

## 5-Alkylresorcinols from *Merulius incarnatus*

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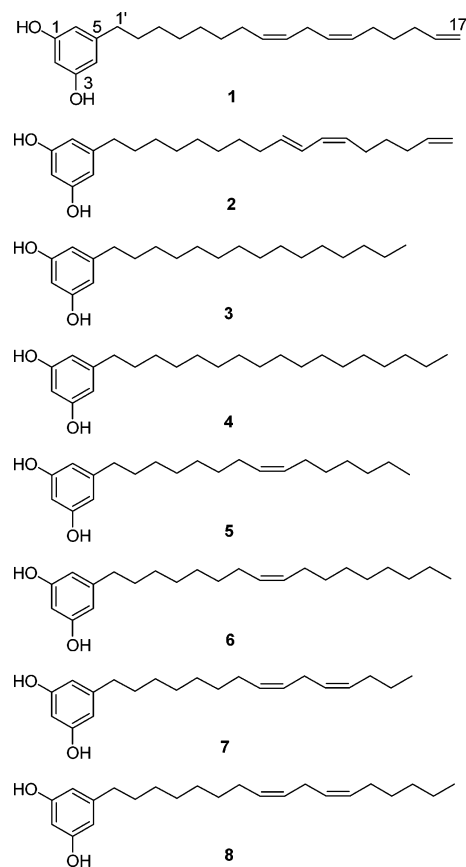
Two new, 5-heptadeca-8'*Z*,11'*Z*,16-trienylresorcinol (**1**) and 5-heptadeca-9'*E*,11'*Z*,16-trienylresorcinol (**2**), and six known 5-alkylresorcinols (**3–8**) were isolated from the mushroom *Merulius incarnatus*. Compound **2** is the first 5-alkylresorcinol derivative that contains a *trans*–*cis* conjugated double bond system. Compounds **1**, **2**, **5**, **6**, **7**, and **8** were found to inhibit methicillin-resistant *Staphylococcus aureus* (MRSA) with IC<sub>50</sub> values of 2.5, 15, 9.5, 8.0, 5.0, and 6.5 µg/mL, respectively. Compound **1** was also active against leishmania, with an IC<sub>50</sub> value of 3.6 µg/mL, and showed no cytotoxicity in our Vero cell test up to a concentration of 25 µg/mL. The structures of these isolates were determined by spectroscopic data including 1D and 2D NMR.

Mushrooms have been an integral part of human life for thousands of years. Polypore mushrooms are members of the Aphyllophorales, a group of morphologically complex, terrestrial Basidiomycetes, and are considered by many authors as a major source of compounds having antimicrobial, antiviral, antifungal, anticancer, cardiovascular, antiinflammatory, antioxidant, nematocidal, immunostimulating, and other activities.<sup>1,2</sup> In our effort to isolate new antimicrobial compounds from polypores, 92 mushroom samples were collected from Canada, North Carolina, and Mississippi. Preliminary screening showed that the crude EtOH extract of *Merulius incarnatus* exhibited 100% growth inhibition against methicillin-resistant *Staphylococcus aureus* (MRSA) at 200 µg/mL, with an IC<sub>50</sub> determined as 20 µg/mL in the secondary test.

*Merulius incarnatus* Schweinitz 1822 (Corticiaceae), also known as *Phlebia incarnata* Nakesone & Bursdall 1994, is a polypore mushroom occurring on dead logs and stumps of hardwoods, particularly those of white oak, beech, maple, and birch in the Southeastern United States. Although the secondary metabolites from *Merulius* (*Phlebia*) species such as *Merulius tremellosus* (syn. *Phlebia tremellosa*) and *Phlebia radiata* have been investigated before,<sup>3–9</sup> the chemical composition of *M. incarnatus* has not been reported.

Bioassay-guided fractionation of the crude extract led to an active fraction with an IC<sub>50</sub> value of 15 µg/mL. This fraction showed one spot on a normal-phase TLC plate, but this was a mixture of several compounds, and even repeated HPLC separation did not afford any pure compound. Eventually, argentation chromatography<sup>10</sup> followed by HPLC led to two new (**1** and **2**) and six known 5-alkylresorcinols (**3–8**). Compound **2**, which contained a *trans*–*cis* conjugated double bond system, was purified by recycling HPLC. All of the isolates were evaluated for their activity against methicillin-resistant *Staphylococcus aureus* (MRSA). The structure elucidation of new compounds, some observations of their biological activity, and a brief discussion of structure–activity relationships are described herein.

