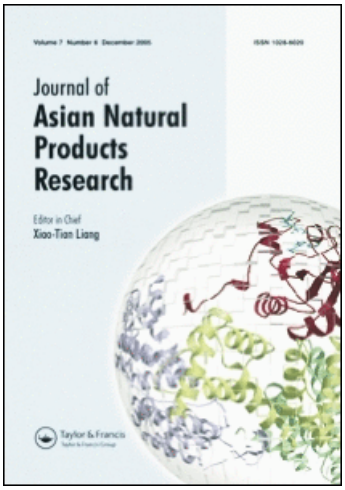

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Three new metabolites from *Botrytis cinerea*

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Three new metabolites, γ-abscisolactone (**1**), botrytisic acids A (**3**) and B (**4**) were isolated from the fermentation broth of *Botrytis cinerea* TB-3-H8. Their structures were elucidated on the basis of MS, IR, UV, and NMR spectroscopic data. Compound **2** was isolated from natural resource for the first time. The structure of **1** was further confirmed by single-crystal X-ray diffraction (**CCDC-265897**).

Keywords: abscisic acid; *Botrytis cinerea*; γ-abscisolactone; botrytisic acids A and B

1. Introduction

It was reported that *Botrytis cinerea* could produce natural abscisic acid (ABA), an efficient plant hormone [1]. The natural ABA was industrially produced by the fermentation of this fungus in China [2]. As a part of ongoing investigations on the analogs of ABA, threes new metabolites were found, which was helpful for the metabolism mechanism of ABA in *B. cinerea*. In this paper, we present the isolation and structure elucidation of these new metabolites. **1** was sesquiterpene lactone, while **3** and **4** were irregular sesquiterpenoid acids that exhibited a previously undescribed carbon skeleton (Figure 1). Meanwhile, a derivative of ABA (**2**) was isolated from natural resource for the first time, which was reported to be a product of ABA by chemical synthesis [3]. The structures of these new metabolites were elucidated by means of their spectroscopic data, mainly HR-ESI-MS and NMR spectra (1D and 2D NMR). The structure of **1** was also confirmed by X-ray diffraction.

2. Results and discussion

Botrytis cinerea TB-3-H8 was grown on self-made medium in 15t fermentor for 15 days. In the fermentation broth, three new metabolites (**1**, **3**, and **4**) and one known metabolite (**2**) were produced under the fermenting condition.

γ-Abscisolactone (**1**) was obtained as colorless crystal. The molecular formula was established as $C_{15}H_{16}O_4$ according to HR-ESI-MS at *m/z* 259.0970 $[M - H]^-$, indicating eight degrees of unsaturation in the molecule. The IR spectrum showed strong absorption bands for α,β-unsaturated γ-lactone (1782, 1760 cm^{-1}) and hydroxyl group (3410 cm^{-1}). Inspection of ^{13}C NMR spectrum with the assistance of DEPT experiment suggested the presence of three olefinic methines, five olefinic quaternary carbons, one carbonyl carbon, one ester carbonyl carbon, and one quaternary carbon. So eight degrees of unsaturation were assigned to two C=O groups, four C=C double bonds, and two rings.

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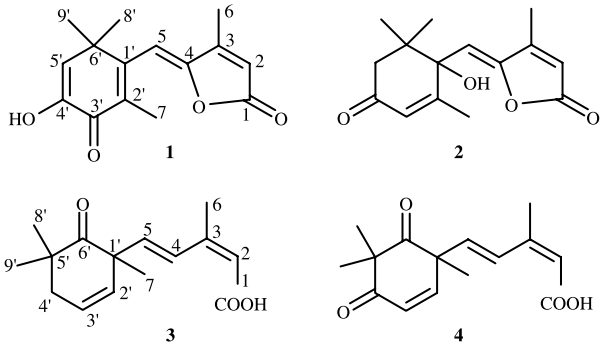

Figure 1. Structures of **1**–**4**.

The chemical shifts of **1** distributing in ^{1}H and ^{13}C NMR spectra were very similar to those of ABA [4,5]. HSQC spectrum allowed the assignment of all protonated carbons as shown in Table 1. The structure of **1** was elucidated by the HMBC correlations using four characteristic methyls as the starting points.

