Inhibition of Fungal Sphingolipid Biosynthesis by Rustmicin, Galbonolide B and Their New 21-Hydroxy Analogs

Guy H. Harris*, Ali Shafiee, M. Angeles Cabello, James E. Curotto, Olga Genilloud, Kent E. Göklen, Myra B. Kurtz, Mark Rosenbach, Peter M. Salmon, Rosemary A. Thornton, Deborah L. Zink and Suzanne M. Mandala

Department of Natural Products Drug Discovery, Merck Research Laboratories, R80Y-340, P.O.Box 2000, Rahway, New Jersey 07065, U.S.A.

(Received for publication March 30, 1998)

The mode of action of the known antifungal macrolides rustmicin (**1**) and galbonolide B (**2**) has been determined to be the inhibition of sphingolipid biosynthesis. A large scale fermentation and isolation process was developed for production of large quantities of rustmicin. New 21-hydroxy derivatives of both compounds were isolated from pilot scale fermentations and were also produced by biotransformation of rustmicin and galbonolide B.

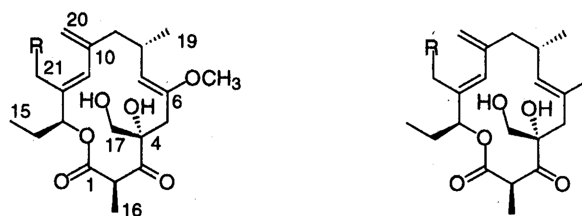
Natural products are a rich source of sphingolipid biosynthesis inhibitors. The sphingofungins[1,2], lipoxamycin[3], myriocin[4] and viridiofungins[5,6] inhibit serine palmitoyltransferase, the first committed enzyme of sphingolipid synthesis. Fumonisin B1[7] and australifungin[8,9] inhibit sphinganine *N*-acyltransferase (ceramide synthase). All of these natural products have antifungal activity. These early steps of sphingolipid synthesis have mammalian counterparts and thus are less attractive as targets for antifungal therapy. The first fungal-specific enzyme in sphingolipid biosynthesis is inositol phosphoceramide (IPC) synthase. It catalyzes the transfer of phosphoinositol from glycerophosphatidylinositol to the C1 hydroxyl of ceramide to produce inositol phosphoceramide which is further elaborated in fungi. We have targeted the fungal specific steps of sphingolipid synthesis in our natural product screening program with the goal of obtaining a broad spectrum, fungicidal agent without mammalian toxicity. In this report we demonstrate that the known antifungal macrolides rustmicin (**1**) and galbonolide B (**2**) inhibit fungal sphingolipid biosynthesis. Their new 21-hydroxy derivatives are also described.

Experimental

General Methods

$^{1}H/^{13}C$ NMR spectra were recorded at 25°C in C_6D_6, unless otherwise noted, at either 100/400 or 125/500 MHz on a Varian Unity spectrometer equipped with a Nalorac inverse detection microprobe. Chemical shifts are reported downfield from TMS and spectra were referenced to the solvent peak. UV spectra were recorded on a Beckman model DU70 spectrophotometer. IR spectra were recorded as a thin film on a ZnSe multiple internal reflectance crystal using a Perkin Elmer model 1750 spectrophotometer. MS were recorded on a JEOL SX-102A (electron impact, EI, 90 eV) mass spectrometer. Exact mass measurements were performed at high resolution (HR-EI) using perfluorokerosene (PFK) as an internal standard. Optical rotations were measured on a Perkin Elmer model 241 polarimeter.

Fig. 1. Structures of compounds **1**~**4**.



1 R=H **2** R=H

3 R=OH **4** R=OH

Micromonospora sp. (MA 7094), UV Mutant (MA 7186)

The isolation, taxonomy and shake flask fermentation of these cultures is described in the accompanying paper[10].

Pilot Scale Fermentation of MA 7186

MA 7186 was grown through three seed stages in ATCC+BE+BP medium of the following composition per liter: glucose monohydrate (CPC) 11 g; soluble starch (Difco) 20 g; yeast extract (8005, Springer) 5 g; NZ Amine A (Sheffield) 5 g; beef extract paste (Difco) 3 g; peptone (Difco) 5 g; $CaCO_3$ 1 g. The pH was adjusted to 7.0 before addition of the $CaCO_3$. Approximately 2 ml/liter of antifoam (UCON LB625, Union Carbide) was added to medium used in fermentors to minimize foaming. The medium was sterilized at 121~123°C for 35 minutes. The first seed stage (4 days) was conducted in 250 ml shake flasks (50 ml medium, 1 ml frozen vegetative mycelium as inoculum), and the second seed stage (2 days) was conducted in 2 liter shake flasks (500 ml medium, 10 ml inoculum). Both shake-flask stages were run at 28°C, 200 rpm on an orbital shaker with 2″ throw. The third seed stage (2 days) was conducted in 300 liter fermentors (180 liter medium, 2 liter inoculum). The fermentors were run at 28°C, 0.5~0.7 psi, 50~100 slpm, 150~250 rpm.

