Oridamycins A and B, Anti-*Saprolegnia parasitica* Indolosesquiterpenes Isolated from *Streptomyces* sp. KS84

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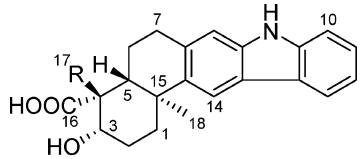
Received January 25, 2010

Oridamycins A (**1**) and B (**2**) were isolated from the fermentation broth of *Streptomyces* sp. strain KS84 as selective anti-*Saprolegnia parasitica* antibiotics. Their structures were elucidated as pentacyclic indolosesquiterpenes by the combination of NMR and spectroscopic analyses. The absolute configuration of **1** was determined by ROESY analyses after the advanced Mosher analysis. Compound **1** exhibited anti-*S. parasitica* activity with an MIC value of 3.0 μg/mL, but was much less active against the phytopathogenic fungus *Phoma* sp. and the yeast *Saccharomyces cerevisiae*.

Saprolegnia spp. is one genus of water molds, with characteristics of both fungi and algae. A series of morphological alterations including sporangial formation, sporulation, and the extension of vegetative hyphae are similar to those of fungi.[1] Taxonomically, the genus *Saprolegnia* belongs to the division Oomycota. *Saprolegnia* spp. infect freshwater fish and ova, creating a condition referred to as saprolegniasis, and can be easily detected by visible white patches of filamentous mycelia on fish skin. Saprolegniasis causes severe damage in commercial aquacultures, including those of salmon, trout, and sea eels, and in noncommercial aquariums. *Saprolegnia* infects the surface of the fish body after an invasion of epidermal tissues, causing epidermal damage and cellular necrosis. Severe *Saprolegnia* infections can be fatal. In the past, malachite green, an organic dye, had been used to prevent *Saprolegnia* infections in aquacultures.[2] However, this practice has been banned worldwide because malachite green is toxic to human cells and induces liver tumor formation.[3,4] Therefore, there is an urgent need for developing novel alternative compounds to control saprolegniasis.

In the course of discovering anti-*S. parasitica* compounds from microorganism extracts, we found an extract that was more active against *S. parasitica* than against the fungus *Phoma* sp. or the yeast *Saccharomyces cerevisiae*. In the work described herein, we show the isolation, structure elucidation, and biological activity of two novel indolosesquiterpenes, oridamycins A (**1**) and B (**2**), which are the first indolosesquiterpenes from prokaryotes.



1: R=Me
2: R=CH_2OH

Results and Discussion

The bacterial source of these compounds was isolated from a soil sample collected in Uji, Kyoto, Japan, and was identified as a *Streptomyces* sp. by 16S rDNA phylogenetic analysis. The fermentation of the strain was performed for 168 h in starch−casein medium at 30 °C with agitation and aeration. The filtrate of this culture (8 L) broth was extracted with EtOAc, followed by a series of multistep fractionation procedures, resulting in the isolation of two anti-*Saprolegnia* compounds, named oridamycins A (**1**, 6.4 mg) and B (**2**, 2.0 mg).

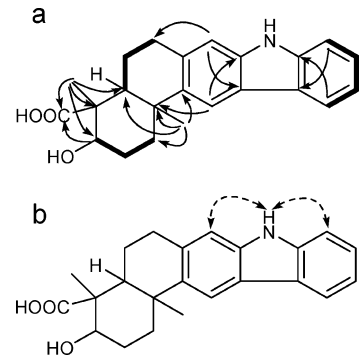


Figure 1. (a) Summary of 2D NMR analyses: (bold lines) ^{1}H−^{1}H COSY correlations; (arrows) key HMBC correlations. (b) ROE correlations to determine the position of the NH group in DMSO-d_6.

