Botcinins E and F and Botcinolide from *Botrytis cinerea* and Structural Revision of Botcinolides

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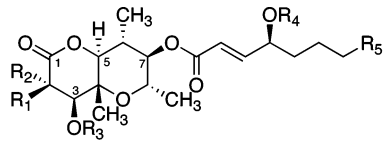
Botcinins E and F were isolated together with the known botcinolide. The structures of botcinins E and F were determined to be 3-*O*-deacetylbotcinin A (**5**) and 3-*O*-deacetyl-2-*epi*-botcinin A (**6**), respectively, by spectroscopic methods and chemical conversion. The structure of botcinolide was revised on the basis of spectroscopic data and chemical conversion. Botcinolide was originally reported as a nine-membered lactone (**7**), but the revised structure is the seco acid of botcinin E (**13**). Thus botcinolide is renamed botcinic acid, and homobotcinolide is renamed botcineric acid. Reinvestigation of the spectroscopic data reported for all botcinolide analogues indicates that 4-*O*-methylbotcinolide and 3-*O*-acetyl-2-epibotcinolide are the same as a methyl ester of botcinic acid (**13a**) and botcinin A (**1**), respectively, and that 2-epibotcinolide may be the same as botcinin E (**5**). Compounds **5**, **6**, and **13** showed weak antifungal activity against *Magnaporthe grisea*, a pathogen of rice blast disease.

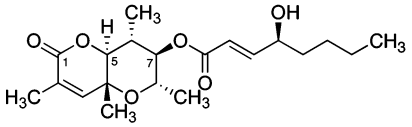
Botrytis cinerea is a well-known pathogen of a number of commercial plants and produces many structurally diverse metabolites, such as botrydials,[1−7] mycosporines,[8−10] botrylactone,[11,12] abscisic acid,[13] cinereain,[14] and botcinolides.[15−18] Recently, we reported the isolation of botcinins A−D (**1**−**4**), which show antifungal activity against *Magnaporthe grisea*, a pathogen of rice blast disease, from the neutral fraction of a culture filtrate of a strain (AEM 211) of *B. cinerea*.[19] The botcinins' structures are comprised of a unique bicyclic unit and a fatty acyl portion. Our continuing search for new botcinins among metabolites in an acidic fraction obtained from the culture filtrate of the same fungus resulted in isolation of three anisaldehyde-positive substances. Two of them were determined to be 3-*O*-deacetylbotcinin A (**5**) and 3-*O*-deacetyl-2-*epi*-botcinin A (**6**), named botcinins E and F, respectively, and one was identified as botcinolide.[15,16] However the chemical behavior of botcinolide was not in accordance with the reported structure. Further analysis through chemical reactions allowed us to revise the structure.

Botcinolide (**7**) was first reported in 1993.[15] The structure was elucidated by extensive spectroscopic studies, suggesting that botcinolide had a nine-membered lactone ring and a fatty acyl portion.[15,16] The relative stereochemistry, except for C-4′, was proposed on the basis of the NOE correlations, the coupling constant, and molecular modeling.[16] To date, homobotcinolide (**8**), 4-*O*-methylbotcinolide (**9**), 3-*O*-acetyl-5-*O*-methylbotcinolide (**10**), 2-epibotcinolide (**11**), and 3-*O*-acetyl-2-epibotcinolide (**12**) have been reported as botcinolide analogues.[17,18] Their structures were determined only by comparison of their MS and NMR data with those reported for **7**. We carefully reinvestigated the spectroscopic data reported for all botcinolide analogues and revised the structures of 4-*O*-methylbotcinolide and 3-*O*-acetyl-2-epibotcinolide as a result.

In this paper, we report the isolation and structure elucidation of two new botcinin A analogues, botcinins E (**5**) and F (**6**), and the structural revision of botcinolide (**7**) and 2-epibotcinolide (**11**). Furthermore, we discuss the validity of structures of the other botcinolide analogues. The antifungal activities of **5**, **6**, and **13** against *Magnaporthe grisea* are also reported.



