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Blasticidin A Derivatives with Highly Specific Inhibitory Activity toward Aflatoxin Production in *Aspergillus parasiticus*

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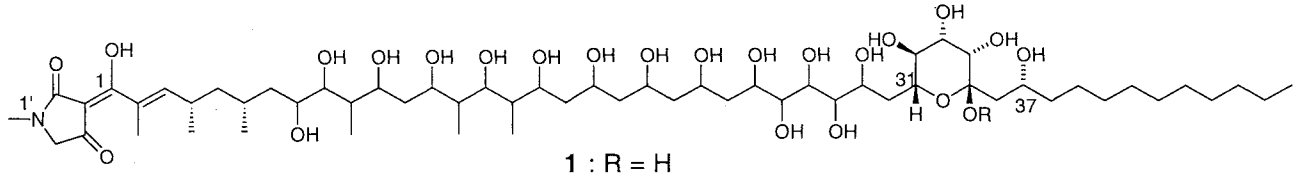
Blasticidin A (**1**), an antibiotic, has strong inhibitory activity toward aflatoxin production by *Aspergillus parasiticus*. We prepared some derivatives of **1** and examined their biological activities. Among them, derivatives **3** and **4** without the tetramic acid moiety of **1** maintained inhibitory activity toward aflatoxin production, but did not show antifungal activity or toxicity. RT-PCR experiments indicated that derivatives **3** and **4** as well as **1** significantly reduced the expression of genes encoding aflatoxin biosynthetic enzymes (*pksA*, *ver-1* and *omtA*) and a regulatory gene (*aflR*) in *A. parasiticus*. These results suggested that derivatives **3** and **4** can inhibit aflatoxin production more specifically than **1** by inhibiting an early step prior to the expression of *aflR* in the pathway of aflatoxin biosynthesis.

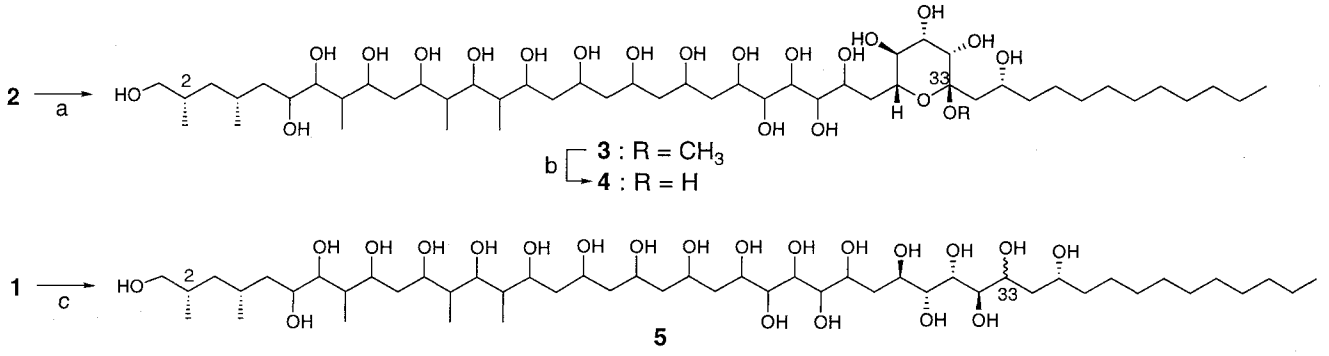
Aflatoxins, a group of mycotoxins, are potent carcinogens in mammals and can be found as contaminants in a wide variety of food and feed commodities.[1] A specific inhibitor for aflatoxin biosynthesis may be a good candidate for a useful drug to protect foods and feeds from aflatoxin contamination, and is expected to depress aflatoxin contamination without incurring rapid spread of drug-resistant strains. During the course of our studies on specific inhibitors of aflatoxin production in *Aspergillus parasiticus*, we have recently reported the isolation and structures of aflastatin A and B,[2~5] and the structure of blasticidin A (**1**).[6,7] These compounds are all *Streptomyces* metabolites and have similar unique structures, which are tetramic acid derivatives with long, highly oxygenated alkyl chains. They inhibit aflatoxin production by *A. parasiticus* at low concentrations without essentially affecting the fungal mycelial weight in liquid cultures.[2] We have recently found that aflastatin A reduces the expression of *aflR* encoding a key regulatory protein involved in aflatoxin biosynthesis (SAKURADA *et al.*, unpublished data). Therefore, aflastatin A is suggested to inhibit a very early step in the aflatoxin biosynthetic pathway. However, the molecular mechanism of aflastatins or **1** for inhibition of aflatoxin production has not yet been verified.

