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Isolation and Characterization of Gibbestatin B, an Inhibitor of Gibberellin-induced Expression of α-Amylase, and Gibbestatin C from Streptomycetaes

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The plant hormone, gibberellin, exerts various physiological effects in plants such as promotion of seed germination, stem elongation and flower initiation. In cereal seeds, gibberellin (GA) and abscisic acid (ABA) regulate the expression of various hydrolases production and seed germination. For example, α-amylase is produced in response to GA in cereal aleurone layers.[1] This system is a good model for the study on GA signal transduction at molecular level and has been used widely for researching the biochemical events during GA signal transduction.[2,3] However, the molecular mechanisms of GA action are still unclear. We intend to isolate inhibitors of GA signal transduction from the microbial metabolites, because these inhibitors would be useful tool in elucidating the mechanism of GA signal transduction. From the results of screening for the inhibitor of GA action on the expression of α-amylase, *Streptomyces* sp. C39 was found to produce an active compound, gibbestatin B (**1**) and a new related compound, gibbestatin C (**2**). **1**, renamed as gibbestatin B, has been reported as an antibiotic 2-11-B from *Streptomyces* sp. 2-11.[4] However, the biological activities and stereochemistry of antibiotic 2-11-B have not been reported. This report deals with the fermentation, isolation, structure determination and biological activity of **1** and **2**.

The strain C39 was cultured in the medium consisting of glucose 1.0%, soluble starch 1.5%, soybean meal 0.4%, peptone 0.1%, meat extract 0.1%, yeast extract 0.1% and corn steep liquor 0.1% (pH 7.2) for 2 days at 28°C. This seed culture (200 ml) was inoculated into a 30-liter jar fermenter containing 17 liters of the same medium mentioned above. Fermentation was carried out for 48 hours at 28°C with aeration (0.8 v/v/m) under constant agitation (250 rpm). The fermentation broth (30 liter) was centrifuged to remove the mycelia, and the supernatant was adjusted to pH 4.0 with 6 N HCl and passed through the DIAION HP-20 column (3 liter). The column was washed with the distilled water and eluted with MeOH. This eluate was evaporated, and then extracted with EtOAc at pH 4.0. The organic layer was dried over Na_2SO_4 and evaporated to give an oily residue (2.1 g). The oily residue was purified by silica gel column chromatography with a $CHCl_3$-MeOH solvent system. Active fractions containing **1** and **2** were further purified by a silica gel column ($CHCl_3$-MeOH), ODS MPLC (60% MeOH) and Sephadex LH-20 column chromatography ($CHCl_3$: MeOH=1 : 1). **1** (96 mg) and **2** (82 mg) were isolated as pale yellow powders. The physicochemical properties of **1** and **2** are as follows. **1**: MP >240°C (decomp.); $[\alpha]_D$ −12.4° (*c* 2.05, MeOH); UV λ_{max} (MeOH) nm 237 (29000), 287 (22000), 321 (10000); IR ν_{max} (KBr) 3398, 2973, 2931, 1663, 1612, 1581 cm^{-1}; HRFAB-MS *m/z* 387.1795 calcd. for $C_{22}H_{27}O_6$ (Δ−1.2 mmu); ^1H and ^{13}C NMR data (Table 1). **2**: MP >240°C (decomp.); $[\alpha]_D$ −69.0° (*c* 0.28, MeOH); UV λ_{max} (MeOH) nm 220 (52000), 255 (23500), 286 (22000), 339 (15700); IR ν_{max} (KBr) 3445, 2975, 2934, 1668, 1613, 1582 cm^{-1}; HRFAB-MS *m/z* 479.2079 calcd. for $C_{28}H_{31}O_7$ (Δ−1.0 mmu); ^1H and ^{13}C NMR data (Table 1).

The chemical structure of **1** was established by 2D NMR analyses. It agreed with the reported planner structure of an antibiotic 2-11-B which was elucidated as a methyl ester of antibiotic 2-11-B. Furthermore, the NMR data and specific rotation of a methyl ester of **1** are completely identical to those of the methyl ester of antibiotic 2-11-B. Thus, **1** was identified as antibiotic 2-11-B. The NMR signals of **1** were assigned on the basis of DQF-COSY and HMBC spectra (Table 1, Fig. 2). The vicinal coupling constant of 9.4 Hz between H-20 and H-12 indicated that the configuration between H-20 and H-12 is an *anti* form. NOESY spectrum of **1** displayed cross peaks from a diene proton (H-11) to a methine proton (H-20) and methyl protons (H-21), and from a methine proton (H-12) to an olefinic proton (H-10). The configuration of protons (H-16 and H-17) on epoxy ring was determined to be *trans* by the NOESY correlation between methyl protons (H-18) and an epoxy proton (H-16). However, the stereochemistry of epoxy ring was not elucidated from the NOESY spectrum. As a result, the relative stereochemistry of **1** except for epoxy ring was established as shown in Fig. 1.

