

Cerebrosides of the Halotolerant Fungus *Alternaria raphani* Isolated from a Sea Salt FieldWenliang Wang,<sup>†,‡</sup> Yi Wang,<sup>†,‡</sup> Hongwen Tao,<sup>†</sup> Xiaoping Peng,<sup>†</sup> Peipei Liu,<sup>†</sup> and Weiming Zhu<sup>\*†</sup>

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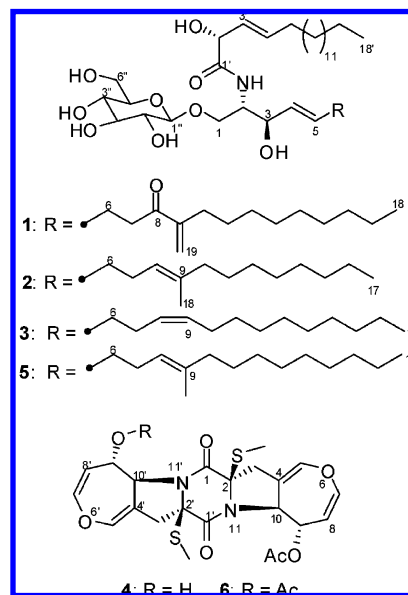
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In order to search for structurally novel and bioactive natural compounds from marine-derived fungi, a halotolerant fungal strain (THW-18) identified as *Alternaria raphani* was isolated from sediment collected in the Hongdao sea salt field. From the ethyl acetate extract of *Alternaria raphani*, three new cerebrosides, alternarosides A–C (**1–3**), and a new diketopiperazine alkaloid, alternarosin A (**4**), together with 15 known compounds were isolated and identified by spectroscopic and chemical methods, as well as X-ray crystal diffraction analysis. Compounds **1–4** showed weak antibacterial activity against *Escherichia coli*, *Bacillus subtilis*, and *Candida albicans* with MIC values ranging from 70 to 400  $\mu\text{M}$ .

Halotolerant microorganisms are those microbes that are able to grow well in variable salinity. The marine environment is the major source of the halotolerant microorganisms. Some halotolerant microorganisms living in extreme environments might activate silent genes and induce unique biosynthetic pathways.<sup>1</sup> Therefore, investigating the secondary metabolites of halotolerant microbes isolated from hypersaline ecological niches may increase the chances of finding bioactive compounds with novel structures. In order to screen halotolerant “talented strains”<sup>2</sup> and find structurally novel and bioactive secondary metabolites, we are continuing to isolate and identify microbes from hypersaline environments. As a result, a marine-derived halotolerant fungal strain (THW-18), authenticated as *Alternaria raphani*, was isolated from the sediments collected in the Hongdao sea salt field, Qingdao, China. Only five metabolites, tetramic acid, alternariol, altenuene, altertoxin I, and tenuazonic acid, have been previously identified from this fungus.<sup>3,4</sup>

The EtOAc extract of *A. raphani*, showing significant cytotoxicity against the mouse *cdc2* mutant cell line (tsFT210), was subjected to flash column chromatography over silica gel, Sephadex LH-20 chromatography, and HPLC separation to afford three new cerebroside compounds, alternarosides A–C (**1–3**), and a new diketopiperazine alkaloid, alternarosin A (**4**), together with 15 known compounds, cerebroside C (**5**),<sup>5,6</sup> bisdethiobis(methylthio)acetylaranotin (**6**),<sup>7</sup> cerebroside D,<sup>5</sup> acetylaranotin,<sup>8</sup> *N*-acetyltyramine,<sup>9</sup> *cyclo*-(Tyr-Pro),<sup>10</sup> (2*E*,2*R*)-3 $\beta$ ,5 $\alpha$ -dihydroxy-23-methylergosta-7,22-dien-6-one,<sup>11,12</sup> (2*E*,2*R*)-3 $\beta$ ,5 $\alpha$ ,9 $\alpha$ -trihydroxyergosta-7,22-dien-6-one,<sup>12</sup> (2*E*,2*R*)-23-methylergosta-7,22-diene-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol,<sup>13</sup> cerevisterol,<sup>14</sup> 6 $\beta$ -methoxyergosta-7,22-diene-3 $\beta$ ,5 $\alpha$ -diol,<sup>14</sup> ergosterol peroxide,<sup>15</sup> ergosterol,<sup>16</sup> alterperyleneol,<sup>17</sup> and altertoxin I.<sup>18,19</sup> The new compounds exhibited weak antibacterial activity, but did not show cytotoxicity against P388, A-549, BEL-7402, and HL-60 cells ( $\text{IC}_{50} > 50 \mu\text{M}$ ) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity ( $\text{IC}_{50} > 500 \mu\text{M}$ ).