Compound **1** (20 mg) was obtained as an orange-colored oil and was shown to have a molecular formula of C<sub>23</sub>H<sub>34</sub>O<sub>2</sub> by HRTOFMS ([M – H]<sup>–</sup> *m/z* 341.2505, calcd 341.2481), which was consistent with <sup>13</sup>C NMR and DEPT spectra. The 23 carbons were characterized by <sup>13</sup>C NMR and DEPT-135 spectra as an olefinic methylene, 11 methylenes, eight olefinic methines, and three olefinic quaternary carbons. <sup>1</sup>H–<sup>1</sup>H COSY, HMQC, and HMBC data showed that **1** was 5-alkylresorcinol with a terminal double bond in the side chain. Two additional double bonds were present, isolated by a



methylene group ( $\delta$  2.77). The presence of HMBC correlations between  $\delta$  1.46 (HC-14') and the two olefinic carbons at  $\delta$  139.1 (C-16') and 130.0 (C-12') indicated that there were three methylene groups between the terminal (C16'–C17') and C11'–C12' double bonds. The configuration of the double bonds at the 8' and 11' positions were determined as *Z* by comparison with the <sup>13</sup>C NMR data of the known 5-heptadeca-8'*Z*,11'*Z*-dienylresorcinol.<sup>11</sup> The allylic carbon signals of C-7', C-10', and C-13' ( $\delta$  27.5, 25.8, and 26.9) of **1** were coincidental with those of this known compound ( $\delta$  27.2, 25.6, and 27.2). This assignment was also consistent with other known compounds such as 3-heptadec-8'*Z*-enyl-1,2-dimethoxybenzene versus its 8'*E* isomer,<sup>12,13</sup> where the allylic carbons of the 8'*Z* isomer were shown at  $\delta$  27.2, as compared with those of the 8'*E* isomer ( $\delta$  32.6).

Compound **2** (3 mg) was obtained as an orange-colored oil and was shown to have the same molecular formula as compound **1**,

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i.e.,  $C_{23}H_{34}O_2$  by HRTOFMS ( $[M - H]^-$   $m/z$  341.2501, calcd 341.2481). Compound **2** also contained a terminal double bond. Analogously with **1**, the same HMBC correlations between  $\delta$  1.45 (HC-14') and the two olefinic carbons at  $\delta$  138.6 (C-16') and 129.1 (C-12') were present, indicating the same structural fragment of three methylene groups separating the terminal and the other two double bonds. In contrast with **1**, the nonterminal double bonds in the side chain were assigned to a *trans*-*cis* conjugated system by analysis of  $^1H$ - $^1H$  COSY, HMQC, and HMBC spectra and the coupling constants. The double bond at the 11' position was determined to be *Z* by the coupling constant of the two vinylic protons ( $J = 10.8$  Hz), and the allylic carbon signal of C-13' was shown at  $\delta$  26.8, consistent with the allylic carbon signals of 3-heptadec-8'-Z-enyl-1,2-dimethoxybenzene ( $\delta$  27.2). The double bond at the 9' position was determined to be *E* as the large coupling constant of the two vinylic protons ( $J = 15.0$  Hz), and the allylic carbon signal (C-8') appeared at  $\delta$  32.6, consistent with the allylic carbon signal of 3-heptadec-8'-E-enyl-1,2-dimethoxybenzene ( $\delta$  32.6). The  $^1H$ - $^1H$  COSY signals of C-10' and C-11' indicated that it was a conjugated system. The isolation of metabolite **2** with a conjugated system of double bonds, isomeric to **1**, is rather unique for 5-alkylresorcinols and to our knowledge has not been reported in the literature. Although lipids with *cis*-*trans* conjugated systems of double bonds have been isolated from various plants and animals, e.g., punicic acid from pomegranate seeds (*Punica granatum*),<sup>14</sup> compounds from seed oils of numerous plants,<sup>15</sup> or bombykol from silkworm moth (*Bombyx mori*),<sup>16</sup> they have, to our knowledge, not been reported in mushrooms. Even in the large group of natural phenolic lipids, the *cis*-*trans* conjugated compounds are rather rare.<sup>17,18</sup> It is now well proven that *cis*-*trans* conjugated lipids are formed from their unconjugated precursors by the action of corresponding enzymes known as acyl-lipid desaturases (FADX).<sup>14</sup> Chemical isomerization of isolated double bond systems to conjugated ones is not an easy process, and it is rather unlikely that isomerization of this type occurred during extraction and chromatographic isolation procedures. Our attempts to isomerize compound **1** to **2** with silica gel (including silica gel impregnated with silver nitrate) and various solvents were all unsuccessful, and formation of compound **2** as an artifact was excluded. The structures of known compounds (**3**–**8**) were determined by HRTOFMS and comparison of spectroscopic data with similar data in the literature.<sup>11,12</sup>

