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New Secondary Metabolites from an Endophytic Fungus in *Porodaedalea pini*

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Abstract: Eight benzenoids including three new phenols, piniphenols A–C (**1**–**3**), along with five known compounds, 4-vinylphenol (**4**) and 4-hydroxybenzaldehyde (**5**), 2-methoxy-2-(4'-hydroxyphenyl)ethanol (**6**), 2,3-dihydroxypropyl acetate (**7**), and phenol (**8**) were isolated from ethyl acetate layer of liquid fermentation with *Porodaedalea pini* (Hymenochaetaceae) BCRC 35384. All structures were established by spectral analysis and comparison with the literature data. Of these isolates, **1** showed moderate NO inhibitory activity with an IC_{50} value of 60.0 μM. Compound **1** also showed the androgen receptor (AR) antagonism with the IC_{50} value of 0.42 μM. To the best of our knowledge, this is the first report on benzenoid metabolites from the genus *Porodaedalea*.

Keywords: *Porodaedalea pini*; fungus; benzenoids; NO inhibitory activity; androgen antagonism.

1. Introduction

Endophytes are microorganisms that live in the internal tissues of their host without causing any apparent disease symptoms. Instead, endophytes affect their hosts in a positive way including growth enhancement and protection against pathogens and feeding damage [1–3]. An endophytic fungus, *Porodaedalea pini* (Brot.) Murrill (*Phellinus pini*) (Hymenochaetaceae), internal strain number BCRC 35384, was isolated from the *Pinus* species (Pinaceae). *P. pini* (Brot.) Murrill (*Phellinus pini*), which causes tree diseases commonly known as “red ring rot” or “white speck”, plays an important forest disturbance agent and a key role in habitat formation for several forest animals [4]. The investigation

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regarding the secondary constituents and biological activities of an endophytic fungus, *P. pini* BCRC 35384 have never been reported. Nitric oxide (NO) is a mediator of the inflammatory response involved in host defense. In the course of our search for potential diverse secondary metabolites from natural fungal sources and to further understanding of the minor metabolites of the genus *Porodaedalea*, we examined the EtOAc extract of *P. pini*, which showed inhibitory activity on lipopolysaccharide (LPS)-induced nitric oxide (NO) release in RAW 264.7 murine macrophages, as determined by our primary screening (approximately 95% inhibition at a concentration of 10 μg/mL). Investigation of the bioactive metabolites of the active EtOAc extract from the fungus *P. pini* led to the isolation of two new compounds, piniphenol A (**1**) and piniphenol B (**2**), together with one compound which was isolated from a natural resource for the first time, but have previously been synthesized, namely piniphenol C (**3**), together with five known compounds (Figure 1). The structures of these isolates were established by means of spectral experiments. The isolation, structure elucidation, and inhibitory effects of some isolates on NO production by macrophages are described herein..

2. **Materials and Methods**

2.1. General Experimental Procedure

All melting points were determined on a Yanaco micro melting apparatus (Yanaco, Kyoto, Japan) and were uncorrected. Optical rotations were measured on a Jasco P-2000 polarimeter (Jasco, Kyoto, Japan), UV spectra were obtained with a Jasco-V-530 UV/vis spectrophotometer (Jasco, Kyoto, Japan), and IR spectra (ATR) were acquired with a Mattson Genesis IITM FTIR spectrophotometer (Mattson Genesis, Mattson, Germany). 1D (^{1}H, ^{13}C, DEPT) and 2D (COSY, NOESY, HSQC, HMBC) NMR spectra, were recorded on a Varian Germini-200 spectrometer, Varian Mercuryplus-400 spectrometer, Varian VNMRS-600 spectrometer (Varian, Inc. Vacuum Technologies, MA, USA) operated at 200, 400, 600 MHz (1H) and 50, 100, 150 MHz (13C). Low-resolution mass spectra were obtained with POLARIS Q Thermo Finnigan (Thermo Fisher Scientific, Chicago, USA), Water ZQ 4000 (Waters, Massachusetts, USA), and VG Quattro GC/MS/MS/DS (Waters, Massachusetts, USA) mass spectrometers. HRESIMS were recorded on a Bruker APEX II mass spectrometer (Bruker, Karlsruhe, Germany). Silica gel (70-230 and 230-400 mesh; Silicycle, Quebec, Canada) was used for column chromatography (CC), and silica gel 60 F254 (Merck, Darmstadt, Germany) and RP-18 F254S (Merck, Darmstadt, Germany) were used for TLC and prep. TLC. Respectively, visualised with $Ce_2(SO_4)_3$ aq solution. Further purification was performed by medium-performance liquid chromatography (MPLC) (ceramic pump: VSP-3050; EYELA, Kyoto, Japan).