	R_1	R_2	R_3	R_4	R_5
1:	CH_3	H	Ac	H	CH_3
2:	CH_3	H	Ac	H	$(CH_2)_2CH_3$
3:	H	CH_3	Ac	H	$(CH_2)_2CH_3$
5:	CH_3	H	H	H	CH_3
5a:	CH_3	H	Ac	Ac	CH_3
6:	H	CH_3	H	H	CH_3



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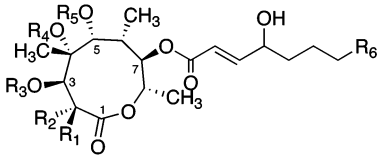
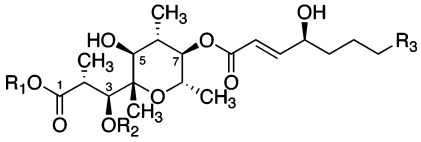


	R_1	R_2	R_3	R_4	R_5	R_6
7:	H	CH_3	H	H	H	CH_3
8:	H	CH_3	H	H	H	$(CH_2)_2CH_3$
9:	H	CH_3	H	CH_3	H	CH_3
10:	H	CH_3	Ac	H	CH_3	CH_3
11:	CH_3	H	H	H	H	CH_3
12:	CH_3	H	Ac	H	H	CH_3



	R_1	R_2	R_3
13:	H	H	CH_3
13a:	CH_3	H	CH_3
14:	CH_3	Ac	CH_3
15:	H	H	$(CH_2)_2CH_3$

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Botrytis cinerea AEM 211 was cultured on a malt extract medium, without shaking, at 24 °C for 14 days in the dark. The metabolites in the culture filtrate were extracted with ethyl acetate and separated into neutral and acidic fractions. The acidic fraction was purified by chromatographic separations to afford three anisaldehyde-positive compounds (**5**, **6**, and **13**) in respective yields of 0.4, 0.5, and 0.6 mg/L.

Compound **5** was isolated as an amorphous solid. The molecular formula of $C_{20}H_{32}O_7$ was established by HRFABMS and ^{13}C NMR data, requiring five degrees of unsaturation. The ^{13}C NMR spectrum of **5** in CD_3OD displayed 20 carbon resonances: five owing to methyls, three to methylenes, seven to sp^3 methines (five of which were oxygenated), two to sp^2 methines, one to an oxygenated quaternary carbon, and two to carbonyl carbons. The ^1H NMR spectrum of **5** in $CDCl_3$ was very similar to that of botcinin A (**1**), indicating that **5** was a botcinin A analogue. Two resonances at δ_C 20.6 and 170.1 due to the acetoxyl in the ^{13}C NMR spectrum of **1** and a resonance at δ_H 2.12 due to the acetyl methyl protons in the ^1H NMR spectrum of **1** disappeared in the ^{13}C and ^1H NMR spectra of **5**, respectively. Moreover, a resonance at δ_H 5.40 due to the oxygenated methine proton H-3 of **1** shifted upfield to δ_H 4.14 in the ^1H NMR spectrum of **5**. These data indicated that **5** was a deacetyl derivative of **1**. To confirm the presumption, **5** was acetylated using acetic anhydride and pyridine, affording diacetyl derivative (**5a**), which was identical in all respects to the compound obtained by acetylation of **1**.[19] Thus **5** was identified as 3-*O*-deacetylbotcinin A, and the absolute stereochemistry was assigned as 2*R*, 3*S*, 4*R*, 5*S*, 6*R*, 7*R*, 8*S*, 4′*S* on the basis of the absolute configuration of botcinin A.[19] 3-*O*-Deacetylbotcinin A (**5**) was named botcinin E.

Compound **6** was obtained as an amorphous solid. The molecular formula of $C_{20}H_{32}O_7$ was the same as that of **5**. The ^{13}C NMR spectral data of **6** were very similar to those of **5** except for some differences in chemical shifts. In the NOE experiments, irradiation of H-5 produced NOE enhancement of the H-3 and H-7 resonances, irradiation of the 4-methyl protons caused NOE enhancement of the H-2, H-6, and H-8 resonances, and irradiation of H-6 produced NOE enhancement of the H-8 resonance, indicating that **6** is the C-2 epimer of **5**. From the biogenetic viewpoint, compounds **5** and **6** may be biosynthesized from the same intermediate, suggesting that **5** and **6** have the same absolute configurations except for C-2. Thus, **6** was determined to be 3-*O*-deacetyl-2-*epi*-botcinin A and was named botcinin F.