Alternaroside A (**1**) was obtained as a colorless, amorphous powder. Its molecular formula was suggested as  $\text{C}_{43}\text{H}_{77}\text{NO}_{10}$  according to its HRESIMS at  $m/z$  768.5662 [ $\text{M} + \text{H}$ ]<sup>+</sup>. The 1D NMR spectra of **1** indicated the presence of aliphatic chain(s), an amide linkage, and a sugar residue, which strongly suggested a cerebroside structure for **1** (Table 1). Except for terminal double-bond signals ( $\delta_{\text{H/C}}$  6.09 and 5.79/124.5,  $\text{CH}_2$ ) and a carbonyl signal ( $\delta_{\text{C}}$  200.7, qC) instead of an allylic methyl signal ( $\delta_{\text{H/C}}$  1.54/15.7,  $\text{CH}_3$ ) and a methine signal ( $\delta_{\text{H/C}}$  5.09/123.5, CH), respectively, the 1D NMR spectra of **1** were similar to those of cerebroside C (**5**).<sup>5,6</sup>



Analysis of the  $^1\text{H}$ – $^1\text{H}$  COSY spectrum led to the identification of an isolated proton spin-system corresponding to the C-1–C-7 subunit of **1**. HMBC correlations from H-6, H-7, and H-19 to the carbonyl suggested that the carbonyl linked C-7 and C-9 (Figure 1). Methanolysis of **1** afforded methyl 2-hydroxyoctadeca-3-enoate, which was identified by GC-MS ( $t_{\text{R}}$  18.61 min,  $m/z$  312 [ $\text{M}$ ]<sup>+</sup>, Figure S13), and methyl glucopyranosides ( $[\alpha]_{\text{D}}^{25} +75$ , ESIMS  $m/z$  195 [ $\text{M} + \text{H}$ ]<sup>+</sup>),<sup>20</sup> indicating that **1** contained a  $\text{C}_{18}$ -sphingosine and a glucose moiety. ESIMS/MS showed key fragments at  $m/z$  588.6 [ $\text{M} + \text{H} - 180$ ]<sup>+</sup>, 570.6 [ $588.6 - \text{H}_2\text{O}$ ]<sup>+</sup>, 290.3 [ $570.6 - 280.3$ ]<sup>+</sup>, and 280.3 [ $570.6 - 290.3$ ]<sup>+</sup> (Figures S10 and S16). The loss of  $m/z$  280.3 ( $\text{C}_{18}\text{H}_{32}\text{O}_2$ ) and the fragments at  $m/z$  280.3 ( $\text{C}_{18}\text{H}_{34}\text{NO}$ ) and 290.3 ( $\text{C}_{19}\text{H}_{32}\text{NO}$ ) confirmed that the fatty acid unit and sphingosine unit were 2-hydroxyoctadec-3-enoic acid and 2-amino-1,3-dihydroxy-9-methyleneoctadec-4-en-8-one, respectively. Thus the constitution of **1** was established. Acidic hydrolysis of the methyl glucopyranosides afforded D-glucose, which was identified by directly comparing the specific rotation ( $[\alpha]_{\text{D}}^{25} +49$ ) and  $R_f$  value (0.30 and 0.35/ $\text{CHCl}_3$ –MeOH– $\text{H}_2\text{O}$ , 7:3:0.5) with those of standard D/L-glucose ( $[\alpha]_{\text{D}}^{25} +54/-56$ ).<sup>21</sup> The NMR signals of the anomeric proton and carbon at  $\delta_{\text{H/C}}$  4.12 (d,  $J = 7.8$ )/103.5 (CH) suggested the  $\beta$ -configuration of the glucoside. The coupling constants of  $J_{3',4'}$  (15.1 Hz) and  $J_{4,5}$  (15.6 Hz) suggested the *E*-configuration of the 3',4'- and 4,5-double bonds. The specific rotation of the corresponding methyl 2*R*-hydroxyoctadec-3-enoate

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**Table 1.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR Data for **1–3** (600, 150 MHz, DMSO- $d_6$ , TMS,  $\delta$  ppm)