The production medium was KHC-14, of the following composition per liter: dextrin (Stadex 60K, A.E. Staley) 25 g; primary yeast (Champlain) 14 g; tomato paste (Heinz) 4 g; $CoCl_2 \cdot 6H_2O$, 5 mg; antifoam (UCON LB625, Union Carbide), 2 ml. The pH was adjusted to 7.2, and the medium was sterilized at 122~123°C for 30~35 minutes. The production fermentations were conducted in 800 liter fermentors (600 liter medium, 25 liter inoculum). The fermentors were initially run at 28°C, 3445 Pa, 50 slpm, 100 rpm, with the pressure, airflow, and agitator speed increased (to 10330 Pa, 550 slpm, and 350 rpm respectively) during the fermentation to maintain a minimum dissolved oxygen concentration of 50% of atmospheric saturation. After 100 hours of cultivation, the pH was kept below 6.3 with H_2SO_4. The fermentation was harvested after 152 hours, with titers of **1** between 45 and 95 mg/liter.

Pilot Scale Isolation of Crude Mixture of 1~4

Whole broth (2869 liter) of MA 7186 fermentation described above containing 126 g of **1** was chilled to 2~6°C and the pH adjusted from 6.59 to 5.49 by addition of conc. H_2SO_4. The broth was then extracted with methanol (2831 liter) with stirring for 1 hour and the solution again chilled. The MeOH/broth extract was harvested through a disk-stack centrifuge at 11.3 liter/minute to yield a clarified extract (5148 liter). The clarified methanol extract was adjusted to pH 5.6 with H_3PO_4 and charged at 8.7 liter/minute onto a column of Mitsubishi SP207 (V_b=246 liter) which had been previously equilibrated with a solution of methanol-25 mM NH_4OAc, 1:1 (v/v) pH 5.5. The column was washed with methanol-NH_4OAc buffer, 1:1 (v/v), 189 liter and then MeOH-buffer, 73:27 (v/v), 1476 liter. Compounds **1~4** were eluted from the resin at 10.2 liter/minute with MeOH (1230 liter). Fractions containing compounds **1~4** were concentrated *in vacuo* to 30.2 liter and contained 92 g (73%) of **1**.

Isolation of 1 and 2

SP207 rich cut described above (5.5 liters) was applied to a silica gel 60 (E. Merck, 230~400 mesh, V_b=10.5 liters) which had been equilibrated with hexane-EtOAc-H_2O (75:25:1). The column was eluted with the same solvent (8 liters) at 400 ml/minute collecting 1 liter fractions. Fractions 21~25 contained **2** and fractions 49~63 contained **1**. Fractions 49~63 were combined and conc. *in vacuo* to a syrup which was dissolved in MeOH (final vol. = 565 ml). This solution was diluted with an equal volume of methanol (565 ml) and then H_2O (1100 ml) was added with vigorous stirring at room temp. Crystallization occurred upon standing at −20°C. The crystals were collected by filtration, washed with H_2O and dried to yield **1** (10.1 g). **2** (9.2 g) was similarly obtained from fractions 21~25.

Isolation of 3 and 4

SP207 rich cut described above (2 liters) was applied to a silica gel 60 (E. Merck, 230~400 mesh, 1 kg) which had been equilibrated with hexane-EtOAc-H_2O (75:25:1). The column was eluted with the same solvent (8 liters) at 200 ml/minute. The column was then sequentially eluted with hexane-EtOAc-H_2O (67:33:1, 8 liters) and hexane-EtOAc-H_2O (50:50:1, 4 liters). All fractions were 250 ml except 1~4 which were 1 liter. Fractions 40~48 contained crude **4** and fractions 49~64 contained **3**. A portion (50%) of crude compound **4** was subjected to preparative RP HPLC on C8, 9.4×250 mm, MeOH-25 mM NH_4OAc, pH 4.5 (60:40) at 3.5 ml/minute, 40°C. Fractions containing **4** were combined, desalted by extraction into CH_2Cl_2 and dried to yield **3** (19.5 mg). A portion (50%) of crude **3** was similarly purified to yield **3** (33.2 mg).

21-Hydroxyrustmicin (3)

NMR assignments are shown in Table 2. HREI-MS m/z (rel. intensity, empirical formula): 378.2028 (0.7, $C_{21}H_{30}O_6$, M-H_2O), 364 (7.3, $C_{20}H_{28}O_6$, M-CH_3OH), 346.1774 (15.0, $C_{20}H_{26}O_5$), 302.1864 (16.2, $C_{19}H_{26}O_3$), 284.1766 (21.0, $C_{19}H_{24}O_3$), 263.1613 (39.5, $C_{16}H_{23}O_3$), 245.1536 (67.9, $C_{16}H_{21}O_2$), 165.0990 (67.7, $C_{10}H_{13}O_2$), 125.0574 (34.3, $C_7H_9O_2$), 120.0932 (67.0, C_9H_{12}), 105.0699 (73.4, C_8H_9). UV: λ_{max} (MeOH) = 235 nm. IR (ZnSe): 3451, 2964, 2931, 1708 (C=O), 1669, 1247, 1078, 1043 cm^{-1}. $[\alpha]_D^{25} = -263°$ MeOH (c, 1.13).

