*-dimethylelaiophylin (**3**) displayed antimicrobial activities against Gram-positive bacteria and some fungi (Table 2) but were not active against *Candida albicans*. The cytotoxic effect was determined with L929 mouse fibroblast cells, K562 human leukemia cells and HeLa cell cultures (IC_{50} 0.7−2.4 μg/mL). In comparison to elaiophylin (**1**) isolated from the same microbial sources, **2** and **3** displayed somewhat lower cytotoxicity (Table 3).

Experimental Section

General Experimental Procedures. IR spectra were recorded on a Shimadzu FTIR spectrometer. High-resolution

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Table 1. ^{13}C and ^{1}H NMR Chemical Shifts of 11-*O*-Monomethylelaiophylin (**2**) and 11,11′-*O*-dimethylelaiophylin (**3**) (500 MHz, CD_3OD, δ in ppm relative to internal TMS); The Numbers Represent the Lower-Half Portion of the Molecules, and the Upper-Half Data for **2** and **3** Are Identical with That Shown for **3** in This Table

	2		**3**	
carbon	δ_H (m, *J* in Hz)	δ_C	δ_H (m, *J* in Hz)	δ_C
1	–	170.2 s	–	170.2 s
2	5.74 (d, 15.2)	122.6 d	5.73 (d, 15.2)	122.6 d
3	6.93 (dd, 15.2, 11.1)	146.9 d	6.99 (dd, 15.2, 11.1)	146.9 d
4	6.16 (dd, 15.1, 11.1)	132.5 d	6.15 (dd, 15.1, 11.1)	32.6 d
5	5.67 (dd, 15.1, 9.8)	146.1 d	5.67 (dd, 15.1, 9.8)	146.2 d
6	2.58 (m)	42.7 d	2.58 (m)	42.9 d
7	5.03 (dd, 10.3, 2.0)	78.3 d	5.12 (dd, 10.4, 2.0)	78.5 d
8	1.95 (m)	37.8 d	1.87 (m)	38.4 d
9	3.51 (m)	71.8 d	3.51 (m)	70.9 d
10	1.72 (m)	44.0 d	2.05 (q, 6.9)	39.3 d
11	–	100.9 s		104.5 s
12	1.12 (m)	39.0 t	1.31 (m)	35.5 t
	2.33 (dd, 13.1, 4.5)		2.35 (dd, 13.1, 4.5)	
13	3.94 (dt, 10.7, 4.7)	71.0 d	3.85 (dt, 10.7, 4.7)	71.2 d
14	1.16 (m)	49.8 d	1.31 (m)	48.7 d
15	3.51 (m)	69.1 d	3.51 (m)	69.1 d
16	1.13 (d, 6.4)	19.5 q	1.18 (d, 6.4)	19.3 q
17	1.05 (d, 6.7)	15.8 q	1.04 (d, 6.7)	15.1 q
18	0.94 (d, 6.6)	10.3 q	0.94 (d, 6.6)	10.3 q
19	0.98 (d, 6.9)	7.1 q	0.94 (d, 6.8)	7.6 q
20	1.46 (m); 1.66 (m)	20.3 t	1.46 (m); 1.66 (m)	20.3 t
21	0.87 (t, 6.5)	9.5 q	0.86 (t, 6.5)	9.5 q
22	5.04 (d, br, 3.6)	94.9 d	5.02 (d, br, 3.5)	94.9 d
23	1.62 (dd, 12.7, 4.9)	33.7 t	1.62 (dd, 12.7, 4.9)	33.7 t
	1.94 (dd, 12.7, 3.8)		1.92 (dd, 12.7, 3.8)	
24	3.90 (m)	67.0 d	3.90 (m)	67.0 d
25	3.53 (m)	72.4 d	3.52 (m)	72.5 d
26	3.91 (m)	68.2 d	3.90 (m)	68.0 d
27	1.19 (d, 6.8)	17.3 q	1.18 (d, 6.8)	17.3 q
28	3.04 (s, 1.5H[a])	46.9 q	3.04 (s, 3H)	46.9 q

[a] Apparently 1.5 H due to the symmetry of the molecule.

Table 2. Diameter of Inhibition Zone (mm) Caused by 50 μg of **1**, **2**, or **3** in the Agar Plate Diffusion Assay

	diameter of inhibition zone (mm)		
test organism	1	2	3
Bacillus subtilis ATCC 6633	22	18	23
Staphylococcus aureus 134/94	22	22	13
Enterococcus faecium 1528	22	23	17
Mycobacterium sp. SG 987	15	17	15
Candida albicans	0	0	0

Table 3. Cytotoxicity of **1**, **2**, and **3** against Cell Cultures of L-929, K-562, and HeLa Cells

	IC_{50} (μg/mL)		
compound	L-929	K-562	HeLa
11,11′-*O*-dimethylelaiophylin (**3**)	2.4	1.3	2.2
11-*O*-monomethylelaiophylin (**2**)	1.2	0.7	1.2
elaiophylin (**1**)	0.3	0.2	0.3

EI and FAB mass spectra (3-nitrobenzyl alcohol as matrix) were recorded on an AMD-402 instrument of BE geometry equipped with direct inlet system (AMD Intectra Harpstedt, Germany). Electrospray MS and CID-MS/MS were recorded by use of a triple quadrupole mass spectrometer Quattro (VG Biotech, Altrincham, England). NMR spectra of **2** and **3** were recorded in 20 mM solution in CD_3OD on a Bruker Avance DRX 500 instrument. COSY, HSQC, and HMBC spectra were recorded using pulsed field gradients rather than phase cycling for coherence pathway selection.

