Sch 54445: A New Polycyclic Xanthone with Highly Potent Antifungal Activity Produced by *Actinoplanes* sp.

Min Chu,* Imbi Truumees, Ronald Mierzwa, Joseph Terracciano, Mahesh Patel, David Loebenberg, James J. Kaminski, Pradip Das, and Mohindar S. Puar

Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, New Jersey 07003

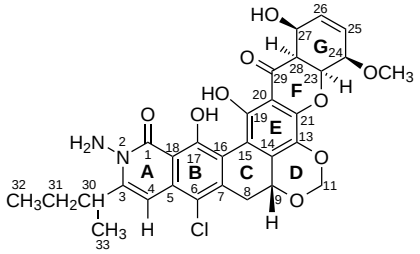
Received November 22, 1996[⊗]

A novel antifungal agent, Sch 54445, was isolated from the fermentation broth of an *Actinoplanes* species. Sch 54445 was identified as a polycyclic xanthone related to the albofungin family of compounds on the basis of analyses of spectroscopic data. As a broad-spectrum antifungal agent, Sch 54445 exhibits highly potent activities against various yeasts and dermatophytes with MIC values ~0.000 38 μg/mL.

The clinical cases of opportunistic mycotic infections associated with acquired immune deficiency syndrome (AIDS) have increased tremendously in the last two decades.[1] Some antifungal drugs, such as polyene antibiotics and azole analogues, are currently used in antifungal therapies with certain limitations due to various side effects.[2,3] Therefore, the development of novel, more effective, and less toxic broad-spectrum antifungal agents is required for the treatment of both common and rare fungal infectious diseases.[4] In our continued search for new antifungal natural products, a novel secondary metabolite, Sch 54445 (**1**, Figure 1), was discovered from the fermentation culture broth of an *Actinoplanes* sp. (SCC 2314, ATCC 55600), which was collected in Tarlac on the Philippine Islands.[5] Compound **1** possesses a polycyclic xanthone structural feature and exhibits very potent antifungal activity against a variety of fungal pathogens including yeasts, dermatophytes, and *Aspergillus*. In this paper, the isolation, structure elucidation, and biological activity of **1** will be presented.

The isolation of **1** was accomplished by three consecutive LH-20 size-exclusion gel chromatographies with a CH_2Cl_2-MeOH solvent mixture in three different ratios to afford pure **1** as yellow-brown needles; mp = 201−203 °C dec; $[\alpha]^{23}_D = -558°$ (*c* 0.2, $CHCl_3$). The IR spectrum of **1** indicated the presence of hydroxyl, conjugated amide carbonyl, and γ-pyrone functionalities due to strong absorption bands at 3450, 1640, and 1570 cm^{-1}, respectively. UV absorptions at 215, 251, 270 (sh), 326, and 393 nm further suggested the presence of a polycyclic xanthone moiety. The molecular weight of **1** was determined to be 596 on the basis of mass spectral data that showed a protonated molecular ion at *m/z* 597 $(M + H)^+$ in Cs^+ liquid SIMS. A chlorine-containing ion cluster was observed (the ratio of intensity for *m/z* 597 and 599, 3:1). The molecular formula was deduced as $C_{30}H_{29}N_2O_9Cl$ by means of high-resolution Cs^+ liquid SIMS [*m/z* 597.1609 $(M + H)^+$, Δ5.1 mmu], together with ^1H and ^{13}C NMR data (Table 1). Elemental analysis of **1** also provided confirmatory evidence for the above formula (*anal.* C 60.04%, H 4.87%, N 4.70%, Cl 5.87%, calcd for $C_{30}H_{29}N_2O_9Cl$, C 60.05%, H 4.79%, N 4.44%, Cl 5.46%).

The ^1H NMR spectrum of **1** contained resonances for a methyl triplet at δ 0.98, a methyl doublet at δ 1.33, a methylene AB multiplet at δ 1.60 and 1.80, and a methine multiplet at δ 3.45 indicating the presence of a *sec*-butyl group. COSY data confirmed the CH_3CH_2-



1

Figure 1. Proposed structure of Sch 54445 (**1**).