21-Hydroxygalbonolide B (4)

NMR assignments are shown in Table 2. HREI-MS m/z (rel. intensity, empirical formula): 380.2187 (12.7, $C_{21}H_{32}O_6$), 362.2096 (8.6, $C_{21}H_{30}O_5$), 279.1926 (10.9, $C_{17}H_{27}O_3$), 261.1847 (26.3, $C_{17}H_{25}O_2$), 243.1396 (24.8, $C_{16}H_{19}O_2$), 225.1125 (50.3, $C_{12}H_{17}O_4$), 204.1520 (75.8, $C_{14}H_{20}O$), 187.1462 (100, $C_{14}H_{19}$). UV: λ_{max} (MeOH) = 235 nm. IR (ZnSe): 3452, 2968, 2878, 1708 (C=O), 1456, 1381, 1250, 1018 cm^{-1}. $[\alpha]_D^{25} = -218°$ MeOH (c, 0.75).

Biotransformation of **1** and **2** and Isolation of **3** and **4**

Streptomyces sp. (MA 7165) was grown at 27°C in 250 ml Erlenmeyer flasks containing 50 ml of a medium consisting of dextrose 0.1%, dextrin 1.0%, beef extract 0.3%, ardamine pH 0.5%, NZ amine type E 0.5%, $MgSO_4 \cdot 7H_2O$ 0.005%, K_2HPO_4 0.037%, $CaCO_3$ 0.05%, pH 7.1 before autoclaving. After 40 hours 2 ml of this seed culture was transferred to each of 10 ~ 250 ml Erlenmeyer flasks containing 50 ml of a biotransformation medium consisting of glucose 2%, soya meal 0.5%, yeast extract 0.5%, NaCl 0.5%, MES 0.98%, pH 7.0 before autoclaving. After 40 hours the cells were harvested by centrifugation at 3750 rpm for 15 minutes. The cell pellet was washed with 0.1 M MES buffer, pH 5.5. Washed cells (10 g wet weight) were suspended in 0.1 M MES buffer, pH 5.5 (final volume 30 ml) and **1** or **2** (1 mg), dissolved in 0.1 ml MeOH, was added to the cell suspension. After 17 hours the cell suspension was extracted with an equal volume of MeOH.

The MeOH extract (52 ml) of the biotransformation culture to which **1** was added was concentrated *in vacuo* to remove MeOH and the solution extracted with CH_2Cl_2. RP HPLC of the CH_2Cl_2 extract on C8 (5 μm, 9.4 × 250 mm) using a mobile phase consisting of MeOH - 0.025 M NH_4OAc pH 4.5, 75 : 25 (v/v) at a flow rate of 4.0 ml/minute yielded **3** (340 μg, Rt = 12.8 minutes). Compound **4** was similarly purified from a biotransformation culture, to which **2** had been added, except that a mobile phase consisting of MeOH - 0.025 M NH_4OAc pH 4.5, 72 : 28 (v/v) at 4.0 ml/minute was used. This yielded **4** (1 mg, Rt = 16.0 minutes).

Antifungal Strains

Candida albicans MY1055, *Cryptococcus neoformans* MY2062, *Candida tropicalis* MY1012 and *Aspergillus fumigatus* MF5668 were obtained from the Merck Culture Collection, Rahway, N.J.

Sphingolipid Synthesis

Fungal cells were labeled with ^3H-inositol in microtiter plates and counts incorporated into sphingolipids and phosphatidylinositol were distinguished by alkaline methanolysis, as previously described[11].

Antifungal Assays

MICs were determined by microtiter broth dilution assay in Difco Yeast Nitrogen Base medium containing 2% glucose (YNBD) with fungi inoculated at $OD_{600} = 7 \times 10^{-4}$ (*ca.* 1×10^4 yeast cells or conidia/ml). Serial 2-fold dilutions of inhibitors were made from 200 μg/ml; the MIC value was the lowest concentration of inhibitor which prevented visible growth after 24 hours at 37°C (48 hours for *C. neoformans* strains).

Results and Discussion

Initial Isolation and Identification of Antifungal Components from Culture MA 7084

Methanol extracts of *Micromonospora* sp. culture MA 7094 were found to inhibit sphingolipid biosynthesis and exhibited potent antifungal activity, especially against *Cryptococcus neoformans* and non-*albicans* *Candida* species. A survey of a portion of this extract using gradient RP HPLC revealed one major antifungal zone, containing two components with a UV λ_{max} at 235 nm, and several, slightly more polar minor zones. The major antifungal zone was confirmed to inhibit sphingolipid biosynthesis. To identify the compound responsible for this activity, the methanol extract was concentrated *in vacuo* to remove the methanol and the remaining aqueous layer was extracted with CH_2Cl_2. Two active components corresponding to the major activity zone were purified from the CH_2Cl_2 layer using silica gel column chromatography and preparative reverse phase RP HPLC.

