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Isolation and Characterization of Two New Antifungal Antibiotics from a Basidiomycete

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Two novel antibiotics, Sch 484129 (**1**) and Sch 484130 (**2**), were isolated from the fermentation broth of a fungal culture, which was identified as a Basidiomycete. The new antibiotics were obtained by ethyl acetate extraction followed by reversed phase HPLC purification. Structure elucidation of **1** and **2** was accomplished by spectroscopic data analyses. Derivatizations of the major component **1** were performed in order to provide definitive structural information. Both components were identified as glycolipids and displayed antifungal activity against *Saccharomyces* and *Aspergillus* strains.

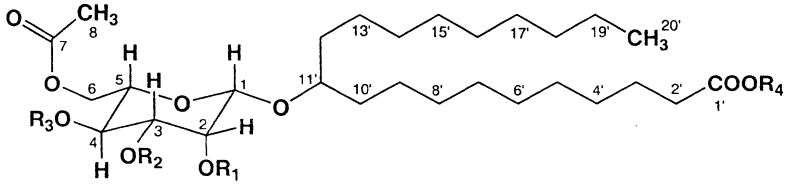
During the course of searching for novel antifungal agents potentially with new mechanisms of actions, thousands of extracts from our culture collection were screened. As a result of the screening process, two new antibiotics, Sch 484129 (**1**) and Sch 484130 (**2**), were discovered from the fermentation broth of a fungal strain. The producing microorganism (HAM-240) was identified as a Basidiomycete based on taxonomic information. The structural characterization of **1** and **2** revealed that these two glycolipids are congeners with a sugar moiety linked to a twenty-carbon arachidic acid unit as shown in Figure 1. This paper describes the fermentation, isolation, structure determination and biological activity of **1** and **2**.

Materials and Methods

Instrumental Analysis

The optical rotations were measured on a JASCO DIP-140 digital polarimeter (Japan Spectroscopic Co.). UV

Fig. 1. Structures of Sch 484129 (**1**), Sch 484130 (**2**), Sch 484138 (**3**) and Sch 484141 (**4**).



Sch 484129 (**1**): R_1=$COCH_2COOH$ R_2=H R_3=H R_4=H
Sch 484130 (**2**): R_1=H R_2=$COCH_2COOH$ R_3=H R_4=H
Sch 484138 (**3**): R_1=$COCH_2COOCH_3$ R_2=Ac R_3=Ac R_4=H
Sch 484141 (**4**): R_1=$COCH_2COOCH_3$ R_2=Ac R_3=Ac R_4=CH_3

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spectra were recorded on a Hewlett Packard '8450A' UV-vis spectrophotometer. LC/MS data were produced on a TSQ-7000 Finnigan triple quadrapole mass spectrometer with the Hewlett Packard HP-1090 HPLC system. High resolution FAB-MS were measured on a JOEL HX110A double focusing mass spectrometer using a 3-nitrodenzyl alcohol (NBA) matrix. Both ^{1}H and ^{13}C NMR spectra were obtained from a Varian XL-400 instrument operating at 400 and 100 MHz, respectively.

Fermentation

Stock culture was maintained as frozen whole broth at −80°C in a final concentration of 10% glycerol. The inoculum medium contained the following ingredients (g/liter): proteus peptone, 5; sodium chloride; 5; KH_2PO_4, 5; yeast extract, 3; cerelose, 20; soybean grits, 5; antifoam, 1 ml, tap water to 1 liter. The pH was adjusted to 7.2 prior to autoclaving. A 250 ml Erlenmeyer flask containing 70 ml of this medium was inoculated with 2.0 ml of the stock culture. The flasks were incubated at 24°C on a rotary shaker (New Brunswick Scientific) at 250 rpm for 96 hours. This seed culture (2.5 ml) was used to inoculate another 250 ml Erlenmeyer flask containing 70 ml of the same seed medium and the flask was incubated under the same above conditions for 96 hours.

Five percent of the second germination was used to inoculate the fermentation medium containing (g/liter): sucrose, 30; glucose, 10; K_2HPO_4, 3; yeast extract, 3; corn step powder, 3; artificial sea salts, 0.277 and tap water to 1 liter. The pH was adjusted to 7.5 prior to autoclaving. The fermentation was carried out in a 2 liters Erlenmeyer flask containing 350 ml of the fermentation medium. The flasks were incubated at 24°C on a rotary shaker at 250 rpm for 120 hours.