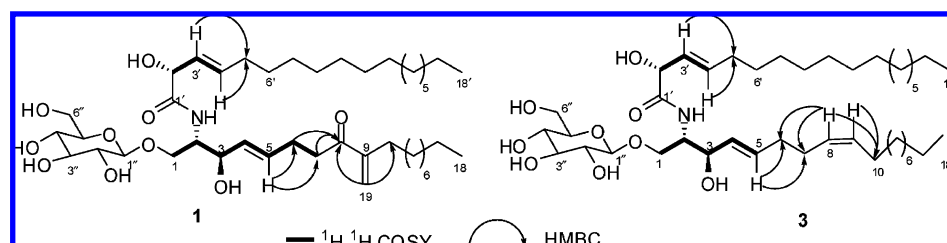
no.	<b>1</b>		<b>2</b>		<b>3</b>	
	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (J in Hz)
1	68.5, CH <sub>2</sub>	3.94, m; 3.51, dd (10.2, 4.1)	68.6, CH <sub>2</sub>	3.94, m; 3.51, dd (10.6, 4.1)	68.6, CH <sub>2</sub>	3.95, m; 3.51, dd (10.1, 4.1)
2	52.9, CH	3.78, m	52.9, CH	3.78, m	52.9, CH	3.78, m
3	70.5, CH	3.98, m	70.5, CH	3.97, m	70.5, CH	3.97, m
4	131.3, CH	5.40, dd (15.6, 6.9)	130.9, CH	5.37, dd (15.6, 6.9)	131.0, CH	5.36, dd (15.6, 6.9)
5	130.0, CH	5.58, dt (15.6, 6.4)	130.9, CH	5.57, dt (15.6, 6.2)	130.7, CH	5.56, dt (14.2, 6.2)
6	26.6, CH <sub>2</sub>	2.16, m	27.4, CH <sub>2</sub>	1.94, m	32.0, CH <sub>2</sub>	1.97, m
7	36.7, CH <sub>2</sub>	2.73, t (7.6)	32.1, CH <sub>2</sub>	1.94, m	28.6, CH <sub>2</sub>	1.97, m
8	200.7, qC		123.5, CH	5.09, br t (6.2)	129.5, CH	5.38, "t"-like (3.3)
9	147.9, qC		134.9, qC		130.2, CH	5.38, "t"-like (3.3)
10	30.3, CH <sub>2</sub>	2.15, m	39.1, CH <sub>2</sub>	1.94, m	28.7, CH <sub>2</sub>	1.97, m
11	28.0, CH <sub>2</sub>	1.23, m	27.3, CH <sub>2</sub>	1.23, m	31.9, CH <sub>2</sub>	1.23, m
12–14	29.1, CH <sub>2</sub>	1.23, m	29.1, CH <sub>2</sub>	1.23, m	29.0, CH <sub>2</sub>	1.23, m
15	29.1, CH <sub>2</sub>	1.23, m	28.7, CH <sub>2</sub>	1.23, m	28.9, CH <sub>2</sub>	1.23, m
16	31.3, CH <sub>2</sub>	1.23, m	22.1, CH <sub>2</sub>	1.23, m	31.3, CH <sub>2</sub>	1.23, m
17	22.1, CH <sub>2</sub>	1.23, m	13.9, CH <sub>3</sub>	0.85, t (6.9)	22.1, CH <sub>2</sub>	1.23, m
18	13.9, CH <sub>3</sub>	0.85, t (6.9)	15.7, CH <sub>3</sub>	1.54, br s	13.9, CH <sub>3</sub>	0.85, t (6.8)
19	124.5, CH <sub>2</sub>	6.09, br s; 5.79, br s				
1'	172.0, qC		172.0, qC		172.0, qC	
2'	71.9, CH	4.30, br t (5.1)	71.9, CH	4.30, br t (4.6)	71.9, CH	4.30, br t (5.1)
3'	129.0, CH	5.44, dd (15.1, 5.1)	129.0, CH	5.44, dd (15.4, 5.3)	129.0, CH	5.43, dd (15.1, 5.5)
4'	131.0, CH	5.67, dt (15.1, 6.9)	130.9, CH	5.68, dt (15.4, 6.4)	130.9, CH	5.68, dt (15.1, 6.9)
5'	31.7, CH <sub>2</sub>	1.93, dt (6.9, 6.8)	31.6, CH <sub>2</sub>	1.94, m	31.7, CH <sub>2</sub>	1.97, m
6'–15'	29.1, CH <sub>2</sub>	1.23, m	29.0, CH <sub>2</sub>	1.23, m	29.0, CH <sub>2</sub>	1.23, m
16'	31.3, CH <sub>2</sub>	1.23, m	31.3, CH <sub>2</sub>	1.23, m	31.3, CH <sub>2</sub>	1.23, m
17'	22.1, CH <sub>2</sub>	1.23, m	22.1, CH <sub>2</sub>	1.23, m	22.1, CH <sub>2</sub>	1.23, m
18'	13.9, CH <sub>3</sub>	0.85, t (6.6)	13.9, CH <sub>3</sub>	0.85, t (6.9)	13.9, CH <sub>3</sub>	0.85, t (6.8)
1''	103.5, CH	4.12, d (7.8)	103.5, CH	4.11, d (7.8)	103.5, CH	4.12, d (7.8)
2''	73.4, CH	2.96, m	73.4, CH	2.96, m	73.4, CH	2.96, m
3''	76.6, CH	3.14, m	76.5, CH	3.14, m	76.5, CH	3.14, m
4''	70.0, CH	3.05, m	70.0, CH	3.04, m	70.0, CH	3.04, m
5''	76.9, CH	3.09, m	76.9, CH	3.08, m	76.9, CH	3.09, m
6''	61.1, CH <sub>2</sub>	3.67, br dd (11.0, 6.0) 3.46, ddd (11.0, 6.0, 5.0)	61.0, CH <sub>2</sub>	3.66, br dd (9.7, 6.0) 3.43, ddd (9.7, 6.0, 5.5)	61.0, CH <sub>2</sub>	3.67, ddd (11.3, 5.9, 1.9) 3.43, ddd (11.3, 5.9, 5.0)
NH		7.39, d (9.1)		7.39, d (9.7)		7.39, d (9.6)
OH-3		4.97, m		4.95, d (5.5)		4.95, d (5.5)
OH-2'		5.78, d (5.0)		5.78, d (5.0)		5.78, d (4.6)
OH-2''		4.99, m		4.98, d (4.6)		4.99, d (4.6)
OH-3''		4.93, m		4.92, d (5.0)		4.92, d (5.0)
OH-4''		4.94, m		4.94, d (4.6)		4.93, d (5.0)
OH-6''		4.53, t (6.0)		4.52, t (6.0)		4.52, t (5.9)