Table 1. ^1H, ^{13}C, and NOESY Data for **1**[a−c]

position	^{13}C (δ)	^1H (δ)	NOESY
1	164.3 s		
2		4.87 br. s (NH_2)[d]	H-30
3	150.4 s		
4	100.0 d	6.80 s	H-30, H-31, H-33
5	133.5 s		
6	116.6 s		
7	148.0 s		
8	33.2 t	2.68_{ax} dd (12.9, 14.7)	$H\text{-}8_{eq}$
		3.82_{eq} dd (4.8, 14.7)	$H\text{-}8_{ax}$, H-9
9	72.2 d	4.78 dd (4.8, 12.9)	$H\text{-}8_{eq}$, $H\text{-}11_{ax}$
11	90.5 t	5.22_{ax} d (5.9)	H-9, $H\text{-}11_{eq}$
		5.50_{eq} d (5.9)	$H\text{-}11_{ax}$
13	132.9 s		
14	130.9 s		
15	109.4 s		
16	114.6 s		
17	155.5 s	13.44 br. s (OH)[d]	
18	109.7 s		
19	151.7 s	11.84 s (OH)[d]	
20	106.7 s		
21	137.9 s		
23	77.7 d	4.49 dd (3.7, 14.2)	H-24, H−27, H-28
24	71.7 d	4.15 dd (3.7, 8.4)	H-23, H-25, OCH_3-24
25	133.0 d	6.00 dddd (1.8, 1.8, 3.7, 10.1)	H-24, H-26
26	124.1 d	5.95 dd (1.8, 10.1)	H-25, H-27
27	67.5 d	4.60 br. d (8.5)	H-23, H-26, OH-27
28	46.5 d	3.45 dd (8.5, 14.2)	H-23
29	200.4 s		
30	35.4 d	3.45 m	NH_2-2, H-4, H-33
31	28.5 t	1.60, 1.80 AB, m	H-32
32	11.2 q	0.98 t (7.4)	H-30, H-31
33	18.7 q	1.33 d (6.8)	H-30
24-OCH_3	58.0 q	3.62 s	H-24

[a] Recorded at 400 MHz in $CDCl_3$, chemical shift (δ) in ppm from TMS. [b] Multiplicity was determined by DEPT data. [c] Coupling constants in parentheses (Hz). [d] Exchangeable with CD_3OD.

[⊗] Abstract published in *Advance ACS Abstracts,* April 15, 1997.

S0163-3864(96)00737-9 CCC: $14.00

Table 2. HMBC Data for **1**

proton	two-bond correln	three-bond correln	four-bond correln
NH_2-2		C-1, C-3	
H-4	C-3	C-6, C-18, C-30	C-1, C-17
H-8_{ax}[a]	C-9	C-6, C-16	C-1, C-17
H-8_{eq}[a]	C-9	C-6, C-16	C-5
H-9	C-8	C-13, C-15	C-19, C-21
H-11_{ax}		C-9, C-13	
H-11_{eq}		C-9, C-13	
OH-17	C-17	C-16, C-18	
OH-19[b]	C-19	C-15, C-20	C-29
H-23	C-24, C-28	C-27, C-2 9	
H-24	C-23, C-25	C-26, C-28, OCH_3-24	C-29
H-25	C-24	C-23, C-27	
H-26	C-27	C-24, C-28	C-23
H-27	C-26, C-28	C-25, C-29	
H-28	C-23, C-27, C-29	C-24	
H-30	C-3, C-31, C-33	C-4, C-32	
CH_2-31	C-30, C-32	C-3, C-33	
CH_3-32	C-31	C-30	
CH_3-33	C-30	C-3, C-31	

[a] The 5-bond correlations of H-8_{ax} and H-8_{eq} to C-21 were observed. [b] A four-bond correlation of OH-19 to C-29 was observed as a weak cross peak.

(CH_3)CH moiety due to the correlations of these proton signals. Two sets of doublet of doublets at δ 2.68 and 3.82 from a methylene group were connected to a doublet of doublets methine signal at δ 4.78, indicating a CH_2CHO group based on analyses of COSY data. A dioxymethylene moiety (OCH_2O) was also observed as an AB spin system at δ 5.22 and 5.50. A 1,4-disubstituted cyclohexene ring was assigned because of the cross couplings between four methine protons at δ 3.45, 4.15, 4.49, and 4.60 and two vinyl protons at δ 5.95 and 6.00 in the COSY spectrum. In addition, a singlet at δ 3.62 revealed the presence of a methoxyl group, and an aromatic proton singlet at δ 6.80 was observed. Two methine proton signals overlapped at δ 3.45 on the basis of HETCOR data as well as the proton integration measurements.

