Identification of Elaiophylin Skeletal Variants from the Indonesian *Streptomyces* sp. ICBB 9297

Yan Sheng,[†] Phillip Lam,[†] Salmah Shahab,[‡] Dwi Andreas Santosa,[‡,§] Philip J. Proteau,[†] T. Mark Zabriskie,[†] Taifo Mahmud[†,] *

[†]Department of Pharmaceutical Sciences, College of Pharmacy, Oregon State University, Corvallis, Oregon, 97331-3507, U.S.A.; [‡]Indonesian Center for Biodiversity and Biotechnology, ICBB-Complex, JI. Cilubang Nagrak No. 62, Situgede, Bogor 16115, Indonesia; [§]Department of Soil Science and Land Resources, Faculty of Agriculture, Bogor Agricultural University, Bogor, Indonesia.

ABSTRACT

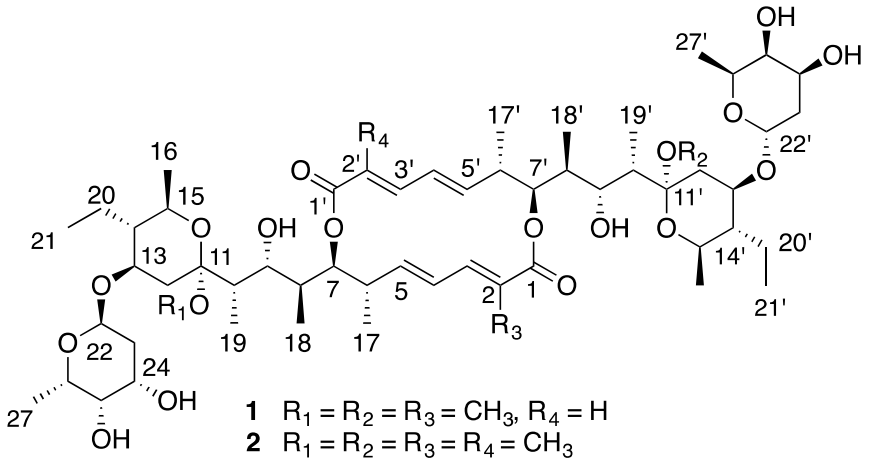
Four new elaiophylin macrolides (**1**-**4**), together with five known elaiophylins (**5**-**9**), have been isolated from cultures of the Indonesian soil bacterium *Streptomyces* sp. ICBB 9297. The new compounds have macrocyclic skeletons distinct from those of the known dimeric elaiophylins in that one or both of the polyketide chains contain(s) an additional pendant methyl group. Further investigations revealed that **1** and **2** were derived from **3** and **4**, respectively, during isolation processes. Compounds **1**-**3** showed comparable antibacterial activity to elaiophylin against *Staphylococcus aureus*. However, interestingly, only compounds **1** and **3**, which contain a pendant methyl group at C-2, showed activity against *Mycobacterium smegmatis*, whereas compound **2**, which has two pendant methyl groups at C-2 and C-2′, and the known elaiophylin analogues (**5**-**7**), which lack pendant methyl groups at C-2 and/or C-2′, showed no activity. The production of **3** and **4** in strain ICBB 9297 indicates that one of the acyltransferase (AT) domains in the elaiophylin polyketide synthases (PKSs) can recruit both malonyl-CoA and methylmalonyl-CoA as substrates. Bioinformatic analysis of the AT domains of the elaiophylin PKSs revealed that the ela_AT7 domain contains atypical active site amino acid residues, distinct from those conserved in malonyl-CoA- or methylmalonyl-CoA-specific ATs.

Indonesia is recognized as a biodiversity hotspot in the world with a high level of endemic species and microorganisms.[1] The biodiversity and species richness of the various ecosystems in this archipelagic country represent a wealth of untapped resources for pharmaceutical, agricultural and environmental applications. However, many of them are currently threatened by climate change, rapid economic growth, and human activities.[2,3]

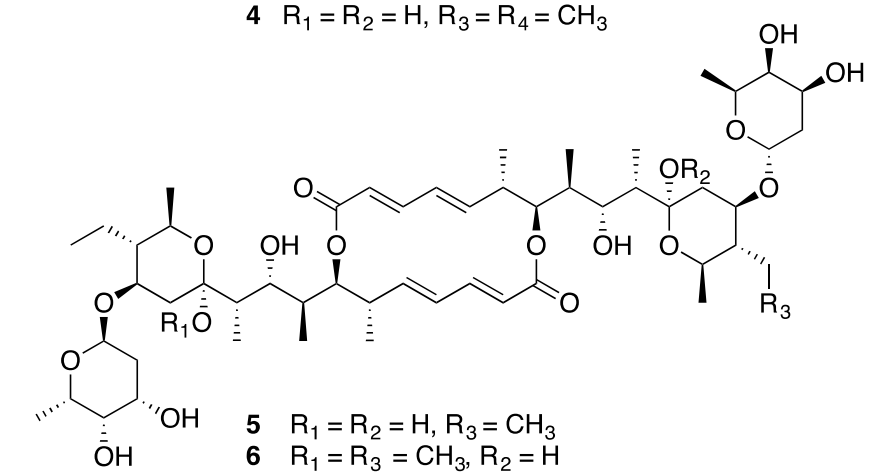
In our continuing efforts to discover new bioactive natural products from Indonesian microorganisms, we investigated the active metabolites of a soil bacterium, *Streptomyces* sp. ICBB 9297, isolated from an agricultural field soil sample collection in Jatiroto, East Java, Indonesia. Bioassay-guided separation of the culture broths of strain ICBB 9297 and purification led to the isolation of four new elaiophylin analogues (**1**-**4**), together with five known elaiophylins: elaiophylin (**5**),[4] 11-*O*-monomethylelaiophylin (**6**),[5] 11,11′-*O,O*-dimethylelaiophylin (**7**),[5] efomycin G (**8**),[6] and 11,11′-*O,O*-dimethyl-14′-deethyl-14′-methylelaiophylin (**9**).[7]

Elaiophylin (**5**, also known as azalomycin and gopalamycin) is a glycosylated macrodiolide antibiotic produced by several *Streptomyces* strains.[4,8-11] This unusual C_2-symmetric diolide has been reported to have various biological activities, such as antibacterial,[4,5,7,11] cytocidal,[5] and immunosuppressive activities,[12] and enhances rumen fermentation efficiency.[13] The antibacterial activity of elaiophylin has been proposed to result from its ability to form stable, long-lasting cation-selective ion channels in the lipid bilayer membranes.[14]

Herein, we describe the isolation, structure elucidation, and antibacterial activities of the new elaiophylin analogues (**1**-**4**), two of which were demonstrated to be isolation artifacts, as well as a bioinformatic analysis of acyltransferase domains in the elaiophylin polyketide synthases (PKSs) that give rise to these new compounds.