Aflastatins and **1** have antimicrobial activity against some microorganisms such as *Saccahromyces cerevisiae*, other than aflatoxin inhibitory activity.[2,8,9] It is not clear whether their target as antibiotics is the same as that as inhibitors of aflatoxin production. Studies on the structure-activity relationship of aflastatins and **1** are very important to develop effective inhibitors with high specificity for aflatoxin production in aflatoxigenic fungi, but we have little information on such relationships. This paper describes the preparation of blasticidin A derivatives (**3**, **4**



1 : R = H
2 : R = CH_3

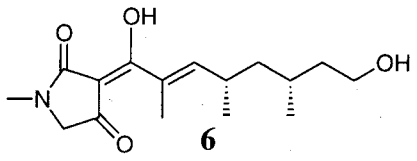
Fig. 1. Procedures for the preparation of derivatives **3**, **4** and **5**.



(a) O_3; $NaBH_4$. (b) 3 M HCl. (c) O_3; $NaBH_4$.

and **5**), which lack the tetramic acid moiety of **1**, and their biological activities. The biological activities of methyl glycoside of **1** (**2**) and fragment molecules of **1** (**6**, **7**, and **8**) and the effects of **1**, **3**, **4** and **5** on the expression of some genes responsible for aflatoxin biosynthesis are also described.

Procedures for the preparation of derivatives **3**, **4** and **5** are summarized in Fig. 1. Treatment of **1** with 5% HCl-MeOH afforded methyl glycoside of **1** (**2**). Derivative **3** was obtained by ozonolysis of **2**, followed by $NaBH_4$ reduction. Treatment of **3** with 3 M HCl afforded **4**. On the other hand, **1** was oxidized with ozone, and followed by $NaBH_4$ reduction to prepare **5**. Crude **5** was acetylated and purified by a reverse-phase HPLC, and deacetylation of the peracetate afforded **5**. The ^{13}C-NMR spectrum of the obtained **5** indicated that the ketone group at C-33 in the ozonolysis product of **1** was completely reduced, but the sample was a mixture of structurally related compounds, which were probably diastereomers at C-33 produced by the reduction. Since it was difficult to separate the diastereomers, this mixture was used as an assay sample. Compounds **6** and **8** have been prepared as fragment molecules of **1** previously.[6] Compound **7** was obtained by deacetylation of peracetate of **7**, which has also been prepared previously.[6]



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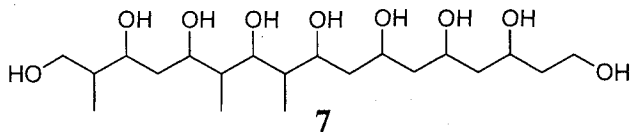

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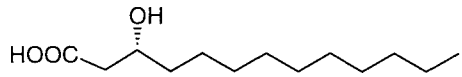

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Inhibitory activity of compounds **1**~**8** toward aflatoxin production in *A. parasiticus* NRRL2999 was examined by an agar dilution method.[2] Antifungal activity of these compounds against *S. cerevisiae* was also tested by a paper disc method. The resulting IC_{50} and MID values are listed in Table 1. Derivative **2** showed about the same activity as **1** toward aflatoxin production, suggesting that the free hydroxyl group at C-35 in **1** is not essential for the activity. Derivatives **3** and **4** maintained relatively strong inhibitory activity toward aflatoxin production, whereas they did not show antifungal activity against *S. cerevisiae* even at a dose of 1000 μg/disc. These activities of **3** and **4** indicated that the tetramic acid moiety of **1** is essential for its antifungal activity, but not for its inhibitory activity toward aflatoxin production. Derivative **5** showed weaker activity toward aflatoxin production than **3** or **4**, suggesting that the tetrahydropyran ring of **1** is important for the activity. On the other hand, all small fragment molecules of **1** (**6**, **7** and **8**) showed neither inhibitory activity toward aflatoxin

Table 1. Biological activities of blasticidin A and its derivatives.