The molecular formula of **13** ($C_{20}H_{34}O_8$) was obtained by HRFABMS and ^{13}C NMR data. The spectroscopic data were completely identical to those reported for botcinolide (**7**).[15,16] Since the optical rotation of botcinolide had not been reported, it was measured and found to be identical with that of **13**, indicating that botcinolide and **13** were identical. In the course of our purification, the behavior of **13** was not consistent with expected properties for the reported structure. For example, a remarkable tailing of the peak of **13** was produced when analyzed by HPLC without addition of acetic acid to the solvent, suggesting the existence of an acidic function. Furthermore, treatment of **13** with diazomethane gave a monomethyl ester derivative (**13a**), whose ^1H NMR spectrum showed a new methoxy resonance at δ_H 3.65 (3H, s), providing evidence for the presence of a carboxyl group. Thus, the earlier reported structure of botcinolide was not correct and its structure should be revised. The molecular formula of **13** had one more H_2O than that of botcinin E (**5**). The $^1H-^1H$ COSY of **13** revealed the three spin systems of $-C(2)CH_3-C(3)H-$, $-C(5)H-C(6)HCH_3-C(7)H-C(8)HCH_3-$, and the acyl portion, which are the same as in botcinin E. If one allows for the existence of a carboxyl group, compound **13** would be a seco acid of botcinin E. The ^{13}C NMR spectrum of **13**, including DEPT, is similar to that of **5**, except for the chemical shifts of the resonances of C-1, C-4, C-5, and C-6. In the ^{13}C NMR spectrum of **13**, C-1, C-4, C-5, and C-6 resonances are observed 2.8 ppm downfield, 1.9 ppm downfield, 7.2 ppm upfield, and 2.5 ppm downfield, respectively, compared to that of **5**. These shifts are due to the disappearance of the lactone ring. To verify this speculation, we treated **13** with acetic anhydride and pyridine to afford an acetylated and lactonized product, which was identical in all respects to **5a**. Thus the structure of botcinolide was revised from **7** to **13**, and the absolute configuration was assigned as 2*R*, 3*S*, 4*S*, 5*S*, 6*S*, 7*R*, 8*S*, 4′*S*. The name botcinolide is associated with the lactone, and thus botcinolide is renamed botcinic acid.

Although botcinins E (**5**) and F (**6**) are neutral metabolites, they were isolated from the acidic fraction together with botcinic acid (**13**), the seco acid of botcinin E, as described above. In addition, although neutral polar substances could be found in the acidic fraction by the present extraction procedure, the polarities of botcinins E and F are not high enough that they can be separated into the acidic fraction. This suggests that they exist as an equilibrium mixture of seco acid and lactone in the culture filtrate and that lactonization of a part of the seco acid in the acidic fraction occurs during extraction and purification. Thus, the seco acid of botcinin F is probably present somewhere in the acidic fraction of this fungus.

The ^1H and ^{13}C NMR data in $CDCl_3$ for botcinin E (**5**) were almost identical to those reported for 2-epibotcinolide (**11**)[18] in the same solvent except for three ^{13}C and one ^1H resonance. The resonance at δ_C 77.2 in the reported data could not be distinguished from the resonances of $CDCl_3$ in our spectrum. Two resonances at δ_C 174.0 and 165.8 in our spectrum have been reported at δ_C 173.2 and 170.1, respectively. The latter chemical shift δ_C 170.1 for C-1′ may be mistaken because it should be observed around 166 ppm. The resonace at δ_H 7.02 in our data corresponded with the resonance at δ_H 7.91 in the reported data, whose chemical shift may be typed incorrectly. Further investigation is needed before identifying 2-epibotcinolide to be the same as botcinin E.

Thus, the structures of 4-*O*-methylbotcinolide[18] and 3-*O*-acetyl-2-epibotcinolide[18] should be revised. 4-*O*-Methylbotcinolide (**9**) must be a methyl ester of botcinic acid (**13a**) because C-4 and the oxygen atom attached to C-4 were a part of the tetrahydropyrane ring in the corrected structure of botcinolide. The reported ^1H NMR data for 4-*O*-methylbotcinolide in C_6D_6 were identical to those of **13a** in the same solvent. Thus, the structure of 4-*O*-methylbotcinolide is revised from **9** to **13a**. In the course of reinvestigation we found the NMR data for 3-*O*-acetyl-2-epibotcinolide (**12**) to be completely identical to those of botcinin A (**1**). Therefore, the structure of 3-*O*-acetyl-2-epibotcinolide is revised from **12** to **1**.