2.2. Microorganism Material

The fungus used in this study was isolated from the *Pinus* sp., and identified as *P. pini* on the basis of the rDNA internal transcribed spacer (ITS) gene sequence. The strain is preserved with the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (FIRDI), under the ID No. BCRC 35384.

2.3. Cultivation and Preparation of the Fungal Strain

The fungus used in this study was isolated from the *Pinus* sp., and identified as *P. pini* on the basis of the rDNA internal transcribed spacer (ITS) gene sequence. 21-Day-old colonies of the *P. pini* strain on PDA medium in 9-cm Petri dish were cut into the bottle and blended for 30 seconds with 200 mL distilled water to prepare the fungal inoculum for liquid fermentation. Each 500 mL flask contained 200 mL liquid cultural media (ingredients: corn starch 30 g, corn steep liquor 10 g, yeast extract 5 g, and sea salt 2 g in 1L distilled water, pH 6) were added 10 mL fungal inocula and incubated at 25°C for three weeks on a rotary shaker at the speed of 100 circles/min. A total of 16 L fungal fermented broth were harvested and then filtrated to remove fungal mycelium.

2.4. Fermentation and Isolation

The liquid fermentate of *P. pini* BCRC 35384 (16 L) was partitioned with EtOAc, and the EtOAc-layer (3.54 g) was afforded. The H_2O layer was further extracted with *n*-BuOH, and got the *n*-BuOH-layer. The active EtOAc-soluble fraction was subjected to MPLC (SiO_2; *n*-hexane–EtOAc (10:1 to 5:1) to produce 14 fractions (Frs. 1~14). Fr. 5 (26.2 mg) was subjected to MPLC (SiO_2; *n*-hexane–EtOAc 7:1) to yield **3** (2.6 mg) and **4** (2.1 mg). Fr. 9 (9.6 mg) was subjected to MPLC (SiO_2, *n*-hexane–acetone (10:1)) to yield **1** (2.5 mg). Fr. 11 was further separated with prep. TLC (H_2O–MeOH (1:1)) to obtain **5** (R_f 0.44; 18.4 mg). Fr. 14 (3.4 g) was subjected to Sephadex LH-20 (eluted with MeOH), gained eight fractions (Frs. 14-1~14-8). Fr. 14-3 (1.1 g) was then subjected to MPLC (SiO_2, *n*-hexane–EtOAc (4:1)) to yield 13 subfractions (Frs. 14-3-1~14-3-13). Fr. 14-3-5 was further purified by HPLC (CH_2Cl_2–acetone (40:1) in 35 min.) to gain **2** (1.5 mg). Fr. 14-3-10 was subjected to MPLC (SiO_2; CH_2Cl_2–MeOH (60:1)) to produce 15 subfractions (Frs. 14-3-10-1~14-3-10-15). Fr. 14-3-10-6 was further separated with prep. TLC (H_2O–acetonitrile (1:1)) to obtain **6** (R_f 0.47; 0.3 mg). Fr. 14-3-13 (283 mg) was subjected to MPLC (RP-18, H_2O–MeOH (3:1)) to produce 15 subfractions (Frs. 14-3-13-1~14-3-13-15). Fr. 14-3-13-2 was then subjected to MPLC (SiO_2, CH_2Cl_2–acetone (5:1)) to yield **7** (1.9 mg). Fr.14.5 (395 mg) was further separated with prep. TLC (CH_2Cl_2–MeOH (4:1)) to obtain **8** (R_f 0.29; 1.6 mg).