( $[\alpha]_{\text{D}}^{20} -42$ ) indicated the *R*-configuration at C-2' ( $[\alpha]_{\text{D}}^{25} -45.3$ ).<sup>22</sup> The values of  $\delta_{\text{C}-1-\text{C}-5}$  and  $\delta_{\text{C}-1'-\text{C}-4'}$  and the specific rotation ( $[\alpha]_{\text{D}}^{20} -11$ ) of **1** were close to those of cerebroside C (**5**) ( $[\alpha]_{\text{D}}^{20} -8$ ), suggesting that **1** shared the same *2S,2'R,3R*-configuration with **5**. Thus, the structure of **1** is proposed as (*2R,3E*)-2-hydroxy-*N*-[(*2S,3R,4E*)-1- $\beta$ -D-glucopyranosyloxy-3-hydroxy-9-methylene-8-oxooctadec-4-en-2-yl]octadec-3-enamide.

The molecular formula of alternaroside B (**2**) was determined to be C<sub>42</sub>H<sub>77</sub>NO<sub>9</sub> by HRESIMS at  $m/z$  740.5666 [M + H]<sup>+</sup>. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of **2** were almost the same as those of cerebroside C (**5**),<sup>5,6</sup> except for subtle differences in the chain length of the sphingosine unit. The ESIMS/MS showed key fragments at  $m/z$  560.6 [M + H - 180]<sup>+</sup>, 542.6 [560.6 - H<sub>2</sub>O]<sup>+</sup>, 524.6 [542.6 - H<sub>2</sub>O]<sup>+</sup>, 281.3 [560.6 - 279.3]<sup>+</sup>, 280.3 [560.6 - 280.3 or 524.6 - 244.3]<sup>+</sup>, and 262.3 [280.3 - H<sub>2</sub>O or 524.6 - 262.3]<sup>+</sup> (Figures S11 and S17). The loss of  $m/z$  279.3 (C<sub>18</sub>H<sub>33</sub>NO) and the fragments

at  $m/z$  280.3 (C<sub>18</sub>H<sub>34</sub>NO) and 262.3 (C<sub>18</sub>H<sub>32</sub>N) corresponded to a sphingosine unit with a methylated odd-carbon straight chain and a hydroxyoctadecenoyl moiety. The coincidence of NMR data from C-1 to C-10 and the similar specific rotation to that of cerebroside C (**5**)<sup>6</sup> supported the configuration of the sphingosine unit as (*2S,3R,4E,8E*). Methanolysis of **2** also afforded methyl 2*R*-hydroxyoctadec-3-enoate and methyl D-glucopyranosides. Accordingly, the structure of **2** was established as nor-cerebroside C, i.e., (*2R,3E*)-2-hydroxy-*N*-[(*2S,3R,4E,8E*)-1- $\beta$ -D-glucopyranosyloxy-3-hydroxy-9-methylheptadec-4,8-dien-2-yl]octadec-3-enamide.

Alternaroside C (**3**) was obtained as a colorless, amorphous powder, and HRESIMS at  $m/z$  740.5656 [M + H]<sup>+</sup> suggested the same molecular formula as for **2**. The NMR spectra of **3** were quite similar to those of **5** except for the substitution of an olefinic methine resonance ( $\delta_{\text{H/C}}$  5.38/130.2) for the vinyl methyl signal ( $\delta_{\text{H/C}}$  1.54/15.7) and the olefinic quaternary carbon signal ( $\delta_{\text{C}}$  134.8).

