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ISOLATION, STRUCTURE ELUCIDATION, AND MUTAGENICITY OF FOUR ALTERNARIOL DERIVATIVES PRODUCED BY THE MANGROVE ENDOPHYTIC FUNGUS No. 2240

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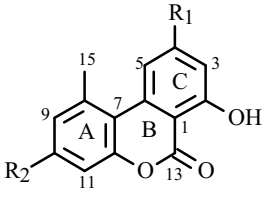
*A new alternariol derivative, 2240B (**1**), together with alternariol (**2**), alternariol 4,10-dimethyl ether (**3**), and alternariol 4-methyl ether (**4**), was isolated from the ethyl acetate extract of the liquid medium GYT of No. 2240, the mangrove endophytic fungus from the South China Sea Coast. The structure of compound **1** was unambiguously elucidated as alternariol 4-methyl-10-acethyl ester by spectra including one/two-dimensional NMR, HREIMS, IR, and UV. The structures of compounds **2**–**4** were also established by spectroscopic analyses and comparison with related literature data. The anticancer tests showed that compounds **2** and **4** had strong activities against KB and KBv200 cells with IC_{50} values of 3.17, 3.12, and 4.82, 4.94 μg/mL, while compounds **1** and **3** exhibited weak activities against the two kinds of tumor lines with IC_{50} values of more than 50 μg/mL.*

Key words: alternariol derivatives, mangrove endophytic fungus, alternariol 4-methyl-10-acethyl ester, mutagenicity, KB tumor lines, KBv200.

Endophytic fungi, residing almost ubiquitously inside the fresh healthy tissue of plants, have been accepted as a big but nearly untapped microbial reservoir that can be expected to provide a wide variety of structurally unique and/or biologically potent natural products [1]. The mangrove habitat has been proved to be a rich source of new fungal species. As the structurally novel and biologically active secondary metabolites were found, the mangrove endophytic fungi have gained increased attention in the last decade. In our search for secondary metabolites of endophytic fungi, many bioactive and/or novel compounds were isolated [2-8]. Alternariol was a mycotoxin with mutagenic properties, which had been widely reported [9, 10]. In this paper, we reported the isolation, structure elucidation, and mutagenic properties against tumor lines (KB and KBv200) of four alternariol derivatives produced by the mangrove endophytic fungus No. 2240 from the South China Sea Coast. The structural identification for the new compound **1**, alternariol 4-methoxy-10-acetoxy ester, was detailed below. The structures of three known compounds including alternariol (**2**), alternariol 4,10-dimethyl ether (**3**), and alternariol methyl ether (**4**) were also established by spectroscopic analyses and comparison with related literature data [11-13].



1 - 4

1: R_1 = OMe, R_2 = Ac; **2:** $R_1 = R_2$ = OH

3: $R_1 = R_2$ = OMe; **4:** R_1 = OMe, R_2 = OH

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TABLE 1. NMR Data of 2240B (**1**) ($CDCl_3$, δ, ppm, J/Hz)

C atom	δ_C (DEPT)	δ_H	HMBC
1	99.79 (C)		
2	165.27 (C)		
3	99.87 (CH)	6.61 (d, J = 2.5)	C-1,2
4	166.42 (C)		
5	105.70 (CH)	7.31 (d, J = 2.0)	C-1,3,4,6,7
6	137.05 (C)		
7	115.62 (C)		
8	138.23 (C)		
9	122.57 (CH)	6.95 (d, J = 2.5)	C-7,10
10	150.61 (C)		
11	109.41 (CH)	7.04 (d, J = 2.5)	C-7,9,10,12
12	152.19 (C)		
13	164.96 (C)		
14	55.75 (CH_3)	3.92 (s)	C-4
15	25.66 (CH_3)	2.84 (s)	C-7,8,9
16	21.12 (CH_3)	2.34 (s)	C-17
17	168.83 (C)		
2-OH		11.89 (s)	C-1,2

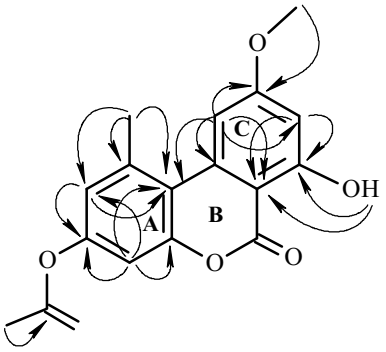

Fig. 1. The selective correlations of HMBC for 2240B (**1**).

Compound **1**, named 2240B, was isolated as a white amorphous powder and had a molecular formula $C_{17}H_{14}O_6$ requiring eleven double-bond equivalents, as deduced from the molecular-ion peak at *m/z* 314.0781 (M^+) in the HREIMS. The numbers of hydrogen and carbon atoms observed in the ^1H and ^{13}C NMR spectra were in agreement with this molecular formula (Table 1).