Organisms and Culture Conditions. The producing *Streptomyces* strains HKI-0113 and HKI-0114 were obtained from the strain collection of the Hans-Knöll-Institute of Natural Products Research Jena. An inoculum culture was grown for 48 h (28 °C, rotary shaker 180 rpm; glass bottles 500 mL each containing 50 mL medium) on a liquid medium composed of (g/L) glucose (15), soya flour (15), NaCl (5), $CaCO_3$ (1), KH_2PO_4 (3), distilled water, pH 7.0. The main culture was carried out in a 20 L fermenters for 96 h at 28 °C on a medium composed as follows (g/L): glucose (20), soya flour (20), NaCl (5), $CaCO_3$ (3), distilled water, pH 8.1 (aeration 1:1).

Extraction and Isolation. After 96 h the mycelium was separated from the culture broth and suspended in 5 L of ethanol. The extract was concentrated in vacuo, and the aqueous residue (500 mL) was reextracted with ethyl acetate. The extract was dried over Na_2SO_4 and evaporated to dryness (16 g crude product). Purification of the three elaiophylins (**1**–**3**) was carried out by column chromatography on Sephadex LH-20 (MeOH; column 6 × 40 cm). The fractions containing elaiophylins (monitored by TLC and antibacterial activity) were pooled and subjected to preparative TLC (Merck; silica gel 60, aluminum sheets, $CHCl_3$; run 3 times). The three zones staining blackish by 3% vanillin/concentrated H_2SO_4 (R_f 0.3 (**1**), 0.4 (**2**), and 0.5 (**3**)) were eluted by $CHCl_3$/MeOH (1:1). Final purification was accomplished by preparative TLC on silica gel RP_{18} aluminum sheets (Merck, acetonitrile/H_2O (83:17)). The zones with R_f 0.6 (**1**), 0.7 (**2**), and 0.8 (**3**), respectively, were eluted by $CHCl_3$/MeOH (1:1) and dried. TLC proved to be the most effective method of purification of **2** and **3** since during preparative HPLC in the presence of weakly acidic eluent such as trifluoroacetic acid, both compounds were hydrolyzed markedly to yield **1**. Otherwise, a solution of **1** in MeOH was stable over four weeks and no formation of methyl or dimethyl derivatives was detected. It should be mentioned that 11-*O*-monomethylelaiophylin (**2**) was never reported as a product of chemical synthesis, in contrast to 11,11′-*O*-dimethylelaiophylin which is easily obtainable as the sole reaction product of **1** with methanol in the presence of mild acidic catalysts.[5]

11-*O*-Monomethylelaiophylin (2): Colorless microcrystalline solid (MeOH), mp 176–177 °C. IR (KBr) λ_{max}: 1350, 1376, 1611, 1633 (C=C), 1698 (CO), 2690, 3440 (OH) cm^{-1}. FABMS (matrix: 3-nitrobenzyl alcohol): *m/z* 1061 $[M + Na]^+$; 541; 389. For ^{1}H and ^{13}C NMR data see Table 1.

11,11′-*O*-Dimethylelaiophylin (3): Colorless microcrystalline solid (MeOH), mp 177–178 °C. IR (KBr) λ_{max}: 1347, 1378, 1633 (C=C), 1696 (CO), 2965, 3435 (OH) cm^{-1}. FABMS (matrix 3-nitrobenzyl alcohol) *m/z* 1075 $[M + Na]^+$; 663, 603, 577, 541, 389. For ^{1}H and ^{13}C NMR data see Table 1.

Cell Cultures and Measurements of Cytotoxicity.[10] The adherent mouse fibroblast cell line L-929 was cultured in Eagle's MEM (Gibco) with 0.35 mg/mL sodium bicarbonate, 100 units/mL penicillin/100 μg/mL streptomycin, 10 mM HEPES, and 10% heat-inactivated FBS at 37 °C in culture flasks. The adherent cells were harvested at the logarithmic growth phase after trypsination using 0.05% trypsin in phosphate-buffered saline (PBS) containing 0.02% EDTA.

The nonadherent human leukemia cell line K-562, was cultured in RPMI 1640 medium (Gibco), supplemented with 100 units/mL penicillin/100 μg/mL streptomycin and 10% FBS in culture flasks (Greiner).

L-929 and K-562 cells were inoculated with 1×10^4 cells in 0.1 mL culture medium, containing $NaHCO_3$ without HEPES, per well of the 96-well microplates (Costar). The plates were previously prepared with dilutions of the test compounds in 0.1 mL medium. The microplates were kept for 72 h at 37 °C in a humidified atmosphere containing 5% CO_2.

The adherent human cell line HeLa was cultured in MEM Eagle (SIGMA) with 100 units/mL penicillin/100 μg/mL streptomycin, 10% FBS (Gibco), and 2 mM L-glutamine (Gibco) in vented culture flasks. The adherent cells were harvested during the logarithmic growth phase after trypsination with 0.4% trypsin in PBS containing 0.02% EDTA. These cells were seeded with 2.5×10^4 cells in 0.1 mL culture medium per well of the 96-well microplates (Costar). HeLa cells were preincubated 48 h without the test compound. The dilutions of the compounds were carried out on the monolayer of HeLa cells after preincubation time.

After incubation, the monolayer of the adherent L-929 and HeLa cells were fixed by glutaraldehyde and stained with a

0.05% solution of methylene blue for 15 min. After washing the stain was eluted by 0.2 mL of 0.33 N HCl in the wells. The optical densities were measured at 630 nm in a Dynatech MR 7000 microplate reader (Dynatech, Denkendorf, Germany).

After incubation, the K-562 cells were analyzed using an electronic cell analyzer system CASY 1 (Schärfe System, Reutlingen, Germany) and software CASYSTAT for determination of IC_{50} values.[6] The IC_{50} values were determined by integrated software CASYSTAT.

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