The most potent of the two compounds was identified as the antifungal macrolide rustmicin (galbonolide A), **1**. $^1H/^{13}C$ NMR, MS, UV spectral data and optical rotation for this isolate were identical to that reported

Table 1. Comparison of ^{13}C NMR chemical shift for **3** with galbonolide B and neorustmicin A.

3 (C_6D_6)	Galbonolide B[13] (C_6D_6)	Δ (3-galbonolide B)	**3** ($CDCl_3$)	Neorustmicin A[14] ($CDCl_3$)	Δ (3-rustmicin)
209.3	209.0	0.3	210.3	207.5	2.8
168.7	168.7	0	169.1	171.7	−2.6
144.2	144.2	0	143.7	144.4	−0.7
137.3	137.0	0.3	138.1	135.2	2.9
135.4	135.1	0.3	134.8	133.6	1.2
128.5	128.7	−0.2	129.1	133.0	−3.9
128.2	128.1	0.1	127.7	125.8	1.9
116.5	116.5	0	116.7	115.4	1.3
83.8	84.3	0.5	82.4	82.4	0
81.0	80.8	0.2	81.5	81.3	0.2
68.1	68.1	0	67.9	65.9	2.0
50.2	50.0	0.2	50.7	46.2	4.5
45.9	45.8	0.1	45.7	45.7	0
41.6	41.7	−0.1	41.9	45.1	−3.2
33.4	33.2	0.2	33.7	34.9	−1.2
26.4	26.4	0	25.9	25.3	0.6
19.6	19.5	0.1	19.8	21.6	−1.8
18.9	18.8	0.1	18.6	17.5	1.1
15.8	15.8	0	15.2	15.2	0
15.0	15.2	−0.2	14.5	12.9	1.6
10.0	9.9	0.1	10.0	10.1	−0.1

for both rustmicin[12] and galbonolide A[13]. Spectral data for the second component was identical to that reported for galbonolide B, **2**. The structure for this isolate of **2** was confirmed from extensive NMR (COSY, HMQC and HMBC) and X-ray crystallographic data. ^{13}C NMR data (Table 1) for this isolate are consistent with that reported for galbonolide B[13], but not with that reported for neorustmicin A[14]. The structure proposed for neorustmicin A by ABE, *et al.* is identical to that of galbonolide B. However it has been suggested that neorustmicin A is incorrect and more likely analogous to a minor galbonolide[15]. The data on this isolate of **2** support this suggestion. The absolute stereochemistry of both **1** and **2** were later determined by total synthesis[16,17].

Large Scale Isolation of 1 and 2 from Culture MA 7186

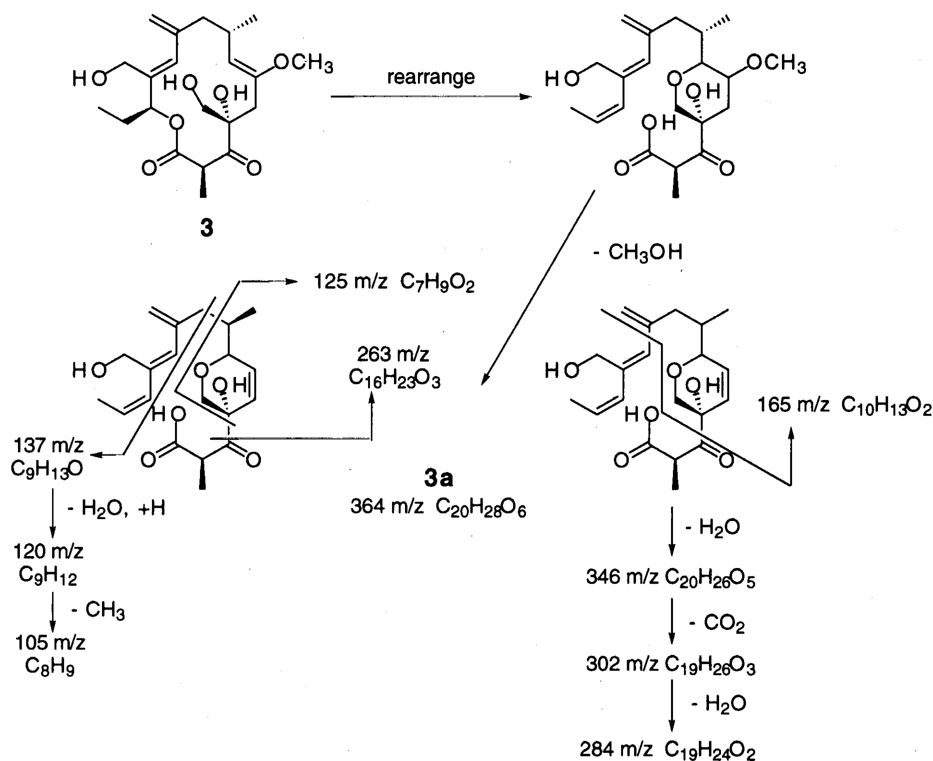
The original culture (MA 7094) yielded approximately 10 mg/liter of both **1** and **2**. Supply of sufficient quantities of **1** and **2** to support *in vivo* antifungal evaluation and derivative preparation required higher fermentation titers. Titers of **1** were increased 10-fold in shake flask fermentation by UV mutation of MA 7094, to yield culture MA 7186, combined with media optimization[10]. Fermentation of MA 7186 in 800 liter fermentors yielded 45~95 mg/liter of **1**. Both compounds, as well as numerous minor components, were recovered from the methanol extract of the culture by adsorption onto SP207 and elution with methanol. The concentrated SP207 rich cut was stored at −20°C and both **1** and **2** were stable indefinitely under these conditions. Purification of **1** required separation from an equivalent amount of **2** and from numerous minor components. This was accomplished using either low pressure silica gel column chromatography or preparative RP HPLC with a methanol or acetonitrile mobile phase buffered with NH_4OAc at pH 5.5. Following either separation, both **1** and **2** could be crystallized from acetonitrile-buffer mixtures.