*2.4.1. Piniphenol A (**1**)*

Colorless oil; $[\alpha]_D^{25}$ ± 0 (*c* 0.13, MeOH); UV λ_{max} (MeOH) (log ε): 225 (3.67), 276 (3.04) nm; IR ν_{max} (ATR): 3345 (OH), 1614, 1517, 1454 (aromatic ring) cm^{-1}; ESIMS *m/z* 217 $[M+Na]^+$; HRESIMS *m/z* 217.0835 $[M+Na]^+$ (Calcd for $C_{11}H_{14}NaO_3$: 217.0841); ^1H NMR ($CDCl_3$, 400 MHz) δ_H: 1.73 (1H, dddd, *J* = 12.4, 10.0, 7.2, 6.2 Hz, H-3'b), 1.94 (1H, dddd, *J* = 13.3, 8.6, 6.2, 2.0 Hz, H-4'b), 2.20 (1H, dddd, *J* = 13.3, 10.0, 6.0, 5.2 Hz, H-4'a), 2.35 (1H, dddd, *J* = 12.4, 8.6, 7.2, 6.0 Hz, H-3'a), 3.41 (3H, s, OCH_3-5'), 4.92 (1H, br s, OH-1, D_2O exchangeable), 5.00 (1H, t, *J* = 7.2 Hz, H-2'), 5.22 (1H, dd, *J* = 5.2, 2.0 Hz, H-5'), 6.79 (2H, d, *J* = 8.4 Hz, H-2, H-6), 7.21 (2H, d, *J* = 8.4 Hz, H-3, H-5); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C: 32.5 (C-4'), 32.6 (C-3'), 54.9 (C-7), 79.1 (C-2'), 105.5 (C-5'), 115.2 (C-2, C-6), 127.5 (C-3, C-5), 134.5 (C-4), 155.0 (C-1).

*2.4.2. Piniphenol B (**2**)*

Colorless oil; $[\alpha]_D^{25}$ −36.2 (*c* 0.083, MeOH); UV λ_{max} (MeOH) (log ε): 225 (3.67), 276 (3.04) nm; IR ν_{max} (ATR): 3310 (OH), 1604, 1454 (aromatic ring) cm^{-1}; ESIMS *m/z* 217 $[M+Na]^+$; HRESIMS *m/z* 217.0844 $[M+Na]^+$ (Calcd for $C_{11}H_{14}NaO_3$: 217.0841); ^1H NMR ($CDCl_3$, 600 MHz) δ_H: 1.98 (1H, m, H-4'b), 2.05 (1H, m, H-3'b), 2.10 (1H, m, H-3'a), 2.22 (1H, m, H-4'a), 3.43 (3H, s, OCH_3-5'), 4.78 (1H, br s, OH-1, D_2O exchangeable), 4.96 (1H, dd, *J* = 9.6, 6.0 Hz, H-2'), 5.07 (1H, d, *J* = 5.4 Hz, H-5'), 6.80 (2H, d, *J* = 8.7 Hz, H-2, H-6), 7.25 (2H, d, *J* = 8.7 Hz, H-3, H-5); ^{13}C NMR ($CDCl_3$, 150 MHz) δ_C: 32.9 (C-4'), 33.9 (C-3'), 54.7 (C-7), 82.6 (C-2'), 105.3 (C-5'), 115.2 (C-2, C-6), 128.0 (C-3, C-5), 135.4 (C-4), 154.9 (C-1).

*2.4.3. Piniphenol C (**3**)*

Colorless oil; $[\alpha]_D^{25}$ – 6.38 (*c* 0.017, $CHCl_3$); UV λ_{max} (MeOH) (log ε): 206 (3.79), 229 (3.91), 280 (3.33) nm; IR ν_{max} (ATR): 3333 (OH), 1613, 1513 (aromatic ring) cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ_H: 2.05, (1H, m, H-8b), 2.26 (1H, m, H-8a), 3.42 (1H, m, H-7), 4.50 (1H, br s, OH-1, D_2O exchangeable), 6.75 (2H, dd, *J* = 8.7, 2.6 Hz, H-2, H-6), 7.09 (2H, d, *J* = 8.7, 2.6 Hz, H-3, H-5); ^{13}C NMR ($CDCl_3$, 150 MHz) δ_C: 26.0 (C-8), 47.8 (C-7), 115.1 (C-2, C-6), 127.8 (C-3, C-5), 137.0 (C-4), 153.8 (C-1).

2.5. NO Production and Cell Viability Assay

Mouse macrophage cell line (RAW 264.7) was obtained from Bioresource Collection and Research Center (BCRC 60001) and cultured at 37 ºC in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco), 4.5 g/L glucose, 4 mm glutamine, penicillin (100 units/mL), and streptomycin (100 mg/mL) in a humidified atmosphere in a 5% CO_2 incubator. The cells were treated with 10, 25, and 50 mm natural products in the presence of 1 mg/mL

Whitish LPS (Sigma-Aldrich) for 20 h. The concentration of NO in culture supernatants was determined as nitrite, a major stable product of NO, by Griess reagent assay [5], and cell viabilities were determined using the MTT assay as described in [6].