The ^{13}C NMR data (Table 1) indicated the presence of two methyl (δ 11.2 and 18.7), a methoxy (δ 58.0), two methylene (δ 28.5 and 33.2), two methine (δ 35.4 and 46.5), four oxymethine (δ 67.5, 71.7, 72.2, and 77.7), a dioxymethylene (δ 90.5), and three olefinic methine carbons (δ 100.0, 124.1, and 133.0). The data also revealed a total of 13 aromatic/olefinic quaternary carbons (δ 106.7−155.5), an amide carbonyl (δ 164.3), and a conjugated carbonyl carbon (δ 200.4). All multiplicity assignments were supported by DEPT and HETCOR experiments.

The precise connectivities of **1** were established by interpretation of HMBC data summarized in Table 2. The presence of a *sec*-butyl group was confirmed by the observation of couplings of H-30 to C-31, C-32, and C-33. Furthermore, this carbon chain appeared to attach to the A ring at C-3 because of the correlations of H-30 to C-3 and C-4, H-31 to C-3, and H-33 to C-3. Since NH_2 protons correlated to C-1 and C-3, the primary amino functionality was connected to the amide nitrogen on the A ring. HMBC data showed that H-4 coupled to C-1, C-3, C-6, C-17, C-18, and C-30. These correlations not only supported previous assignments but also indicated the presence of the lactam A ring in conjunction with the highly substituted aromatic B ring. The OH signal at δ 13.44 was coupled with C-16, C-17, and C-18; therefore, this OH was located at C-17 on the B ring. A downfield chemical shift of OH-17 suggested a hydrogen bond to the amide carbonyl oxygen at C-1. Correlations of methylene H-8_{ax} and H-8_{eq} to C-5, C-6, C-9, C-16, and C-17 further revealed that the B ring had a fused-ring junction with the C ring through C-7 and C-16. Therefore, the chlorine atom was assigned to the only remaining aromatic quaternary carbon at C-6. Correlations of the dioxymethylene H-11 to C-9 and C-13 and the oxymethine H-9 to C-8, C-13, C-15, C-19, and C-21 allowed assignments to extend from C to D and E rings. In addition, the OH singlet at δ 11.84 exhibited cross peaks to C-15, C-19, and C-20. These couplings indicated the position of this OH at C-19 on the E ring. A four-bond coupling of OH-19 to C-29 was also observed. This evidence in combination with a downfield chemical shift of OH-19 indicated a hydrogen bond between OH-19 and the C-29 carbonyl oxygen and further suggested that a carbonyl group was connected to the E ring at C-20. Thus, the assignments for the A−E rings of structure **1** were completed.

HMBC data revealed that the H-24 signal (δ 4.15) correlated to C-23, C-25, C-26, and C-28, the H-27 doublet (δ 4.60) correlated to C-25, C-26, and C-28, and the H-28 signal (δ 3.45) correlated to C-23, C-24, and C-27. These data were consistent with COSY experiments indicating the formation of a 1,4-substituted cyclohexene, which is designated as the G ring. Other correlations of H-23 (δ 4.49), H-25 (δ 6.00), and H-26 (δ 5.95) to their vicinal carbons from C-23 to C-28 also supported the assignments for the G ring. Furthermore, the OCH_3 signal at δ 3.62 cross-linked to C-24, while H-24 coupled to the methoxy carbon, indicates the position of OCH_3 group at C-24. In addition, a coupling of H-28 to C-29 was observed; therefore, the carbonyl group should connect to C-28 on the G ring. C-28 was the only nonoxygenated aliphatic methine carbon assignable for a carbonyl attachment. Since both E and G rings appeared to have two open ends to join together, a cyclic formation was a logical connection in order to match unsaturation of the molecule. Therefore, a dihydropyrone was assigned between the E and G rings as the F ring to complete the structural elucidation for **1**.

The stereochemistry of **1** at H-9 was determined on the basis of analyses of the circular dichroic (CD) and NOESY spectral data in comparison with related compounds. The CD spectrum of **1** showed a positive cotton effect at the 220−270 nm region and two negative cotton effects at the 200−220 and 270−380 nm regions, which is very similar to the CD curve of albofungol, a degradation product from albofungin reported in literature.[6] Therefore, the same stereochemistry at H-9 for **1** was assigned as axial (β-position) accordingly since the absolute stereochemistry of albofungol was well established.[6−8] In addition, NOESY and difference NOE experiments supported the above assignment by observation of correlations from H-8_{eq} to H-9 and from H-11_{ax} to H-9, which were further inspected by using a three-dimensional model of **1** generated from computer simulation as shown in Figure 2.