The IR spectrum of **1** showed absorptions for a chelated hydroxyl (3086 cm^{-1}), two carbonyl groups (1757, 1671 cm^{-1}), Me groups (2942, 1351 cm^{-1}), and an aromatic skeleton (1625, 1604, 1593 cm^{-1}). The ^1H NMR spectrum of **1** exhibited signals for two Me groups at δ 2.34 and 2.84 (each, s), a OMe group at δ 3.92 (s), two pairs of meta-positioned aromatic protons at δ 6.61 and 6.95 (each, d, J = 2.5), δ 7.05 and 7.31 (each, d, J = 2.0), and a chelated phenolic OH group at δ 11.89 (s). No correlation in the ^1H-^1H COSY spectrum also proved the presence of meta-positioned aromatic protons. In the ^{13}C NMR spectrum, there were 17 carbon atom signals, including eight olefinic carbon atom signals (δ 99.79, 99.87, 105.70, 109.41, 115.62, 122.57, 137.05, 138.23), four olefinic carbon atom bearing oxygen signals (δ 150.61 152.19 165.27, 166.42), two carbonyl signals (δ 164.96, 168.83), two methyl signals (δ 21.12, 25.66), and a methyl bearing oxygen signal (δ 55.75).

The eleven unsaturation equivalents, twelve olefinic carbon atoms, and two carbonyl groups indicated that the compound had three rings. All of the above informations showed that **1** might be a coumarin compound with two benzene rings. The HMQC spectrum exhibited correlations between protons and carbons that verified that the signals at δ 6.61, 7.31, 6.95, and 7.04 belong to the olefinic protons respectively correlating with the carbon signals at δ 99.87, 105.70, 122.57, and 109.41. Analysis of the HMBC data established the overall structure of **1** (Fig. 1); in particular, the multiple correlations between H-5, H-9, H-11, H-15, and C-7 showed that the connection point between rings A and B was C-7. The correlations from H-15 to C-7, C-8, and C-9 located the Me at the C-8 (Ar-C) position of ring A. The correlation between H-14 and C-4 placed the MeO at the C-4 position of ring **C**; OH correlated with C-2 and C-1, showing the OH at the C-2 (Ar-C, δ 165.27) position of ring **C**. The correlation between H-16 and C-17 indicated the presence of the acetyl group. Taken together, the NMR, IR, and MS data established the structure of **1** as alternariol 4-methyl-10-acethyl ester.

The IR and NMR data of compounds **2**-**4** were similar to those of **1**, so compounds **1**-**4** were of the same molecular skeleton. In the ^{1}H NMR spectrum of **2,** a Me group at δ 2.77 (s), two pairs of meta-positioned aromatic protons at δ 6.44 and 7.33 (each, d, J = 2.1), δ 6.69 and 6.78 (each, d, J = 2.4), and three phenolic OH groups at δ 9.48 (br, 2s) and 11.89 (s) were observed. The ^{13}C NMR spectrum of **2** exhibited signals for a methyl group (δ 26.70), eight olefinic carbon atoms (δ 100.14, 102.79, 103.59, 106.12, 111.62, 119.21, 140.38, 140.39), four olefinic carbon atoms bearing oxygen atoms (δ 154.92, 160.08, 166.72, 166.80), and a carbonyl group (δ 166.81). Further comparison of the ^{1}H NMR spectrum of **2** with that of **1** showed that Ac at C-10 and MeO at C-4 in **1** was replaced by two hydroxyl groups at δH 9.48 (br, 2s) in **2**. The EIMS of **2** showed a molecular ion peak at *m/z* 258, which was identical with the molecular weight of alternariol. Major IR absorptions corresponding to the major functional groups were at 3448 (OH), 3185 (intermolecular hydrogen-bonded OH), 2974, 1352 (CH_3), 1662 (lactone shifted upfield due to H bonding with OH), and 1580, 1515, 1465 cm^{-1} (aromatic skeleton). According to the literature data [11], the structure of compound **2** was established as alternariol.

The IR spectrum of **3** exhibited the presence of major functional groups, a chelated hydroxyl (3117 cm^{-1}), a Me group (2974, 1354 cm^{-1}), a carbonyl group (1639 cm^{-1}), and an aromatic skeleton (1600, 1573, 1555 cm^{-1}). The ^{13}C NMR spectrum of **3** showed 16 signals, attributable to a methyl group (δ 24.65), two methoxyl groups (δ 54.81, 56.42), a carbonyl group (δ 167.84), eight olefinic carbon atoms (δ 101.27, 105.08, 106.91, 107.11, 111.29, 125.44, 137.45, 138.99), and four olefinic carbon atoms bearing oxygen (δ 152.67, 159.32, 159.92,164.18). The ^{1}H NMR spectrum of **3** revealed signals for a Me group at δ 2.75 (s), two OMe groups at δ 3.53 (s) and 3.97 (s), a phenolic OH group at δ 11.66 (s), and two pairs of meta-positioned aromatic protons at δ 6.31 and 6.70 (each, d, J = 2.1), δ 6.35 and 6.63 (each, d, J = 2.4). Compared with the ^{1}H NMR spectrum of **2**, that of **3** showed that two MeO groups at δ 3.53 (s) and 3.97 (s) in **3** respectively replaced two OH groups δ 9.48 (br, 2s) at C-4 and C-10 in **2**. By mp analysis and further comparison with literature data [12], the structure of compound **3** was identified as alternariol 4,10-dimethyl.