The structures of the other botcinolide analogues are also discussed below. The ^1H NMR data for 3-*O*-acetyl-5-*O*-methylbotcinolide (**10**)[18] in $CDCl_3$ are very similar to those for **13a** in the same solvent. A new resonance due to acetyl methyl protons appears in the ^1H NMR spectrum of 3-*O*-acetyl-5-*O*-methylbotcinolide, and a resonance due to the methine proton (H-3) attached to the acetoxyl shifts downfield from the ^1H NMR spectrum of **13a**. Thus the structure of 3-*O*-acetyl-5-*O*-methylbotcinolide should be revised from **10** to **14**. Compound **14** is a methyl ester of 3-*O*-acetyl botcinic acid. Homobotcinolide[17] differs from **7** only in the length of the fatty acyl portion. Hence, the structure of homobotcinolide should be revised from **8** to **15**, and homobotcinolide is renamed botcineric acid.

In an antifungal assay, compounds **5**, **6**, and **13** showed weak antifungal activity, all with MICs of 100 μM, against *Magnaporthe grisea*. Botcinins B (**2**) and D (**4**) were the most active (both with MIC 12.5 μM) among the compounds tested so far.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Horiba SEPA-200 polarimeter. UV spectra were recorded with a Hitachi U-2001 spectrophotometer, and IR spectra with a JASCO FT/IR 7000 spectrometer. NMR spectra were measured with a JEOL

Table 1. ^{1}H and ^{13}C NMR Data (500 and 125 MHz) for **5**, **6**, and **13**

	5				**6**		**13**	
position	δ_C[a]	δ_H (J in Hz)[a]	δ_C[b]	δ_H (J in Hz)[b]	δ_C[b]	δ_H (J in Hz)[b]	δ_C[a]	δ_H (J in Hz)[a]
1	177.4		174.0		172.9		180.2	
2	39.5	3.21 dq (9.4, 7.3)	38.4	3.05 dq (9.9, 7.4)	42.2	2.54 dq (9.2, 7.4)	39.7	2.74 dq (7.1, 2.3)
2-CH_3	10.5	1.15 d (7.3)	10.3	1.28 d (7.4)	16.0	1.47 d (7.4)	17.4	1.32 d (7.1)
3	75.0	4.09 d (9.4)	74.0	4.14 d (9.9)	78.7	3.58 d (9.2)	77.7	3.57 d (2.3)
4	78.1		c		74.0		80.0	
4-CH_3	11.6	1.19 s	11.0	1.24 s	8.9	1.28 s	14.9	1.23 s
5	79.6	3.95 d (11.0)	78.4	3.70 d (11.0)	79.5	3.54 d (11.5)	72.4	3.78 d (10.8)
6	36.8	2.22 ddq (11.0, 10.8, 6.2)	35.6	2.18 ddq (11.0, 10.5, 6.2)	35.3	2.12 ddq (11.5, 10.3, 6.2)	39.3	1.88 ddq (10.8, 10.5, 6.4)
6-CH_3	13.9	1.03 d (6.2)	13.7	1.06 d (6.2)	13.5	1.05 d (6.2)	14.7	0.98 d (6.4)
7	77.9	4.51 dd (10.6, 9.9)	76.2	4.53 dd (10.5, 9.6)	76.6	4.50 dd (10.3, 9.6)	78.4	4.34 dd (10.5, 9.8)
8	69.5	3.79 dq (9.9, 6.2)	68.4	3.73 dq (9.6, 6.2)	68.2	3.75 dq (9.6, 6.2)	69.3	3.61 dq (9.8, 6.2)
8-CH_3	18.6	1.08 d (6.2)	18.2	1.12 d (6.2)	18.2	1.12 d (6.2)	18.1	1.00 d (6.2)
1′	167.6		165.8		165.7		167.7	
2′	119.9	6.06 dd (15.6, 1.6)	119.1	6.07 dd (15.6, 1.6)	119.1	6.07 dd (15.6, 1.6)	120.1	6.03 dd (15.6, 1.8)
3′	154.1	7.03 dd (15.6, 4.6)	151.8	7.02 dd (15.6, 4.6)	151.8	7.02 dd (15.6, 4.6)	153.6	6.99 dd (15.6, 4.8)
4′	71.6	4.26 m	71.1	4.34 m	71.1	4.34 m	71.6	4.24 m
5′	37.2	1.50−1.63 m	36.4	1.54−1.67 m	36.4	1.54−1.67 m	37.2	1.49−1.62 m
6′	28.7	1.33−1.47 m	27.4	1.31−1.48 m	27.4	1.31−1.45 m	28.7	1.29−1.46 m
7′	23.6	1.33−1.47 m	22.5	1.31−1.48 m	22.5	1.23−1.43 m	23.6	1.29−1.46 m
8′	14.3	0.93 t (7.3)	13.9	0.91 t (7.1)	13.9	0.92 t (7.1)	14.3	0.90 t (7.1)

