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Antimycobacterial Pimarane Diterpenes from the Fungus *Diaporthe* Sp.

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Abstract—Two new pimarane diterpenes, diaportheins A (**1**) and B (**2**), were isolated from a culture broth of the fungus *Diaporthe* sp. BCC 6140. Diaporthein B (**2**) strongly inhibited the growth of *Mycobacterium tuberculosis* with the MIC value of 3.1 μg/mL, while diaporthein A (**1**) showed only mild activity (MIC value of 200 μg/mL).

The incidence of tuberculosis infection has rapidly increased, it is estimated that approximately one-third of the worlds population is infected with *Mycobacterium tuberculosis* and that seven to eight million new cases of tuberculosis occur each year.[1] Development of new drugs for use against the emerging multidrug resistant strains of *M. tuberculosis* is therefore urgently needed. In the course of our continuing search for biologically active substances from plants and microorganisms,[2] we have screened extracts from a number of Thai plants and fungi, and those with interesting biological activities are routinely subjected to chemical exploration. We report herein the isolation and characterization of two new antitubercular pimarane diterpenes, diaportheins A (**1**) and B (**2**), from the culture broth of the fungus *Diaporthe* sp. BCC 6140.

Structural Elucidation

Diaporthein A (**1**) possessed a molecular formula of $C_{20}H_{30}O_6$ (the ESITOF mass spectrum exhibited an accurate mass of *m/z* 389.1971 $[M+Na]^+$, $\Delta+3.1$ mmu). The IR spectrum of **1** exhibited a broad OH stretching at 3425 cm^{-1}. Analyses of ^1H, ^{13}C, DEPT135 and HMQC NMR data revealed that diaporthein A (**1**) contained 20 carbons including three singlet methyl, six methylene, four methine, and seven quaternary carbons. NMR spectral data also demonstrated that **1** possessed a methylene connected to an oxygen (at δ_H 3.36 and 3.97, and δ_C 68.3), an sp^2 methylene (at δ_H 5.04, and δ_C 111.5), two olefinic protons (at δ_H 5.85, and δ_C 146.4; and at δ_H 5.99, and δ_C 133.3), and two methines connected to an oxygen atom (at δ_H 4.63, and δ_C 73.3; and at δ_H 3.86, and δ_C 67.3). The presence of 20 carbons in **1** together with three singlet methyls and a number of sp^3 methylene protons implied that diaporthein A (**1**) possesses a diterpenoid skeleton. ^1H–^1H COSY spectrum of **1** revealed the H-1 to H-3 partial structure and also correlations between H-11 and H-12, and between H-15 and H-16. The connections between rings A, B and C in diaporthein A (**1**) were unambiguously assigned by HMBC correlations. Key long ranged ^1H-^{13}C correlations were observed from both H-18 and H-19 to C-3, C-4, C-5; H-18 to C-19; H-19 to C-18; H-20 to C-1, C-5, C-9 and C-10; H-17 to C-12, C-13, C-14 and C-15; H-16 to C-13; H-15 to C-12 and C-13; H-14 to C-7, C-9, C-12 and C-15; H-11 to C-10, and H-7 to C-6, C-8 and C-9. The downfield shift of the methylene H-20 (at δ_H 3.36 and 3.97, and δ_C 68.3) suggested that diaporthein A (**1**) possessed an ester or ether linkage at this oxygenated methylene, and the HMBC correlation from H-20 to a quaternary C-6 (at δ_C 105.9) finally established a hemiacetal unit in **1**. Complete assignment of protons and carbons in **1** is shown in Table 1. The NOESY spectrum was extremely informative concerning the assignment of

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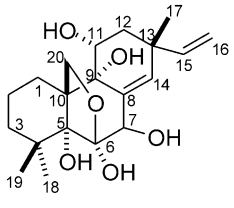
Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data ($CDCl_3$) of diaportheins A (**1**) and B (**2**)