**Figure 1.** Key  $^1\text{H}$ - $^1\text{H}$  COSY and HMBC correlations of **1** and **3**.

**Table 2.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR Data for **4** (600, 150 MHz, DMSO- $d_6$ , TMS,  $\delta$  ppm)

no.	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (J in Hz)	no.	$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$
1	163.9, qC		1'	164.3, qC	
2	71.0, qC		2'	69.8, qC	
3	39.2, CH <sub>2</sub>	3.25, dt (15.1, 2.3) 2.96, br d (15.1)	3'	38.9, CH <sub>2</sub>	3.13, dt (15.1, 2.3) 2.94, br d (15.1)
4	111.0, qC		4'	111.1, qC	
5	137.0, CH	6.78, t (2.3)	5'	136.5, CH	6.68, t (2.3)
7	139.9, CH	6.45, dd (8.2, 2.3)	7'	137.8, CH	6.29, dd (8.3, 2.3)
8	105.3, CH	4.72, dd (8.2, 2.3)	8'	110.4, CH	4.80, dd (8.3, 2.3)
9	71.1, CH	5.62, dt (8.3, 2.3)	9'	71.1, CH	4.38, tt (7.3, 2.3)
10	59.6, CH	4.96, br d (8.3)	10'	63.0, CH	4.75, br d (7.3)
2-SCH <sub>3</sub>	14.0, CH <sub>3</sub>	2.21, s	2'-SCH <sub>3</sub>	14.1, CH <sub>3</sub>	2.20, s
9-O COCH <sub>3</sub>	20.6, CH <sub>3</sub>	1.98, s	9'-OH		5.29, d (7.3)
9-O COCH <sub>3</sub>	169.4, qC				

**Table 3.** MIC Values of Antibacterial Activities against *Escherichia coli*, *Bacillus subtilis*, and *Candida albicans* for Compounds **1–4** ( $\mu\text{M}$ )

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
<i>Escherichia coli</i>	130	135	135	203
<i>Bacillus subtilis</i>	130	68	135	203
<i>Candida albicans</i>	260	270	270	406

These data combined with the same ESIMS/MS pattern as that of **2** (Figures S12 and S18) indicated a hydroxyoctadecenoyl moiety and a 2-amino-1,3-dihydroxyoctadec-4,8-diene moiety. A  $^1\text{H}$ – $^1\text{H}$  COSY experiment showed the connections from C-1 to C-6 and from C-2' to C-4'. HMBC correlations from H-5 ( $\delta$  5.56) and H-8 ( $\delta$  5.38) to C-6 ( $\delta$  32.0), C-7 ( $\delta$  28.6), and C-10 ( $\delta$  28.7) located the second double bond at C-8/C-9 in **3** (Figure 1). Methanolysis of **3** gave methyl 2R-hydroxyoctadec-3-enoate and methyl D-glucopyranosides. The “t”-like multiplet of two olefinic protons ( $\delta$  5.38) and the chemical shifts of C<sub>7</sub> and C<sub>10</sub> ( $\delta$  28.7 and 28.9, respectively) indicated the Z-configuration of the 8,9-double bond.<sup>23</sup> Thus, the structure of **3** was established as (2R,3E)-2-hydroxy-N-[(2S,3R,4E,8Z)-1- $\beta$ -D-glucopyranosyloxy-3-hydroxyoctadec-4,8-dien-2-yl]octadec-3-enamide.

Alternarosin A (**4**) was obtained as a colorless, amorphous powder. Its molecular formula was determined as C<sub>22</sub>H<sub>24</sub>N<sub>2</sub>O<sub>7</sub>S<sub>2</sub> according to its HRESIMS peak at  $m/z$  515.0917 [M + Na]<sup>+</sup>. Diagnostic IR absorption peaks were observed for hydroxy and amide carbonyl groups at 3418 and 1648 cm<sup>-1</sup>, respectively. Its 1D NMR spectra revealed three carbonyls, four quaternary carbons (two sp<sup>2</sup>), 10 methines (six sp<sup>2</sup>), two methylenes, and three methyl groups (Table 2). Except for the absence of an acetyl group, the 1D NMR spectra of **4** were quite similar to those of bisdethiodi(methylthio)acetylaranotin (**6**),<sup>7</sup> implying that **4** was the 9- or 9'-deacetyl derivative of **6**. This conclusion was further confirmed by the HMBC correlations between 9'-OH ( $\delta_{\text{H}}$  5.29, s) and C-8' ( $\delta_{\text{C}}$  110.4, CH), C-9' ( $\delta_{\text{C}}$  71.1, CH), and C-10' ( $\delta_{\text{C}}$  63.0, CH). Acetylation of **4** with acetic anhydride in pyridine afforded compound **6**, and the absolute configuration of **6** was determined by single-crystal X-ray diffraction analysis.<sup>24</sup> Thus, the structure of **4** was elucidated as 9'-O-deacetyl bisdethiobis(methylthio)acetylaranotin.