The relative stereochemistry of the G ring was established by studies of NOESY and difference NOE data. The NOE correlations of H-23 to H-24 and H-28, H-28 and to H-23 and H-27 clearly suggested that these

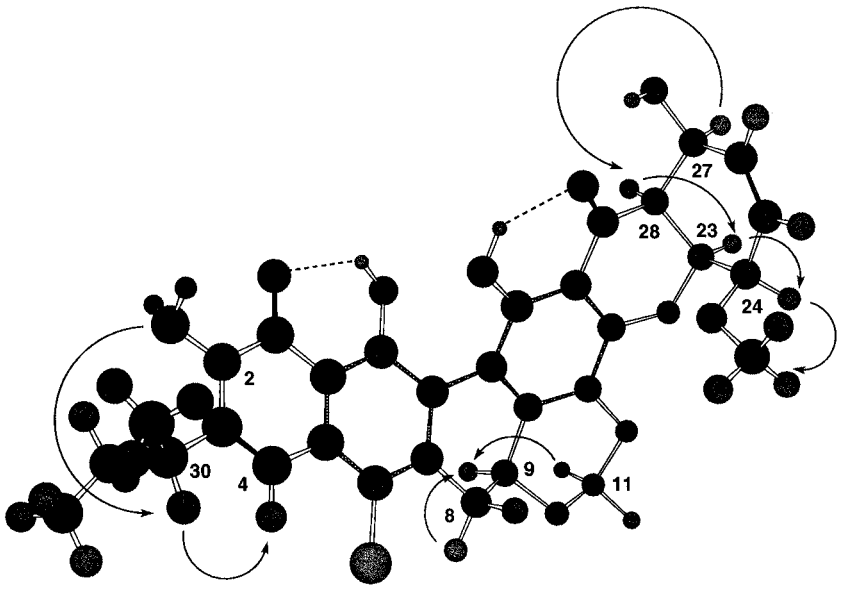


Figure 2. Some important NOE correlations of **1** observed from NOESY and difference NOE experiments.

four methine protons have an *all-cis* configuration on the G ring. The methoxy group at C-24 was also confirmed by NOESY data showing a correlation between the H-24 and OCH_3 signals.

Heats of formation for three conformational G ring isomers of **1** were calculated using the AM 1 molecular orbital approximation method. An isomer of **1** in which the G ring adopts a pseudoboat conformation was the most stable one, $H_f = -255.2$ kcal/mol. Isomers of **1** in which the G ring adopts a pseudochair conformation, $H_f = -252.7$ kcal/mol, and a pseudotwistboat conformation, $H_f = -250.2$ kcal/mol, were less stable by 2.5 and 5.0 kcal/mol, respectively. As depicted in Figure 2, only the lowest energy pseudoboat conformation orients the four methine protons of the G ring in a pseudoaxial position. The stereochemistry of H-23, H-24, H-27, and H-28 on the G ring related to H-9 (*syn* or *anti* configuration), however, was unable to be determined by NMR methods due to the remote distance between the G ring and H-9. In addition, since the CD spectrum of **1** was not quite comparable to albofungin because of the saturation of a double bond at C-23 and C-28 in the γ-pyrone F ring that changed the planar chromophore of **1** in comparison to albofungin, the relative stereochemistry of the G-ring versus H-9 could not be established by a CD correlation study. The *anti* configuration of four protons on the G ring versus H-9, however, was proposed on the basis of the analysis of optical rotation data. Large negative values of optical rotation for **1** $[\alpha]^{23}_D = -558°$ ($CDCl_3$) and albofungin $[\alpha]^{26}_D = -670°$ (DMF) suggests the same configuration for both compounds.[9]

The antifungal activity of **1** against various yeasts and dermatophytes is shown in Table 3. The potency of **1** was superior to its related compounds, Sch 42137[10] and albofungin, against most yeasts and dermatophytes. More impressively, **1** demonstrated better activity than both Sch 42137 and albofungin (MIC 0.025 μg/mL) against *Aspergillus*, which is a clinically important pathogen of fungal infections. Like its related compounds, **1** also displayed *in vivo* toxicity with an LD_{50} = 1 mg/kg iv in mice.