1 $R_1 = R_2 = R_3 = CH_3$, $R_4 = H$
2 $R_1 = R_2 = R_3 = R_4 = CH_3$
3 $R_1 = R_2 = R_4 = H$, $R_3 = CH_3$
4 $R_1 = R_2 = H$, $R_3 = R_4 = CH_3$



5 $R_1 = R_2 = H$, $R_3 = CH_3$
6 $R_1 = R_3 = CH_3$, $R_2 = H$
7 $R_1 = R_2 = R_3 = CH_3$
8 $R_1 = R_2 = R_3 = H$
9 $R_1 = R_2 = CH_3$, $R_3 = H$

RESULTS AND DISCUSSION

The EtOAc extract of strain ICBB 9297, which was identified as a *Streptomyces* sp. based on its 16S rRNA gene sequence analysis, exhibited activity against the Gram-positive bacterium *Staphylococcus aureus* ATCC 12600. The extract was then subjected to C_{18} SPE fractionation followed by silica gel and Sephadex LH-20 column chromatography to yield an active fraction. HPLC and (+)-ESI-MS analyses of this fraction showed that it contained a mixture of macrolide

natural products related to the elaiophylins (Figure 1, Peaks A-L). Isolation and comparison of the ^{1}H NMR spectra of the major products with those previously reported for elaiophylin analogues revealed that peaks B, F, and J represented elaiophylin (**5**), 11-*O*-methylelaiophylin (**6**) and 11,11′-*O,O*-dimethylelaiophylin (**7**), respectively. Inspection of the (+)-ESI-MS data for the other products revealed that peak K (**1**) had an *m/z* value of 1089 ($[M+Na]^{+}$), which was different from the molecular masses of any known elaiophylins, suggesting that this compound was a new elaiophylin analogue.

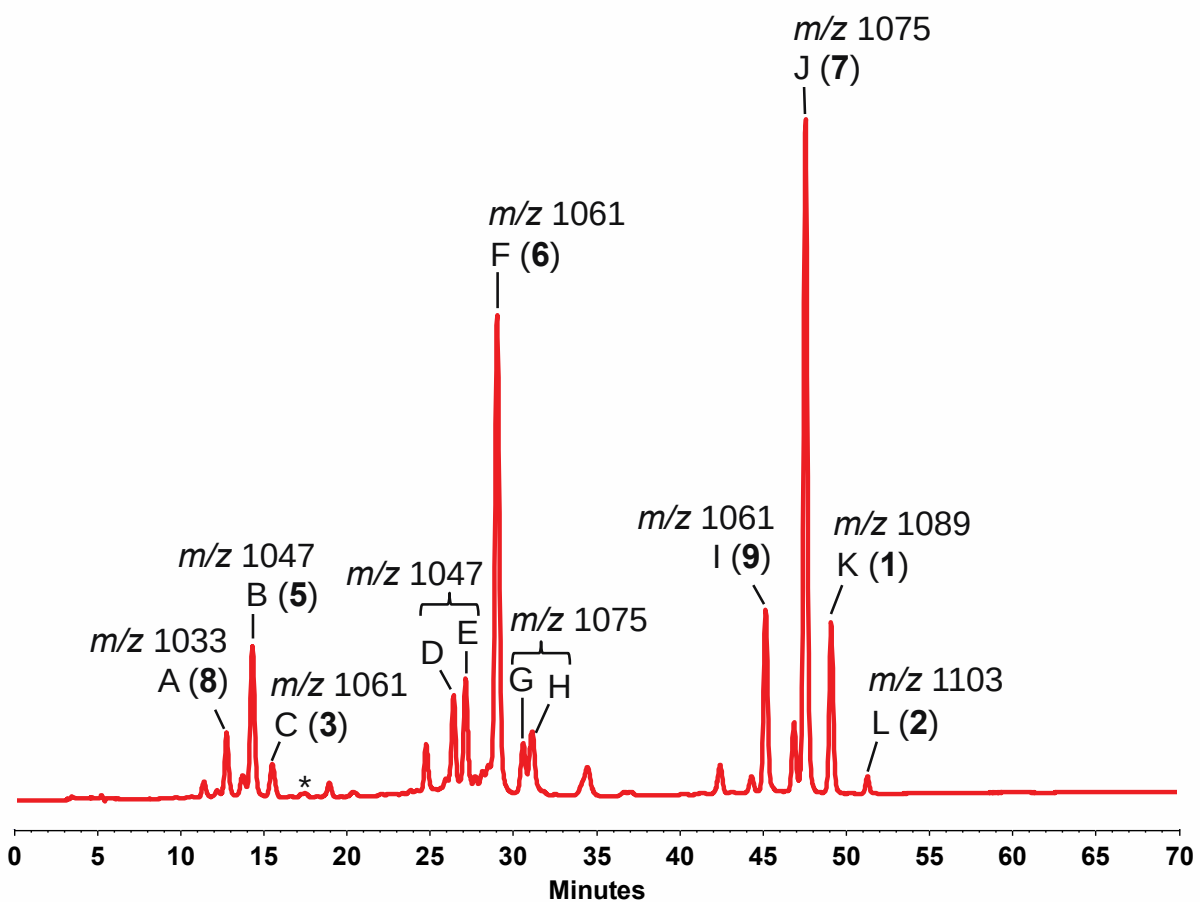


Figure 1. HPLC profile of elaiophylin mixture. Due to its very low yield, the peak for compound **4** (*m/z* 1075) is not labeled but is indicated with an asterisk. The *m/z* values represent the $[M+Na]^{+}$ ions of the compounds.

Compound **1** was isolated as a white powder and the molecular formula was determined as $C_{57}H_{94}O_{18}$ by HR-TOF-MS analysis. The ^{1}H NMR spectrum of **1** showed close similarity to that of **7** except that an additional singlet for a methyl group was observed at 1.78 ppm (Table 1).[5] Moreover, proton signals in the olefinic region of **1** are more complex than that of **7**, indicating the loss of symmetry in the structure, presumably due to the presence of the additional pendant methyl group. ^{13}C and DEPT-135 NMR spectra of **1** showed the presence of two non-protonated carbon signals at 102.8 ppm and 126.6 ppm and two carbonyl signals at 167.4 ppm and 168.0 ppm. These are different from compound **7**, which has a single hemiketal carbon and one carbonyl.