Acetylation of **1**

To a mixture of **1** (15 mg) and pyridine (0.2 ml) in CH_2Cl_2 (5 ml) and MeOH (0.1 ml) solution was added acetic anhydride (0.2 ml), and then stirred at room temperature overnight. After removal of solvent, the crude material was purified on semi-preparative HPLC (YMC PVA-Sil 20×250 mn column with a guard column, 1~3% MeOH in *n*-BuCl with a linear gradient in 20 minutes, then 3~50% linear gradient in 15 minutes, 12 ml/minute, UV detection at 220 nm) to afford 5 mg of pure Sch 484138 (**3**) (see Table 2 for NMR spectra data). The esterification of carboxylic acid at C-11 was observed in **3** probably due to the presence of MeOH in the solution. LC/MS (APCI+) data: *m/z* 717 $(M+H)^+$. NMR spectral data: see Table 2.

Esterification-Acetylation of **1**

To a solution of **1** (~80 mg) in dry CH_2Cl_2 (8 ml) was added freshly prepared CH_2N_2 in Et_2O solution (2 ml) at room temperature, and then stirred for 1 hour until the color of solution turned from yellow to colorless. To the reaction mixture was added pyridine (1 ml) and acetic anhydride (0.75 ml), and stirred at room temperature for 3 hours. After the removal of solvent, the residue was purified by semi preparative PVA-Sil HPLC with the same conditions described above. About 60 mg of pure Sch 484141 (**4**) was obtained as colorless waxy material. LC/MS (APCI+) data: *m/z* 731 $(M+H)^+$. NMR spectral data: see Table 2.

Producing Microorganism

The microorganism was isolated from a soil sample in a mountainous area near Seattle, Washington, USA. Taxonomic characteristics of the producing microorganism were determined by cultivation of the strain on modified media described by RAWN[1] and HUNTER-CEVERA[2]. Glucose/yeast extract (GYE) agar contained the following ingredients (g/liter): glucose, 10; yeast 2; agar, 15 and distilled water to 1 liter. Rose Bengal agar (Becton Dickenson Microbiology Systems) contained the following ingredients (g/liter): papaic digest of soybean meal, 5; dextrose, 10; KH_2PO_4, 1; $MgSO_4$, 0.5; Rose Bengal, 0.05; agar, 15; chloramphenicol, 0.1 and distilled water to 1 liter. On GYE mycelia was medium white with hyphae growing in a fan like pattern. Hyphae growing on Rose Bengal agar grouped to form medium white mycelial cords.

Micromorphological characteristics were observed after incubation at 24°C for 10 days on glucose/yeast extract agar. By light microscopy, the producing microorganism formed clamp connections on vegetative and aerial hyphae (Figure 2a and 2b). Hyphae were hyaline and septate. Conidiogenous cells produced were club shape with 7 sterigmata (Figure 3a and 3b). Conidia were single celled, ovoid to lunate, smooth (Figure 4), and appeared to be mounted asymmetrically on sterimata. On the basis of these morphological characteristics and terminology from BARNETT, HUNTER[3] and TALBOT[4], the strain was assigned to the phylum Basidiomycota, class Basidiomycete.

Results and Discussion

Isolation

The fermentation broth (8 liters) was extracted with ethyl acetate at harvest pH. The crude extract was obtained as brown colored gummy residue (~800 mg) after rotary evaporation *in vacuo*. The residue was purified by semi-

Fig. 2. Light micrographs of well developed clamp connections on septate hyphae of the producing organism.