The new isolates **1–4** were evaluated for cytotoxicity against P388 and HL-60 cancer cells with the SRB method,<sup>25</sup> and the A549 and BEL-7402 cancer cells with the MTT methods.<sup>26</sup> Their antimicrobial activities against *Escherichia coli*, *Bacillus subtilis*, and *Candida albicans* were also evaluated by an agar dilution method.<sup>27</sup> Compounds **1–4** were also evaluated for their DPPH radical scavenging activity.<sup>28</sup> Compounds **1–4** showed very weak antimicrobial activities against *Escherichia coli*, *Bacillus subtilis*, and *Candida albicans* with MIC values from 70 to 400  $\mu\text{M}$  (Table 3), while they showed no cytotoxic effect on the four cancer cell lines (IC<sub>50</sub> > 100  $\mu\text{M}$ ) and no DPPH radical scavenging activity (IC<sub>50</sub> > 500  $\mu\text{M}$ ).

## Experimental Section

**General Experimental Procedures.** Specific rotations were obtained on a JASCO P-1020 digital polarimeter. UV spectra were recorded on a Beckman DU 640 spectrophotometer. NMR data were recorded on a JEOL JNM-ECP 600 spectrometer using TMS as internal standard, and chemical shifts were recorded as  $\delta$  values. ESIMS was measured on a Q-TOF ULTIMA GLOBAL GAA076 LC mass spectrometer. Vacuum-liquid chromatography (VLC) utilized silica gel H (Qingdao Marine Chemical Factory, Qingdao, China). Semipreparative HPLC was performed using an ODS column [YMC-pack ODS-A, 10  $\times$  250 mm, 5  $\mu\text{m}$ , 4 mL/min]. The GC-MS system consisted of an Agilent 6890 gas chromatograph and an Agilent 5973 mass selective detector in the electron-ionization mode.

**Fungal Material.** The fungus THW-18 was isolated from sediment collected in the Hongdao sea salt field, Qingdao, China. According to its morphological characteristics and 18S rRNA sequences (Supporting Information; GenBank GQ354822), THW-18 was identified as *Alternaria raphani* by Prof. Chengxiang Fang, China Center for Type Culture Collection. The voucher specimen was deposited in our laboratory at  $-80$  °C. The producing strain was prepared on potato dextrose agar slants and stored at 4 °C.

**Fermentation and Extraction.** *A. raphani* was grown under static conditions at 20 °C for 45 days in 250 L conical flasks containing the liquid medium (300 mL/flask, pH 7.0) composed of mannitol (20 g/L), maltose (20 g/L), glucose (10 g/L), monosodium glutamate (10 g/L), KH<sub>2</sub>PO<sub>4</sub> (0.5 g/L), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.3 g/L), yeast extract (3 g/L), corn steep liquor (1 g/L), and sea salt (100 g/L). The fermented whole broth (75 L) was filtered through cheese cloth to be separated into filtrate and mycelia. The filtrate was concentrated under reduced pressure to about 25% the original volume and then extracted three times with equal volumes of EtOAc to give an EtOAc solution, while the mycelia were extracted three times with acetone. The acetone solution was concentrated under reduced pressure to afford an aqueous solution. The aqueous solution was extracted three times with equal volumes of EtOAc to give another EtOAc solution. The EtOAc extracts were combined and concentrated under reduced pressure to give a crude extract (42 g).

**Purification.** The extract (42 g), showing a cytotoxic effect on K562 cells at 100  $\mu\text{g}/\text{mL}$ , was separated into five fractions on a silica gel VLC column using step gradient elution with CHCl<sub>3</sub>–petroleum ether (0–100%) and then with MeOH–CHCl<sub>3</sub> (0–50%). Fraction 2 (1.5 g) was further separated into seven subfractions by Sephadex LH-20, eluting with MeOH–CHCl<sub>3</sub> (1:1). Ergosterol (175 mg) was obtained from fraction 2-1 after recrystallization from MeOH–CHCl<sub>3</sub>. Fraction 3 (1.8 g) was subjected to Sephadex LH-20, eluting with MeOH–CHCl<sub>3</sub> (1:1), to afford six subfractions. Subfraction 3-2 was (121 mg) further purified by semipreparative HPLC (70% MeOH–H<sub>2</sub>O, 4.0 mL/min) to yield **4** (6.2 mg), **6** (42 mg), and acetylaranotin (26 mg). Ergosterol peroxide (48 mg) was obtained from subfraction 3-3 after recrystallization from MeOH–CHCl<sub>3</sub>. The mother liquor of fraction 3-3 was purified by semipreparative HPLC (gradient elution 70–100% MeOH, 4.0 mL/min) to afford 6 $\beta$ -methoxyergosta-7,22-diene-3 $\beta$ ,5 $\alpha$ -diol (7.3 mg) and (22E,24R)-23-methylergosta-7,22-diene-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol (8.9 mg). Fraction 4 (8.5 g) was separated into 17 subfractions by Sephadex LH-20, eluting with MeOH–CHCl<sub>3</sub> (1:1). Subfractions 4-3 and 4-4 were combined and purified by semipreparative HPLC (gradient elution 92–100% MeOH, 4.0 mL/min) to afford **1** (22.8 mg), **2** (16.2 mg), **3**