Table 1. ^{1}H NMR Data (700 MHz) for Compounds **1**-**4** in DMSO-d_6

	1	**2**	**3**	**4**		**1**	**3**
No.	δ_H, mult. (*J* in Hz)	δ_H, mult. (*J* in Hz)	δ_H, mult. (*J* in Hz)	δ_H, mult. (*J* in Hz)	No.	δ_H, mult. (*J* in Hz)	δ_H, mult. (*J* in Hz)
2					2'	5.62, d (15.3)	5.63, d (15.4)
3	6.66, d (11.6)	6.66, d (11.5)	6.64, d (11.6)	6.63, d (11.1)	3'	6.85, dd (15.1, 11.3)	6.83, dd (15.0, 11.3)
4	6.20 dd (14.6, 12.0)	6.23, dd (14.6, 11.9)	6.19, dd (14.5, 11.8)	6.21, dd (14.8, 11.2)	4'	6.07, dd (14.8, 11.3)	6.05, dd (14.7, 11.3)
5	5.56, dd (14.6, 9.9)	5.58, dd (14.6, 9.9)	5.55, dd (14.8, 10.1)	5.56, dd (15.1, 9.7)	5'	5.67, dd (14.9, 10.0)	5.65, dd (14.8, 9.9)
6	2.54, m	2.56, m	2.54[a]	2.55[a]	6'	2.47, m	2.47, m
7	5.15, d (10.5)	5.13, d (10.5)	5.10, d (10.5)	5.10, d (10.2)	7'	5.11, d (10.4)	5.08, d (10.4)
8	1.70, m	1.72, m	1.77[a]	1.77[a]	8'	1.73, m	1.80[a]
9	3.32[a]	3.33[a]	3.79[a]	3.79[a]	9'	3.32[a]	3.79[a]
10	1.89, q (6.9)	1.90, q (6.7)	1.58[a]	1.58[a]	10'	1.89, q (6.9)	1.58[a]
12ax	1.08[a]	1.08[a]	0.97[a]	0.96[a]	12'ax	1.08[a]	0.97[a]
12eq	2.39, dd (13.3, 3.9)	2.39, dd (13.0, 3.9)	2.26, brd	2.26, dd (11.9, 3.8)	12'eq	2.39, dd (13.3, 3.9)	2.26, brd (8.5)
13	3.70[a]	3.70, td (10.7, 4.7)	3.80[a]	3.81[a]	13'	3.70[a]	3.8[a]
14	1.05[a]	1.04[a]	1.06[a]	1.06[a]	14'	1.05[a]	1.06[a]
15	3.40, m	3.40, dq	3.77[a]	3.78[a]	15'	3.40, m	3.77[a]

		(10.3, 5.9)					
16	1.11, d (5.7)	1.11, d (6.1)	1.04, d (5.7)	1.04, d	16'	1.11, d (5.7)	1.04, d (5.7)
17	0.97, d (6.1)	0.97, d (6.4)	0.98, d (4.7)	0.97, d (6.4)	17'	0.96, d (6.2)	0.98, d (4.7)
18	0.85, d (6.8)	0.89, d (6.9)	0.80, b	0.83, d (7.0)	18'	0.87, d (6.8)	0.81, (6.7)
19	0.82, d (6.4)	0.83, d (6.9)	0.86, d (6.7)	0.86, d (7.0)	19'	0.82, d (6.4)	0.86, d (6.7)
20a	1.60, m	1.59, m	1.61[a]	1.60[a]	20'a	1.60, m	1.61[a]
20b	1.36[a]	1.35[a]	1.37[a]	1.37[a]	20'b	1.36[a]	1.37[a]
21	0.77, t (7.4)	0.78, t (7.5)	0.79, t (7.4)	0.79, t (7.2)	21'	0.77, t (7.4)	0.79, t (7.4)
22	4.90, brs	4.90, d (3.0)	4.92, d (2.9)	4.92, d (2.5)	22'	4.90, brs	4.92, d (2.9)
23a	1.80[a]	1.79[a]	1.81[a]	1.81[a]	23'a	1.80[a]	1.81[a]
23b	1.38[a]	1.38[a]	1.40[a]	1.40[a]	23b	1.38[a]	1.40[a]
24	3.72[a]	3.72[a]	3.74[a]	3.74[a]	24'	3.72[a]	3.74[a]
25	3.37[a]	3.37[a]	3.38[a]	3.38[a]	25'	3.37[a]	3.38[a]
26	3.73[a]	3.73[a]	3.75[a]	3.75[a]	26'	3.73[a]	3.75[a]
27	1.06, d (6.3)	1.05, d (6.4)	1.07, d (6.3)	1.07, d (6.3)	27'	1.06, d (6.3)	1.07, d (6.3)
11-OMe	2.96, s	2.95, s			11'-OMe	2.94, s	
11-OH			5.42, brs	[b]	11'-OH		5.42, brs
9-OH	4.43, d (8.0)	4.43, d (7.4)	4.47, d (8.4)[c]	[b]	9'-OH	4.44, d (8.8)	4.48, d (7.4)[c]
24-OH	4.56, brs	4.54 brs	4.54, brs	4.50, brs	24'-OH	4.56, brs	4.54
25-OH	4.32, brs	4.28, d (3.8)	4.30, brs	4.27, brs	25'-OH	4.32, brs	4.30
2-Me	1.78, s	1.75, s	1.78, s	1.74, s	2'-Me		

[a]Multiplicity patterns were unclear due to signal overlap. [b]Signal not observed. [c]These signals are interchangeable.

Table 2. ^{13}C NMR Data (175 MHz) for Compounds **1-3** in DMSO-d_6

	1	**2**	**3**		**1**	**3**
No.	δ_c, type	δ_c, type	δ_c, type	No.	δ_c, type	δ_c, type
1	168.0, C	168.2, C	168.0, C	1'	167.4, C	167.4, C
2	126.6, C	126.7, C	126.6, C	2'	120.2, CH	120.2, CH
3	138.6, CH	138.6, CH	138.4, CH	3'	145.1, CH	145.0, CH

4	127.7, CH	126.7, CH	127.7, CH	4'	129.6, CH	129.6, CH
5	143.8, CH	144.5, CH	143.6, CH	5'	145.8, CH	145.6, CH
6	41.6, CH	41.5, CH	41.5, CH	6'	41.2, CH	41.2, CH
7	76.2, CH	76.2, CH	76.0, CH	7'	76.0, CH	75.7, CH
8	36.9, CH	37.2, CH	36.4, CH	8'	37.0, CH	36.4, CH
9	68.1, CH	68.2, CH	69.5, CH	9'	68.1, CH	69.5, CH
10	37.6, CH	37.6, CH	43.0, CH	10'	37.6, CH	43.0, CH
11	102.8, C	102.8, C	99.2[a], C	11'	102.8, C	99.3[a], C
12	33.7, CH_2	33.7, CH_2	36.6, CH_2	12'	33.7, CH_2	36.6, CH_2
13	68.2, CH	68.2, CH	68.3, CH	13'	68.2, CH	68.3, CH
14	47.2, CH	47.2, CH	48.0, CH	14'	47.2, CH	48.0, CH
15	66.9, CH	66.9, CH	65.8, CH	15'	66.9, CH	65.8, CH
16	19.0, CH_3	19.0, CH_3	19.1[b], CH_3	16'	19.0, CH_3	19.2[b], CH_3
17	15.9, CH_3	15.8, CH_3	15.8, CH_3	17'	15.5, CH_3	15.5, CH_3
18	10.2, CH_3	10.0, CH_3	9.8, CH_3	18'	10.0, CH_3	9.7, CH_3
19	7.3, CH_3	7.2, CH_3	7.0, CH_3	19'	7.3, CH_3	7.0, CH_3
20	18.8, CH_2	18.8, CH_2	18.8, CH_2	20'	18.8, CH_2	18.8, CH_2
21	8.8 CH_3	8.8 CH_3	8.8 CH_3	21'	8.8 CH_3	8.8 CH_3
22	92.5, CH	92.5, CH	92.5, CH	22'	92.5, CH	92.5, CH
23	32.7, CH_2	32.7, CH_2	32.7, CH_2	23'	32.7, CH_2	32.7, CH_2
24	66.4, CH	64.9, CH	65.0, CH	24'	66.4, CH	65.0, CH
25	70.3, CH	70.3, CH	70.3, CH	25'	70.3, CH	70.3, CH
26	65.0, CH	66.4, CH	66.4, CH	26'	65.0, CH	66.4, CH
27	17.2, CH_3	17.1, CH_3	17.2, CH_3	27'	17.2, CH_3	17.2, CH_3
11-OMe	45.6[c], CH_3	45.6, CH_3		11'-OMe	45.7[c], CH_3	
2-Me	12.26, CH_3	11.9, CH_3	12.2, CH_3	2'-Me		

[a,b,c] Signals with the same superscripts are interchangeable.