The molecular formula of oridamycin A (**1**) was determined as $C_{23}H_{25}NO_3$ by its HRFABMS spectrum. The IR spectrum implied the presence of a carboxylic acid group (1610 cm^{-1}) and hydroxy group (3405 cm^{-1}). An initial analysis of the ^{1}H NMR spectrum of **1** showed that there were six aromatic protons, an oxymethine proton, and two aliphatic methyl groups. Analyses of ^{13}C NMR, DEPT, and HMQC spectra indicated the presence of an oxymethine group, an aliphatic methine group, four methylene groups, and nine quaternary carbons (one carbonyl, six aromatic, and two aliphatic carbons). Furthermore, four successively coupled aromatic protons and two isolated aromatic protons suggested the presence of two aromatic rings, one of which was 1,2-disubstituted and the other of which was 1,2,4,5-tetrasubstituted. A ^{1}H−^{1}H COSY analysis in the aliphatic region revealed two partial structures, C-1 to C-3 and C-5 to C-7 (Figure 1a, bold lines), both of which are connected by HMBC correlations from two methyl groups, H-18/C-1, C-5, C-14a and H-17/C-3, C-4, C-5 (Figure 1a, arrows). Further HMBC correlations of H-3, H-5, and H-17/C-16 indicated that the carboxylic acid group was connected to C-4. Further HMBC correlations of H-8/C-7 and H-14/C-15 revealed that the bicyclic moiety was conjugated to an aromatic ring involving the singlet aromatic protons H-8 and H-14. The remaining functional group from the molecular formula was an NH group. This amine group was clearly located between two benzene rings, bridging either C-8a and C-9a or C-13a and C-13b. To determine the whole planar structure of **1**, a ROESY spectrum was acquired in DMSO-d_6. This experiment

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10.1021/np1000522
Published on Web 04/06/2010

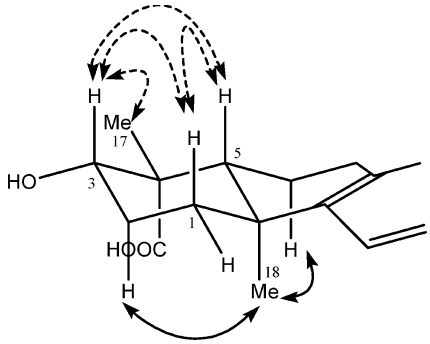


Figure 2. Key ROE correlations. Solid and dashed arrows indicate ROE correlations on the α- and *β*-faces, respectively.

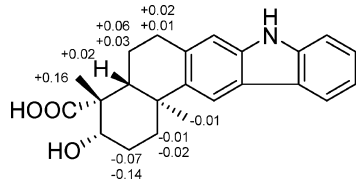


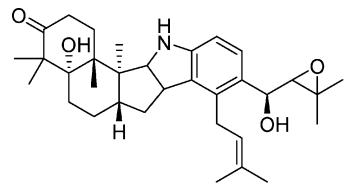
Figure 3. Results of the advanced Mosher's method. $\Delta\delta$ ($\delta_S - \delta_R$) values were obtained from the ^{1}H NMR and COSY spectra of (*R*)- and (*S*)-MTPA esters.

yielded several cross-peaks including δ 10.86 (NH)/δ 7.03 (H-8) and δ 10.86 (NH)/δ 7.35 (H-10), indicating that the position of the amine group was as described in Figure 1b. Thus, the planar structure of **1** was determined to be that of a pentacyclic indolosesquiterpene.

The relative configuration of the bicyclic moiety in **1** was determined by the ROESY spectrum in CD_3OD (Figure 2). ROESY correlations between H-2α and H-18 and between H-6α and H-18 indicated that H-2α, H-6α, and H-18 were located on the same α-face, while H-1*β*, H-3, H-5, and H-17 were on the *β*-face. The absolute configuration at C-3 was determined by applying the advanced Mosher's method to the secondary hydroxyl group.[5] Compound **1** was treated with (*S*)-(+)- and (*R*)-(−)-MTPA chloride to afford (*R*)-(+)- and (*S*)-(−)-MTPA esters (**3** and **4**), respectively. After the purification of each derivative, all ^{1}H NMR chemical shifts in **3** and **4** were assigned by a combination of COSY and HMQC analyses. A consistent distribution of positive and negative $\Delta\delta$ values around C-3 (Figure 3) revealed that the absolute configuration of C-3 was *S*, thereby resulting in the configurational assignment of 4*R*, 5*R*, and 15*S*.

The molecular formula of oridamycin B (**2**) determined as $C_{23}H_{25}NO_4$ by its HRFABMS spectrum revealed an extra oxygen compared to **1**. The characteristic UV absorptions at 215, 237, 261, and 300 nm and IR spectrum (1635, 3385 cm^{-1}) implied that **2** had a similar structure to that of **1**. Indeed, the appearance of an oxymethylene signal (δ 3.92 and 4.09) and the lack of a methyl group in the ^{1}H NMR spectrum of **2** suggested that the methyl group in **1** was replaced by a hydroxymethyl group. HMBC correlations between H-17α (δ 3.92)/C-3, C-4, and C-5 suggested that the hydroxymethyl group was bound to C-4. The remaining structure of **2** was the same as that of **1**, as elucidated by standard 1D and 2D NMR analyses. Also, all relative configurations in **2** were the same as those of **1** by ROESY analysis.