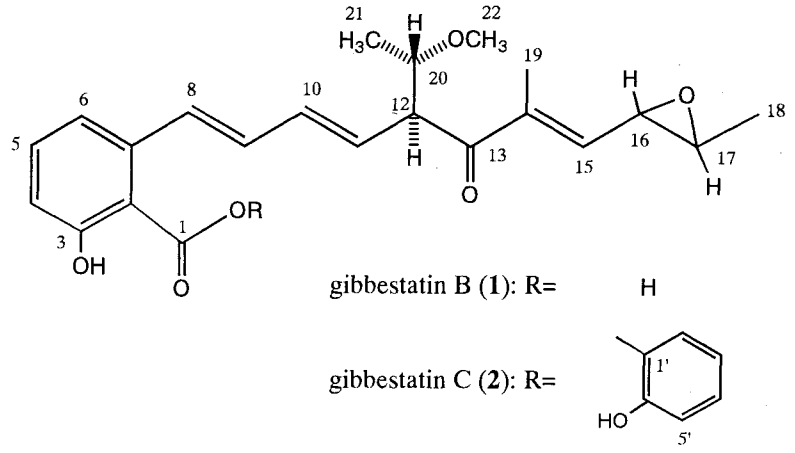
The structure of **2** was mainly determined by 2-D NMR experiments and by comparison with NMR signals of **1**. DQF-COSY spectrum of **2** indicated the connectivities from an olefinic proton (H-8) to a methine proton (H-12) and from an olefinic proton (H-15) to methyl protons (H-18). It

Table 1. ^{1}H, ^{13}C NMR data of **1** and **2**.

	1			**2**		
No.	δ_C	δ_H		δ_C	δ_H	
1	177.8 (s)			177.3 (s)		
2	118.4 (s)			119.1 (s)		
3	162.5 (s)			161.8 (s)		
4	116.8 (d)	6.72	d (8.1)	116.5 (d)	6.71	d (7.9)
5	132.5 (d)	7.2	dd (8.1, 7.4)	132.2 (d)	7.14	dd (7.9, 7.6)
6	118.4 (d)	6.97	d (7.4)	118.2 (d)	6.92	d (7.6)
7	142.1 (s)			141.1 (s)		
8	135.8 (d)	7.68	d (15.5)	135.6 (d)	7.46	d (15.4)
9	129.8 (d)	6.51	dd (15.5, 10.4)	129.4 (d)	6.36	dd (15.5, 10.5)
10	136.7 (d)	6.31	dd (15.2, 10.4)	137.6 (d)	6.19	dd (15.1, 10.5)
11	129.7 (d)	5.52	dd (15.2, 9.4)	128.8 (d)	5.25	dd (15.1, 9.4)
12	57.9 (d)	3.90	t (9.4)	57.7 (d)	4.00	t (9.4)
13	202.9 (s)			202.9 (s)		
14	141.6 (s)			139.9 (s)		
15	140.3 (d)	6.23	dd (8.2, 0.7)	143.4 (d)	6.75	dd (8.4, 0.7)
16	56.8 (d)	3.48	dd (8.2, 2.0)	58.4 (d)	4.41	dd (8.4, 2.7)
17	57.6 (d)	3.08	qd (5.2, 2.0)	70.5 (d)	4.11	qd (6.4, 2.7)
18	17.8 (q)	1.37	d (5.2)	20.1 (q)	1.26	d (6.4)
19	12.3 (q)	1.92	d (0.7)	12.7 (q)	1.88	d (0.6)
20	80.0 (d)	3.71	qd (9.4, 5.2)	79.4 (d)	3.79	qd (9.4, 6.1)
21	17.1 (q)	1.12	d (5.2)	17.2 (q)	1.09	d (6.1)
22	57.2 (q)	3.19	s	57.2 (q)	3.24	s
1'				143.4 (s)		
2'				133.9 (d)	7.89	d (7.6)
3'				116.5 (d)	6.38	dd (7.8, 7.6)
4'				133.9 (d)	7.10	dd (8.4, 7.8)
5'				113.4 (d)	6.60	d (8.4)
6'				151.1 (s)		

All spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C sepctrum in CD_3OD, coupling constant (Hz).

Fig. 1. Structure of gibbestatin B (**1**) and C (**2**).



gibbestatin B (**1**): R= H

gibbestatin C (**2**): R=

Fig. 2. HMBC and NOESY correlations of **1**.