In the HMBC spectrum, significant correlations of four methyls via ^{2}J and ^{3}J were observed between H-6/C-2, H-6/C-3, H-6/C-4, H-7′/C-1′, H-7′/C-2′, H-7′/C-3′, H-8′(9′)/C-1′, H-8′(9′)/C-5′, and H-8′(9′)/C-6′, indicating the existence of three fragments as shown in Figure 2. The connections of these three fragments were determined by the HMBC correlations of H-5 with C-4 and C-1′, and the unique mobile proton (δ_H 6.36, s) showing the HMBC correlations to C-3′, C-4′, and C-5′, which suggested the hydroxyl group was attached to C-4′. Lastly, the ester group attaching to C-2 was determined by the HMBC correlation of H-2 to C-1, and the loading site of oxygen atom was determined by considering the absence of H-4 signal and the downfield of C-4 compared with the corresponding signal of ABA [4,5].

A single-crystal X-ray diffraction analysis further confirmed the structure of **1** (Figure 3). So **1** was elucidated as (4-*cis*)-5-[2,6,6-trimethyl-3-oxo-4-hydroxyl-1,4-cyclohexadien-1-yl]-3-methyl-γ-2,4-penadienelactone, named γ-abscisolactone.

Compound **2** was identified by the comparison of its NMR spectral data with the literature data [3]. To our knowledge, **2** was isolated from natural resource for the first time, although it has been synthesized from ABA in alkaline condition [3].

Botrytisic acid A (**3**) was obtained as yellow oil. A $[M + Na]^{+}$ at *m/z* 271.1306 in the HR-ESI-MS corresponded to the molecular formula $C_{15}H_{20}O_3$ with six degrees of unsaturation. The IR spectrum showed absorption bands for α,β-unsaturated carboxyl group (3000–2500 and 1683 cm^{-1}) and keto carbonyl group (1705 cm^{-1}). Fifteen carbons were characterized by ^{13}C NMR and DEPT experiment as four methyls, one methylene, five olefinic methines, one olefinic quaternary carbon, two quaternary carbons, one carbonyl carbon, and one carboxyl carbon. So the degrees of unsaturation were assigned to two C═O groups, three C═C double bonds, and one ring.

The assignment of protons and their corresponding carbons were also determined by HSQC spectrum (Table 1). ^{1}H NMR spectrum indicates the presence of two fragments, (*trans*)—CH═CH— (I) and (*cis*)—CH═CH—CH_2— (II). H-4 and H-5 were in a *trans* configuration since $J_{H\text{-}4/H\text{-}5} = 16.2$ Hz, indicating the existence of fragment I. Identically, H-2′ and H-3′ were in a *cis* configuration since $J_{H\text{-}2'/H\text{-}3'} = 10.0$ Hz, and the unique methylene was attached to C-3′ by considering the coupling relationship of H-3′ and H-4′, which suggested the existence of fragment II. The structure of **3** was deducted by the HMBC correlations using these two fragments as the starting points. The protons of

Table 1. ^{1}H and ^{13}C NMR[a] spectral data for **1**–**4**.

Position	**1**		**2**		**3**		**4**	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	168.3		167.2		171.2		170.0	
2	118.1	6.07 (s)	116.1	6.02 (s)	116.7	5.61 (s)	117.9	5.76 (s)
3	151.6		151.2		153.1		151.7	
4	154.2		155.2		126.4	7.52 (d, 16.2)	128.1	7.64 (d, 16.2)
5	104.9	5.87 (s)	110.5	5.32 (s)	141.6	6.04 (d, 16.2)	139.5	6.14 (d, 16.2)
6	12.0	2.29 (s)	12.1	2.18 (d, 1.32)	21.3	1.92 (s)	21.1[d]	2.02 (s)
1′	154.5		79.8		51.2		51.6	
2′	131.7		162.9		132.0	5.70 (d, 10.0)	149.5	6.94 (d, 10.5)
3′	181.8		126.4	5.89 (s)	125.5	5.88 (dt, 10.0, 4.2)	127.1	6.37 (d, 10.5)
4′	144.9		197.3		38.6	2.22 (β, dd, 4.2, 1.2) 2.19 (α, d, 4.2)	200.8	
5′	125.6	6.11 (s)	50.0	2.43 (β, d, 17.2)	44.0		58.7	
6′	39.4		42.5	2.38 (α, d, 17.2)	216.1		210.1	
7′	13.9	1.91 (s)	19.4	2.01 (d, 1.32)	26.0[b]	1.23 (s)	25.3	1.48 (s)
8′	27.6	1.29 (s)	24.7	1.13 (s)	25.9[b]	1.06[c] (s)	21.1[d]	1.33[e] (s)
9′	27.6	1.29 (s)	22.7	1.10 (s)	26.0[b]	1.04[c] (s)	26.4	1.32[e] (s)
HO		6.36 (s)		4.00 (s)				

[a] ^{1}H and ^{13}C NMR spectra were measured in $CDCl_3$ at 600 and 150 MHz, respectively. All protons and carbons were assigned by the HSQC and HMBC spectra.
[b–e] Interchangeable signals.