Compoud	Inhibitory activity for aflatoxin production[a] IC_{50} (μM)	Antifungal activity[b] MID[c] (μg/disc)
1	0.04	1
2	0.05	10
3	0.6	>1000
4	0.4	>1000
5	10	>1000
6	>100	>1000
7	>100	>1000
8	>100	>1000

[a] In *Aspergillus parasiticus*.
[b] Against *Saccharomyces cerevisiae*.
[c] Minimum dose where inhibitory zone was observed.

Table 2. Effects of **1**, **3** and **4** on aflatoxin production by *Aspergillus parasiticus* in liquid culture.

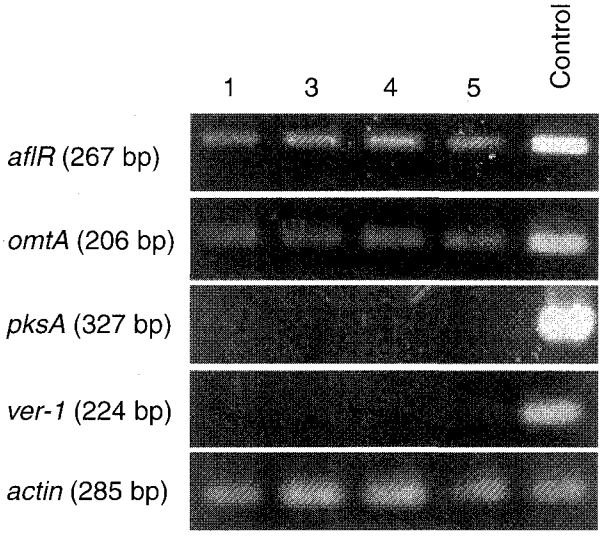
Compoud	Conc. (μM)	Mycelial dry weight (m±S.D., mg/10ml)[b]	Aflatoxin conc.[a] (m±S.D., μg/ml)[b]
control		23.8±0.9	16.6±0.6
1	1.0	11.5±2.1	0.1±0.01
3	10	21.5±0.6	0.7±0.2
4	10	22.1±1.0	0.3±0.1
5	100	21.8±2.1	0.5±0.1

[a] Total amount of aflatoxin B_1, B_2, G_1 and G_2.
[b] n = 3.

production nor antifungal activity. It was known that **1** reduced the hyphal extension rate of *A. parasiticus* on the agar plate by about 50% of control at a concentration of 0.4 μM together with causing some morphological changes to afford dense and elevated colonies.[7] In the case of **3** and **4**, however, they showed no reduction of the hyphal extension rate of the fungus and did not cause any morphological changes at a concentration of 10 μM. When **1** was intraperitoneally administered to mice at a dose of 80 mg/kg, 80% of the mice was dead. On the other hand, no mice was dead by administration of **3** or **5** at the same dose.

Next, we examined if **1**, **3**, **4** or **5** affects the expression of genes involved in the aflatoxin biosynthesis. *A. parasiticus* was cultured in a liquid medium with or without **1**, **3**, **4** or **5** for 2 days. The culture broth was filtered to obtain the filtrate and mycelia, which were used for aflatoxin and RNA extraction, respectively. As shown in Table 2, aflatoxin production was almost completely inhibited by **1**, **3**, **4** or **5** at a concentration of 1.0, 10, 10, or 100 μM, respectively. At that time, **1** delayed the mycelial growth of the fungus similarly to aflastatin A (SAKURADA *et al.*, unpublished data), while little or no effect of **3**, **4** or **5** on the mycelial growth was observed (Table 2). We analyzed the mRNA levels of *pksA*, *ver-1*, *omtA* and *aflR* by RT-PCR. When **1**, **3**, **4** or **5** was added to the culture, the expression of these four genes was reduced in all cases (Fig. 2). PKSA, VER-1 and OMTA are aflatoxin biosynthetic enzymes involved in the conversion steps from hexanoyl CoA to norsolorinic acid, from versicolorin A to demethylsterigmatocystin and from sterigmatocystin to *O*-methylsterigmatocystin, respectively.[10,11] AFLR induces expression of each of these three genes.[12~15] Therefore,

Fig. 2. Effects of **1**, **3**, **4** and **5** on the expression of genes involved in the aflatoxin biosynthesis in *Aspergillus parasiticus*.