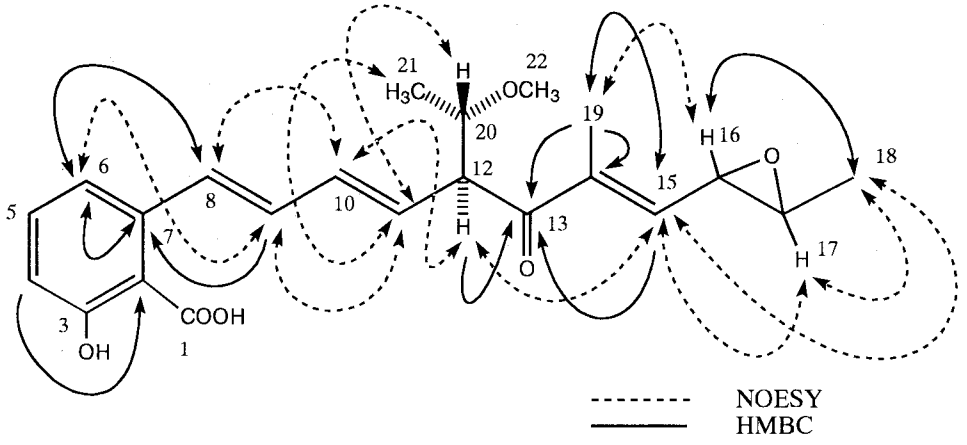
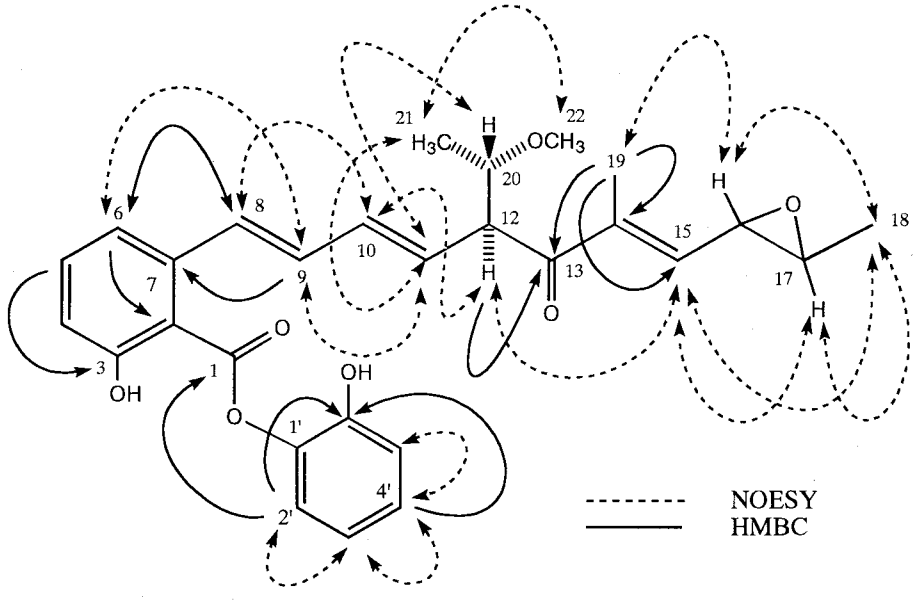


Fig. 3. HMBC and NOESY correlations of **2**.



also showed that the two benzene rings were a 1,2-disubstituted and a 1,2,3-trisubstituted system, respectively. The HMBC spectrum displayed the correlation between H-2′ and C-1. The connections from C-6 to C-8 and from C-12 to C-15 were also confirmed by HMBC correlations. The other correlations observed in HMBC spectrum and the assignments of NMR signals of **2** were shown in Fig. 3 and Table 1, respectively. The configuration between H-12 and H-20 were elucidated to be an *anti* form by the vicinal coupling constant (9.4 Hz). The NOE correlations of **2** were the same as observed in **1** (Fig. 3). It showed that the configuration of protons on epoxy ring would be a *trans* form and also that the stereochemistry at C-12 and C-20 positions would be the same as those of **1**. However, the stereochemistry of epoxy ring on **2** also could not be determined by NOESY spectrum. Thus, the stereostructure of **2** except for epoxy ring was determined as shown in Fig. 1.

1 inhibited GA-induced α-amylase expression in the deembryonated rice and barley with an IC_{50} of 25 to

50 ppm. A complete inhibition was observed at 100 to 200 ppm. On the other hand, **2** did not show inhibitory activity at 200 ppm. **1** and **2** showed no antimicrobial activities against gram negative and positive bacteria, yeast and fungi at 100 ppm (MIC, agar dilution method using bouillon agar for bacteria and Sabouraud agar for yeast and fungi). Furthermore, protein synthesis of tobacco plant was not inhibited by **1** at 200 ppm. The detailed biological activities of **1** will be published elsewhere.

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