Several indolosesquiterpenes such as polyalthenol,[6] suaveolindole,[7] greenwayodendrine,[8] polyavolinamide,[9] polyveoline,[10] and 3-farnesylindole derivatives[11,12] have been reported from plants. Until recently, no indolosesquiterpenes had been isolated from bacteria, although indoloditerpenes, such as paspalicine,[13] paxilline,[14] janthitrems,[15] lolitrems,[16] and penitrems,[17] have been isolated from fungi. Sespendole[18] (**5**) was isolated from the filamentous fungus *Pseudobotrytis terrestri* as an inhibitor of lipid droplet synthesis in macrophages[19] and was the first metabolite with an indolosesquiterpene core obtained from a microorganism. Lecanindole D (**6**) and three congeners, lecanindoles A−C, are additional indolosesquiterpenes isolated from the terrestrial fungus *Verticillium lecanii* 6144.[20] Compound **6** exhibited potent and selective agonist activity at the progesterone receptor. The novel compounds (**1** and **2**) from the broth of a *Streptomyces* sp. in this study are the first indolosesquiterpenes isolated from a prokaryote. A biosynthetic study of sespendole by feeding experiments with acetate, anthranilic acid, and tryptophan revealed that the indolosesquiterpene core was constructed by a cyclization after the condensation of a farnesyl residue derived from the mevalonate pathway with an anthranilate-derived indole-3-glycerol phosphate.[21] In the current study, feeding experiments with anthranilic acid and tryptophan resulted in a broth that did not contain the desired compounds (data not shown). This inconclusive result prevents any conclusions regarding the biosynthesis of the indole units in **1** and **2**.



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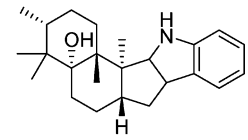

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The biological activity of compounds **1** and **2** was assessed with *S. parasitica*, *Phoma* sp., and *S. cerevisiae* assays. Compound **1** exhibited an anti-*S. parasitica* activity with an MIC value of 3.0 *μ*g/mL. It was much less active toward *Phoma* sp. (242 *μ*g/mL) and exhibited no activity against *S. cerevisiae* even at a concentration of 1000 *μ*g/mL. Compound **2**, the 17-hydroxymethyl analogue of **1**, exhibited 100-fold lower activity against *S. parasitica* compared to that of **1**. These results demonstrate the efficacy of **1** as a selective anti-*S. parasitica* antibiotic.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 343 digital polarimeter in MeOH. UV absorptions were measured on a Jasco V-630 spectrophotometer. IR spectra were recorded on a Shimadzu FT/IR-8600 spectrometer. ^{1}H and ^{13}C NMR and all 2D NMR spectra were recorded on a JEOL ECA600 NMR spectrometer at 293 K for both compounds. ^{1}H and ^{13}C NMR chemical shifts were referenced to the solvent peaks: δ_H 3.30 and δ_C 49.0 for CD_3OD and δ_H 2.49 and δ_C 40.0 for DMSO-d_6. ESI mass spectra were obtained on an Applied Biosystem API-3200 triple quadrupole mass spectrometer using a MeCN/H_2O with 0.1% AcOH solvent system.

Isolation of Oridamycins A and B. *Streptomyces* sp. KS84 was isolated from a soil sample collected in Uji City, Kyoto, Japan. The taxonomy of the strain was determined by a 16 rDNA analysis (accession no. AB550852). *Streptomyces* sp. KS84 was deposited in a culture collection of the National Institute of Advanced Industrial Science and Technology (AIST), Japan (accession no. FERM AP-21858). The fermentation was performed in starch−casein medium (1.0% starch, 0.03% casein, 0.2% NaCl, 0.2% K_2HPO_4, 0.005% $MgSO_4$, 0.002% $CaCO_3$, 0.001% $FeSO_4$, pH 7.2, 10 L) for 168 h at 30 °C with agitation and aeration. The filtrate of this culture broth was extracted with EtOAc. The active EtOAc extract was separated by ODS chromatography with aqueous MeCN. The 50% MeCN eluate was purified by reversed-phase HPLC (Cosmosil ARII; 10 × 250 mm) with a 30%−60% MeCN gradient to afford oridamycins A (**1**, 6.4 mg) and B (**2**, 2 mg).