Total RNA was extracted from fungal mycelia incubated for 2 days with or without **1**, **3**, **4** or **5** at a concentration of 1.0, 10, 10 or 100 μM, respectively. Expression of *pksA*, *ver-1*, *omtA*, *aflR* and actin genes was analyzed by RT-PCR.

reduction of the *aflR* expression may critically affect the aflatoxin production. These results suggest that all of these compounds may inhibit an early step prior to the expression of *aflR* in the pathway of aflatoxin biosynthesis similarly to aflastatin A.

In conclusion, we have found inhibitors highly specific for aflatoxin production in *A. parasiticus* through the studies on the structure-activity relationship of blasticidin A, and showed that the inhibitors as well as blasticidin A may inhibit a very early step in aflatoxin production.

Experimental

Preparation of **3** and **4**

A solution of **1** (50 mg) in 5% HCl-MeOH (5.0 ml) was stirred at room temperature for 1 hour. The reaction mixture was purified by reverse-phase HPLC (column: Capcell Pak C_{18}, 10×250 mm, Shiseido; mobile phase: gradient elution of 66~90% MeOH in water containing 0.5% diethylamine in 10 minutes; flow rate: 3 ml/minute) to afford **2** (26.5 mg). **2**: HR-FABMS (positive, NBA matrix) *m/z* 1222.7213 $(M+Na)^+$ (Calcd for $C_{59}H_{109}NO_{23}Na$, 1222.7288); $[\alpha]_D^{25}$ +3.1° (*c* 0.1, DMSO); δ_H (DMSO-d_6, 500 MHz) 5.46 (H-3), 3.93 (H-13), 3.89 (H-19), 3.89 (H-11), 3.88 (H-37), 3.82 (H-29), 3.82 (H-27), 3.80 (H-23), 3.78 (H-21), 3.78 (H-17), 3.65 (H-25), 3.62 (H-31), 3.56 (H-33), 3.56 (H-8), 3.43 (H-28), 3.43 (H-15), 3.41 (H-34), 3.31 (H-5′), 3.27 (H-9), 3.25 (H-26), 3.18 (H-32), 3.03 (H-54), 2.70 (H-6′), 2.49 (H-4), 2.12 (H-30a), 1.89 (H-6), 1.85 (H-24a), 1.79 (H-36a), 1.69 (H-48), 1.63 (H-14), 1.61 (H-10), 1.55 (H-20), 1.53 (H-22), 1.53 (H-18), 1.51 (H-16), 1.48 (H-30b), 1.48 (H-12a), 1.42 (H-36b), 1.35 (H-24b), 1.33 (H-12b), 1.33 (H-5a), 1.30 (H-38), 1.26 (H-39), 1.23 (H-40, 41, 42, 43, 44, 45, 46), 1.22 (H-7), 0.93 (H-5b), 0.88 (H-49), 0.86 (H-50), 0.84 (H-51), 0.84 (H-47), 0.78 (H-53), 0.67 (H-52); δ_C (DMSO-d_6, 125 MHz) 191.2 (C-1), 189.3 (C-4′), 174.4 (C-2′), 139.5 (C-3), 135.2 (C-2), 99.8 (C-3′), 99.8 (C-35), 75.9 (C-15), 74.5 (C-9), 73.9 (C-26), 73.4 (C-17), 73.2 (C-34), 71.6 (C-11), 71.2 (C-32), 71.2 (C-28), 71.0 (C-13), 70.6 (C-33), 70.4 (C-31), 69.7 (C-27), 69.7 (C-25), 68.3 (C-37), 68.3 (C-29), 68.2 (C-23), 68.0 (C-8), 67.8 (C-21), 67.8 (C-19), 55.9 (C-5′), 47.1 (C-54), 44.7 (C-5), 44.5 (C-22), 42.5 (C-7), 41.8 (C-20), 41.8 (C-14), 41.4 (C-36), 41.4 (C-18), 41.0 (C-24), 39.7 (C-10), 38.2 (C-16), 38.0 (C-38), 36.1 (C-30), 34.7 (C-12), 31.3 (C-45), 29.9 (C-4), 29.1 (C-44), 29.1 (C-43), 29.1 (C-42), 29.1 (C-41), 28.7 (C-40), 28.5 (C-6′), 26.1 (C-6), 25.1 (C-39), 22.1 (C-46), 21.4 (C-49), 20.9 (C-50), 14.0 (C-47), 13.2 (C-48), 10.5 (C-52), 8.7 (C-51), 6.0 (C-53).