Alkylresorcinols have been found to have multiple biological activities, including antimicrobial,<sup>19,20</sup> molluscicidal,<sup>21</sup> and antitumor<sup>22,23</sup> properties. Compounds isolated from *M. incarnatus* were tested against the most common opportunistic infection pathogens, i.e., *Candida albicans*, *C. glabrata*, *C. krusei*, *Cryptococcus neoformans*, methicillin-resistant *Staphylococcus aureus*, *Mycobacterium intracellulare*, and *Aspergillus fumigatus*. Compounds **3** and **4**, containing saturated side chains, were inactive. The other compounds showed moderate to strong inhibition of MRSA. Compound **1** was the most active, with an  $IC_{50}$  of 2.5  $\mu g/mL$ . Compounds **2**, **5**, **6**, **7**, and **8** were moderately active, with  $IC_{50}$  values of 15, 9.5, 8.0, 5.0, and 6.5  $\mu g/mL$ , respectively, as compared to ciprofloxacin ( $IC_{50}$  0.1  $\mu g/mL$ ). Compound **6** also showed moderate activity against *Mycobacterium intracellulare*, with an  $IC_{50}$  of 10  $\mu g/mL$  (ciprofloxacin,  $IC_{50}$  0.4  $\mu g/mL$ ), and compound **7** showed weak activity against *Cryptococcus neoformans*, with an  $IC_{50}$  of 20  $\mu g/mL$  (amphotericin B,  $IC_{50}$  0.75  $\mu g/mL$ ). Compound **1** was also active in the anti-leishmanian assay, with an  $IC_{50}$  value of 3.6  $\mu g/mL$  and  $IC_{90}$  value of 7.0  $\mu g/mL$ , as compared to penatmidine, with an  $IC_{50}$  of 0.6  $\mu g/mL$  and  $IC_{90}$  of 1.9  $\mu g/mL$ , and was not cytotoxic in the Vero cell test at concentrations up to 25  $\mu g/mL$ .

The results of this research provide indications of several structure-activity relationships: (1) an increase in the number of double bonds in the side chain (**3**–**5**, **5**–**7**, **4**–**6**, **6**–**8**, **8**–**1**)

increases anti-MRSA activity, (2) the length of the side chain (**3**–**4**, **5**–**6**, **7**–**8**) has little effect on the activity, (3) the change of *cis*-*cis* methylene interrupted double bonds in the side chain to conjugated *trans*-*cis*-configuration (**1**–**2**) greatly decreases the activity.

## Experimental Section

**General Experimental Procedures.** UV spectra were obtained using a Hewlett-Packard 8452A spectrophotometer. IR spectra were obtained using a Bruker Tensor 27 FTIR. NMR spectra ( $^1H$ ,  $^{13}C$ , COSY, HMQC, HMBC) were recorded on a Bruker DRX 400 or 600 spectrometer operating at 400 or 600 MHz for  $^1H$  and 100 or 125 MHz for  $^{13}C$ , in  $CDCl_3$  or  $CD_3OD$ , using residual solvent peaks as internal references. The HRTOFMS data were acquired by direct injection into an Agilent MSD TOF in ESI negative mode. HPLC was performed using a Waters Delta Prep 400. Recycling HPLC was performed using a recycling preparative HPLC LC-9101, JAIGEL-1H+2H 2ea column (20  $\times$  600 mm), with  $CHCl_3$  as mobile phase at a flow rate of 3.5 mL/min.