Minor Components Produced by Culture MA 7186

Numerous minor components related to **1** and **2** were produced by both MA 7094 and mutant MA 7186. Only those more polar than **1**, as judged by RP HPLC and silica gel elution, inhibited sphingolipid biosynthesis and had antifungal activity. The two most abundant of these were isolated from the large scale SP207 rich cut described above using silica gel chromatography followed by preparative RP HPLC.

The structure of the first minor component, 21-hydroxyrustmicin (**3**), was determined from analysis of NMR (^1H, ^{13}C, COSY, HMQC and HMBC) and MS

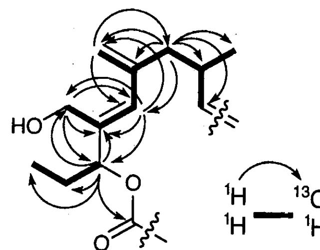
Fig. 2. EI-MS fragmentation of compound **3**.



data. A strong ion at 419 *m/z* [M+Na^+] was observed in the ESI-MS of **3** indicating a molecular weight of 396. A molecular ion for **3** was not observed in the HREI-MS (Figure 2) however ions at 378 *m/z* [M−H_2O] and 364 *m/z* [M−CH_3OH] (**3a**) were of sufficient intensity for HR-MS measurements. This information and the observation of 21 resonances in the ^{13}C NMR spectrum established a molecular formula of $C_{21}H_{32}O_7$ for **3**. Fragment ions in the HREI-MS (Figure 2) derived from **3a** containing C-21 are 16 mass units greater than the corresponding ions in the HREI-MS of **1** consistent with hydroxylation in this portion of the molecule. The ^1H NMR spectrum of **3** is very similar to that of **1** except that the C-21 methyl group at 1.69 ppm is replaced by a pair of AB doublets at 4.126 and 4.488 ppm. Critical COSY and HMBC correlations for this portion of **3** are shown in Figure 3 and complete NMR assignments are summarized in Table 2. **3** produced by MA 7186 was identical to **3** produced by biotransformation of **1** establishing the absolute stereochemistry of the natural product.

The structure of the second minor component, 21-hydroxygalbonolide B (**4**), was similarly determined. In contrast to **3**, a molecular ion was observed in the HREI-MS of **4** (found 380.2187, calc 380.2199) corresponding to the molecular formula of $C_{21}H_{32}O_6$. Fragmentation of between C-8 and C-9 yielded the same 137 *m/z* ion for the C-9 to C-15 hydroxylated portion of the molecule that was observed in the HREI-MS of **3** (Figure 2). The NMR data for the left side of **4** was very similar to that of **3** (Table 2). **4** produced by MA 7186 was identical to **4** produced by biotransformation of **2** establishing the absolute stereochemistry of the natural product.

Fig. 3. HMBC correlations for compound **3**.



Biotransformation of 1 and 2

Random screening of *Streptomyces* sp. cultures for the ability to biotransform **1** and **2** yielded a culture (MA

Table 2. ^{1}H and ^{13}C NMR assignments for **3** and **4** in C_6D_6.