Detailed analysis of 2D NMR data (COSY, HSQC, HMBC, and TOCSY) revealed that the additional methyl group attaches to the C-2 olefinic carbon. The proton signal of this methyl group shows HMBC correlations with the C-2 quaternary olefinic carbon (δ_C 126.6), the C-1 carbonyl carbon (δ_C 168.0), and C-3 (δ_C 138.6) (Figure 2). Due to the absence of the proton at C-2 in compound **1**, the signal for H-3 appeared as a doublet (δ_H 6.66), instead of as the doublet of doublets seen for **7**.[5] The C-2 methyl group substitution mainly affects the chemical environment of the macrodiolide ring, resulting in different chemical shifts between the upper and the lower

parts of the ring structure. On the other hand, signals for the polyketide side chains, the hemiketal rings and the sugar moieties from both sides of the molecule are almost identical. The coupling constants of the protons and the ROESY correlations are all consistent with the published data for known elaiophylins, indicating they all share the same relative configuration.

The core structures of all known elaiophylins are constructed from two linear polyketide chains via C_2-symmetric dimerization. Although there are some variations at C-14 and C-14′, where the substituent can be a methyl or an ethyl group, as well as at C-11 and C-11′ (hydroxy or methoxy groups) the polyketide backbones that form the macrodiolide ring of known elaiophylins are all identical.[4,5] Therefore, the identification of **1**, in which one of the polyketide backbones has an additional pendant methyl group at the C-2 position, is rather unusual. This may indicate that either the polyketide synthase (PKS) proteins that are responsible for elaiophylin biosynthesis have relaxed substrate specificity or there is a post-PKS tailoring enzyme that catalyzes the *C*-methylation of the polyketide backbone. In either case, there was a high likelihood that another analogue with pendant methyl groups on both polyketide chains was present in the mixture. This prompted us to reexamine the HPLC and MS profiles of the elaiophylin mixture, and as a result we discovered a peak (peak L, Figure 1) with an *m/z* of 1103 $[M+Na]^+$, which is 14 daltons higher than **1**. As the compound appeared to be produced in a low yield, the strain was grown in a 20-L culture and compound **2** (1.7 mg) was purified by successive chromatography experiments.

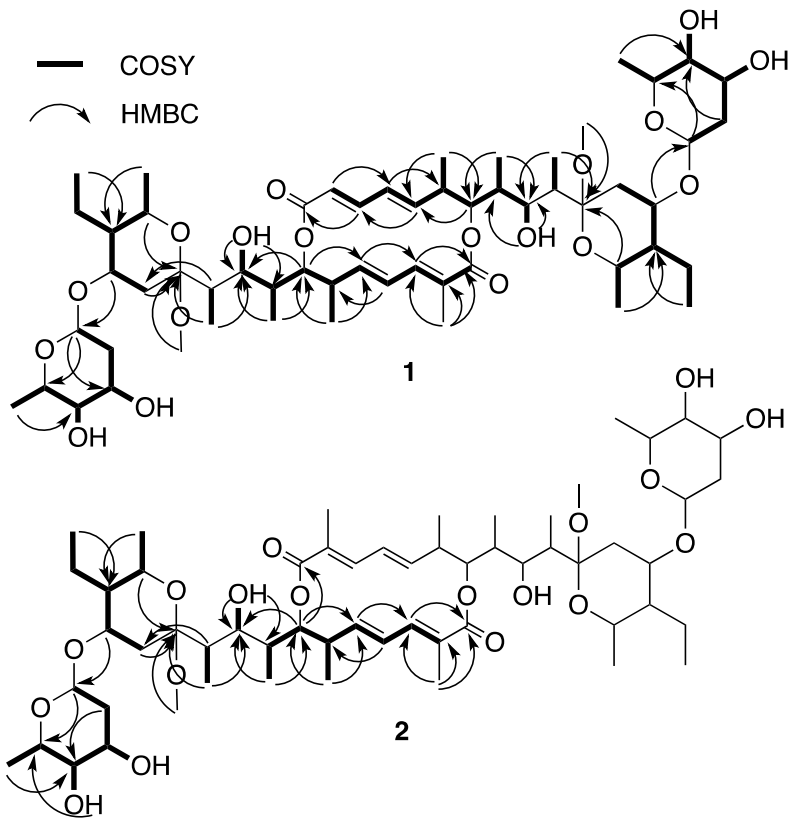


Figure 2. Selected HMBC and COSY correlations of **1** and **2**.

Compound **2** was isolated as a white powder and the molecular formula was determined to be $C_{58}H_{96}O_{18}$. Compared to the ^{1}H NMR spectrum of **1**, fewer signals were observed in that of **2**, particularly those of the olefinic protons. The rest of the spectrum was similar to that of **1**, including the presence of a methyl singlet at 1.75 ppm. On the basis of the MS data for **2** and the number of protons and carbons in its ^{1}H, ^{13}C, and DEPT-135 spectra, it was postulated that **2** has a *C*$_2$-symmetric structure. Further analysis of the 2D NMR (COSY, HSQC, HMBC) data for **2** confirmed the presence of additional pendant methyl groups at C-2 and C-2′. The HMBC correlation between H-7 (or H-7′) (δ_H 5.13) and C-1 (or C-1′) (δ_C 168.2) signifies the connection between the two linear polyketide chains (Figure 2). The characteristic doublet for H-3 (and H-3′) (δ_H 6.66 ppm) and the quaternary olefinic carbon for C-2 (and C-2′) (δ_C 126.7 ppm) were both

observed. The coupling constants and the ROESY correlations of **2** are consistent with those of **1**, indicating that compound **2** has the same relative configuration as compound **1**.

As shown in the HPLC chromatogram (Figure 1), the elaiophylin mixture was composed of three clusters of compounds. The major peaks from each cluster, peaks B (**5**), F (**6**) and J (**7**), have been identified as known elaiophylin analogues. Upon examination of their chemical structures, it is postulated that the first cluster consists of compounds that have free hemiketal hydroxy groups (11-OH and 11′-OH). The second cluster consists of elaiophylins with the C-11 hydroxy replaced by a methoxy group, whereas the third cluster consists of compounds that have two methoxy groups, at C-11 and C-11′. Examination of the minor components of the clusters also showed these correlations, where similar compounds differing only in the number of methoxy groups at the C-11/11′ positions were found in every cluster. For example, peak I, which was identified as 11,11′-*O,O*-dimethyl-14′-deethyl-14′-methylelaiophylin (**9**), is a dimethoxy analogue of efomycin G (**8**; peak A). This observation suggested that peak C (**3**), with a molecular mass of 14 daltons higher than peak B (**5**), is a new elaiophylin analogue. Thus, isolation and chemical characterization of this compound was pursued.

