

Article

New and Cytotoxic Components from *Antrodia camphorata*

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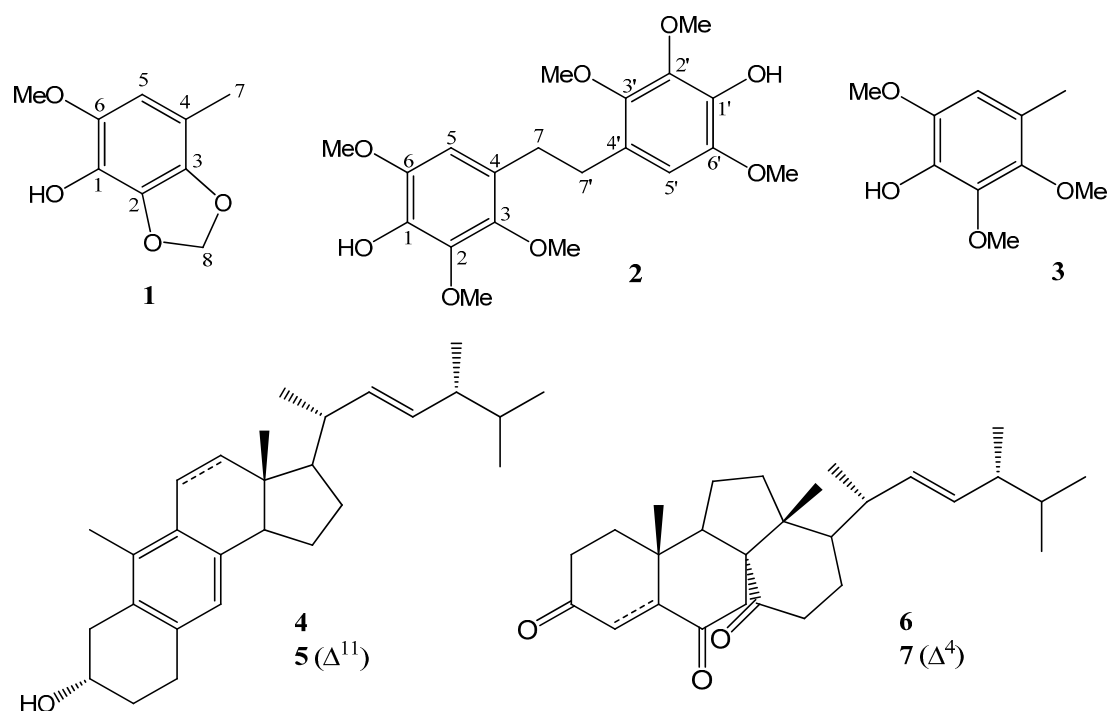
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Abstract: The solid-state cultured products of *Antrodia camphorata* as health foods has been blooming for the past few decades in Taiwan. In continuing our studies on the chemical constituents of the solid-state cultured products of this fungus, 6-methoxy-4-methyl-2,3-(methylenedioxy)phenol (**1**) and 4,4'-(ethane-1,2-diyl)bis(2,3,6-trimethoxyphenol)(**2**) together with 2,3,6-trimethoxy-4-methylphenol (**3**), 1(10→6)*abeo*-ergosta-5,7,9,22-tetraen-3 α -ol (**4**), citreanthrasteroid B (**5**) and dankasterones A (**6**) and B (**7**) were purified by a series of column chromatography. Their structures were elucidated by spectral data analysis. For bioactivity assay, compounds **4–7** showed significant cytotoxicity toward murine colorectal CT26 and human leukemia K562 cancer cell lines with IC₅₀ values ranging from 6.7 to 15.3 μ M and from 12.5 to 23.1 μ M, respectively.

Keywords: *Antrodia camphorata*; phenylmethanoid; dihydrostilbene; *abeo*-ergostane; cytotoxicity