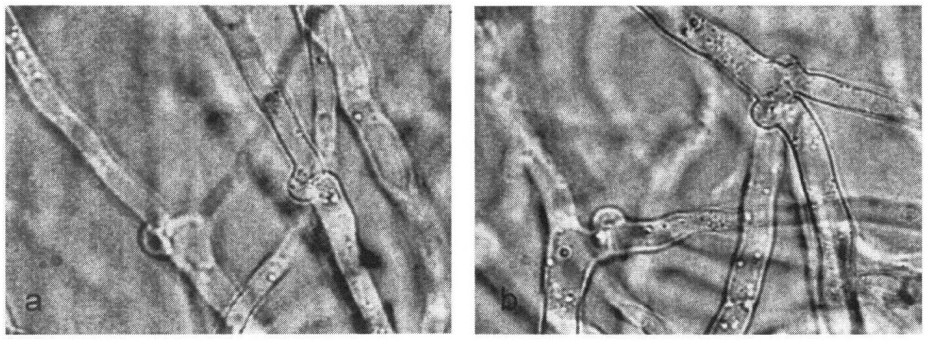


Fig. 3. Photomicrograph of conidiogenous cells with conidia situated symmetrically on 7 sterigmata.

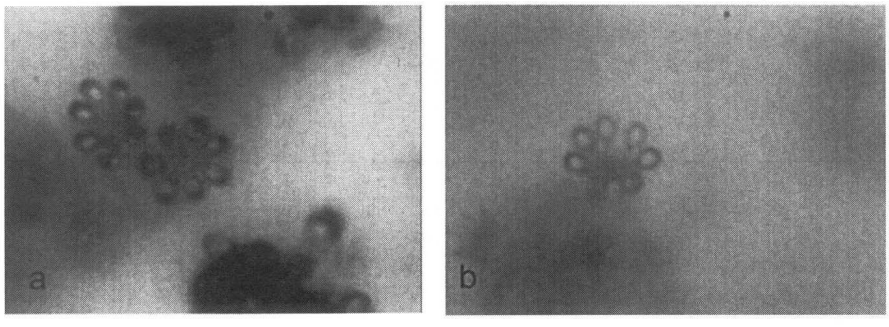
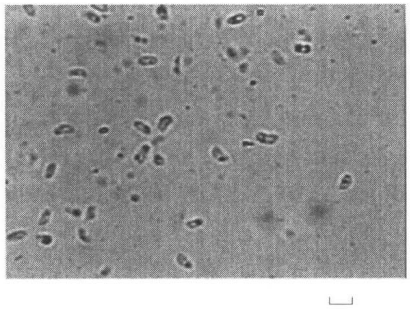


Fig. 4. Conidia of the producing organism.



Bar represents 5 μm.

preparative reversed-phase HPLC (YMC ODS 20×250 mm column with a guard column, S-5, 120 A, 5~50% CH_3CN in H_2O with linear gradient in 20 minutes, then 50~100% CH_3CN/H_2O) in 20 minutes, and hold 100% CH_3CN for 10 minutes, 12 ml/minute, UV=220 nm, ~100 mg each injection) to afford pure **1** (~29 mg) and **2** (10 mg as colorless oil with optical rotation $[\alpha]_D^{22}$ −28.93° (*c* 0.5, MeOH), and −21.55° (*c* 0.1, MeOH), respectively. Both compounds are soluble in EtOAc, MeOH, CH_3Cl and DMSO, insoluble in hexane and H_2O.

Structure Elucidation

The LC/MS data of **1** using positive atmospheric pressure chemical ionization (APCI+) interface showed the molecular ion M^+=618 and water adduct ion $(M+H_2O)^+$ at *m/z* 636, respectively. The molecular weight of **1** was also confirmed by APCI-mode data that showed the deprotonated molecular ion $(M-H)^-$ at *m/z* 617 and dimer ion $(2M-H)^-$ at *m/z* 1235. The molecular formula of **1** was found to be $C_{31}H_{54}O_{12}$ based on HRFAB-MS data (Calcd: *m/z* 641.3513 for $C_{31}H_{54}O_{12}Na$. Found: *m/z* 641.3531). The

Table 1. NMR spectral data of Sch 484129 and Sch 484130[a].