The ^{1}H NMR spectrum of **3** was almost identical to that of **1,** except that it lacked signals for methoxy groups shown in **1** (δ_H 2.94, 2.96 ppm). Additionally, it showed an extra signal for a hydroxy group at δ_H 5.43 ppm. Detailed analysis of HR-ESI-MS and 1D and 2D NMR spectra of **3** confirmed that **3** has the same macrolide ring as **1**, but has hemiketals at C-11 and C-11′. In addition, we have also isolated a small amount of compound **4** (Figures 1 and S22), which, based on its mass and ^{1}H NMR spectra, is postulated to be a symmetric version of **3**.

The absolute configuration of elaiophylin (**5**) was established by X-ray crystallography,[15] chemical degradation,[16] and total synthesis.[17-19] Elaiophylin (**5**), isolated from strain ICBB 9297,

showed $[\alpha]^{23}_D$ values of -52 (*c* 1.0, $CHCl_3$) and -51 (*c* 1.0, MeOH), which are comparable to those reported in the literature, $[\alpha]^{25}_D$ -46.9 (*c* 0.83, $CHCl_3$)[9] and $[\alpha]^{25}_D$ -42.4 (*c* 1.0, MeOH).[20] The closely related compounds efomycin G (**8**) and SNA-4606-1 have also been reported to have similar $[\alpha]^{25}_D$ values of -43.2 (*c* 1.0, MeOH) and -44.8 (*c* 0.5, MeOH), respectively.[20] Interestingly, 11,11′-*O,O*-dimethylelaiophylin (**7**) and 11,11′-*O,O*-dimethyl-14′-deethyl-14′-methylelaiophylin (**9**), which bear methoxy groups at C-11 and C-11′, showed opposite sign $[\alpha]^{25}_D$ values of +18.5 (*c* 1.0, MeOH) and +21.6 (*c* 0.59, MeOH), respectively.[5,7] Consistent with this observation, compounds **1** and **2**, both of which bear methoxy groups at C-11 and C-11′, showed positive $[\alpha]^{22}_D$ values of +39 (***c*** 0.10, MeOH) and +36 (***c*** 0.06, MeOH), respectively, whereas compound **3**, which has hydroxy groups (instead of methoxy groups) at C-11 and C-11′, showed a negative $[\alpha]^{22}_D$ value of -29 (*c* 0.33, MeOH). Combined the results indicate that the elaiophylin analogues from strain ICBB 9297 have the same absolute configuration as that of the reported elaiophylins.

Recently, it has been reported that the C-11 and C-11′ methoxy groups in certain elaiophylins are a result of a non-enzymatic event that occurs during purification and isolation processes.[7] When allowed to stand for several days in MeOH, compound **5** was found to convert gradually to **6** and **7**. By the same token, compounds **1** and **2** may be derived non-enzymatically from compounds **3** and **4**, respectively, as MeOH was also used during their purification. To investigate if this is the case, we repeated the experiments using a freshly prepared culture. The EtOAc extract was prepared and then fractionated by silica gel and Sephadex LH-20 chromatography and HPLC without the use of MeOH during the entire purification process. As shown in Figure 3, the fresh mixture, which had no contact with MeOH, only contained a cluster of “hydroxy” elaiophylins, whereas those that were exposed to MeOH for 3 h or overnight

showed the presence of a "monomethoxy" elaiophylin cluster (Figure 3B) and mono- and dimethoxy elaiophylin clusters (Figure 3C), respectively, confirming that compounds **1** and **2** are experimental artifacts of compounds **3** and **4**.

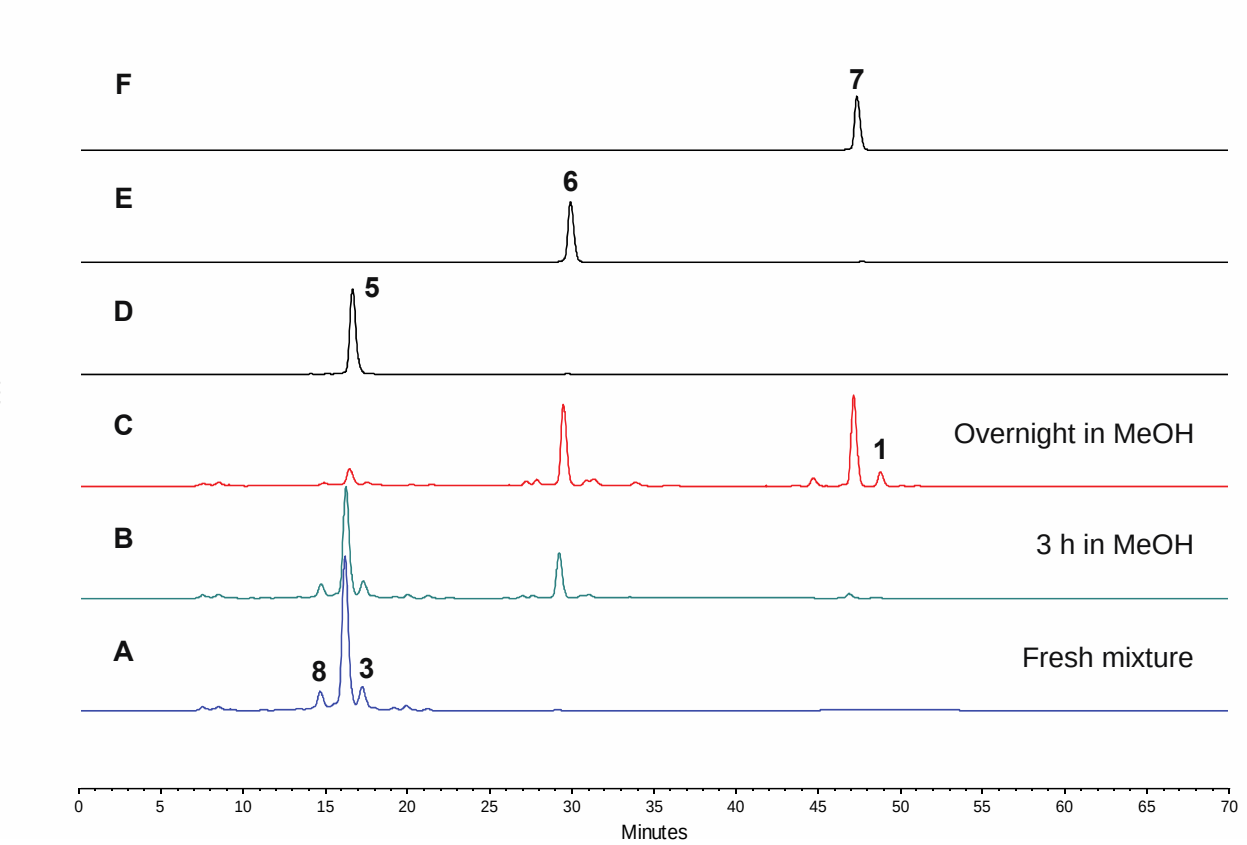


Figure 3. HPLC analysis of elaiophylin mixtures. **A**, Fresh elaiophylin mixture which had no contact with MeOH; **B**, Elaiophylin mixture in MeOH after 3 h at room temperature; **C**, Elaiophylin mixture in MeOH after standing overnight at room temperature; **D**, pure compound **5**; **E**, pure compound **6**; **F**, pure compound **7.**