Position	δ ^{1}H		δ ^{13}C	
	3	**4**	**3**	**4**
1	—	—	169.2	169.1
2	3.712 (q, 7.0)	3.776 (q, 6.5)	50.2	49.4
3	—	—	207.7	208.5
4	—	—	83.6	84.5
5	2.485 (d, 14.9) 2.274 (d, 14.9)	2.674 (d, 14) 1.852 (d, 14)	33.1	41.0
6	—	—	148.8	128.2
7	4.671 (d, 9.4)	5.166 (br d, 9.5)	121.8	136.3
8	2.93 (m)	2.430 (m)	30.1	33.0
9	2.354 (dd, 7.7, 13.2) 2.112 (dd, 2.9, 13.2)	2.198 (br d, 12) 2.110 (dd, 7.5, 13)	45.6	45.0
10	—	—	143.4	143.3
11	6.117 (br s)	6.117 (br s)	131.7	131.0
12	—	—	139.2	139.8
13	5.226 (t, 7.3)	5.230 (br t, 7.0)	78.9	78.5
14	1.67 (m, 2H)	1.680 (m, 2H)	27.5	27.8
15	0.837 (t, 7.0)	0.858 (t, 7.5)	10.3	10.3
16	1.382 (d, 7.0)	1.408 (d, 7.0)	14.8	15.3
17	3.572 (dt, <1, 12.0) 3.342 (dd, 3.1, 11.9)	3.468 (br t, 10.5) 3.276 (d, 11.5)	67.5	67.8
18	3.172 (s)	1.639 (br s)	56.3	19.1
19	0.908 (d, 7.0)	0.844 (d, 6.5)	20.5	19.4
20	4.908 (br s) 4.829 (br s)	4.925 (br s) 4.805 (br s)	118.4	118.4
21	4.488 (dd, 12.6) 4.126 (dd, 12.7)	4.558 (d, 12.5) 4.102 (d, 12.5)	60.3	60.5

7165) capable of converting both compounds to a more polar product. The polarity of the products suggested that the biotransformation might involve hydroxylation. Small quantities of each were isolated and characterized. Molecular ions 16 mass units higher than the starting compound were observed in the MS of both products consistent with hydroxylation. The site of hydroxylation was determined to be C-21 from analysis of ^{1}H NMR and MS data and comparison of the natural products produced by culture MA 7186 with the biotransformation derived compounds showed them to be identical. Epimerization of the C-2 methyl group under biotransformation conditions is unlikely and therefore the stereochemistry of **3** and **4** should be identical to that of **1** and **2** respectively.

We speculate that the hydroxylation of **1** and **2** at C-21 by culture MA 7165 is carried out by a cytochrome P-450 type enzyme. Cytochrome P-450 has been shown to be responsible for the hydroxylation of numerous organic compounds in microbial biotransformation studies.[18] The timing and role of C-21 hydroxylation relative to the biosynthesis of **1** and **2** by culture MA 7186 is interesting. Hydroxylation after synthesis of **1** and **2** by a cytochrome P-450 mediated process is likely, but hydroxylation of a common precursor of **1**~**4** cannot be excluded.

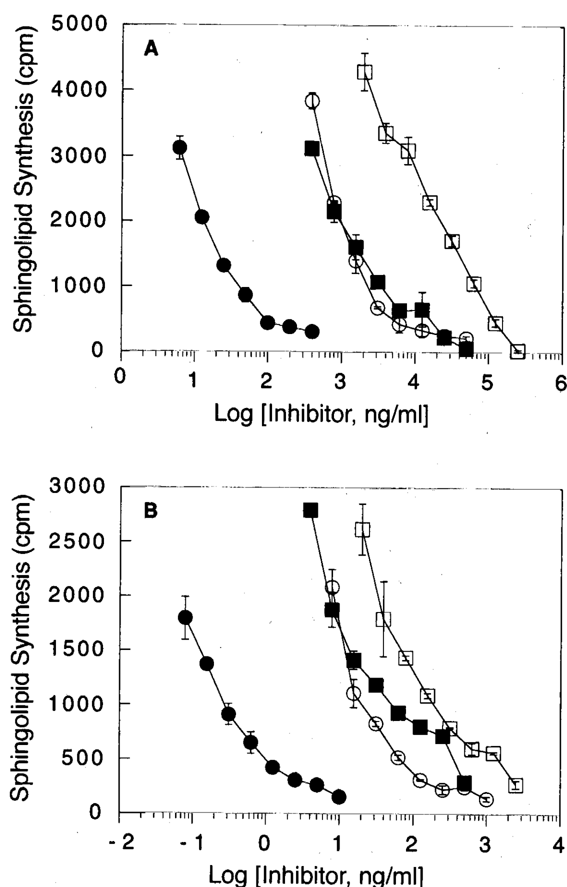
Biological Activity

Inhibition of Sphingolipid Synthesis

Compounds **1** and **2** were first isolated for their growth inhibitory activity against plant pathogenic fungi[19,20]. Investigation into their mode of action indicated that they did not destabilize membranes, or inhibit the synthesis of chitin, DNA or RNA, but the mechanism of their antifungal activity was not determined. In the course of screening for fungal specific inhibitors of sphingolipid synthesis, we isolated **1** and **2**. The effects of **1** and **2**, and related minor components **3** and **4**, on ^{3}H-inositol incorporation into the deacylation-resistant sphingolipids of *C. albicans* and *C. neoformans* are shown in Figure 4. None of the compounds affected phosphatidylinositol synthesis, but all of them inhibited sphingolipid synthesis with varying levels of potency. **1** was the most potent compound with an approximate IC_{50} of