**Mushroom Material.** Mushrooms, *Merulius incarnatus*, were collected in the fall of 2001 and 2002 at Duke Forest in Durham, North Carolina, from fallen oaks (*Quercus* sp.) and hickories (*Carya* sp.). After collection, the wood residues were carefully removed, and the freshly collected mushrooms were soaked with 95% EtOH. The voucher specimen No. 41/2002 is located in the Department of Pharmacognosy at the University of Mississippi.

**Extraction and Isolation.** The freshly collected sample (~67 g) of *M. incarnatus* was soaked with 95% EtOH for two weeks, and the extract was evaporated under reduced pressure to yield 19 g of oil. The crude extract was partitioned between water and ethyl acetate. The ethyl acetate fraction that tested active against MRSA was subjected to further fractionation. Flash column chromatography with a gradient of hexane/ethyl acetate was used for fractionation, followed by  $CHCl_3$ /MeOH/HOAc systems to obtain the active fraction ( $IC_{50}$  15  $\mu g/mL$ ). This fraction (290 mg) showed one single spot on normal-phase silica TLC plates in several solvent systems, while reversed-phase C-18 TLC showed this fraction to contain several components. HPLC was applied and even repeated injection did not afford pure compounds. Argention chromatography was applied, and four fractions (I, II, III, and IV) were obtained. Compounds **3** (7 mg), **4** (4 mg), **5** (6 mg), and **6** (7 mg) were isolated from the first fraction by HPLC, and compounds **7** (3 mg) and **8** (32 mg) were isolated from fraction II. The single peak collected from the third fraction proved to be a mixture according to NMR analysis. Recycling HPLC was used for further purification. In recycling HPLC, the sample passes through the detector and is reloaded to the column without collection. The sample (12 mg) was injected and showed small amounts of impurities. The first two cycles were made to remove these impurities from the system. The main peak was then automatically recycled from cycle 3, and two peaks were resolved at the 37th cycle. The major peak was collected as compound **2** (3 mg). Compound **1** (20 mg) was isolated from fraction IV.

**5-Heptadeca-8'Z,11'Z,16-trienylresorcinol (1):** oil; UV (MeOH)  $\lambda_{max}$  (log  $\epsilon$ ) 232 (3.50), 278 (3.13) nm; IR (film)  $\nu_{max}$  3344, 3009, 2927, 2854, 1599, 1155  $cm^{-1}$ ;  $^1H$  NMR ( $CDCl_3$ , 400 MHz)  $\delta$  6.25 (2H, d,  $J = 2.0$  Hz, H-4, H-6), 6.17 (1H, t,  $J = 2.0$  Hz, H-2), 5.82 (1H, ddt,  $J = 16.8, 10.0, 6.4$  Hz, H-16'), 5.36 (4H, m, H-8', H-9', H-11', H-12'), 5.01 (1H, dd,  $J = 16.8, 2.0$  Hz, H-17'a), 4.95 (1H, br d,  $J = 10.0$  Hz, H-17'b), 2.77 (2H, dd,  $J = 6.0, 6.0$  Hz, H-10'), 2.45 (2H, t,  $J = 8.0$  Hz, H-1'), 2.05 (6H, m, H-7', H-13', H-15'), 1.53 (2H, m, H-2), 1.46 (2H, tt,  $J = 7.2, 7.2$  Hz, H-14'), 1.35–1.29 (8H, m, H-3', H-4', H-5', H-6');  $^{13}C$  NMR ( $CDCl_3$ , 100 MHz)  $\delta$  156.6 (2C, C-1, C-3), 146.5 (C, C-5), 139.1 (CH, C-16'), 130.5 (CH, C-11'), 130.0 (CH, C-12'), 128.6 (CH, C-9'), 128.2 (CH, C-8'), 114.8 (CH<sub>2</sub>, C-17'), 108.4 (2CH, C-4, C-6), 100.5 (CH, C-2), 36.1 (CH<sub>2</sub>, C-1'), 33.6 (CH<sub>2</sub>, C-15'), 31.3 (CH<sub>2</sub>, C-2'), 29.9–29.1 (5CH<sub>2</sub>, C-3', C-4', C-5', C-6', C-14'), 27.5 (CH<sub>2</sub>, C-7'), 26.9 (CH<sub>2</sub>, C-13'), 25.9 (CH<sub>2</sub>, C-10'); HRTOFMS  $m/z$   $[M - H]^-$  341.2505 (calcd for  $C_{23}H_{33}O_2$ , 341.2481).

