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A New Antifungal Sterol Sulfate, Sch 601324, from *Chrysosporium* sp.

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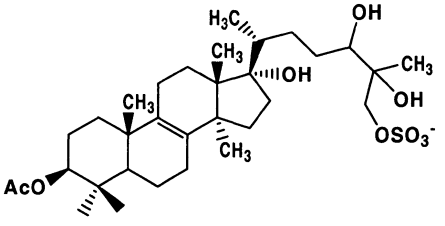
Due to increasing of drug resistance from infectious fungal pathogens, searching for novel antifungal agents with different modes of action is continuously important in new drug development. An enzyme (1,3)-β-D-glucan synthase is one of the key enzymes in the synthesis of fungal cell wall. Three types of antifungal natural products with inhibition activity of cell wall (1,3)-β-D-glucan synthase have been discovered, including lipopeptides (pneumocandins and echinocandins), glycolipids (papulacandins) and terpenoids (enfumafungin, ascosteroside, ergokonin A, and arundifungin).[1] Among these antifungal agents, caspofungin acetate (MK-0991), a semi-synthetic analogue of pneumocandins, has shown promising antifungal application in clinical trials, and has been launched as a parenteral antifungal agent.[2] In the course of our continuing search for novel antifungal agents, we have isolated a novel antifungal agent Sch 601324 (**1**), from a fungal culture *Chrysosporium* sp. (Family: Onygenaceae). Sch 601324 was identified as a sterol sulfate, structurally related to arundifungin (**2**).[3,4] In this paper, we describe the isolation and structure elucidation of **1** using high resolution ESMS and extensive NMR spectroscopic analysis.

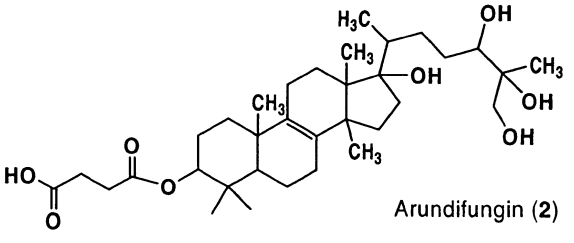
Fermentation studies were carried out in shake flasks. Stock cultures were maintained as frozen whole broths at −80°C in a final concentration of 10% glycerol. The inoculum medium contained: Proteus Peptone, 5 g; NaCl, 5 g; KH_2PO_4, 5 g; Yeast Extract, 3 g; Cerelose, 20 g; Soybean Grits, 5 g; Antifoam, 1 ml; Tap H_2O 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 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, as above for 96 hours.

Five percent of the second germination was used to inoculate the fermentation medium containing: Oat Flour, 20 g; Soy Flour, 20 g; Yeast Extract, 2 g, Corn Step Powder, 5 g; K_2HPO_4, 11 g; KH_2PO_4; 4 g; and Tap H_2O to 1 liter. The fermentation was carried out in a 2-liter 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 hrous.

The fermentation culture broth (~8 liters) was stirred with 400 g of NaCl and 16 liters of acetonitrile. The organic layer was separated and dried in vacuum. The salt in the extract was further removed by a solid phase extraction (SPE) method. Extract was absorbed onto the polymeric resin, CG161 (~100 ml) and the NaCl salt was washed out with water (200 ml). The absorbed organic material was removed from the resin with 150 ml of 80% acetonitrile (ACN). After drying in vacuum, 983 mg of organic material was obtained. The organic material was fractionated on an HPLC semi-preparative ODS-A column (YMC, 120Å, S-7, 2 cm×25 cm). The column was eluted with a gradient of ACN-H_2O: 1~40% ACN in 50 minutes, then 40% isocratic for 20 minutes, and then 40~100% ACN in another 50 minutes, with a flow rate of 15 ml/minute. Fractions were collected (13 ml/fraction) by a fraction



Sch601324 (**1**)



Arundifungin (**2**)

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collector. Pure **1** (~1.0 mg) was obtained with each 150 mg injection at retention time ~63 minutes as white amorphous powder. The procedure was repeated eight times, and all pure fractions containing **1** were combined to yield 7 mg of material.