#	Sch 484129		Sch 484130	
	^{1}H(δ)	^{13}C (δ)	^{1}H(δ)	^{13}C (δ)
1	5.18 s	98.79 d[c]	5.04 s	100.93 d
2	6.12 s	74.49 d	4.79 s	70.61 d
3	4.36 s	73.89 d	5.64 dd, J=10	79.25 d
4	4.34 s	69.09 d	4.72 t, J=10	66.00 d
5	4.03 m	75.69 d	4.07 m	75.80 d
6	4.76, 5.03 m,m	64.97 t	4.76, 4.97 m,m	65.07 t
7	--	170.81 s	--	171.12 s
8	2.10 s	20.85 q	2.02 s	21.15 q
9	--	167.90 s	--	168.46 s
10	3.85 q, J=15[b]	42.93 t	3.80 br.s	43.37
11	--	170.14 s	--	170.35
1'	--	176.01 s	--	176.35 s
2'	2.52 t, J=6	34.94 t	2.53 t, J=6	35.36 t
3'	1.81 m	25.72 t	1.80 m	26.06 t
4'-8'	1.22-1.33 m	29.57-34.09 t	1.23-1.30 m	29.98-30.58 t
9'	1.58 m	25.61 t	1.46 m	26.03 t
10'	1.68 m	35.30 t	1.70 m	34.96 t
11'	3.95 m	79.76 d	3.93 m	80.32 d
12'	1.65 m	34.39 t	1.62 m	35.83 t
13'	1.53 m	25.60 t	1.38 m	25.96 t
14'-17'	1.21-1.45 m	29.57-34.09 t	1.22-1.35 m	29.98-30.58 t
18'	1.23 m	32.15 t	1.22 m	32.49 t
19'	1.22 m	22.96 t	1.24 m	23.31 t
20'	0.88 t, J=6	14.30 q	0.88 t, J=6	14.64 q

a. Recorded on 400 MHz (^{1}H) & 100 MHz (^{13}C) at 25°C in pyridine-d_5, respectively.
b. Multiplicity was determined by DEPT data.
c. Coupling constants in Hz.

^{13}C NMR spectral data of **1** (Table 1) indicated the presence of four carbonyl resonances at δ 167.90~176.01. These signals represented either carboxylic acid or ester functionality due to the lack of unsaturation and nitrogen in the molecule. The observation of one anomeric carbon at δ 98.79, one oxygenated methylene at δ 64.97 and five oxygenated methine carbons at δ 69.09~79.76 suggested the presence of a sugar unit. A total of eighteen aliphatic methylenes and one methyl carbon indicated a long lipophilic carbon chain. The ^{1}H NMR spectrum of **1** (Table 1) was consistent with ^{13}C NMR data showing several oxy-methine/methylene proton signals from the sugar unit, and numerous aliphatic methylene multiplets and a methyl triplet from the carbon chain. In addition a sharp singlet at δ 2.10 represented an acetate group.

Extensive 2D-NMR studies for all detailed assignments of **1** focused on both itself and its acetylation/esterification derivative **3** & **4** in order to obtain additional information and better quality of spectra for structural assignments (see Table 2). Two partial structures A and B of **4** were established based on HMQC-TOCSY data as shown in Figure 5. As depicted in Figure 6, the 3-bond correlation of H-2 to C-9 observed in **1** revealed the attached of malonic acid group at position-2 on the sugar ring. The acetyl group was connected to the oxy-methylene at position-6 due to the coupling of H-6 to C-7. The observation of correlation between H-1 and C-11; permitted the assignment of arachidic acid unit to the oxy-methylene at position-1. This assignment was also supported by 3-bond correlation of H-11′ to C-1 obtained from an HMBC experiment of **4**. The linkage of oxygen to arachidic acid at position-11′ was proposed based on the couplings of H-11′ to C-10′ and C-12′. This connectivity of the C-11′ on the arachidic acid chain was further confirmed using NMR simulation modeling by computer. The relative stereochemistry of **1** was established by coupling constant calculation and NOE experiments of **4**. The NOE correlations of H-1 to H-2, H-3 and H-5 indicated that these four protons have the same α-orientation, and the H-4 has the β-orientation due to the lack of NOE coupling to adjacent protons as shown in

Table 2. NMR spectral data of Sch 484138 and Sch 484141[a].