Table 3. *In Vitro* Activity of **1** and Related Compounds against Various Fungi

fungi[a]	no. of strains	Geometric mean MICs (mg/mL) in various media		
		1	Sch 42137	albofungin
Y (SDB)	12	~0.00038	~0.125	~0.00055
D (SDB)	7	0.00764	~0.125	0.0359
A (SDB)	6	0.025	b	0.125
Y (EMEM)	9	0.5−6.4	16	~0.125

[a] Y = yeast (SDB) = six strains of *Candida albicans*; two strains each *C. tropicalis*, C. *stellatoidea*, and *C. parapsilosis*. D = dermatophytes (SDB) = two strains each of *Trichophyton mentagrophytes*, *T. rubrum*, and *T. tonsurans*; 1 strain of *Microsporum canis*. A = aspergillus (SDB) = two strains of *Aspergillus flavus*; 1 strain of *A. niger*; and 1 strain of *A. fumgatus*. Y = yeast (EMEM) = six strains of *C. albicans*; two strains of *C. tropicalis*; 1 strain of *C. stellatoidea*. SDB = Sabouraud dextrose broth, pH = 5.7. EMEM = Eagle's minimum essential medium, pH = 7.0. [b] Not tested.

Experimental Section

General Experimental Procedures. The melting point was recorded on a MEL-TEMP apparatus and is uncorrected. Optical rotation was measured on a Perkin-Elmer 243B polarimeter. IR and UV spectra were obtained using a Nicolet FTIR model 10-MX and Hewlett-Packard '8450A' UV−vis spectrophotometer, respectively. Cs^+ liquid SIMS data were produced by VG ZAB-SE mass spectrometer in a glycerol−thioglycerol matrix. Both ^1H and ^{13}C NMR spectra were obtained using Varian XL-400 instruments operating at 400 and 100 MHz, respectively.

Organism and Fermentation. The microorganism was isolated from soil from the town of Tarlac on the Philippine Island of Luzon with egg white media (egg whites 3.0 g, Dow Corning AF-2 1 mL, agar 15.0 g, and 1 L of tap H_2O). It was identified as an *Actinoplanes* sp. and deposited in the American Type Culture Collection (ATCC 55600). The fermentation was initiated by the production of an inoculum, which was produced in two stages with inoculum medium A (beef extract 3 g/L, tryptone 5 g/L, yeast extract 5 g/L, cerelose 1 g/L, soluble starch 24 g/L, $CaCO_3$ 2 g/L, tap H_2O 1 L, antifoam 1 mL/L, presterilization pH 7.0). Inoculum preparation was carried out for large-scale fermentation

(8 L). Two and a half mL of freshly thawed whole broth were used to inoculate 70 mL of medium A. The 250 mL Erlenmeyer flasks were incubated at 30 °C for 96 h on a shaker at 250 rpm having a 2 in. throw.

A 250 mL Erlenmeyer flask containing 70 mL of sterile inoculum medium A was inoculated using 5% of the first stage inoculum. The procedure for the first inoculum stage was followed.

A 2 L Erlenmeyer flask containing 500 mL of sterile fermentation medium B (soluble starch 15 g/L, sucrose 5 g/L, dextrose 5 g/L, soy peptone 7.5 g/L, corn steep liquor 5 mL/L, K_2HPO_4 1.5 g/L, NaCl 0.5 g/L, mineral solution 10 mL/L, tap H_2O 1 L, presterilization pH 7.0) was inoculated using 5% of second-stage inoculum. The 2 L Erlenmeyer flask was incubated at 30 °C for 96 h on a shaker at 250 rpm having a 2 in. throw. After 24 h, 50 g wet weight of washed sterile XAD-16 resin was added to the 2 L flask, and fermentation was continued for the remaining 72 h. The antifungal activity was monitored during the course of fermentation by disking on *Candida albicans* agar diffusion plates.

Isolation. The XAD-16 resin (800 mL), which was introduced during the fermentation process to stabilize and absorb the antifungal active components, was separated from the culture broth (8 L) by paper filtration. After removal of inactive filtrate, XAD-16 resin was thoroughly washed with water. The active resin was extracted with EtOAc (2 × 2 L). The EtOAc solution was dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo*. The residue (1.9 g), which was redissolved in CH_2Cl_2–MeOH (7:3), was loaded on a Sephadex LH-20 column and eluted with CH_2Cl_2–MeOH (7:3). The combined antifungal active fractions (1 g) were further chromatographed on two consecutive LH-20 columns by a CH_2Cl_2–MeOH solvent mixture with two different ratios (1:1 and 3:7), respectively. Pure **1** (30 mg) was obtained as a yellow-brown solid. In this process, some restrictions should be noted. The antifungal components were unstable in aqueous media; therefore, the culture broth was extracted as soon as it was harvested, and only organic solvent was utilized in isolation. Degradation of **1** occurred by using reversed-phase chromatography. In addition, silica gel column was not suitable for the separation because of observed decomposition under acidic conditions.