As described above, the identification of compounds **1**-**4**, in which one or both of the polyketide backbones has an additional pendant methyl group at the C-2/C-2′ position(s), is rather unusual. This may be due to relaxed substrate specificity of the PKSs responsible for

elaiophylin biosynthesis. The elaiophylin biosynthetic gene clusters have been identified in *Streptomyces* sp. DSM4137[21] and NRRL 30748.[22] The proposed 63.2 kb gene cluster contains 24 individual open reading frames (ORFs) including five type I polyketide synthase genes (orf 7-11), which are responsible for the biosynthesis of the polyketide backbone (Figure 4).[22] In modular type I PKS systems, extension of the polyketide chains occurs through repeated condensation of acyl-CoA thioesters. Generally, each module is responsible for one chain elongation cycle. The acyltransferase (AT) domains, which catalyze the transfer of acyl groups from CoA thioesters to the phosphopantetheine arms of the acyl carrier proteins (ACPs), are responsible for the selection of the extender units. Based on the sequence of the active site residues in AT domains, the substrate specificity can be predicted with a high confidence level.[23,24]

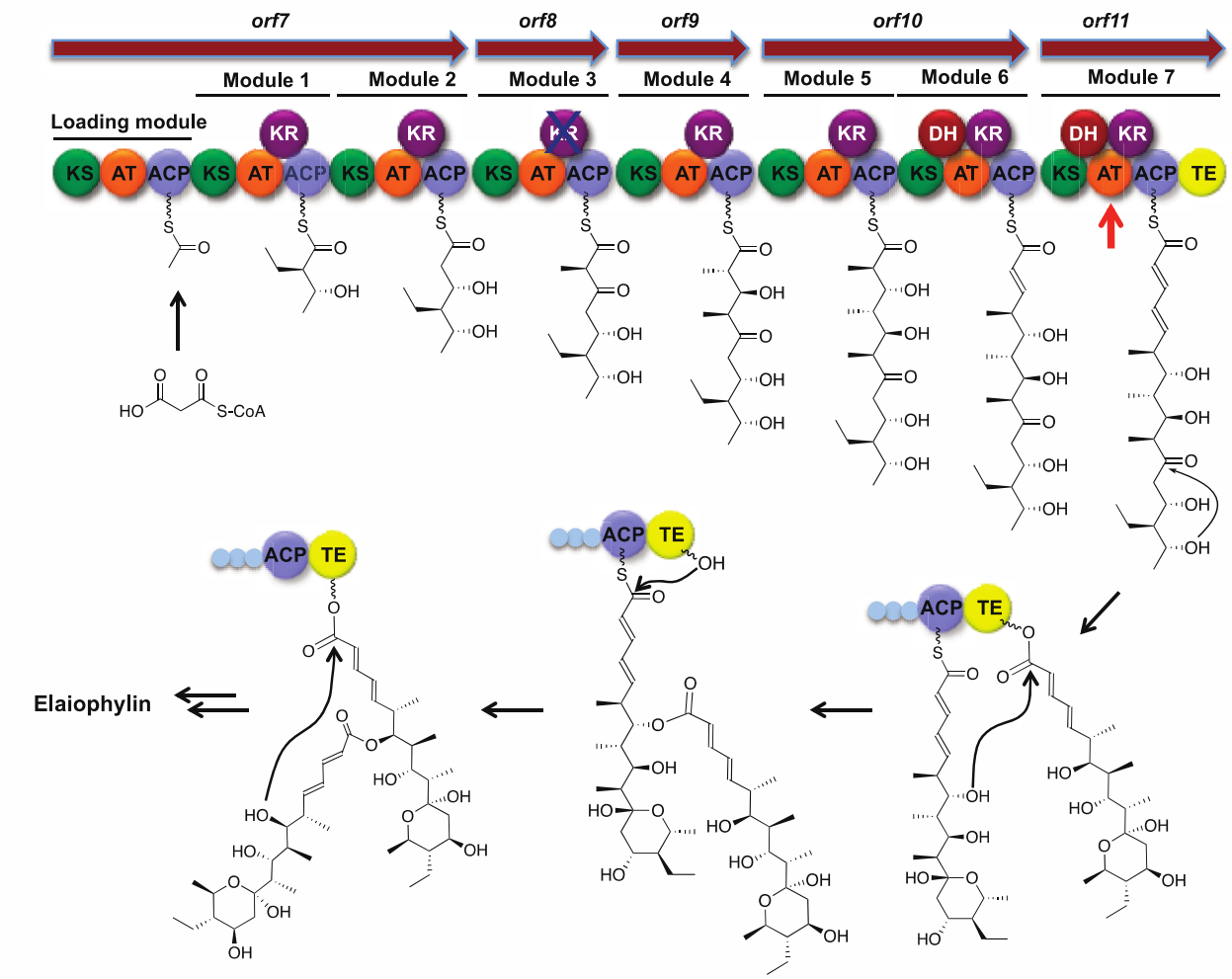


Figure 4. A proposed elaiophylin PKS assembly line. The PKS gene cluster organization was adapted from Haltli[22] with some modifications and the dimerization mechanism was adapted from Zhou et al.[25] However, the absolute configurations of the intermediates are different from those depicted in both reports,[22,25] but are consistent with the configuration reported for elaiophylin.

In elaiophylin biosynthesis, the polyketide backbone starts with an acetyl unit, which is derived from KS_Q domain-catalyzed decarboxylation of a malonyl thioester.[26] Subsequently, the growing polyketide backbone is passed through seven extension modules which specifically

recruit one ethylmalonate (module 1), three malonates (modules 2, 6, 7) and three methylmalonates (modules 3, 4, 5) to reach the full length of elaiophylin polyketide backbone (Figure 4). The formation of the symmetrical diolide has recently been proposed to take place via three catalytic steps: 1) coupling between the TE-bound and the ACP-bound monomers to give a linear dimer attached to the ACP, 2) transfer of the linear dimer to the vacant TE active site, and 3) regioselective cyclization of the linear dimer to give a symmetrical diolide backbone (Figure 4).[25]

The discovery of compounds **3** and **4** with additional pendant methyl group(s) in the macrocyclic ring structure indicates that the last module of the elaiophylin PKS system (module 7) can use not only malonyl-CoA but also methylmalonyl-CoA as extender units. However, based on the yields of the products, malonyl-CoA appears to be the preferred substrate. Careful analysis of the conserved active site residues of the AT domains within the elaiophylin PKSs revealed that except for AT7, all other ATs have a conserved motif and active side residues that match perfectly with those in other AT domains with known specificity (Tables 3 and S1).[23] Interestingly, instead of having the typical conserved 'HAFT' motif (residues 198-201) for malonyl-CoA specific ATs, the ela_AT7 domain has an unusual 'IAAH' motif (Table 3).[23,24,27] A previous study using computational modeling and site-specific mutagenesis concluded that amino acid residue 200 plays a crucial role in controlling substrate selection by AT domains.[24] However, instead of phenylalanine commonly found in malonyl-CoA-specific ATs, the ela_AT7 domain contains alanine at this position. This may explain its relaxed substrate specificity, but it does not explain why it still prefers malonyl-CoA as substrate. Also, the motif differs from the previously reported hybrid motif (HASH) in the epothilone PKS, epo_AT3 (Table 3).[28]

Nevertheless, Ala200 is also found in the loading modules of the erythromycin and megalomicin PKS systems,[23] which recognize propionyl-CoA as the substrate.