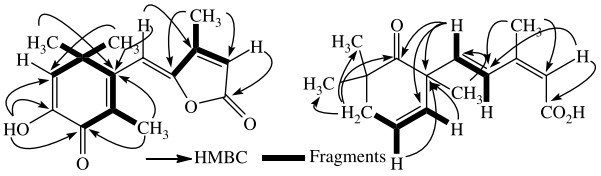

Figure 2. Key HMBC correlations of compounds **1** and **3**.

the unique ethylene in fragment II show HMBC correlations with C-5′, C-6′, C-8′, and C-9′; H-2′ shows HMBC correlations with C-7′, C-1′, and C-6′. These HMBC correlations demonstrated the existence of a six-membered ring. Meanwhile, the HMBC correlations were observed in the 2,4-pentadienoic acid side chain via ^{2}J and ^{3}J such as H-6/C-2, H-6/C-3, H-6/C-4, and H-2/C-1, and the connection of this side chain to the ring was determined by the significant HMBC correlations of H-5 to C-1′, C-2′, and C-6′.

The structure of compound **3** was deduced by the above analysis as (2-*cis*,4-*trans*)-5-(1,5,5-trimethyl-6-oxo-2-cyclohexen-1-yl)-3-methyl-2,4-pentadienoic acid, named botrytisic acid A. To our knowledge, this carbon skeleton has never been described in the literature.

Botrytisic acid B (**4**) was obtained as yellow oil. The HR-ESI-MS data showed the molecular formula of **4** as $C_{15}H_{18}O_4$ that required seven degrees of unsaturation. The IR spectrum showed absorption bands for carboxyl group (2982–2450, 1682 cm^{-1}), carbonyl group (1715 cm^{-1}). The UV absorption maximum at 266 nm was very approximate to that of **3** ($\lambda_{max} = 267$ nm), which demonstrated that **4** should have the same side chain to **3**. The ^{1}H NMR spectrum of **4** was close to that of **3**, except for the absence of the characteristic methylene signals of H-3′ and the appearance of a carbonyl signal at δ_c 200.8 in ^{13}C NMR spectrum. In HMBC spectrum, the carbonyl group correlated with H-2′, H-3′, and H-8′(9′).

Depending on the above analysis, **4** was deduced to be 4′-oxo derivative of **3**, named botrytisic acid B.

3. Experimental

3.1 General experimental procedures

Melting points were determined with an XRC-1 microscope type and are uncorrected. UV spectra were recorded on a Perkin–Elmer Lambda 35 and IR spectra were recorded on a Perkin–Elmer Spectrum One FT-IR Spectrometer. ESI-MS was recorded on a Finnigan LCQ^{DECA} mass spectrometer while HR-ESI-

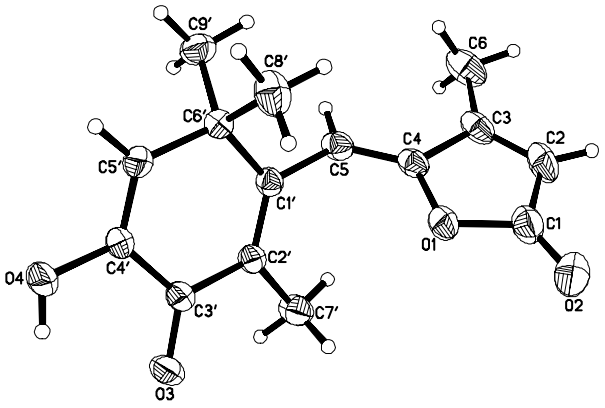

Figure 3. The ORTEP drawing of **1**.

MS in Bruker Bio TOF III^{Q} mass spectrometer. All NMR spectra were measured with a Bruker Avance 600 spectrometer at 600 MHz for ^{1}H-NMR and 150 MHz for ^{13}C NMR in $CDCl_3$ using TMS as an internal standard.

X-ray diffraction data for **1** were collected at 298 K on a Siemens P4 diffractometer equipped with a graphite monochromator, MoKα ($\lambda = 0.71073$ Å), to a maximum θ of 27.00. The structure was solved by direct methods and refined by full-matrix least squares using SHELXS-97 [6]. All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were refined in calculated positions. The programs Siemens XSCANS, Siemens SHELXTL, and Siemens SHELXL-97 were utilized for the data collection, reduction, and refinement, respectively [7].