From high-resolution negative ESI-MS, the molecular formula of **1** was established as $C_{32}H_{53}O_9S$ (Found 613.3409; calcd. 613.3415 for $[M]^-$) suggesting one sulfate group in the molecule (performed on a PE Sciex QSTAR mass spectrometer; two internal standards, taurocholic acid and nitrazepan, were used in the negative ion ESI-HR-MS measurements). The structure of **1** was further elucidated by extensive NMR data analysis. Thirty two carbons were observed in the ^{13}C NMR spectrum (Table 1), suggesting a triterpene skeleton with two additional carbons. Highly overlapped proton signals in ^1H NMR (Table 1) can be interpreted and assigned to their adjacent carbons by HSQC analysis. The triterpene skeleton was mainly determined by interpretation of HMBC data. The significant 2- or 3-bond H–C correlations were observed through the following seven methyl groups: H_3-19 (δ 0.95) to C-1 (δ 34.8), C-5 (δ 50.1), C-9 (δ 135.4), and C-10 (δ 36.4); H_3-18 (δ 0.72) to C-12 (δ 25.0), C-13, C-14 (δ 49.2 and 49.7), and C-17 (δ 84.1); H_3-28 (δ 1.12) to C-8 (δ 133.2), C-13, C-14, and C-15 (δ 31.1); H_3-29 (δ 0.83) and H_3-30 (δ 0.82) to C-3 (δ 79.9), C-4 (δ 37.4), and C-5; H_3-21 (δ 0.85) to C-17, C-20 (δ 42.6), and C-22 (δ 29.9); H_3-27 (δ 0.97) to C-24 (δ 75.5), C-25 (δ 73.1), and C-26 (δ 70.7) (see Figure 1). These long range correlations combined with carbon and APT data allow us to determine the boldfaced fragments shown in Figure 1. The fragment pattern is a typical pattern of 3-hydroxyl-lanostanoid skeleton. This was confirmed by the following connectivity obtained from ^1H-^1H COSY and HSQC-TOCSY (Figure 1): H_2-2 (δ 1.59) to H-3 (δ 4.36); H-5 (δ 1.06) to H_2-6 (δ 1.47, 1.64); H_2-1 (δ 1.18, 1.71) to H_2-2; H_2-6 to H_2-7 (δ 2.02); H_2-11 (δ 2.01) to H_2-12 (δ 1.35, 2.19); H_2-15 (δ 1.24, 1.62) to H_2-16 (δ 1.87).

After the ring skeleton was determined, the structure of the side chain was established by the same strategy. From HMBC correlations mentioned previously, C-24 was adjacent to a quaternary oxygenated carbon (C-25), which was substituted with a terminal methyl group (C-27) and an oxygenated methylene carbon (C-26). The connectivity of H_2-22, H_2-23, and H-24 was further resolved by HSQC-TOCSY (see Figure 1). Thus the full skeleton was concluded.

An acetate and a sulfate groups remained to be assigned to two of the four oxygen positions. The acetate was assigned to 3-*O* position and the sulfate to C-26 position based on the following evidence. Chemical shift of C-3 (δ

Table 1. NMR spectral data for compound **1** in DMSO-d_6[a].

C/H no.	^1H (δ)	^{13}C (δ)
1α	1.18, m	34.8 t
1β	1.71, m	
2	1.59, m	23.8 t
3	4.36, dd, J = 7.7, 8.6	79.9 d
4		37.4 s
5	1.06, dd J = 12.7, 1.8	50.1 d
6	1.47, m	17.5 t
	1.64, m	
7	2.02, m	25.4 t
8		133.2 s
9		135.4 s
10		36.4 s
11	2.01, m	20.4 t
12	2.19, m	25.0 t
	1.35, dd J = 12.9, 8.7	
13		49.7 s*
14		49.2 s*
15	1.24, m	31.1 t
	1.62, m	
16	1.87, m	39.5 t
17		84.1 s
18	0.72, s	18.4 q
19	0.95, s	18.7 q
20	1.46, m	42.6 d
21	0.85, d, *J* = 6.6	14.4 q
22	0.96, m	29.9 t
	1.74, m	
23	0.94, m	29.2 t
	1.61, m	
24	3.32, m	75.5 d
25		73.1 s
26	3.55, d, *J* = 10.6	70.7 t
	4.72, t, *J* = 10.6	
27	0.97, s	21.4 q
28	1.12, s	26.6 q
29	0.83, s	27.7 q
30	0.82, s	16.4 q
CO_2CH_3	2.00, s	21.4 q
CO_2CH_3		170.1 s
24-OH	4.12 bs	

[a] Recorded on a Varian Unity 500 NMR instrument at 500 MHz for ^1H and 125 MHz for ^{13}C, using standard Varian pulse sequence programs (VNMR Version 6.1 Software). δ in ppm; *J* in Hz.