	Sch 484138		Sch 484141	
#	^{1}H(δ)	^{13}C (d)	^{1}H(δ)	^{13}C (δ)
1	4.67 s	97.26 d[c]	4.66 s	97.38d
2	5.44 d, J=3[b]	70.35 d	5.43 d, J=3	70.32 d
3	5.05 dd, J=3,10	71.15 d	5.04 dd, J=3,10	71.10 d
4	5.19 t, J=10	66.34 d	5.18 t, J=10	66.25 d
5	3.63 m	72.19 d	3.62 m	72.22 d
6	4.15 dd, J=3,12	62.84 t	4.13 dd,J=3,12	62.74 t
	4.25 dd, J=4,12	--	4.24 dd,J=6,12	--
7	--	170.72 s	--	170.56 s
8	2.08 s	20.72 q	2.07 s	20.68 q
9	--	165.92 s	--	165.90 s
10	3.47, 3.56 ABd, J=9	41.17 t	3.45, 3.54 ABd, J=16	41.14 t
11	--	166.60 s	--	166.42 s
1'	--	176.65 s	--	174.22 s
2'	2.36 t, J=8	33.52 t	2.30 t, J=8	34.05 t
3'	1.62 m	24.90 t	1.61 m	24.91 t
4'-8'	1.19-1.38 m	29.80-28.86 t	1.22-1.30 m	29.10-29.77 t
9'	1.19-1.38 m	25.24 t	1.22-1.30 m	25.19 t
10'	1.46 m	34.47 t	1.48 m	34.51 t
11'	3.59 t, J=6	80.29 d	3.58 t, J=6	80.44 d
12'	1.41 m	33.81 t	1.42 m	33.71 t
13'	1.19-1.38 m	24.68 t	1.22-1.30 m	25.00 t
14'-17'	1.19-1.38 m	29.80-28.86 t	1.22-1.30 m	29.10-29.77 t
18'	1.19-1.38 m	31.92 t	1.22-1.30 m	31.89 t
19'	1.19-1.38 m	22.68 t	1.22-1.30 m	22.66 t
20'	0.88 t, J=8	14.10 q	0.87 t, J=8	14.08 q
3-CH_3	2.02 s	20.51 q	2.00 s	20.48 q
3-CO	--	170.19 s	--	170.13 s
4-CH_3	2.05 s	20.69 q	2.03 s	20.66 q
4-CO	--	169.56 s	--	169.50 s
11-OCH_3	3.78 s	52.53 q	3.76 s	52.46 q
1'-OCH_3	--	--	3.65 s	51.39 q

a. Recorded on 400 MHz (^{1}H) and 100 MHz (^{13}C) at 25°C in $CDCl_3$, respectively.
b. Multiplicity was determined by DEPT data.
c. Coupling constants in Hz.

Fig. 5. Partial structure assignments of **4** by HMQC-TOCSY data.

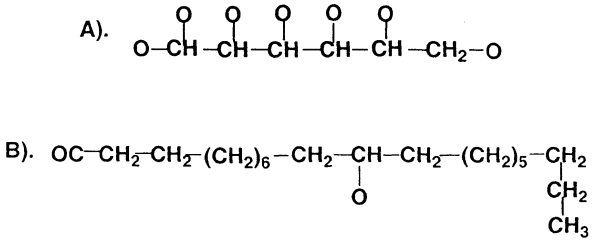


Figure 7. Thus, the structure of **1** was proposed. However, the stereochemistry of C-11′ could not be determined by NOE experiments due to the free rotation of O–C11′ bond.

The molecular weight of **2** was found to be the same as **1**, 618, based on the LC/MS data that showed a protonated molecular ion at *m/z* 619 $(M+H)^+$. The molecular formula was determined to be $C_{31}H_{54}O_{12}$ by HRFAB-MS data (Calcd: *m/z* 641.3513 for $C_{31}H_{54}O_{12}Na$. Found: *m/z* 641.3484). Both ^{1}H and ^{13}C NMR spectra of **2** (Table 1) were very similar to **1** suggesting that **2** is the isomer of **1**. Further examination of the 2D-NMR spectra of **2** confirmed the above assumption, and indicated that only difference was the attachment of malonic acid on the sugar ring. As shown in Figure 6, the 3-bond correlation of H-3 to C-9 led to the assignment of malonic acid at position-3 on

Fig. 6. Some important HMBC correlations observed in **1**, **2** and **4**.