[a] In CD_3OD solution. [b] In $CDCl_3$ solution. [c] The resonance could not be distinguished from the solvent.

JNM-ECP 500 spectrometer. Chemical shifts were referenced to $CDCl_3$ (δ_H 7.26, δ_C 77.0) and CD_3OD (δ_C 49.0). Mass spectra were obtained with a JEOL AX505HA spectrometer (direct probe). *p*-Nitrobenzyl alcohol was the matrix used for FABMS. In EIMS, the ionization voltage was 70 eV. In CIMS, the reaction gas was isobutane. HPLC was carried out with a Cosmosil 5C_{18}-AR column (Nacalai Tesque, 10 × 250 mm), a flow rate of 1.0 mL/min, and detection at 220 nm. Merck Kieselgel 60 F_{254} was used for the TLC. The spots on TLC plates were detected by spraying anisaldehyde-sulfuric acid reagent on the plate and heating it at 110 °C for 20 min. The spray reagent was prepared freshly before use by adding 1 mL of concentrated H_2SO_4 to a solution of 0.5 mL of *p*-anisaldehyde in 50 mL of acetic acid.

Fermentation and Extraction. The fungus[19] was grown without shaking at 24 °C for 14 days in the dark in 500 mL conical flasks (50) containing liquid medium (200 mL/flask) composed of glucose (30 g/L), peptone (3 g/L), the extract from 50 g/L of malt, and H_2O. Metabolites were extracted from the culture filtrate with EtOAc (3 × 10 L) after adjusting the pH to 2.0 with 6 M HCl. The EtOAc solution was extracted with 1 M $NaHCO_3$ (2 × 0.5 volume). The $NaHCO_3$ solution was acidified to pH 2.0 with 6 M HCl and extracted with EtOAc. The EtOAc solution was dried over Na_2SO_4 and concentrated to dryness to give a residue (5.2 g).

Isolation. The residue was subjected to silica gel partition column chromatography (100 g; impregnated with 60 mL of 0.1 M HCO_2H; 32 × 250 mm), with 1000 mL (200 mL × 5) each of 10, 20, 30, 40, and 60% EtOAc in *n*-hexane saturated with 0.1 M HCO_2H as eluent. The second fraction of 20% EtOAc in *n*-hexane was purified by silica gel column chromatography (22 × 105 mm). Elution was performed with 200 mL (40 mL × 5) each of 20, 30, and 40% acetone in *n*-hexane. The fourth and fifth fractions of 20% acetone in *n*-hexane were combined and further purified by Sephadex LH-20 column chromatography (28 × 800 mm) with MeOH as the solvent. Fractions 20−23 were purified by reversed-phase HPLC (60% MeOH) to yield compounds **5** and **6** (0.4 and 0.5 mg/L, t_R 50 and 55 min, respectively). The second fraction of 30% EtOAc in *n*-hexane was subjected to silica gel column chromatography (16 × 100 mm), with 400 mL (80 mL × 5) each of 20, 30, and 40% acetone in *n*-hexane as eluent. The third fraction of 30% acetone in *n*-hexane was purified by reversed-phase HPLC (70% MeOH in 1% AcOH) to yield compound **13** (0.6 mg/L, t_R 22 min).

Botcinin E (5): amorphous solid; $[\alpha]^{25}_D$ −69 (*c* 0.23, EtOH); UV λ_{max} (log ϵ) 212 (4.03) nm; IR (KBr) ν_{max} 3430, 2940, 1721, 1700 cm^{-1}; NMR data, see Table 1; FABMS *m*/*z* 385 $[M + H]^+$; HRFABMS *m*/*z* 385.2243 (calcd for $C_{20}H_{33}O_7$, 385.2226).