Oridamycin A (1): pale yellow solid; $[\alpha]^{20}_D$ +92 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 216 (4.34), 238 (4.46), 262 (4.11), 300 (4.11) nm; IR (film) ν_{max} 3405, 2880, 1610 cm^{-1}; ^{13}C and ^{1}H NMR data, see Table 1; HR-FABMS *m/z* 362.1743 [M − H]$^-$ (calcd for $C_{23}H_{24}NO_3$, 362.1762).

Oridamycin B (2): pale yellow solid; $[\alpha]^{20}_D$ +110 (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ε) 216 (4.70), 238 (4.78), 261 (4.45), 300 (4.43)

Table 1. NMR Spectroscopic Data (500 MHz, CD_3OD) of Oridamycins A (**1**) and B (**2**)

	oridamycin A (**1**)				oridamycin B (**2**)	
no.	δ_C	δ_H (J in Hz)	HMBC[a]	ROESY	δ_C	δ_H (J in Hz)
1α	40.0	2.57, dt (13.6, 3.6)	3, 5	1β, 2α, 2β, 3, 14, 18	39.7	2.60, dt (13.0, 3.5)
1β		1.58, dt (4.1, 13.6)	18	1α, 2β, 3, 5		1.59, m
2α	30.3	2.30, dq (3.6, 13.6)	3, 15	1α, 2β, 18	29.8	2.41, dq (4.1, 13.0)
2β		1.90, dq (13.6, 3.6)	15	1α, 1β, 2α, 3		1.95, m
3	79.1	3.22, dd (12.2, 4.6)	16, 17	1β, 2β, 5, 17	73.3	3.73, dd (12.4, 4.8)
4	48.7				55.5[b]	
5	54.1	1.51, dd (12.2, 2.3)	3, 4, 6, 7, 15, 16	1β, 3, 6β, 7β, 17	46.3	1.92, dd (12.2, 2.3)
6α	22.5	2.09, dt (5.4, 12.7)	5, 7, 15	6β, 7α, 18	22.1	2.08, m
6β		2.23, m	5, 7, 7a, 15	5, 6α, 7β, 17		2.17, m
7α	34.0	3.06, ddd (16.3, 5.4, 2.3)	5, 6, 7a, 8, 14a	6α, 7β, 8	33.3	3.04, dd (10.3, 4.1)
7β		2.94, ddd (16.7, 12.7, 2.3)	6, 7a, 14a	5, 6β, 7α		
7a	134.5				134.5	
8	111.4	7.03, s	7, 13b	7α, 7β	110.5	7.05, s
8a	140.1				140.0	
9						
9a	142.0				141.9	
10	126.1	7.32, d (8.1)	12, 13a		125.9	7.33, d (7.9)
11	110.7	7.25, dt (1.4, 8.1)	9a, 13		111.3	7.27, dt (1.4, 7.9)
12	119.3	7.05, dt (1.4, 8.1)	13a		119.2	7.06, dt (1.4, 7.9)
13	120.6	7.93, d (8.1)	9a, 12		120.4	7.95, d (7.9)
13a	124.6				124.5	
13b	123.2				123.2	
14	117.5	7.93, s	7a, 8a, 15	1α, 18	117.2	7.95, s
14a	140.3				140.5	
15	39.6				39.2	
16	181.0				179.2[b]	
17α	24.8	1.48, s	3, 4, 5, 16	3, 6β	64.4	3.92, d (11.0)
17β						4.09, d (11.0)
18	24.6	1.26, s	1, 5, 14a, 15	1α, 2α, 6α, 14	25.0	1.31, s

[a] HMBC correlations are from protons stated to the indicated carbon. [b] Chemical shifts were assigned from HMBC data.

nm; IR (film) ν_{max} 3385, 2900, 1635 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HMBC H-1α/C-3, and C-5, H-3/C-16, H-5/C-4, C-6, C-7, C-15, C-16, and C-18, H-7/C-5, C-7a, C-8, and C-14a, H-8/C-7, C-13b, and C-14a, H-10/C-12 and C-13a, H-11/C-9a and C-12, H-12/C-11, C-13a, H-14/C-7a, C-8a, C-13b, and C-15, H-17α/C-3, C-4, C-5, and C-16, H-17β/C-3 and C-5, H-18/C-1, C-5, C-14a, and C-15; ROESY H-1α/H-1β, H-2α, H-2β, H-14, and H-18, H-1β/H-1α, H-2β, H-3, and H-5, H-2α/H-1α, H-2β, and H-18, H-2β/H-1α, H-1β, H-2α, and H-3, H-3/H-1β, H-2β, H-5, H-17α, and H-17β, H-5/H-1β, H-3, H-6β, H-7, H-17α, and H-17β, H-6α/H-6β, H-7, and H-18, H-6β/H-5, H-6α, H-7, H-17α, and H-17β, H-8/H-7, H-14/H-1α and H-18, H-17α/H-3, H-5, H-6β, and H-17β, H-17β/H-3, H-5, H-6β, and H-17α, H-18/H-1α, H-2α, H-6α, and H-14; HR-FABMS *m/z* 378.1729 $[M - H]^-$ (calcd for $C_{23}H_{25}NO_4$, 378.1711).