**5-Heptadec-9'E,11'Z,16-trienylresorcinol (2):** oil; UV (MeOH)  $\lambda_{max}$  (log  $\epsilon$ ) 238 (3.54), 282 (3.18) nm; IR (film)  $\nu_{max}$  3347, 2926, 2854, 1598, 1148  $cm^{-1}$ ;  $^1H$  NMR ( $CD_3OD$ , 600 MHz)  $\delta$  6.25 (1H, dd,  $J = 15.0, 10.8$  Hz, H-10'), 6.11 (2H, d,  $J = 1.8$  Hz, H-4, H-6), 6.07 (1H, t,  $J = 2.4$  Hz, H-2), 5.93 (1H, dd,  $J = 10.8, 10.8$  Hz, H-11'), 5.79 (1H, ddt,  $J = 16.2, 10.2, 6.6$  Hz, H-16'), 5.62 (1H, dt,  $J = 15.0, 7.2$  Hz, H-9'), 5.25 (1H, dt,  $J = 10.8, 7.2$  Hz, H-12'), 4.98 (1H, dd,  $J = 16.2,$

1.8 Hz, H-17'a), 4.93 (1H, dd,  $J = 10.2$ , 1.8 Hz, H-17'b), 2.42 (2H, t,  $J = 7.2$  Hz, H-1'), 2.15 (2H, dt,  $J = 7.2$ , 7.2 Hz, H-13'), 2.06 (4H, m, H-8', H-15'), 1.55 (2H, m, H-2'), 1.45 (2H, tt,  $J = 7.2$ , 7.2 Hz, H-14'), 1.38 (2H, m, H-7'), 1.31 (8H, br s, H-3', H-4', H-5', H-6');  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ , 125 MHz)  $\delta$  158.1 (2C, C-1, C-3), 145.1 (C, C-5), 138.6 (CH, C-16'), 134.4 (CH, C-9'), 129.1 (CH, C-12'), 129.0 (CH, C-11'), 125.8 (CH, C-10'), 113.9 (CH<sub>2</sub>, C-17'), 106.7 (2CH, C-4, C-6), 99.8 (CH, C-2), 35.8 (CH<sub>2</sub>, C-1'), 33.1 (CH<sub>2</sub>, C-15'), 32.6 (CH<sub>2</sub>, C-8'), 31.2 (CH<sub>2</sub>, C-2'), 29.4–29.0 (6CH<sub>2</sub>, C-3', C-4', C-5', C-6', C-7', C-14'), 26.8 (CH<sub>2</sub>, C-13'); HRTOFMS  $m/z$   $[\text{M} - \text{H}]^-$  341.2501 (calcd for  $\text{C}_{23}\text{H}_{33}\text{O}_2$ , 341.2481).

**Biological Evaluation.** Seven opportunistic microorganism strains, including *Candida albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113, methicillin-resistant *Staphylococcus aureus* ATCC 43300, *Mycobacterium intracellulare* ATCC 23068, and *Aspergillus fumigatus* ATCC 90906, were obtained from the American Type Culture Collection (Manassas, VA) and were used in the evaluation of antimicrobial activity. Susceptibility tests were performed using a modified version of the NCCLS method<sup>24–26</sup> for all organisms except *M. intracellulare*, for which the modified Alamar Blue procedure was followed.<sup>27</sup> IC<sub>50</sub> values were determined from the dose–response curves. Amphotericin B was used as positive control against *Candida albicans*, *C. glabrata*, *C. krusei*, *Cryptococcus neoformans*, and *Mycobacterium intracellulare* for comparison. Ciprofloxacin was used as positive control against methicillin-resistant *Staphylococcus aureus* and *Aspergillus fumigatus* for comparison. The highest concentration tested for activity was 20  $\mu\text{g/mL}$ . Antileishmanial activity of compound **1** was determined by the Alamar Blue assay using pentamidine and amphotericin B as standards.<sup>28</sup>

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**Supporting Information Available:**  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of compounds **1** and **2** as well as the chromatogram of the separated compound **2** by recycling HPLC are available free of charge via the Internet at <http://pubs.acs.org>.

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