Table 3. Alignment of Conserved Motifs in the Active Site of AT Domains.

Domain name	Specificity	198	199	200	201
ave_AT5	Malonyl-CoA	H	A	F	H
nid_AT3	Malonyl-CoA	H	A	F	H
epo_AT2	Malonyl-CoA	H	A	F	H
amp_AT18	Malonyl-CoA	H	A	F	H
rif_AT2	Malonyl-CoA	H	A	F	H
ela_LD	Malonyl-CoA	H	A	F	H
ela_AT2	Malonyl-CoA	H	A	F	H
ela_AT6	Malonyl-CoA	H	A	F	H
amp_AT2	Methylmalonyl-CoA	Y	A	S	H
ave_AT1	Methylmalonyl-CoA	Y	A	S	H
rif_AT7	Methylmalonyl-CoA	Y	A	S	H
nid_AT4	Methylmalonyl-CoA	Y	A	S	H
ery_AT4	Methylmalonyl-CoA	Y	A	S	H
ela_AT3	Methylmalonyl-CoA	Y	A	S	H
ela_AT4	Methylmalonyl-CoA	Y	A	S	H
ela_AT5	Methylmalonyl-CoA	Y	A	S	H
nid_AT5	Ethylmalonyl-CoA	T	A	G	H
tyl_AT5	Ethylmalonyl-CoA	T	A	G	H
ela_AT1	Ethylmalonyl-CoA	T	A	G	H
ery_LD	Propionyl-CoA	M	A	A	H
meg_LD	Propionyl-CoA	M	A	A	H
epo_AT3	Flexible	H	A	S	H
ela_AT7	Malonyl-CoA	I	A	A	H

Abbreviations: ave, avermectin; nid, niddamycin; epo, epothilone; amp, amphotericin; rif, rifamycin; ery, erythromycin; tyl, tylactone; meg, megalomicin; ela, elaiophylin.

In order to confirm the AT sequence of *Streptomyces* sp. ICBB 9297, the AT7 domain was PCR amplified and sequenced. The primers were designed based on the published sequence of the elaiophylin PKS from *Streptomyces* sp. NRRL 30748. The genomic DNA of strain ICBB 9297 was used as template and the obtained PCR product was sequenced. The result showed that the ICBB 9297 AT7 domain shares 96% identity to that of *Streptomyces* sp. NRRL 30748 (Figure S23). Multiple amino acid sequence alignments showed that the conserved residues of the two AT domains, including those proposed to be important for substrate specificity, were identical. This finding suggests that strain NRRL 30748 is also able to produce elaiophylin analogues with one or both of the dimeric polyketide chains containing an additional pendant methyl group.

Elaiophylin (**5**) and its derivatives have been reported to exhibit antimicrobial activities against Gram-positive bacteria including several drug-resistant strains.[4,8-10] Similar to many other macrolide antibiotics, this group of compounds is not active against Gram-negative bacteria or fungi.[5,11,29] The antimicrobial activities of the new elaiophylin-like macrolides (**1**-**3**) together with some of the known elaiophylin analogues (**5**-**7**) were evaluated against *S. aureus* ATCC 12600, *Mycobacterium smegmatis* ATCC 14468*, Bacillus subtilis* ATCC 6051, *Escherichia coli* ATCC 11775 and *Pseudomonas aeruginosa* ATCC 9721. Compound **4** was not included due to insufficient material for accurate testing. The minimum inhibitory concentrations (MIC) were determined by microdilution assay at final concentrations ranging from 0.05 μg/mL to 100 μg/mL (Table 4). All compounds (**1**-**3**, **5**-**7**) displayed good activities against *S. aureus* ATCC 12600 with MIC values in the range of 0.78-3.13 μg/mL but did not inhibit the growth of *B. subtilis* ATCC 6051 with a final concentration up to 100 μg/mL. As previously noted, none of the tested compounds exhibited activity against the Gram-negative bacteria *E. coli* and *P.*

aeruginosa (Table 4). Remarkably, the new compounds **1** and **3**, which have only one additional pendant methyl group in the macrocyclic core structure, showed good activities against the Gram-positive bacterium *M. smegmatis* with an MIC value of 6.25 μg/mL, whereas compound **2**, which contains two additional pendant methyl groups, and the known elaiophylins **5**-**7**, which do not have the C-2 and/or C-2′ pendant methyl groups, showed no significant growth inhibition (Table 4, Figure S24). The reason for this disparity in activity toward *M. smegmatis* is unknown and will be the subject of future investigations. Nevertheless, the activities of compounds **1** and **3** against *M. smegmatis* may serve as a platform for the development of new antibiotics against the human pathogen *Mycobacterium tuberculosis*.

Table 4. Antimicrobial Activities (MIC, μg/mL) of Elaiophylin Analogues.

Microorganism	**1**	**2**	**3**	**5**	**6**	**7**	Amp[a]	Apra[a]
Staphylococcus aureus	1.56	3.13	0.78	0.78	1.56	1.56	0.78	NT[b]
Mycobacterium smegmatis	6.25	>100	6.25	>100	>100	>100	>100	0.39
Bacillus subtilis	>100	>100	>100	>100	>100	>100	0.78	NT
Pseudomonas aeruginosa	>100	>100	>100	>100	>100	>100	1.56	NT
Escherichia coli	>100	>100	>100	>100	>100	>100	6.25	NT

[a]Amp = ampicillin, Apra = apramycin; [b]NT, not tested.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Jasco P1010 polarimeter. UV spectra were obtained on an Eppendorf BioSpectrometer kinetic. IR spectra were recorded on a Nicolet IR100 FT-IR spectrophotometer. NMR spectra were recorded in DMSO-d_6 referenced to residual solvent signals (δ_H 2.50 ppm, δ_C 39.52 ppm) on a Bruker

Avance III 700 MHz spectrometer equipped with a 5 mm ^{13}C cryogenic probe. ESI-MS data were recorded on a ThermoFinnigan LCQ Advantage system with a quaternary Rheos 4000 pump (Flux Instrument). High-resolution mass spectrometry was performed in a positive ion mode on an AB SCIEX Triple TOF 5600 mass spectrometer. HPLC was performed using a Shimadzu dual LC-20AD solvent delivery system with a Shimadzu SPD-M20A UV/vis photodiode array detector.