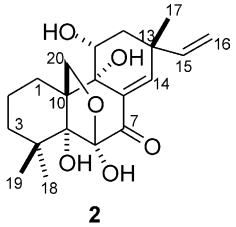
C	Diaporthein A (**1**)		Diaporthein B (**2**)	
	δ_C, multiplicity[a]	δ_H, multiplicity, J in Hz	δ_C, multiplicity[a]	δ_H, multiplicity, J in Hz
1	24.8, t	1.70, m; 1.82, m	25.2, t	1.96, m; 2.03, m
2	18.0, t	1.61,m; 1.67, m	17.6, t	1.62, m; 1.68, m
3	37.8, t	1.22, m; 1.69, m	37.5, t	1.23, m; 1.55, m
4	38.0, s	—	37.3, s	—
5	81.3, s	—	81.9, s	—
6	105.9, s	—	104.1, s	—
7	73.3, d	4.63, d, 2.1	196.2, s	—
8	136.8, s	—	134.7, s	—
9	77.1, s	—	76.2, s	—
10	50.1, s	—	51.1, s	—
11	67.3, d	3.86, dd, 12.0 and 4.2	67.7, d	4.03, dd, 11.7 and 4.1
12	40.4, t	1.71, m; 1.89, m	39.9, t	1.73, m; 2.07, m
13	38.3, s	—	40.1, s	—
14	133.3, d	5.99, dd, 1.6 and 1.7	150.4, d	6.81, d, 1.8
15	146.4, d	5.85, dd, 17.5 and 10.7	144.1, d	5.82, dd, 17.5 and 10.7
16	111.5, t	5.04 (2H), m	113.1, t	5.09 (2H), m
17	25.3, q	1.16, s	25.9, q	1.22, s
18	29.6, q	1.26, s	26.9, q	1.19, s
19	24.2, q	1.44, s	23.7, q	1.45, s
20	68.3, t	3.36, d, 9.6; 3.97, d, 9.6	68.6, t	3.71, d, 10.2; 4.14, d, 10.2

[a]Multiplicity was determined by analyses of DEPT spectra.

stereochemistry in **1**, from which correlations between H-20a (at δ_H 3.97) and H-19 (at δ_H 1.44), H-20b (at δ_H 3.36) and H-11 (at δ_H 3.86), and H-11 and H-17 (at δ_H 1.16) were observed. The coupling constants (H-11, δ_H 3.86, dd, $J=12.0$ and 4.2 Hz) also indicated an axial orientation of H-11. Based upon these spectral data, the structure of diaporthein A (**1**) was identified as an oxygenated pimarane diterpene. Unfortunately, the stereochemistry at C-7 of **1** could not be established based upon available spectral data. However, diaporthein A (**1**) is structurally related to the phytotoxic sphaeropsidins[3–5] and the antibiotics LL-S491β and γ,[6] all of which possess β hydroxyl at C-7. It is more likely that diaporthein A (**1**) also possesses the β hydroxyl group as there is the evidence that **1** also exhibited a positive optical rotation similar to those of sphaeropsidins.[3–5]



1



2

Diaporthein B (**2**) had a molecular formula of $C_{20}H_{28}O_6$ (observed m/z 387.1785 $[M+Na]^+$, Δ +0.1 mmu). The IR spectrum of **2** exhibited a carbonyl stretching at 1704 cm^{-1}, while the ^{13}C NMR data confirmed the presence of a conjugated ketone signal at δ_C 196.2. Generally, the ^{1}H and ^{13}C NMR spectra of **2** were similar to those of diaporthein A (**1**), except the missing of the oxygenated methine signal at δ_H 4.63 in **2** and the replacement from the hydroxyl signal (at δ_C 73.3) in **1** to the carbonyl resonance (at δ_C 196.2) in **2**. Based on these spectral data, diaporthein B (**2**) was assigned as the ketone derivative of diaporthein A (**1**). Analyses of ^{1}H–^{1}H COSY, HMQC and HMBC spectral data allowed complete assignment of protons and carbons in **2** (Table 1). Important HMBC correlations were from H-19 to C-3, C-4 and C-5; H-18 to C-4 and C-5; H-20 to C-1, C-5, C-6, C-9 and C-10; H-17 to C-12, C-13, C-14 and C-15; H-16 to C-13; H-15 to C-12; H-14 to C-7 and C-12; and H-11 to C-13. The NOESY spectrum again demonstrated the correlations between H-20a (at δ_H 4.14) and H-19 (at δ_H 1.45), H-20b (at δ_H 3.71) and H-11 (at δ_H 4.03), and H-11 and H-17 (at δ_H 1.22), thus the stereochemistry of diaporthein B (**2**) was established.