(14.3 mg), **5** (104 mg), and cerebroside D (59 mg). Subfractions 4-6-4-9 were combined and crystallized from MeOH-CHCl<sub>3</sub> (1:1) to yield cerebisterol (255 mg). The mother liquor was further purified by semipreparative HPLC (gradient elution 85-100% MeOH, 4.0 mL/min) to afford (22*E*,24*R*)-3β,5α-dihydroxy-23-methylergosta-7,22-dien-6-one (9.1 mg) and (22*E*,24*R*)-3β,5α,9α-trihydroxyergosta-7,22-dien-6-one (7.1 mg). Subfractions 4-11-4-13 were combined and purified by semipreparative HPLC (gradient elution 45-60% MeOH, 4.0 mL/min) to yield alterperyleneol (4.6 mg) and altertoxin I (7.7 mg). *N*-Acetyltyramine (21 mg) and *cyclo*-(Tyr-Pro) (18 mg) were isolated from subfraction 4-16 by semipreparative HPLC (gradient elution 15-40% MeOH, 4.0 mL/min).

**Methanolysis of 1.** Compound **1** (10 mg) was refluxed with 5% HCl-MeOH (4 mL) for 10 h. The reaction mixture was immediately cooled and extracted with *n*-hexane. The *n*-hexane layer was concentrated to afford methyl 2*R*-hydroxyoctadec-3-enoate (3.2 mg): [α]<sub>D</sub><sup>20</sup> -42 (*c* 0.1, CHCl<sub>3</sub>); GC-MS (30 m × 0.32 mm × 0.25 μm HP-5 MS column: He, 1 mL/min; 40 °C, 2 min, 40-250 °C, Δ 15 °C/min, 250 °C, 10 min); *t*<sub>R</sub> 18.61 min; *m/z* (rel int) 312 (M<sup>+</sup>, 0.6), 253 (M<sup>+</sup> - 59, 98), 151 (5), 137 (9), 123 (21), 109 (46), 95 (75), 83 (56), 81 (55), 57 (100), 43 (38); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 600 MHz) δ 0.88 (3H, t, *J* = 6.8 Hz, H-18), 1.25 (24H, m, H-6-H-17), 2.06 (2H, q, *J* = 6.8 Hz, H-5), 2.88 (1H, d, *J* = 6.0 Hz, 2-OH), 3.80 (3H, 1-OCH<sub>3</sub>), 4.61 (1H, dd, *J* = 6.0, 6.8 Hz, H-2), 4.98 (1H, dd, *J* = 16.5, 6.8 Hz, H-3), 5.88 (1H, dt, *J* = 16.5, 6.8 Hz, H-4). After adding 5 mL of H<sub>2</sub>O, the aqueous MeOH layer was concentrated under reduced pressure to remove residual HCl. The residue was partitioned between H<sub>2</sub>O and EtOAc. The H<sub>2</sub>O layer was evaporated in vacuo, and the residue was purified by a Sephadex LH-20 column with MeOH to yield methyl D-glucopyranosides (2.1 mg): [α]<sub>D</sub><sup>25</sup> +75 (*c* 0.05, MeOH); ESIMS *m/z* 195 [M + H]<sup>+</sup>; *R*<sub>f</sub> 0.50/0.56 (CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O, 7:3:0.5). The obtained methyl D-glucopyranosides (2.1 mg) were refluxed with 2 N HCl in H<sub>2</sub>O (1 mL) for 2 h, and the reaction mixture was neutralized with NaHCO<sub>3</sub> and concentrated in vacuo. The reaction solid was dissolved in MeOH (10 mL), and the solution was concentrated in vacuo to give D-glucose (1.6 mg) as a colorless syrup, which was directly compared with the standard sample by *R*<sub>f</sub> value (0.30 and 0.35/CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O, 7:3:0.5) and [α]<sub>D</sub><sup>25</sup> (+49 vs standard D-glucose +54/L-glucose -56) (*c* 0.1, H<sub>2</sub>O). The same results were also obtained from the methanolysis of **2** and **3**.