Antifungal Assays. Activities were determined in microtiter minimum inhibitory concentration (MIC) tests. Media employed in these tests were Sabouraud dextrose broth (SDB) (Difco, Detroit, MI) and Eagles Minimum Essential Medium with nonessential amino acids, L-glutamine and fetal bovine serum added (EMEM) (Whittaker Bioproducts Inc., Walkerville, MD). Yeasts were grown overnight in SDB at 28 °C with shaking and inocula adjusted in sterile saline using a spectrophotometer at 540 nm. Mycelial fungi were grown in SDB with shaking at 28 °C for 4 days. Compounds were serially diluted in media in 96 well microtiter plates (Falcon, Lincoln Park, NJ). SDB plates were incubated at 28 °C, and plates containing EMEM were incubated at 37 °C under 5% CO_2 and 48 h. MICs in SDB were defined as the lowest concentrations of drug to prevent growth or, in EMEM, the transformation from the yeasts to mycelial phase of growth.

Acknowledgment. The authors are grateful to Mr. Y.-H. Ing for low-resolution mass spectral data, Mr. S. Mittelman for CD and IR data, Mr. G. Torraca for optical rotation and elemental analysis data, and Ms. D. Scott for the preparation of this manuscript.

References and Notes

(1) Greene, S. I. Treatment of Fungal Infections in the Human Immunodeficiency Virus–Infected Individual. In *Antifungal Drug Therapy*; Jacobs, P. H., Nall, L., Eds.; Marcel Dekker: New York, 1990; Chapter 23, p 237.

(2) For a review on antifungal agents, see: Medoff, G.; Kobayashi, G. A.; Scholer, H. J.; Holt, R. J.; Davies, R. R.; Speller, D. C. E. Antifungal Agents. In *Antifungal Chemotherapy*; Speller, D. C. E., Ed.; John Wiley & Sons: New York, 1980; Part I, p 3.

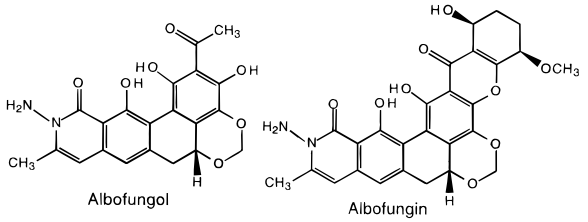
(3) Cauwenbergh, G. *Acta Derm. Venerol.* **1986**, *121*, 147–156.

(4) Janssen, P. A.; Cauwenbergh, G. Antifungal Therapy of the Future. In *Antifungal Drug Therapy*; Jacobs, P. H., Nall, L., Eds.; Marcel Dekker: New York, 1990; Chapter 28, p 315.

(5) The microorganism, *Actinoplanes* sp. SCC 2314, was deposited in the collection of the American Type Culture Collection (ATCC) in Rockville, MD, where it has been assigned accession no. ATCC 55600.

(6) Gurevich, A. I.; Deshko, T. N.; Kogan, G. A.; Kolosov, M. N.; Kudryashova, V. V.; Onoprienko, V. V. *Tetrahedron Lett.* **1974**, 2801–2804.

(7) The CD spectrum of albofungol displayed a positive cotton effect at 220–250 nm region and two negative cotton effects at 200–220, 250–370 nm region. The similar CD spectra of **1** and albofungol could be well explained: both compounds possess the same planar chromophore, which is critical to electric dipole transition measurements in CD spectra.



(8) Onoprienko, V. V.; Kozmin, Y. P.; Kolosov, M. N. *Bioorg. Khim.* **1978**, *4*, 1418–1422.

(9) The value of the optical rotation of **1** should change significantly or even the sign could be changed to a positive number if the stereochemistry of the G ring versus H-9 was in the *anti* configuration. However, it should be noted that in order to establish stereochemistry conclusively without any ambiguity, as well as to assign the configuration of the asymmetric center at C-30 on the butyl group, an X-ray crystallographic study should be conducted. Several attempts unfortunately failed to make a crystal suitable for X-ray analysis. Different approaches, such as derivatization of **1** for X-ray crystallography, are in progress.

(10) Cooper, R.; Truumees, I.; Gunnarsson, I.; Loebenberg, D.; Horan A.; Marquez, J.; Patel, M.; Gullo, V.; Puar, M.; Das, P.; Mittelman, S. *J. Antibiot.* **1992**, *45*, 444–453.

NP960737V