Ozone was passed through a solution of the **2** (140 mg) in absolute MeOH at −78°C for 20 minutes. After removal of excess O_3 by passage of N_2, $NaBH_4$ (50 mg) was added to the solution. The reaction mixture was stirred at room temperature for 2 hours, and the reaction was stopped by adding acetic acid (0.2 ml). After removal of the solvent and boron, a mixture of dry pyridine (10 ml), acetic anhydride (5 ml) and 4-(dimethylamino)pyridine (3 mg) was added to the residue. The reaction mixture was stirred at room temperature for 18 hours, and the reaction was stopped by adding MeOH (10 ml). The solution was extracted with EtOAc (120 ml), and the EtOAc layer was washed with 3 M HCl, sat $NaHCO_3$ solution, brine and distilled water and dried. After evaporation *in vacuo*, the resulting residue was purified by silica gel column chromatography (hexane - EtOAc, 7 : 3) and reverse-phase HPLC (column: Capcell Pak C_{18}, 10×250 mm, Shiseido; mobile phase: 90% CH_3CN in water; flow rate: 3 ml/minute) to afford peracetate of **3**. To a solution of **3** peracetate in absolute MeOH, a small piece of Na was added and stirred at room temperature for 1 hour. After being passed through a Dowex-50W (H^+) column (18×40 mm), the solvent was removed to afford **3** (45.4 mg). **3**: HR-FABMS (positive, glycerol matrix) *m/z* 1073.6710

$(M+Na)^+$ (Calcd for $C_{51}H_{102}O_{21}Na$, 1073.6811); $[\alpha]_D^{26}$ +1.0° (*c* 0.3, CH_3CN); δ_H (pyridine-d_5, 500 MHz) 4.97 (H-25), 4.93 (H-27), 4.71 (H-9), 4.68 (H-23), 4.66 (H-32), 4.63 (H-21), 4.60 (H-11), 4.56 (dd, *J*=9.5, 3.5 Hz, H-31), 4.51 (H-19), 4.50 (H-26), 4.45 (H-15), 4.43 (H-17), 4.33 (H-24), 4.31 (t, *J*=9.5 Hz, H-30), 4.24 (H-6), 4.21 (H-29), 4.18 (H-35), 4.05 (H-13), 4.01 (H-7), 3.80 (dd, *J*=10.0, 5.0 Hz, H-1a), 3.60 (dd, *J*=10.0, 6.5 Hz, H-1b), 3.35 (H-51), 3.18 (H-28a), 2.58 (H-22), 2.49 (H-28b), 2.44 (H-34a), 2.22 (H-34b), 2.18 (H-12), 2.17 (H-8), 2.16 (H-22), 2.16 (H-4), 2.07 (H-16a), 2.04 (H-10), 2.02 (H-20), 1.98 (H-2), 1.91 (H-16b), 1.88 (H-14), 1.86 (H-18), 1.79 (H-5), 1.77 (H-3a), 1.68 (H-36a), 1.66 (H-5), 1.58 (H-36b), 1.54 (H-37), 1.34 (d, *J*=7.0 Hz, H-48), 1.20 (d, *J*=6.5 Hz, H-50), 1.20 (H-38, 39, 40, 41, 42, 43, 44), 1.11 (d, *J*=6.5 Hz, H-46), 1.05 (d, *J*=6.5 Hz, H-47), 1.03 (H-3b), 0.96 (d, *J*=6.5 Hz, H-49), 0.83 (t, *J*=7.0 Hz, H-45); δ_C (pyridine-d_5, 125 MHz) 103.3 (C-33), 78.8 (C-13), 77.4 (C-7), 76.2 (C-24), 76.2 (C-15), 75.4 (C-26), 74.5 (C-11), 74.1 (C-9), 73.1 (C-32), 72.8 (C-29), 72.6 (C-31), 72.4 (C-30), 72.2 (C-23), 71.5 (C-25), 71.2 (C-21), 70.8 (C-27), 70.8 (C-17), 70.6 (C-19), 69.9 (C-6), 67.3 (C-1), 66.9 (C-35), 47.8 (C-51), 45.7 (C-20), 45.7 (C-18), 42.8 (C-16), 42.8 (C-12), 42.1 (C-22), 42.1 (C-5), 41.5 (C-8), 41.5 (C-3), 39.5 (C-14), 39.4 (C-36), 39.4 (C-34), 37.6 (C-28), 36.8 (C-10), 34.0 (C-2), 32.1 (C-43), 30.0 (C-40), 30.0 (C-39), 29.9 (C-41), 29.9 (C-38), 29.5 (C-42), 27.8 (C-4), 26.0 (C-37), 22.9 (C-44), 21.8 (C-47), 18.7 (C-46), 14.3 (C-45), 11.6 (C-49), 8.9 (C-48), 6.1 (C-50).