2.6. Antiandrogen activity (AR) Assay

Human breast carcinoma MDA-MB-453 cells were maintained in RPMI-1640 medium containing penicillin (25 U/mL), streptomycin (25 mg/mL), and 10% fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere. Transfections were performed using the electroporation method with a pPSA-SEAP plasmid (courtesy of Prof. C. H. Wang of Kaohsiung Medical University). Briefly, cells were $1x10^6$ cells were resuspended in 0.5 mL medium with 10 μg plasmid DNA and pipetted into a 0.4-cm electroporation cuvette. Cells were incubated on ice for 5 min, electroporated at 250 V and 960 μF using a gene pulser (Bio-Rad, Hercules, CA), and incubated for 5 more min on ice. Transfected cells were resuspended in growth medium with 10% charcoal-stripped FBS and seeded into a 96-well microplates. After an overnight incubation, cells were treated with DHT (10 nM) and samples of interest for 48 hours. Aliquots of culture media were analyzed for secreted alkaline phosphatase activity using phospha-light reporter chemiluminescence assay kit (Applied Biosystems, USA) and the resulting luminescence was detected using a VICTOR Light 1420 luminescence counter (PerkinElmer, USA) [7].

3. Results and Discussion

3.1 Structure Elucidation

The liquid fermentation of *P. pini* BCRC 35384 was partitioned between EtOAc and water to give EtOAc fraction, which was fractionated by a combination of silica gel, MPLC, semi-preparative normal-phase HPLC, and prep. TLC to furnish eight compounds **1**–**8**, including three new benzenoid derivatives, the structures of which were elucidated by 1D- and 2D-NMR spectra and comparison of their NMR data, IR, and optical rotation values with those of the compounds reported in the literature. Compound **1** was obtained as a colorless oil with $[\alpha]_D^{25}$ ± 0 (c = 0.13, MeOH) and its molecular formula was deduced as $C_{11}H_{14}O_3$ from the HRESIMS data (*m/z* 217.0835 $[M+Na]^+$ (Calcd for $C_{11}H_{14}NaO_3$: 217.0841), implying five degrees of unsaturation. The UV spectrum showed maximal absorptions at 225, 276 nm, suggested the presence of a phenolic skeleton of **1**. Its IR spectrum displayed peaks at 3345 (OH group) and 1614, 1517, 1454 (aromatic ring) cm^{-1}. Analyses of the ^1H NMR spectrum indicated the presence of one phenolic OH group at δ_H 4.92 (1H, br s, OH-1, D_2O exchageable), one saturated furan moiety at δ_H 1.73 (1H, dddd, *J* = 12.4, 10.0, 7.2, 6.2 Hz, H-3'b), 1.94 (1H, dddd, *J* = 13.0, 8.6, 6.2, 2.0 Hz, H-4'b), 2.20 (1H, dddd, *J* = 13.0, 10.0, 6.0, 5.2 Hz, H-4'a), 2.35 (1H, dddd, *J* = 12.4, 8.6, 7.2, 6.0 Hz, H-3'a), 5.00 (1H, t, *J* = 7.2 Hz, H-2'), and 5.22 (1H, dd, *J* = 5.2, 2.0 Hz, H-5'), one non-aromatic OCH_3 group at δ_H 3.41 (3H, s, OCH_3-5'), and an AA'BB' system characteristic signals at δ_H 6.79 (2H, d, *J* = 8.4 Hz, H-2, H-6) and 7.21 (2H, d, *J* = 8.4 Hz, H-3, H-5) ascribed to a 4-substituted phenol. The ^{13}C NMR and DEPT spectra indicated 11 carbons, containing one methyl, two methylenes, six methines, and two quaternary carbons. HMBC spectrum showed correlations between H-3 & 5 (δ_H 7.21) to C-2' (δ_C 79.1) and H-2' (δ_H 4.96) to C-4 (δ_C 135.4), established that the tetrahydrofuran ring was located at C-4. The methoxy group was located at C-5' confirmed by the correlation between methoxy proton (δ_H 3.41) and C-5' (δ_C 105.5). High similarity was observed between the NMR data of **1** and those of 5-(4-methoxyphenyl)tetrahydrofuran-3-yl acetate [8], implying a close structural resemblance. The major differences was the presence of signals for an OH moiety at δ_H 4.92 (1H, br s, OH-1, D_2O exchageable) and one OCH_3 group at δ_H 3.41 (3H, s, OCH_3-5'), attached to C-1 and C-5' in **1**, instead of signals for one OCH_3 moiety at δ_H 3.81 (1H, s, OCH_3-1) and one acetate group in 5-(4-methoxyphenyl)tetrahydrofuran-3-yl acetate (Satterfield et al. 2011). The relative configuration of **1** was proposed on the basis of the NOESY experiment. No NOE correlation (Figure 2) between H-2'/H-5' demonstrated that the H-2' and H-5' are *trans* form. Because of the optical inactivity, **1** was proposed to be racemic. These findings unambiguously established the relative configuration structure of compound **1** as depicted in Figure 1 and assigned to 4-((2*R**,5*R**)-5-methoxytetrahydrofuran-2-yl)phenol and trivially named as piniphenol A.