3.2 *Extraction and isolation*

The fungus, *B. cinerea*, was grown on self-made medium in 15t fermentor for 15 days. The self-made medium was composed of the following medium: starch (20 g l^{-1}), cane sugar (2.0 g l^{-1}), KH_2PO_4 (1.0 g l^{-1}), NH_4NO_3 (2.0 g l^{-1}), soybean powder (10 g l^{-1}), and complex B. Then the fermentation broth was absorbed by the macroreticular resin and eluted with EtOH to give a crude gum. This crude gum (17.0 g) was chromatographed over silica gel (200–300 mesh) with a gradient of petroleum ether to ethyl acetate, then rechromatographed over ODS RP-18 with a gradient of 20–80% methanol to afford compounds **1** (3.4 mg), **2** (12.2 mg), **3** (92.6 mg), and **4** (8.2 mg). The purity of these compounds was detected by HPLC (70% MeOH). A single crystal of **1** was obtained from a mixed solution of MeOH–H_2O (1:1) by solvent evaporation at reduced pressure condition at room temperature.

3.2.1 *γ-Abscisolactone (**1**)*

Colorless crystal, mp 123–125°C; UV $CHCl_3$ λ_{max} nm (log ε): 246 (2.47), 288 (2.44); IR ν_{max} KBr cm^{-1}: 3410, 1782, 1760, 1620, 1209; ^{1}H and ^{13}C NMR spectral data, see Table 1; ESI-MS *m/z*: 259.19 [M − H]$^{-}$; HR-ESI-MS *m/z*: 259.0970 [M − H]$^{-}$ (calcd for $C_{15}H_{15}O_4$, 259.0965).

3.2.2 *Compound **2***

White solid, mp 169.5–170.5°C; UV $CHCl_3$ λ_{max} nm (log ε): 243 (1.66), 279 (1.07); IR ν_{max} KBr cm^{-1}: 3474, 3060, 2991, 2876, 1759, 1748, 1657, 1622, 1610, 1346, 1209, 926, 830; ^{1}H and ^{13}C NMR spectral data, see Table 1; HR-ESI-MS *m/z*: 285.1095 [M + Na]$^{+}$ (calcd for $C_{15}H_{18}NaO_4$, 285.1097).

3.2.3 *Botrytisic acid A (**3**)*

Yellow oil, UV $CHCl_3$ λ_{max} nm (log ε): 267 (0.51); IR ν_{max} neat cm^{-1}: 3073, 3028, 2966–2500, 1705, 1683, 1628, 1596, 1453, 1263, 1236, 1150, 1030, 989; ^{1}H and ^{13}C NMR spectral data, see Table 1; ESIMS *m/z*: 247.1 [M − H]$^{-}$; HR-ESI-MS *m/z*: 271.1306 [M + Na]$^{+}$ (calcd for $C_{15}H_{20}NaO_3$, 271.1305).

3.2.4 *Botrytisic acid B (**4**)*

Yellow oil; UV $CHCl_3$ λ_{max} nm (log ε): 266 (0.55); IR ν_{max} neat cm^{-1}: 2983–2500, 1716, 1683, 1633, 1599, 1456, 1267, 1161, 989, 734; ^{1}H and ^{13}C NMR spectral data, see Table 1; ESIMS *m/z*: 261.0 [M − H]$^{-}$; HR-ESI-MS *m/z*: 285.1078 [M + Na]$^{+}$ (calcd for $C_{15}H_{18}NaO_4$, 285.1097).

3.3 *Single-crystal X-ray crystallography of 1*

The X-ray crystallographic data collection and refinement of **1** (0.60 × 0.24 × 0.22 mm) were conducted on a crystal at 286(2) K. $C_{15}H_{16}O_4$, $Mr = 260.28$, monoclinic, space group $P2(1)/c$, $a = 14.644(3)$ Å, $b = 7.257(1)$ Å, $c = 13.670(2)$ Å, $\beta = 109.60(1)°$, $V = 1368.57(44)$ Å^3, $Z = 4$, $\rho = 1.263$ M g m^{-3}, μ (Mo Kα) = 0.091 mm^{-1}, $F(000) = 552$, GOF = 0.872. A total of 3486 reflections were collected in the range $1.48° \leq \theta \leq 27.00°$, of which 2986 reflections were independent

($R_{int} = 0.0128$), and 1632 reflections with $I > 2.0\sigma(I)$ were used for refinement. The final R indices were $R = 0.0419$, $wR = 0.0939$.

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