To a solution of **3** (43.4 mg) in MeOH, 3 M HCl was added and stirred at room temperature for 5 hours. After the mixture was passed through a Dowex-1 (OH^-) column (18×50 mm), the solvent was removed under reduced pressure to afford **4** (38.6 mg). **4**: HR-FABMS (positive, diethanolamine matrix) *m/z* 1059.6605 $(M+Na)^+$ (Calcd for $C_{50}H_{100}O_{21}Na$, 1059.6655); $[\alpha]_D^{26}$ +1.1° (*c* 0.3, CH_3CN); δ_H (pyridine-d_5, 500 MHz) 5.03 (H-25), 4.98 (H-27), 4.85 (H-29), 4.76 (dd, *J*=9.5, 3.5 Hz, H-31), 4.72 (H-35), 4.70 (H-9), 4.66 (H-23), 4.62 (H-21), 4.60 (H-11), 4.57 (H-26), 4.52 (H-19), 4.48 (H-32), 4.48 (H-17), 4.44 (H-15), 4.39 (t, *J*=9.5 Hz, H-30), 4.30 (H-24), 4.26 (H-6), 4.06 (H-13), 4.02 (t, *J*=4.0 Hz, H-7), 3.81 (dd, *J*=10.0, 5.0 Hz, H-1a), 3.61 (dd, *J*=10.0, 6.5 Hz, H-1b), 3.21 (H-28a), 2.74 (H-34a), 2.60 (H-22a), 2.55 (H-28b), 2.20 (H-12), 2.16 (H-8), 2.15 (H-22b), 2.15 (H-4), 2.14 (H-34b), 2.07 (H-16a), 2.05 (H-10), 2.02 (H-20), 1.99 (H-2), 1.91 (H-16b), 1.89 (H-18), 1.89 (H-14), 1.80 (H-3a), 1.79 (H-5a), 1.67 (H-5b), 1.66 (H-36a), 1.54 (H-37), 1.52 (H-36b), 1.35 (d, *J*=7.0 Hz, H-48), 1.21 (d, *J*=7.0 Hz, H-50), 1.18 (H-38, 39, 40, 41, 42, 43, 44), 1.11 (d, *J*=6.5 Hz, H-46), 1.06 (d, *J*=7.0 Hz, H-47), 1.03 (H-3b), 0.96 (d, *J*=7.0 Hz, H-49), 0.84 (t, *J*=7.0 Hz, H-45); δ_C (pyridine-d_5, 125 MHz) 100.1 (C-33), 78.9 (C-13), 77.4 (C-7), 76.1 (C-24), 76.1 (C-15), 75.1 (C-32), 74.6 (C-11), 74.2 (C-26), 74.2 (C-9), 73.4 (C-30), 72.9 (C-31), 72.2 (C-23), 71.7 (C-29), 71.3 (C-25), 71.3 (C-21), 70.8 (C-17), 70.7 (C-27), 70.7 (C-19), 69.9 (C-6), 69.1 (C-35), 67.3 (C-1), 45.7 (C-20), 45.7 (C-18), 42.9 (C-34), 42.9 (C-12), 42.8 (C-16), 42.2 (C-22), 42.2 (C-5), 41.5 (C-8), 41.5 (C-3), 39.6 (C-36), 39.6 (C-14), 37.5 (C-28), 36.8 (C-10), 34.1 (C-2), 32.1 (C-43), 30.1 (C-41), 30.1 (C-38), 29.9 (C-40), 29.9 (C-39), 29.6 (C-42), 27.9 (C-4), 25.9 (C-37), 22.9 (C-44), 21.8 (C-47), 18.7 (C-46), 14.3 (C-45), 11.6 (C-49), 8.9 (C-48), 6.1 (C-50).