1. Introduction

Antrodia camphorata Wu, Ryvarden & Chang (Polyporaceae), a fungus indigenous to Taiwan, was used by the aborigine as a hepatinica and anti-alcoholic agent initially and has been gradually used as a folk remedy for the treatment of liver cancer and various cardiovascular diseases in the past few decades [1]. The fermentation and development of this fungus have already become one of the major components of the biotechnology industry in Taiwan recently, and some of the chemical entities isolated from the fermented products, e.g., antroquinonol [2] and ergosta-7,9,22*E*-trien-3 β -ol [3], were selected as leads for new drug development. Although over one hundred compounds have been identified from this fungus so far [4–6], new chemical entities are still reported continually by virtue of varied culturing conditions for this fungus. In continuing our investigations on the chemical constituents of the mycelium of *Antrodia camphorata*, one new phenylmethanoid, 6-methoxy-4-methyl-2,3-(methylenedioxy)phenol (**1**), and one new stilbene, 4,4'-(ethane-1,2-diyl)bis(2,3,6-trimethoxyphenol)(**2**), together with 2,3,6-trimethoxy-4-methylphenol (**3**), 1(10→6)*abeo*-ergosta-5,7,9,22-tetraen-3 α -ol (**4**), citreanthrasteroid B (**5**) and dankasterones A (**6**) and B (**7**) (Figure 1) were isolated and characterized on the basis of the spectral analysis. The paper describes the isolation and identification of compounds **1–7** from *A. camphorata* along with their anticancer effects in murine colorectal cancer CT26 cells and human leukemia K562 cells.

Figure 1. Chemical structures of 1–7 isolated in this study.

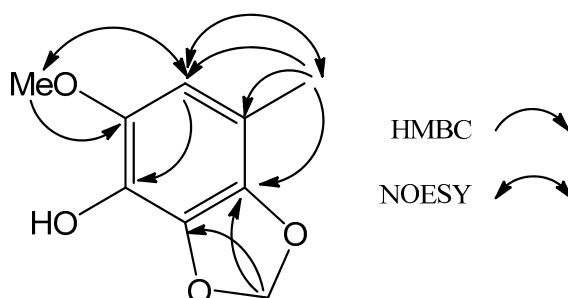
2. Results and Discussion

From the methanolic extracts of the solid-state cultured, fermented dried powder of *A. camphorata*, seven major compounds, including one new phenylmethanoid, **1**, and one new stilbene, **2**, along with a known phenylmethanoid, **3**, and four steroids, **4**–**7**, were isolated by sequential separation on Si-gravity column and normal-phase HPLC. Compound **3** was obtained as an amorphous white solid whose ^1H , ^{13}C -NMR, IR, optical rotation and MS were consistent with those of synthetic 2,3,6-trimethoxy-4-methylphenol [7], and this is the first time Compound **3** has been isolated from a natural resource. Compounds **4** and **5**, two rare 1(10 \rightarrow 6)*abeo*-ergostane-type steroids, were identified as 1(10 \rightarrow 6)*abeo*-ergosta-5,7,9,22-tetraen-3 α -ol (**4**), obtained previously from the stromata of *Epichloe typhina* [8], and citreanthrasteroid B (**5**), isolated from a hybrid bacterial strain, KO 0231 [9]. Compounds **6** and **7**, two uncommon 13(14 \rightarrow 8)*abeo*-ergostane-type steroids, were characterized as respective dankasterones A and B, which were isolated previously from a *Halichondria* sponge-derived fungus, *Gymnascella dankaliensis* [10].

Compound **1** was afforded as a colorless amorphous solid with the molecular formula of $\text{C}_9\text{H}_{10}\text{O}_4$ as established through the analysis of its ^{13}C -NMR and HRESIMS data. The IR absorption peaks of **1** at 3277, 1626 and 1527 cm^{-1} indicated that **1** contained a benzene moiety bearing a hydroxy functionality as reflected in its ^1H - and ^{13}C -NMR data. The ^1H -NMR of **1** exhibited a phenyl proton at δ_{H} 6.27 (s, 1H), a benzene-borne methyl at δ_{H} 2.16 (s, 3H), a benzene-borne methoxyl at δ_{H} 3.82 (s, 3H) and a methylenedioxy group at δ_{H} 5.91 (s, 2H), which were further confirmed by their corresponding six phenyl resonances at δ_{C} 109.0 (d), 118.9 (s), 131.9 (s), 133.6 (s), 135.0 (s) and 136.8 (s), one methyl resonance at δ_{C} 15.8 (q), one methoxyl resonance at δ_{C} 57.2 (q) and one dioxygenated methylene resonance at δ_{C} 101.5 (t) in the ^{13}C -NMR of **1**. Further analysis of the 2D NMR data of **1**, key cross-peaks of δ_{H} 2.16 (H_{3-7})/ δ_{C} 109.0 (C-5), 118.9 (C-4) and 131.9 (C-3), δ_{H} 3.82 (OMe-6)/ δ_{C} 136.8 (C-6),