Preparation of MTPA Esters. A 1.0 mg portion of **1** was treated with (*S*)-(+)-MTPACl in pyridine (100 μL) at room temperature for 2 h, and the reaction mixture was partitioned between H_2O and CH_2Cl_2. The organic layer was purified by reversed-phase HPLC to yield the (*R*)-(+)-MTPA ester **3**. The (*S*)-(−)-MTPA ester **4** was prepared in the same manner as that of **3** except with (*R*)-(−)-MTPACl.

3: ^1H NMR (CD_3OD) 7.96 (H-14), 7.95 (H-13), 7.33 (H-10), 7.27 (H-11), 7.06 (H-8), 7.06 (H-12), 4.80 (H-3), 3.10 (H-7α), 3.03 (H-7β), 2.81 (H-2α), 2.72 (H-1α), 2.21 (H-6β), 2.12 (H-2β), 1.95 (H-6α), 1.77 (H-1β), 1.71 (H-5), 1.21 (H-17), 1.32 (H-18); ESIMS 580 $[M + H]^+$.

4: ^1H NMR (CD_3OD) 7.95 (H-14), 7.95 (H-13), 7.32 (H-10), 7.27 (H-11), 7.06 (H-8), 7.06 (H-12), 4.89 (H-3), 3.12 (H-7α), 3.04 (H-7β), 2.75 (H-2α), 2.70 (H-1α), 2.27 (H-6β), 1.98 (H-2β), 1.98 (H-6α), 1.77 (H-1β), 1.73 (H-5), 1.37 (H-17), 1.31 (H-18); ESIMS 580 $[M + H]^+$.

Anti-*Saprolegnia parasitica* Assay. *S. parasitica* was kindly provided by Dr. K. Hatai, Nippon Veterinary and Life Science University. A spore suspension of *S. parasitica* was prepared by the method described below. The strain was cultured in GY medium (2.5 g yeast extract, 2.5 g glucose, 15.0 g agar, 1 L distilled H_2O) at 16 °C for 7 days. All spore suspensions were used within one week because fungal hyphae start to elongate after spore germination. The spore suspension (100 μL), GY medium (50 μL), and a sample solution (8% MeOH, 50 μL) were mixed in a 96-well microplate and incubated at 16 °C for 24 h. The elongation of fungal hyphae was observed with an inverted microscope. A solution of malachite green (0.25 μg/mL) was used as a positive control.

Anti-*Phoma* sp. Assay. The phytopathogenic fungus *Phoma* sp. was isolated from leaves collected at Ritsumeikan University. The taxonomy of the strain determined by a 18 rDNA analysis showed 99% similarity to the fungus *Phoma* sp. CCF3818 (accession no. FJ430776). The fungus was cultured in Sabouraud agar media (40 g maltose, 10 g peptone, 1 L distilled H_2O, pH 6.0). A piece of the grown agar medium was cut and homogenized to form a mycelial suspension. The method of the anti-*Phoma* sp. assay was the same as that employed with the anti-*S. parasitica* assay apart from the medium (Sabouraud medium) and incubation temperature (30 °C). A solution of cycloheximide (25 μg/mL) was used as a positive control.

Anti-*Saccharomyces cerevisiae* Assay. *S. cerevisiae* was kindly provided by Dr. B. Ono, Ritsumeikan University. A cell suspension of *S. cerevisiae* in distilled water (2.0×10^4 cells/mL, 100 μL) was added to a YPD medium (10 g yeast extract, 20 g peptone, 20 g glucose, pH 6.5, 50 μL) and a sample solution (8% MeOH, 50 μL) and incubated at 30 °C for 24 h. Anti-*S. cerevisiae* activity was estimated by cell counts.

Acknowledgment. We thank Prof. T. Eguchi and Dr. E. Nango, Tokyo Institute of Technology, for measuring HRFABMS spectra.

Supporting Information Available: ^1H, ^{13}C, and 2D NMR spectra for oridamycins A (**1**) and B (**2**). This material is available free of charge via the Internet at http://pubs.acs.org.

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NP1000522