Preparation of **5**

Ozone was passed through a reaction mixture of **1** (100 mg) in absolute MeOH (10 ml) and $NaHCO_3$ (40 mg) at −78°C for 20 minutes. After removal of excess O_3 by passage of N_2, $NaBH_4$ (50 mg) was added to the solution. The reaction mixture was stirred at room temperature for 3 hours, and the reaction was stopped by adding acetic acid (1.0 ml). After removal of the solvent and boron, a mixture of dry pyridine (10 ml), acetic anhydride (5 ml) and 4-(dimethylamino) pyridine (3 mg) was added to the residue. The reaction mixture was stirred at room temperature for 18 hours, and the reaction was stopped by adding MeOH (10 ml). The solution was extracted with EtOAc (120 ml), and the EtOAc layer was washed with 3 M HCl, sat $NaHCO_3$ solution, brine and distilled water and dried. After evaporation *in vacuo*, the resulting residue was purified by silica gel column chromatography (hexane - EtOAc, 3 : 7) and HPLC using the same conditions as used in the purification of **3** peracetate to afford peracetate of **5**. To a solution of **5** peracetate in absolute MeOH, a small piece of Na was added and stirred at room temperature for 1 hour. After being passed through a Dowex-50W (H^+) column (18×40 mm), the solvent was removed to afford **5** (27.6 mg). **5**: FABMS (positive, glycerol matrix) *m/z* 1061 $(M+Na)^+$. In the ^{13}C-NMR spectrum of **5**, each single signal of the carbons involved in the moieties from C-1 to C-22 and from C-37 and C45 was observed and assigned as follows; δ_C (pyridine-d_5, 125 MHz) 78.7 (C-13), 77.2 (C-7), 76.0 (C-15), 74.4 (C-11), 74.0 (C-9), 70.7 (C-17), 70.4 (C-19), 69.8 (C-6), 67.1 (C-1), 45.6 (C-20), 45.6 (C-18), 42.7 (C-12), 42.7 (C-16), 42.1 (C-5), 41.4 (C-8), 41.4 (C-3), 39.5 (C-14), 36.7 (C-10), 34.0 (C-2), 32.0 (C-43), 30.1 (C-41), 30.1 (C-38), 29.9 (C-40), 29.9 (C-39), 29.5 (C-42), 27.8 (C-4), 26.0 (C-37), 22.9 (C-44), 21.8 (C-47), 18.7 (C-46), 14.2 (C-45), 11.6 (C-49), 8.9 (C-48), 6.1 (C-50). More than 15 signals that should be assigned to the carbons from C-22 to C-36 were observed in the spectrum and could not

Table 3. Specific primers and conditions for PCR.