Compound **2** was also assigned as $C_{11}H_{14}O_3$ with $[\alpha]_D^{25}$ –36.2 (c = 0.083, MeOH) by the molecular ion of HRESIMS at *m/z* 217.0844, an isomer of compound **1** with five degrees of unsaturation, showing almost similar IR absorption bands. Comparison of the ^1H and ^{13}C NMR spectra of **2** with those of **1** revealed that these two compounds were structurally very similar. Meanwhile, the ^{13}C NMR spectrum exhibited 11 signals that were clearly classified into six methines, two methylenes, one methyl, and two quaternary carbons based on the HSQC, suggesting **2** might be a benzenoid derivative, similar to **1**, which was corroborated by its 2D NMR spectra involving ^1H, ^1H-COSY, HSQC, HMBC and NOESY (Figure 2). The only difference was that the chemical shift of H-5' of the tetrahydrofuran moiety in this compound was shielded ($\Delta\delta$ 0.15) in comparison to **1**. The shielding effects of H-atoms at C-5' are probably due to the magnetic anisotropic effect of the phenyl ring in **2**. The relative configurations of C-2' and C-5' were assigned by the comparison of the chemical shifts of H-2' and H-5' with literature [8][9], as well as its NOE correlations. The orientation of the two protons at H-2' and H-5' was suggested to be *cis* on the basis of the NMR chemical shifts and NOESY spectrum showed correlation between H-2' and H-5', consistent with the literature concerning *cis*-form tetrahydrofuran ring [8][9]. These findings collectively established the structure of compound **2** as formulated in Figure 1 and assigned to 4-((2*S**,5*R**)-5-methoxytetrahydrofuran-2-yl)phenol and trivially named as piniphenol B.

Compound **3** was also isolated as an optically inactive colorless oil, which easily decomposed. The UV spectrum showed maximal absorptions at 206, 229, 280 nm, suggested the presence of a phenolic skeleton. IR spectrum displayed peaks at 3333 (OH), 1613, 1513 (aromatic ring) cm^{-1}. The ^1H and ^{13}C NMR spectrum of **3** were identical to that of one synthetic compound, (+)-4-hydroxyphenyloxirane [10], with dextrorotatory optical activity $[\alpha]_D^{25}$ + 4.95 (c = 0.8, $CHCl_3$). However, **3** with $[\alpha]_D^{25}$ – 6.38 (c = 0.017, $CHCl_3$) is considered to be an enantiomer of (+)-4-hydroxyphenyloxirane. On the basis of the above spectral evidence, the structure of **3** was elucidated as (–)-4-hydroxyphenyloxirane, named piniphenol C. This is the first report of compound **3** having been isolated from a natural source, although it has been synthesized previously [10][11][12]. The MS of **3** was calculated as *m/z* 136 $[M]^+$, but only the *m/z* 120 $[M]^+$ was found in this study. We predict that the epoxide group of compound **3** might be unstable and changed to the vinyl group, like 4-vinylphenol (**4**) (*m/z* 120 $[M]^+$). The above data indicated that the structure of **3** is (–)-4-hydroxyphenyloxirane, the structure of which was further confirmed by HSQC, NOESY, DEPT, and HMBC experiment.

The known isolates were readily identified by comparison of physical and spectroscopic data (UV, IR, ^1H NMR, ^{13}C NMR, $[\alpha]_D$, and MS) with corresponding literature values, and these included five benzenoids, 4-vinylphenol (**4**) [13], 4-hydroxybenzaldehyde (**5**) [14], 2-methoxy-2-(4'-hydroxyphenyl)ethanol (6) [15], phenol (8) [16], one fatty ester, 2,3-dihydroxypropyl acetate (7) [17].