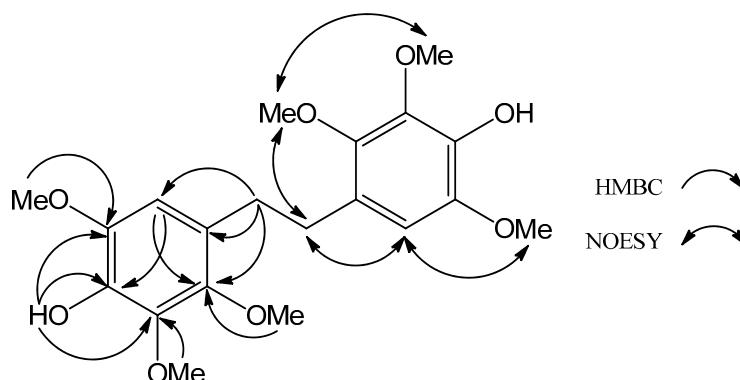
δ_{H} 5.91 (H₂-8)/ δ_{C} 131.9 (C-3) and 135.0 (C-2), δ_{H} 6.27 (H-5)/ δ_{C} 133.6 (C-1) in the HMBC spectrum and mutual-correlated cross peaks of δ_{H} 6.27 (H-5)/ δ_{H} 3.82 (OMe-6) and δ_{H} 2.16 (H₃-7) in the NOESY spectrum (Figure 2) established the locations of all of the functional groups attached to the benzene ring. Thus, **1** was deduced as the shown phenylmethanoid and was named 6-methoxy-4-methyl-2,3-(methylenedioxy)phenol.

Figure 2. Key HMBC and NOESY of **1**.



Compound **2**, obtained as a colorless amorphous solid, was a symmetrical chemical entity as judged from its molecular formula, C₂₀H₂₆O₈, deduced from HRESIMS and only ten resonances in the ¹³C-NMR spectrum. The IR spectrum of **2** confirmed the presence of a hydroxyl group (3410 cm⁻¹) and a benzene ring (1610 and 1503 cm⁻¹). The ¹³C-NMR along with the DEPT of **2** displayed only ten signals (indeed, twenty signals due to symmetry), including one methylene carbon at δ_{C} 31.4, three benzene-borne methoxyl carbons at δ_{C} 56.4, 60.7 and 61.0 and six phenyl carbons at δ_{C} 107.2, 125.2, 137.3, 140.4, 143.2 and 145.2. The ¹H-NMR spectrum showed signals for the methylene group at δ_{H} 2.78 (s, 2H), three methoxyl groups at δ_{H} 3.79 (s, 3H), 3.80 (s, 3H) and 3.91 (s, 3H), one hydroxyl group at δ_{H} 5.45 (brs, 1H) and one phenyl proton at δ_{H} 6.38 (s, 1H). The above assignments indicated that **2** had a stilbene skeleton with three methoxyl and one hydroxyl groups on each benzene ring. The locations of three methoxy, one hydroxy and one phenyl proton were further corroborated by key HMBC interpretations, including δ_{H} 2.78 (H-7, -7')/ δ_{C} 107.2 (C-5, -5'), 125.2 (C-4, -4') and 145.2 (C-3, -3'), δ_{H} 3.79 (OMe-3, -3')/ δ_{C} 145.2 (C-3, -3'), δ_{H} 3.80 (OMe-6, -6')/ δ_{C} 143.2 (C-6, -6'), δ_{H} 3.91 (OMe-2, -2')/ δ_{C} 140.4 (C-2, -2'), δ_{H} 5.45 (OH-1, -1')/ δ_{C} 137.3 (C-1, -1'), 140.3 (C-2, -2'), 143.2 (C-6, -6') and δ_{H} 6.38 (H-5, -5')/ δ_{C} 137.3 (C-1, -1') and 145.2 (C-3, -3'), as well as key NOESY correlations, including δ_{H} 6.38 (H-5, -5')/ δ_{H} 2.78 (H-7, -7') and 3.80 (OMe-6, -6') and δ_{H} 3.79 (OMe-3, -3')/ δ_{H} 2.78 (H-7, -7') and 3.91 (OMe-2, -2') (Figure 3). Accordingly, **1** was determined as the shown dihydrostilbene and was named 4,4'-(ethane-1,2-diyl)bis(2,3,6-trimethoxyphenol). Compound **2** was speculated from the oxidative coupling between two molecules of Compound **3**.

For the anticancer activity, Table 1 shows the IC₅₀ values of compounds **1–7** against murine colorectal cancer CT26 cells and human leukemia K562 cells. Compounds **1–3** exhibited no obvious effect toward CT26 and K562 cells with their IC₅₀ values higher than 20 μM , and **4–7** showed significant cytotoxicity toward murine colorectal CT26 and human leukemia K562 cancer cell lines with IC₅₀ values ranging from 6.7 to 18.2 μM and from 12.5 to 23.1 μM , respectively. At the same condition, the IC₅₀ value of staurosporine against K562 leukemia cells was 16.7 nM.