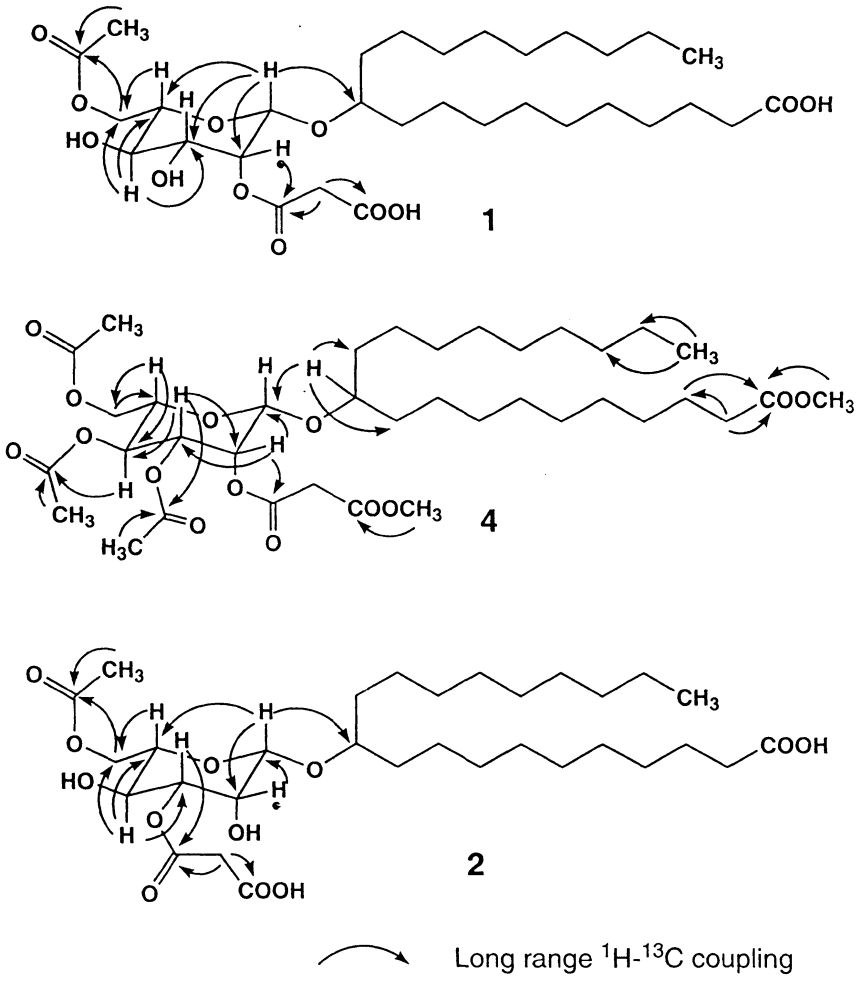
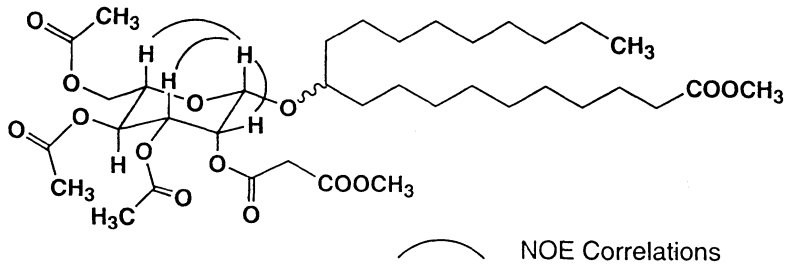


Fig. 7. Stereochemistry determination of **4** by NOE experiment.



the sugar ring. The stereochemistry of **2** was established by NOE experiments as for **1**. Although the stereochemistry of C-11′ was not determined, both **1** and **2** appeared to be single diastereoisomers based on HPLC analyses.

Biological Activity

Both compounds **1** and **2** exhibited antifungal activity against supersensitive *Saccharomyces cerevisiae*

(modification strain of wild-type *S. cerevisiae* obtained by disrupting two efflux pumps, a gene involved in sterol metabolism, and a chitin synthetase gene) with MIC=6.25 and 9.12 μg/ml, weak activity against *Aspergillus* with MIC=25 and 28 μg/ml, respectively. Compounds **1** and **2** were inactive against *Candida albicans* at 100 μg/ml. Acetylation/esterification derivatives **3** and **4** were also tested and were inactive against the above strains.

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