15 ng/ml for inhibition of inositol incorporation in the sphingolipids of *C. albicans* (Figure 4A). Compounds **2** and **3** were 60-fold less potent, and **4** was over 1000-fold less active than **1** at inhibiting sphingolipid synthesis in *C. albicans*. When the compounds were tested against *C. neoformans*, they demonstrated the same rank order of activity, but were considerably more potent at inhibiting sphingolipid synthesis in this pathogen (Figure 4B). *C. neoformans* was remarkably sensitive to **1**, with an approximate IC_{50} of 0.15 ng/ml. Analysis of *in vitro* enzyme activities and the accumulation of sphingolipid intermediates resulted in the identification of the inositol phospoceramide synthase as the target enzyme in the sphingolipid biosynthetic pathway for these inhibitors[21].

Fig. 4. Inhibition of ^{3}H-inositol incorporation into sphingolipids of *C. albicans* (**A**) and *C. neoformans* (**B**) by compounds (**1**) (●), (**2**) (■), (**3**) (○), and (**4**) (□).



Average ^{3}H-inositol incorporation into sphingolipids in the absence of drug treatment was 4080 cpm for A and 3030 cpm for B.

Antifungal Activity

Antifungal activity of the macrolides against clinically relevant human pathogens was measured in a microbroth dilution assay (Table 3). Similar to previous results, compound **1** had moderate antifungal activity against *C. albicans* and was inactive against *A. fumigatus* (Table 3)[12,15]. Additionally, we found that **1** was very active at inhibiting the growth of several non-albicans *Candida* species including *C. tropicalis*, and was extremely potent against *C. neoformans*. The related macrolides were less active than **1** as antifungal agents and, as expected from the sphingolipid inhibition data, *C. neoformans* was the most sensitive organism to this class of compounds. However, the correlation between sphingolipid inhibition and antifungal activity was limited. We have found that activity in the long term antifungal assays is influenced by a combination of factors that include *in vitro* potency against the enzyme, chemical stability of the macrolactone structure, and the activity of multidrug efflux pumps in the fungal pathogens[21].

Rustmicin and the related macrolides described in this report are the third reported class of IPC synthase inhibitors. Aureobasidin A, a cyclic depsipeptide, was recently shown to inhibit the IPC synthase from *Saccharomyces cerevisiae*.[22] Khafrefungin, a 22-carbon linear polyketide acid esterified to an aldonic acid, was discovered because of its activity as an inhibitor of sphingolipid biosynthesis[11]. All three classes of natural products are potent and specific antifungal agents.

Table 3. Antifungal activity by microbroth dilution assay.

Organism (strain number)	MIC (μg/ml)			
	(1)	(2)	(3)	(4)
Candida albicans (MY1055)	6.25	>200	12.5	>200
Candida tropicalis (MY1012)	0.05	200	0.024	0.78
Cryptococcus neoformans (MY2062)	0.0001	12.5	0.1	3.1
Aspergillus fumigatus (MF5668)	>200	>200	>200	>200

Acknowledgements

The authors wish to thank Mr. STUART FORSYTHE for engineering assistance, Dr. TOM CHEN for advice on biotransformation and Ms. WENDY CLAPP-SHAPIRO for assistance with the large scale isolation.