Biological Activities of Pimaranes 1 and 2

While diaporthein A (**1**) demonstrated only weak antimycobacterial activity (with the minimum inhibitory concentration (MIC) value of 200 μg/mL), diaporthein B (**2**) strongly inhibited the growth of *Mycobacterium tuberculosis* with the MIC value of 3.1 μg/mL (Table 2). It should be noted that the structural difference between diaporthein A (**1**) and diaporthein B (**2**) is only at C-7 position (**1** possesses hydroxy group, while **2** contains ketone), and this indicates that the ketone moiety at C-7 in **2** is crucially important for the antitubercular activity. The trend of cytotoxicity against Vero cell line was also similar to that of anti-tubercular activity (Table 2); **1** was less active (IC_{50} value > 50 μg/mL) than **2** (IC_{50} 1.5 μg/mL).

Table 2. Biological activities of diaportheins A (**1**) and B (**2**)

Compd	Antitubercular activity MIC (μg/mL)	Cytotoxicity to Vero cell line IC_{50}, μg/mL
Diaporthein A (**1**)	200[a]	> 50[b]
Diaporthein B (**2**)	3.1[a]	1.5[b]

[a]Triplicate experiments showing the same MIC value.
[b]Typical values from replicate experiments.

Fungal Material, Extraction and Isolation

The fungus *Diaporthe* sp. BCC 6140 was collected from the unidentified wood in 1997 from Songkla Province (Thailand), and identified by Professor E. B. G. Jones; the fungus specimen was deposited at the BIOTEC Culture Collection (registration no. BCC 6140). *Diaporthe* sp. BCC 6140 was cultured in a MEB medium, and then transferred into 250 mL of the same culture medium. The culture was incubated at 25 °C for 38 days, and subsequently harvested for further study. The culture of *Diaporthe* sp. (5 L) was filtered to separate broth and cells. The former was extracted twice with an equal volume of EtOAc, and the combined EtOAc layers were evaporated to dryness, yielding 532 mg of a crude extract. A crude EtOAc extract was subjected to Sephadex LH-20 column (eluted with MeOH) to give five fractions (F1–F5). Fraction F4 was further separated by semi-preparative HPLC (C_{18} reversed phase column and eluted with 35% of MeCN in H_2O) to furnish 4.3 mg of diaporthein A (**1**) and 5.4 mg of diaporthein B (**2**).

Diaporthein A (**1**). Off-white solid; mp 198–200 °C; $[\alpha]_D^{31}$ +25.8° (*c* 0.124, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 203 (4.08) nm; IR (KBr) ν_{max} 3425, 2930, 1652, 1455, 1394, 1048, 1009, 656 cm^{-1}; ESITOF MS *m*/*z* 389.1971 $(M+Na)^+$, calcd for $C_{20}H_{30}O_6Na$ (389.1940); ^1H and ^{13}C NMR see Table 1.

Diaporthein B (**2**). Off-white solid; mp 119–220 °C; $[\alpha]_D^{31}$ +120.1° (*c* 0.106, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 230 (3.97) nm; IR (KBr) ν_{max} 3418, 2927, 1704, 1620, 1454, 1393, 1198, 1043, 1005, 942 cm^{-1}; ESITOF MS *m*/*z* 387.1785 $(M+Na)^+$, calcd for $C_{20}H_{28}O_6Na$ (387.1784); ^1H and ^{13}C NMR see Table 1.

Bioassay Procedures

Antimycobacterial activity was evaluated against *M. tuberculosis* H37Ra employing the Microplate Alamar Blue Assay (MABA).[7] The reference drugs were isoniazid and kanamycin sulfate, showing the MIC values of 0.04–0.09 and 2.0–5.0 μg/mL, respectively. Cytotoxicity was determined according to the colorimetric assay essentially described by Skehan and co-workers.[8] The standard compound was ellipticine exhibiting the activity towards BC-1 and KB cell lines both with the IC_{50} values of 0.3 μg/mL.

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