Gene	Specific primer sequences 5'-3' sense antisense	Amplified fragment size (bp)	PCR condition				
			Preheat	Denature	Annealing	Extention	Cycle
aflR	TTTGCGCCTTTTTGGGTGCA ACTCTTCACCCTGCTTCTTG	267	95°C, 10 min	94°C, 30 sec	50°C, 30 sec	72°C, 30 sec	30
omtA	CTACCAAAGGGCTATTTGACT GTGGGGATGCTTTTGCGAAA	206	95°C, 10 min	94°C, 30 sec	60°C, 30 sec	72°C, 30 sec	50
pksA	ATGTGGTAGTTCTGGACGGA CGTTACTGACACCTACTCCT	327	95°C, 10 min	94°C, 30 sec	50°C, 30 sec	72°C, 30 sec	30
ver-1	TCGTGGTTAACTATGCCCACTC CTCTTCTGGGGTCACGTCTTTC	224	95°C, 10 min	94°C, 30 sec	55°C, 30 sec	72°C, 30 sec	50
β-actin	CTTGACTTCGAGCAGGAGAT TCTGCATACGGTCGGAGATA	285	95°C, 10 min	94°C, 30 sec	50°C, 30 sec	72°C, 30 sec	30

be assigned due to signal overlappings.

Preparation of 7

To a solution of **7** peracetate in absolute MeOH (5 ml), a small piece of Na was added and stirred at room temperature for 1 hour. After being passed through a Dowex-50W (H^+) column (18×40 mm), the solvent was removed to afford **7** (17.5 mg). **7**: HR-FABMS (positive, glycerol matrix) *m/z* 427.2887 $(M+H)^+$ (Calcd for $C_{20}H_{43}O_9$, 427.2907); $[\alpha]_D^{26}$ +0.5° (*c* 0.3, EtOH); δ_H (CD_3OD, 500 MHz) 4.03 (ddd, *J*=10.5, 5.0, 2.0 Hz, H-5), 3.98 (H-13), 3.96 (H-3), 3.95 (H-11), 3.94 (H-9), 3.93 (H-15), 3.69 (t, *J*=6.5 Hz, H-17), 3.64 (dd, *J*=9.5, 1.5 Hz, H-7), 3.60 (dd, *J*=10.5, 6.5 Hz, H-1a), 3.46 (dd, *J*=10.5, 6.5 Hz, H-1b), 1.80 (H-6), 1.73 (H-16a), 1.71 (H-2), 1.69 (H-12a), 1.69 (H-4a), 1.68 (H-10a), 1.67 (H-8), 1.63 (H-16b), 1.62 (H-14), 1.60 (H-12b), 1.60 (H-10b), 1.57 (H-14), 1.52 (H-4b), 0.93 (d, *J*=6.5 Hz, H-20), 0.91 (d, *J*=7.0 Hz, H-18), 0.81 (d, *J*=7.0 Hz, H-19); δ_C (CD_3OD, 125 MHz) 78.6 (C-7), 75.8 (C-9), 74.2 (C-5), 73.2 (C-3), 70.3 (C-11), 70.1 (C-13), 68.8 (C-15), 66.0 (C-1), 60.0 (C-17), 45.5 (C-14), 45.2 (C-12), 43.1 (C-6), 42.6 (C-10), 41.7 (C-2), 40.9 (C-16), 40.2 (C-8), 36.4 (C-4), 11.4 (C-19), 10.9 (C-18), 6.2 (C-20).

Isolation of Total RNA

Each flask containing 10 ml of potato dextrose broth (Difco, USA) was inoculated with 10 μl of spore suspension (1.9×10^5 CFU) and cultured statically for 2 days at 27°C. Cells were harvested by filtration. Total RNA was isolated from the cells by the procedure of SAMBROOK *et al.* using guanidium thiocyanate, followed by centrifugation in cesium chloride solution.[16] RNA samples were treated with DNase (Life Technologies, Inc., USA) for 15 minutes, and 1 μg of total RNA was used for RT-PCR analysis.

RT-PCR

First-strand cDNA was prepared with SUPERSCRIPT™ Preamplification System (Life Technologies, Inc., USA) using random hexamer primers according to the protocol supplied by the manufacturer. The cDNA was used as a template for PCR. PCR primers and conditions for each gene are listed in Table 3. β-Actin was used as a control, and RT-PCR without reverse transcriptase was used to confirm that bands were not derived from the contaminated DNA.

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