3.2 Anti-inflammatory Activity and Antagonism Receptor(AR) Antagonism

The isolates in sufficient amounts (**1**, **2**, **5**, and **7**), were evaluated by examining their inhibitory effects on LPS-induced inducible nitric oxide synthase (iNOS)-dependent NO and IL-6 production in the murine macrophage cell line RAW 264.7 and androgen receptor antagonism. From the results of above tests, the following conclusions can be drawn: Compared to quercetin (IC_{50} value 35.8±1.2 μM), which was used as positive control in this study, piniphenol A (**1**) exhibited NO inhibitory activity with IC_{50} values of 60.0±3.8 μM. And the piniphenol A (**1**) show androgen receptor antagonism with IC_{50} values of 0.42 μM compare with positive control Casodex (IC_{50} value 1.67 ± 0.20 μM). Among the benzenoids analogs piniphenol A with a (2*R**,5*R**)-5-methoxytetrahydrofuran-2-yl group exhibited higher inhibition than its analog **2** with a (2*S**,5*R**)-5-methoxytetrahydrofuran-2-yl group at C-4. Cytotoxic effects were determined using MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay. The high cell viability (> 96%) indicated that the androgen receptor antagonism by active compound **1** did not result from its cytotoxicities.

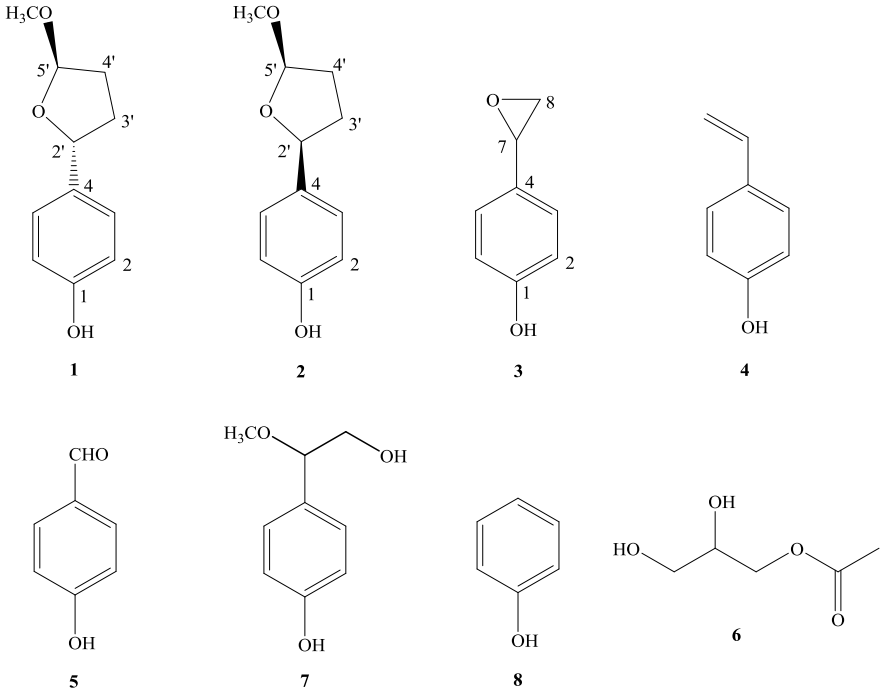

Figure 1. Chemical structures of the compounds (1-8) obtained from the *Porodaedalea pini*.

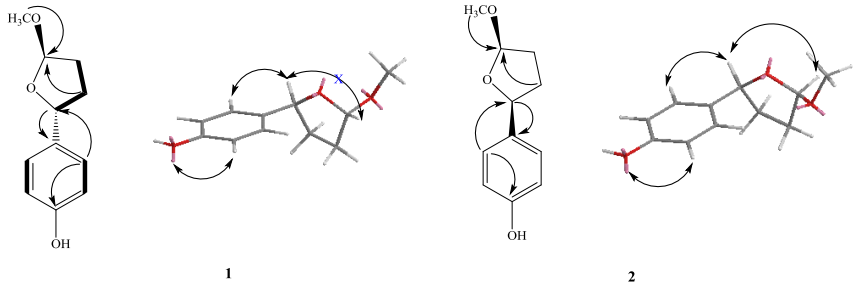

Figure 2. Key COSY (bond line), HMBCs (H→C), and NOESY (H↔H) correlations for compounds **1** and **2**.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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