References

1) VAN MIDDLESWORTH, F. M.; R. A. GIACOBBE, M. LOPEZ, G. GARRITY, J. A. BLAND, K. BARTIZAL, R. A. FROMTLING, J. POLISHOOK, M. ZWEERINK, A. M. EDISON, K. E. WILSON & R. MONAGHAN: Isolation, fermentation, and preliminary biology of sphingofungins, A, B, C and D; A new family of antifungal agents. I. Fermentation, isolation and biological activity. J. Antibiotics 45: 861~867, 1992
2) ZWEERINK, M. M.; A. M. EDISON, G. B. WELLS, W. PINTO & R. L. LESTER: Characterization of a novel, potent and specific inhibitor of serine palmitoyltransferase. J. Biol. Chem. 267: 25032~25038, 1992
3) MANDALA, S. M.; B. R. FROMMER, R. A. THORNTON, M. B. KURTZ, N. M. YOUNG, M. A. CABELLO, O. GENILLOUD, J. M. LIESCH, J. L. SMITH & W. S. HORN: Inhibition of serine palmitoyltransferase activity by lipoxamycin. J. Antibiotics 47: 376~379, 1994
4) MIYAKE, Y.; Y. KOZUTSUMI, S. NAKAMURA, T. FUJITA & T. KAWASAKI: Serine palmitoyltransferase is the primary target of a sphingosine-like immunosuppressant, ISP-1/myriocin. Biochem. Biophys. Res. Comm. 211: 396~403, 1995
5) HARRIS, G. H.; E. T. T. JONES, M. S. MEINZ, M. NALLIN-OMSTEAD, G, L. HELMS, G. F. BILLS, D. ZINK & K. E. WILSON: The isolation and structure elucidation of viridiofungins A, B and C. Tetrahedron Lett. 34: 5235~5238, 1993
6) MANDALA, S. M.; R. A. THORNTON, B. R. FROMMER, S. DREIKORN & M. B. KURTZ: Viridiofungins, novel inhibitors of sphingolipid synthesis. J. Antibiotics 50: 339~343, 1997
7) WANG, E.; W. P. NORRED, C. W. BACON, R. T. RILEY & A. H. MERRILL: Inhibition of sphingolipid biosynthesis by fumonisins. J. Biol. Chem. 266: 14486~14490, 1991
8) MANDALA, S. M.; R. A. THORNTON, B. R. FROMMER, J. E. CUROTTO, W. ROZDILSKY, M. B. KURTZ, R. A. GIACOBBE, G. F. BILLS, M. A. CABELLO, I. MARTIN, F. PELAEZ & G. H. HARRIS: The discovery of australifungin, a novel inhibitor of sphinganine *N*-acyltransferase from *Sporormiella australis*. J. Antibiotics 48: 349~356, 1995
9) HENSENS, O. D.; G. L. HELMS, E. T. T. JONES & G. H. HARRIS: Structure elucidation of australifungin, a potent inhibitor of sphinganine *N*-acyltransferase in sphingolipid biosynthesis from *Sporormiella australis*. J. Org. Chem. 60: 1772~1776, 1995
10) SIGMUND, J. M. & C. F. HIRSCH: Fermentation studies of rustmicin production by a *Micromonospora* sp.: Taxonomy and ferementation. J. Antibiotics 50: 829~836, 1998
11) MANDALA, S. M.; R. A. THORNTON, M. ROSENBACH, J. MILLIGAN, M. G. GARCIA-CALVO & M. B. KURTZ: Khafrefungin, a novel inhibitor of sphingolipid synthesis. J. Biol. Chem. 272: 32709~32714, 1997
12) TAKASU, T.; H. NAKAYAMA, A. SHIMAZU, K. FURIHATA, K. IKEDA, K. FURIHATA, H. SETO & N. OTAKE: Rustmicin, a new macrolide antibiotic active against wheat stem rust fungus. J. Antibiotics 38: 1806~1809, 1985
13) ACHENBACH, H.; A. MÜHLENFELD, U. FAUTH & H. ZÄHNER: Galbonolides A and B – two new non – glycosidic antifungal macrolides from *Streptomyces glabus*. Tetrahedron Lett. 26: 6167~6170, 1985
14) ABE, Y.; H. NAKAYAMA, A. SHIMAZU, K. FURIHATA, K. IKEDA, K. FURIHATA, H. SETO & N. OTAKE: Neorustmicin A, a new macrolide antibiotic active against wheat stem rust fungus. J. Antibiotics 38: 1810~1812, 1985
15) ACHENBACH, H.; A. MÜHLENFELD, U. FAUTH & H. ZÄHNER: The galbonolides. Novel, powerful antifungal macrolides from *Streptomyces galbus* ssp. *eurythermus*. Ann. N.Y. Acad. Sci. 544: 128~140, 1988
16) TSE, B.: Total synthesis of (−)-galbonolide B and the determination of its absolute stereochemistry. J. Am. Chem. Soc. 118: 7094~7100, 1996
17) TSE, B.; C. M. BLAZEY, B. TU & J. BALKOVEC: Determination of the absolute stereochemistry of (−)-galbonolide A. J. Org. Chem. 62: 3236~3241, 1997
18) SARIASLANI, F. S. & C. A. OMER: Actinomycete cytochrome P-450 invovled in oxidative metabolism: biochemistry and molecular biology. Crit. Rev. Plant Sci. 11: 1~16, 1992
19) NAKAYAMA, H.; T. TAKATSU, Y. ABE, A. SHIMAZU, K. FURIHATA, K. IKEDA, K. FURIHATA, H. SETO & N. OTAKE: Rustmicin, a new macrolide antibiotic active against wheat stem rust fungus. Agric. Biol. Chem. 51: 853~859, 1987
20) FAUTH, U.; H. ZÄHNER, A. MÜHLENFELD & H. ACHENBACH: Galbonolides A and B – two non – glycosidic antifungal macrolides. J. Antibiotics 39: 1760~1764, 1986
21) MANDALA, S. M.; R. A. THORNTON, J. MILLIGAN, M. ROSENBACH, M. GARCIA-CALVO, H. G. BULL, G. H. HARRIS, K. ABRUZZO, A. M. FLATTERY, G. J. GILL, G. K. BARTIZAL, S. DREIKORN & M. B. KURTZ: Rustmicin, a potent antifungal agent, inhibits sphingolipid synthesis at inositol phosphoceramide synthase. J. Biol. Chem. 273: 14942~14949, 1998
22) NAGEIC, N. M.; E. E. NAGEIC, J. A. BALTISBERGER, G. B. WELLS, R. L. LESTER & R. C. DICKSON: Sphingolipid synthesis as a target for antifungal drugs. J. Biol. Chem. 272: 9809~9817, 1997