**Chemical Transformation of 4 to 6.** Compound **4** (3 mg) was dissolved in 3 mL of dry pyridine, and Ac<sub>2</sub>O (10 μL) was added under an Ar atmosphere at rt. The mixture was stirred at 40 °C for 1 h, and then 10 mL of H<sub>2</sub>O was added to quench the reaction. The mixture was extracted three times with EtOAc (5 mL each). The EtOAc was evaporated, and the residue was purified by HPLC to afford **6** (1.8 mg): [α]<sub>D</sub><sup>20</sup> -465 (*c* 0.66, CHCl<sub>3</sub>), lit. [α]<sub>D</sub><sup>26</sup> -315.1 (*c* 1, CHCl<sub>3</sub>).<sup>29</sup>

**Alternaroside A (1):** colorless, amorphous powder; [α]<sub>D</sub><sup>20</sup> -11 (*c* 0.6, MeOH); UV (MeOH) λ<sub>max</sub> (log ε) 201 (3.4) nm; IR (KBr) ν<sub>max</sub> 3429, 3155, 2836, 1633, 1567, 1056, 1011, 941 cm<sup>-1</sup>; <sup>1</sup>H NMR and <sup>13</sup>C NMR data, Table 1; HRESIMS *m/z* 768.5662 [M + H]<sup>+</sup> (calcd for C<sub>43</sub>H<sub>78</sub>NO<sub>10</sub>, 768.5625); ESIMS/MS *m/z* 768.7 [M + H]<sup>+</sup>, 750.7 [M + H - H<sub>2</sub>O]<sup>+</sup>, 588.6 [M + H - 180]<sup>+</sup>, 570.6 [588.6 - H<sub>2</sub>O]<sup>+</sup>, 290.3, and 280.3.

**Alternaroside B (2):** colorless, amorphous powder; [α]<sub>D</sub><sup>20</sup> -9 (*c* 0.1, MeOH); UV (MeOH) λ<sub>max</sub> (log ε) 202 (3.2) nm; IR (KBr) ν<sub>max</sub> 3418, 3133, 2819, 1619, 1551, 1048, 1008, 937 cm<sup>-1</sup>; <sup>1</sup>H NMR and <sup>13</sup>C NMR data, Table 1; HRESIMS *m/z* 740.5666 [M + H]<sup>+</sup> (calcd for C<sub>42</sub>H<sub>78</sub>NO<sub>9</sub>, 740.5677); ESIMS/MS *m/z* 740.6 [M + H]<sup>+</sup>, 722.7 [M + H - H<sub>2</sub>O]<sup>+</sup>, 560.6 [M + H - 180]<sup>+</sup>, 542.6 [560.6 - H<sub>2</sub>O]<sup>+</sup>, 524.6 [542.6 - H<sub>2</sub>O]<sup>+</sup>, 281.3, 280.3, and 262.3.

**Alternaroside C (3):** colorless, amorphous powder; [α]<sub>D</sub><sup>20</sup> -4 (*c* 0.1, MeOH); UV (MeOH) λ<sub>max</sub> (log ε) 201 (3.2) nm; IR (KBr) ν<sub>max</sub> 3430, 3126, 2822, 1648, 1571, 1061, 1021, 962 cm<sup>-1</sup>; <sup>1</sup>H NMR and <sup>13</sup>C NMR data, Table 1; HRESIMS *m/z* 740.5656 [M + H]<sup>+</sup> (calcd for C<sub>42</sub>H<sub>78</sub>NO<sub>9</sub>, 740.5677); ESIMS/MS *m/z* 740.6 [M + H]<sup>+</sup>, 722.7 [M + H - H<sub>2</sub>O]<sup>+</sup>, 560.6 [M + H - 180]<sup>+</sup>, 542.6 [560.6 - H<sub>2</sub>O]<sup>+</sup>, 524.6 [542.6 - H<sub>2</sub>O]<sup>+</sup>, 280.3, and 262.3.

**Alternarosin A (4):** colorless, amorphous powder; [α]<sub>D</sub><sup>20</sup> -85 (*c* 1.0, CHCl<sub>3</sub>); UV (MeOH) λ<sub>max</sub> (log ε) 223 (4.1) nm, 285 (3.6) nm; IR (KBr) ν<sub>max</sub> 3418, 3126, 1740, 1648, 1230, 1059, 1031 cm<sup>-1</sup>; <sup>1</sup>H NMR

and <sup>13</sup>C NMR data, Table 2; HRESIMS *m/z* 515.0917 [M + Na]<sup>+</sup> (calcd for C<sub>22</sub>H<sub>24</sub>N<sub>2</sub>O<sub>7</sub>S<sub>2</sub>Na, 515.0923).

**Cerebroside C (5):** colorless, amorphous powder; [α]<sub>D</sub><sup>20</sup> -8 (*c* 0.1, MeOH); <sup>1</sup>H NMR and <sup>13</sup>C NMR data were consistent with those reported in the literature;<sup>6</sup> ESIMS *m/z* 754.8 [M + H]<sup>+</sup>, 736.8 [M + H - H<sub>2</sub>O]<sup>+</sup>.

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**Supporting Information Available:** 1D NMR spectra of **1-4**, X-ray data for **6**, ESIMS/MS of **1-3**, GC-MS of methanolysis products of **1-3**, and bioassay protocols used. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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