Organism Collection and Identification. The strain ICBB 9297 was isolated from a soil sample of an agricultural field in Jatiroto, East Java, Indonesia using a "heating method". The soil sample (0.5 g) was homogenized in NaCl solution (0.85%) and heated at 80 °C for 30 min and spread onto YMG agar plates (4 g yeast extract, 10 g malt extract, 4 g glucose, 20 g agar, 1 L H_2O, pH adjusted to 7.2).[30] The isolate was stored in 20% glycerol at -80 °C. The 16S rRNA gene was amplified from strain ICBB 9297 genomic DNA using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'- TACGGYTACCTTGTTACGACTT-3') as template. The 16S rRNA gene sequence of strain ICBB 9297 was found to have 99% identity over the sequenced region to several *Streptomyces* strains in the NCBI database. The GenBank accession number for the sequence of the 16S rDNA is KT210116.

Fermentation and Isolation. *Streptomyces* sp. ICBB 9297 was grown on a modified Bennett's agar plate [glucose (10 g), yeast extract (1 g), beef extract (1 g), soytone (2 g), metal ion solution [$FeSO_4 \cdot 7H_2O$ (50 mg), $CaCl_2 \cdot 2H_2O$ (90 mg), $MnCl_2 \cdot 4H_2O$ (180 mg), $CoCl_2 \cdot 6H_2O$ (25 mg), $CuSO_4 \cdot 5H_2O$ (25 mg), $ZnSO_4 \cdot 4H_2O$ (25 mg), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (25 mg) in ddH_2O (50 mL)] (1 mL), agar (15 g) in ddH_2O (1 L)] at 30 °C for 3 days. Subsequently, the strain was grown in 15 500-mL Erlenmeyer flasks each containing modified Bennett's liquid

medium[31] (100 mL) on a rotary shaker (200 rpm) at 30 °C for 3 days. The resulting seed culture was transferred to 50 2-L Erlenmeyer flasks each containing the production medium (modified Bennett's) (400 mL) and cultivated on a rotary shaker (200 rpm) at 30 °C for 7 days. The culture was centrifuged and the resulted supernatant was collected and extracted three times with EtOAc. The EtOAc extract (4.88 g) was fractionated by reversed-phase (RP) C_{18} solid phase extraction (SPE) using a stepped solvent gradient (10%, 30%, 60%, 80%, 90%, 100% MeOH-H_2O). Fractions obtained from the 60%, 80%, 90% MeOH-H_2O elutions were pooled and further chromatographed on a silica gel flash column eluting with a stepwise gradient solvent system from 100% $CHCl_3$ to 100% MeOH (2%, 4%, 6%, 8%, 16%, 100%). Fractions from the 6% - 8% $CHCl_3$-MeOH elutions were combined and further separated by Sephadex LH-20 (100% MeOH) and semi preparative reversed-phase HPLC (YMC-Pack ODS-A, 250×10 mm I.D., MeOH-H_2O 85:15 – 90:10 – 100:0 gradient, flow-rate 2.5 mL/min, 254 nm detection) to yield compounds **1** (11.7 mg), **2** (1.7 mg), **3** (4.9 mg), and **4** (< 0.5 mg).

2-Methyl-11,11′-O-dimethylelaiophylin (**1**): white powder; $[\alpha]^{22}_D$ +39 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 254 (4.64) nm; IR ν_{max} (KBr) 3434, 2970, 2935, 1697, 1636, 1612, 1462, 1383, 1223, 1088, 1020, 983, 750 cm^{-1}; ^1H and ^{13}C NMR data, Tables 1 and 2; HRESITOFMS *m/z* 1089.6377 $[M + Na]^+$ (calcd for $C_{57}H_{94}O_{18}Na$, 1089.6338).

2,2′-Dimethyl-11,11′-O,O-dimethylelaiophylin (**2**): white powder; $[\alpha]^{22}_D$ +36 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ε) 258 (4.56) nm; IR ν_{max} (KBr) 3448, 2962, 2925, 2854, 1701, 1638, 1561, 1459, 1378, 1248, 1088, 1020, 981, 746 cm^{-1}; ^1H and ^{13}C NMR data, Tables 1 and 2; HRESITOFMS *m/z* 1103.6531 $[M + Na]^+$ (calcd for $C_{58}H_{96}O_{18}Na$, 1103.6494).

2-Methylelaiophylin (**3**): white powder; $[\alpha]^{22}_D$ -29 (*c* 0.33, MeOH); UV (MeOH) λ_{max} (log ε) 254 (4.84) nm; IR ν_{max} (KBr) 3423, 2971, 2932, 1701, 1636, 1462, 1383, 1222, 1169, 1089,

1018, 983, 752 cm^{-1}; ^{1}H and ^{13}C NMR data, Tables 1 and 2; HRESITOFMS *m/z* 1061.6002 $[M+Na]^+$ (calcd for $C_{55}H_{90}O_{18}Na$, 1061.6025).

2,2′-Dimethylelaiophylin (**4**): white powder; UV (MeOH) λ_{max} (log ε) 254 (4.68) nm; ^{1}H NMR data, Table 1; HRESITOFMS *m/z* 1075.6156 $[M+Na]^+$ (calcd for $C_{56}H_{92}O_{18}Na$, 1075.6181).

Antibacterial Activity Assay. Bacteria were grown on agar plates [YMG[30] or Luria-Bertani (LB)] for 1-2 days at 30 or 37 °C and then added to liquid medium (YMG for *S. aureus, M. smegmatis, B. subtilis* and *P. aeruginosa*, and LB for *E. coli*). After shaking at 200 rpm, 30 °C (37 °C for *E. coli*), for 5-20 h, the cultures were diluted with YMG or LB media to obtain an OD_{600} value ~ 0.1. Bacterial cultures (100 μL) with the desired optical density value were added to the media (50 mL) and then mixed gently. The bacterium-containing media (98 μL) were then added to each well of 96-well plates containing DMSO solutions (2 μL) of the tested compounds in different concentrations. DMSO was used as a negative control, whereas ampicillin or apramycin was used as a positive control. The 96-well plates were placed in incubator for 17 to 48 h. An MTT solution (50 μL, 1 mg/mL) was then added to each well. After standing for 30 min, the color change was observed. All experiments were performed in triplicate. The minimum inhibitory concentration (MIC) values were determined as the lowest concentration required for bacterial growth inhibition.

ASSOCIATED CONTENT

Supporting Information

Table of conserved active site residues in various AT domains, ^{1}H NMR spectra of **1-4**, ^{13}C NMR spectra of **1-3**, COSY, HSQC, HMBC, and ROESY spectra of **1-3**, amino acid alignment of AT7 domains of elaiophylin PKSs, and microdilution assay protocol for *Mycobacterium*

smegmatis. The Supporting Information is available free of charge on the ACS Publications website at DOI:….

AUTHOR INFORMATION

Corresponding Author

*Tel: 1-541-737-9679. Fax: 1-541-737-3999. E-mail: Taifo.Mahmud@oregonstate.edu.

Notes

The authors declare no competing financial interest.

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Table of Contents Graphic and Synopsis

Identification of Elaiophylin Skeletal Variants from the Indonesian *Streptomyces* sp. ICBB 9297

Yan Sheng, Phillip Lam, Salmah Shahab, Dwi Andreas Santosa, Philip J. Proteau, T. Mark Zabriskie, Taifo Mahmud