Diacetylbotcinin E (5a). Compound **5** (0.7 mg) was treated overnight with 40 μL of acetic anhydride and 20 μL of pyridine. The product was purified by HPLC (70% MeOH, 1.0 mL/min), giving **5a** (0.6 mg). Similarly, compound **13** (1.4 mg) was treated with acetic anhydride and pyridine to afford **5a** (1.0 mg): colorless needles; $[\alpha]^{25}_D$ −70 (*c* 0.10, EtOH); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.90 (3H, t, J = 6.9 Hz, H-8′), 1.06 (3H, d, J = 6.4 Hz, 6-CH_3), 1.09 (3H, d, J = 6.0 Hz, 8-CH_3), 1.12 (3H, d, J = 6.9 Hz, 2-CH_3), 1.26 (3H, s, 4-CH_3), 1.28−1.71 (6H, m, H-5′−7′), 2.11 (3H, s, CH_3CO), 2.13 (3H, s, CH_3CO), 2.19 (1H, ddq, J = 11.0, 10.1, 6.0 Hz, H-6), 3.16 (1H, dq, J = 9.6, 7.4 Hz, H-2), 3.67 (1H, dq, J = 9.6, 6.0 Hz, H-8), 3.80 (1H, d, J = 11.0 Hz, H-5), 4.53 (1H, dd, J = 10.6, 9.6 Hz, H-7), 5.41 (1H, d, J = 9.7 Hz, H-3), 5.42 (1H, m, H-4′), 5.94 (1H, dd, J = 15.6, 1.4 Hz, H-2′), 6.90 (1H, dd, J = 5.1, 15.6 Hz, H-3′); EIMS *m*/*z* 468 $[M]^+$ (7), 269 (22), 268 (100), 171 (12), 141 (84), 140 (24), 125 (15), 124 (36), 123 (45), 109 (58), 97 (64), 96 (15), 95 (28), 69 (27).

Botcinin F (6): amorphous solid; $[\alpha]^{25}_D$ −51 (*c* 0.26, EtOH); UV λ_{max} (log ϵ) 212 (4.05) nm; IR (KBr) ν_{max} 3432, 2940, 1721 cm^{-1}; NMR data, see Table 1; FABMS *m*/*z* 385 $[M + H]^+$; HRFABMS *m*/*z* 385.2206 (calcd for $C_{20}H_{33}O_7$, 385.2226).

Botcinic acid (13): amorphous solid; $[\alpha]^{25}_D$ −14 (*c* 0.35, EtOH); FABMS *m*/*z* 403 $[M + H]^+$; UV λ_{max} (log ϵ) 210 (4.03) nm; IR (KBr) ν_{max} 3428, 2936, 1717 cm^{-1}; HRFABMS *m*/*z* 403.2352 (calcd for $C_{20}H_{35}O_8$, 403.2332).

Botcinic acid methyl ester (13a). Compound **13** (0.8 mg) in acetone was treated with ethereal diazomethane to afford **13a** (0.8 mg): colorless needles; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.92 (3H, t, J = 7.1 Hz, H-8′), 0.98 (3H, d, J = 6.2 Hz, 6-CH_3), 1.01 (3H, d, J = 6.2 Hz, 8-CH_3), 1.29−1.46 (4H, m, H-6′, 7′), 1.29 (3H, s, 4-CH_3), 1.36 (3H, d, J = 7.1 Hz, 2-CH_3), 1.49−1.62 (2H, m, H-5′), 1.88 (1H, ddq, J = 10.8, 10.6, 6.2 Hz, H-6), 2.79 (1H, dq, J = 2.1, 7.1 Hz, H-2), 3.56 (1H, d, J = 2.1 Hz, H-3), 3.65 (1H, s, CH_3O), 3.72 (1H, q, J = 6.2 Hz, H-8), 3.89 (1H, d, J = 10.8 Hz, H-5), 4.33 (1H, m, H-4′), 4.40 (1H, dd, J = 10.6, 9.9 Hz, H-7), 6.07 (1H, dd, J = 15.6, 1.6 Hz, H-2′), 6.99 (1H, dd, J = 4.6, 15.6 Hz, H-3′); CIMS *m*/*z* 417 $[M + H]^+$.

Antifungal Assay. The antifungal activities of compounds **5**, **6**, and **13** were determined using the procedures reported previously.[19]

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References and Notes

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