Compound **4** was a light purple amorphous powder. In the ^{13}C NMR spectrum of **4**, there were 12 olefinic carbon atoms signals (δ 98.44, 99.13, 101.58, 103.35, 108.77, 117.55, 137.75, 138.39, 152.59, 158.52, 164.08, 164.63), a carbonyl signal (δ 166.13), a methyl signal (δ 24.95), and a methyl bearing oxygen signal (δ 55.78). The IR spectrum of **4** exhibited the presence of hydroxyl groups (3395, 3325 cm^{-1}), a Me group (2986, 1356 cm^{-1}), a carbonyl group (1650 cm^{-1}), and an aromatic skeleton (1617, 1589, 1568 cm^{-1}). The ^{1}H NMR spectrum of **4** revealed signals for a Me group at δ 2.72 (s), a MeO group at δ 3.90 (s), two pairs of meta-positioned aromatic protons at δ 6.59 and 7.20 (each, d, J = 2.5), δH 6.63 and 6.71 (each, d, J = 2.0), and two phenolic OH groups at δH 10.28, 11.79 (each, s). Comparison of the ^{1}H NMR spectrum of **4** with that of **1** showed Ac at C-10 in **1** was replaced by OH group at δ 10.28 in **4**. According to the related literature data [13], mp 277-279°C, EIMS 272 (M^{+}), and IR and NMR spectra of **4** confirm its chemical structure as alternariol 4-methyl ether.

The anticancer tests showed that compounds **2** and **4** had strong activities against KB and KBv200 cells with IC_{50} values of 3.17, 3.12 and 4.82, 4.94 μg/mL, respectively. Compounds **1** and **3** exhibited weak activities against the two kinds of cancer cells with IC_{50} values of more than 50 μg/mL (Table 2). KB and KBv200 are human epidermoid carcinoma cell lines. KBv200 cells, a classic multidrug resistant cell line expressing high levels of P-gp, were cloned from drug-sensitive parental KB cells by stepwise exposure to increasing doses of vincristine (VCR) and ethylmethane sulfonate (EMS) mutagenesis. Compared with the KB cell line, the KBv200 cell line was resistant to VCR about 100-fold.

TABLE 2. Effect of Compounds **1-4** on the Growth of Two Human Tumor Lines

Compounds	KB (IC_{50}, μg/mL)	KBv200 (IC_{50}, μg/mL)
1	>50	>50
2	3.17	3.12
3	>50	>50
4	4.82	4.94

EXPERIMENTAL

Melting point data were measured on an X-4 melting point machine from Beijing Tac Co. Ltd. Infrared absorption spectra were measured in the region 400-4000 cm^{-1} on an EQUINOX 55 FT-IR Analyzer; the samples were prepared either neat on a KBr plate or as a KBr pellet. ^1H NMR and ^{13}C NMR spectra were measured in deuterated $CDCl_3$ with a Mercury-Plus 500 from Varian Co. Ltd. UV spectra were from a Shimadzu UV-2501PC UV-Visible spectrophotometer. Mass spectra were obtained with a MAT95XP high-resolution mass spectrometer from Thermo Co. Ltd. IC_{50} values were calculated from the cytotoxicity curves (Bliss's software).

Fungal Strain. A fungus strain (No. 2240) was isolated from an estuarine mangrove at the South China Sea Coast and has been deposited in the Department of Applied Chemistry, Zhongshan University, Guangzhou, P. R. China and Department of Biology and Chemistry, City University of Hong Kong, China. This fungus has no spore and its species was unidentified.

Culture Conditions. Starter cultures (from Professor E. B. Gareth and Dr. L. L. P. Vrijmoed) were maintained on cornmeal seawater agar. Plugs of agar supporting mycelial growth were cut and transferred aseptically to a 250 mL Erlenmeyer flask containing 100 mL of liquid medium GYT (glucose 5 g/L, peptone 1 g/L, yeast extract 0.5 g/L, beef extract 0.5 g/L,NaCl 3 g/L). The flask was incubated at 30°C for 40 days.