Fig. 1. 2D NMR correlations of **1**.

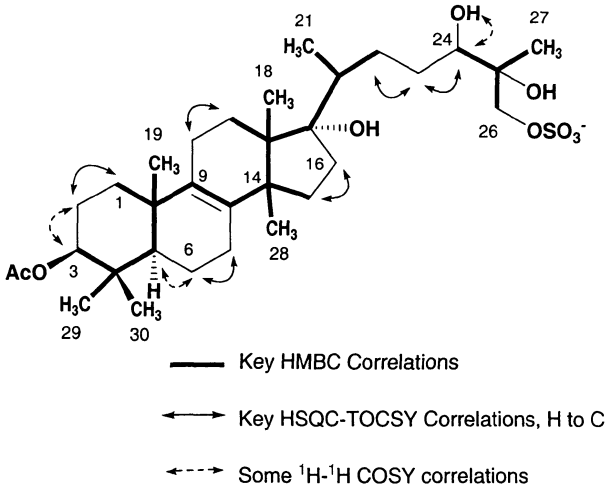
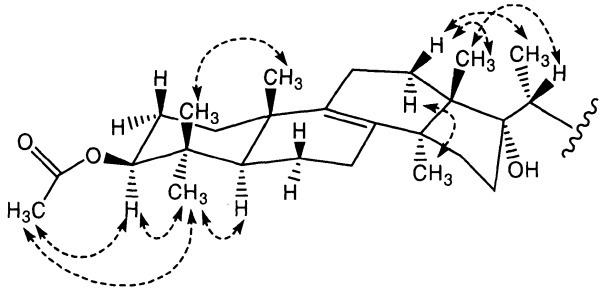


Fig. 2. Key NOE correlations of **1**.



79.9) and H-3 (δ 4.36) are typical for those of 3-β-*O*-Ac lanostanoid comparing to those of the known analogues.[5] In addition, NOE correlations of the acetyl methyl to H-3 and H_3-30 were observed in a ROESY spectrum. Thus, the acetate was assigned to 3-*O* position. Compared to the known analogues, significant downfield shift of chemical shifts of H_2-26 (δ 3.55, 4.72) and C-26 (δ 70.7) have confirmed that the sulfate group is on 26 position.[6,7]

The relative stereochemistry of **1** was determined by the analyses of the ^{1}H-^{1}H coupling patterns (Table 1) and NOE data. Key NOE correlations are shown in Figure 2. Hydroxyl substitution on C-17 was determined as α position as a result of the observation of γ gauge effect on C-12 (δ 25.0, Δ −6 ppm) and NOE correlations of H_3-18 to H-20 and H_3-21. Thus, the structure elucidation of **1** was completed. Sulfated and 17-hydroxyl substituted triterpenes are relatively unusual in microbial kingdom. The skeleton of Sch 601324 is closely related to arundifungin, whose stereochemistry was not addressed.[3,4]

Table 2. Antifungal activity of Sch 601324, MIC-48h (μg/ml).

Strain[c]	SCH601324	Caspofungin (control)
Candida albicans (FLZ-S)	1	<0.06
C.albicans (FLZ-R)	0.5 (1)[a]	<0.06
C.dubliniensis (FLZ-R)	8	<0.06
C.glabrata (FLZ-S)	0.1 (0.5)[a]	<0.06
C.glabrata (FLZ-R)	0.5 (1)[a]	<0.06
C.krusei	2 (16)[a]	0.125
Crypto.neoformans@	16	16
S.cereviseae (S.S.)	<0.06 (4)[a]	NT[b]

[a] MFC-24h in parentheses
[b] NT-Not Tested
[c] FLZ-S: fluconazole sensitive; FLZ-R: fluconazole resistant; S.S.: super sensitive strain

Compound **1** exhibited antifungal activity against various *Candida* strains. The MIC values were shown in Table 2. Standard compound, caspofungin, showed lower MIC values when tested under the same conditions. Sch 601324 did not show any inhibitory effect against tested Aspergillus strains and bacterial strains such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia faecalis*, and *Escherichia coli*.

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