Extraction and Separation of Metabolites. The cultures (200 L) were filtered through cheesecloth. The filtrate was concentrated to 5 L in vacuo below 50°C and extracted three times by shaking with an equal volume of ethyl acetate. The combined organic extracts were applied to a silica gel column, eluting with a gradient of petroleum ether to ethyl acetate to yield compound **1** (15 mg), **2** (12 mg), **3** (8 mg) and **4** (25 mg).

Compound 1 (alternariol 4-methyl-10-acethyl ester), 2240B. White amorphous powder. Mp 197-199°C. EI-MS: 314 (M^+). HREIMS: 314.0781 ($C_{17}H_{14}O_6^+$, calc. 314.0785). ^1H and ^{13}C NMR: Table 1. UV (MeOH): 254 (2.85), 288 (0.59), 299 (0.54), 336 (0.64). IR (KBr): 3086, 2942, 1757, 1671, 1625, 1604, 1593, 1351, 1210, 1151, 1103, 980, 903, 854, 801, 707 cm^{-1}.

Compound 2 (alternariol). Yellowish amorphous powder. EIMS: 258 (M^+), 241 ($[M\text{-}OH]^+$). ^1H NMR (300 MHz, acetone-d_6): δ 2.77 (s, 3H), 6.44 (d, 1H, 2.1), 6.69 (d, 1H, 2.4), 6.78 (d, 1H, 2.4), 7.33 (d, 1H, 2.1), 9.48 (br, s, 2H), 11.91 (s, 1H). ^{13}C NMR (75 MHz, acetone-d_6): δ 26.70 (Me), 100.14 111.61 (2 Ar-C), 102.79 103.59 106.12 119.21 (4 Ar-CH), 140.39 (2 Ar-C), 154.92 160.08 166.72 166.80 166.81 (4 Ar-C-OR, CO). IR (KBr): 3448, 3185, 2974, 1662, 1615, 1580, 1515, 1465, 1421, 1352, 1265, 1253, 1203, 1167, 1128, 1055, 995, 937, 854, 797, 750, 638 cm^{-1}.

Compound 3 (alternariol 4,10-dimethyl ether). Yellowish amorphous powder. Mp 183–185°C. EIMS 286 (M^+). ^1H NMR (300 MHz, $CDCl_3$): δ 2.75 (s, 3H), 3.53 (s, 3H), 3.97 (s, 3H), 6.31 (d, 1H, 2.1), 6.35 (d, 1H, 2.4), 6.63 (d, 1H, 2.4), 6.70 (d, 1H, 2.1), 11.66 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 24.65 (Me), 54.81 56.42 (2 MeO), 105.08 106.91 107.11 125.44 (4 Ar-CH), 101.27 111.29 137.45 138.99 (4 Ar-C), 152.67 159.32 159.92 164.18 167.84 (4 Ar-C-OR, CO). IR (KBr): 3117, 2974, 1639, 1600, 1573, 1555, 1451, 1416, 1354, 1268, 1228, 1165, 1033, 843, 734, 639 cm^{-1}.

Compound 4 (alternariol 4-methyl ether). Light purple amorphous powder. Mp 277–279°C. EIMS 272 (M^+). ^1H NMR (500 MHz, DMSO-d_6): δ 2.72 (s, 3H), 3.90 (s, 3H), 6.59 (d, 1H, 2.5), 6.63 (d, 1H, 2.0), 6.71 (d, 1H, 2.0), 7.20 (d, 1H, 2.5), 10.28 (s, 1H), 11.79 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 24.95 (Me), 55.78 (OMe), 98.44 (Ar-C), 99.13 (Ar-CH), 101.58 (Ar-CH), 103.35 (Ar-CH), 117.55 (Ar-CH), 108.77 (Ar-C), 137.75 (Ar-C), 138.39 (Ar-C), 152.59 158.52 164.08 164.63 166.13 (4 Ar-C-OR, CO). IR (KBr): 3395, 3325, 2986, 1650, 1617, 1589, 1568, 1464, 1424, 1356, 1276, 1231, 1167, 1036, 982, 846, 778, 739, 641 cm^{-1}.

MTT Cytotoxicity Assay. KB and KBv200 cells, obtained from the Chinese Academy of Medical Sciences (Beijing, China), were maintained in RPMI 1640 medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS). All cells were grown in a humidified atmosphere incubator of 5% CO_2 and 95% air at 37°C.

Cells were harvested and seeded in 96-well plates at 3.0×10^3/well for KB and KBv200 in a final volume of 190 μL. After a 24 h incubation, 10 μL cytotoxic agents or compound vehicles were added to each well. After 68 h, 10 μL MTT solution was added to each well. DMSO (100 μL) was added to each well 4 h later. The concentrations required to inhibit growth by 50% (IC_{50}) were calculated from the cytotoxicity curves (Bliss's software). IC_{50} values of compound **1**–**4** see Table 2.

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