Figure 3. Key HMBC and NOESY of **2**.**Table 1.** IC₅₀ values of compounds **1–7** against colorectal cancer CT26 and leukemia K562 cells.

Compounds	IC ₅₀ (μM)	
	CT26	K562
1	>20	>20
2	>20	>20
3	>20	>20
4	15.3	19.9
5	18.2	12.5
6	6.7	>20
7	8.4	23.1

The IC₅₀ value of staurosporine, the positive control, against K562 cells was 16.7 nM.

3. Experimental

3.1. General

Optical rotations were measured on a JASCO DIP-1000 polarimeter (Tokyo, Japan). ¹H and ¹³C-NMR were acquired on a Bruker DMX-500 (Ettlingen, Germany). Low resolution and high resolution mass spectra were obtained using an API4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) and a Synapt High Definition Mass Spectrometry system with an ESI interface and a TOF analyzer (Waters Corp., Manchester, UK), respectively. IR spectra were recorded on a JASCO FT/IR 4100 spectrometer (Tokyo, Japan). TLC was performed using silica gel 60 F₂₅₄ plates (200 μm, Merck, Taipei, Taiwan).

3.2. Fungal Material

Freeze-dried powder of *Antrodia camphorata* was provided by Kang Jian Biotech Corp. Ltd, Nantan, Taiwan R.O.C.

3.3. Extraction and Isolation

The dried powder (2.83 kg) of solid-state cultured *Antrodia camphorata* was extracted with 12 L methanol for three times, and the concentrated residues (323.8 g) were suspended in H₂O and further

partitioned three times with equal volumes of ethyl acetate, then concentrated in vacuum to dryness (131.5 g). The ethyl acetate extract was applied onto an open column with silica gel. The column was eluted with mixtures of *n*-hexane, ethyl acetate and methanol, and each 1 L was collected as one fraction. Fractions 16–33 were combined and evaporated to dryness (1.4 g), which were further purified by HPLC on a semi-preparative Phenomenex Luna Si column (5 μ m, 10 \times 250 mm) with *n*-hexane–ethyl acetate (90:10, *v/v*) as the eluent, 2 mL/min, obtaining **1** (4.6 mg), **2** (6.4 mg) and **3** (8.9 mg). Fractions 92–124 were combined and evaporated to dryness (33.1 g), which were further purified by HPLC on the same column with *n*-hexane–ethyl acetate (50:50, *v/v*) as the eluent, 2 mL/min, obtaining **4** (5.6 mg), **5** (7.4 mg), **6** (10.3) and **7** (9.2 mg).

6-Methoxy-4-methyl-2,3-(methylenedioxy)phenol (**1**): Colorless amorphous solid; IR (neat): ν_{\max} 3277, 3015, 1626, 1527, 1440, 1341, 1208, 1129, 1043, 925 cm^{-1} . $^1\text{H-NMR}$ (CDCl_3 , 400 MHz): δ_{H} 2.16 (3H, s, H-7), 3.82 (3H, s, OMe-6), 5.91 (2H, s, H-8), 6.27 (1H, s, H-6). $^{13}\text{C-NMR}$ (CDCl_3 , 100 MHz): δ_{C} 15.8 (C-7), 57.2 (OMe-6), 101.5 (C-8), 109.0 (C-5), 118.9 (C-4), 131.9 (C-3), 133.6 (C-1), 135.0 (C-2), 136.8 (C-6). ESIMS: $m/z = 183$ $[\text{M}+\text{H}]^+$. HRESIMS: $m/z = 183.0653$ $[\text{M}+\text{H}]^+$ (calcd. for $\text{C}_9\text{H}_{11}\text{O}_4$, 183.0657).

4,4'-(Ethane-1,2-diyl)bis(2,3,6-trimethoxyphenol) (**2**): Colorless amorphous solid; IR (neat): ν_{\max} 3410, 3012, 2956, 2860, 1610, 1503, 1420, 1325, 1248, 1198, 1125, 1076, 970, 880. $^1\text{H-NMR}$ (CDCl_3 , 400 MHz): δ_{H} 2.78 (4H, s, H-7, -7'), 3.79 (6H, s, OMe-3, -3'), 3.80 (6H, s, OMe-6, -6'), 3.91 (6H, s, OMe-2, -2'), 5.45 (2H, brs, OH-1, -1'), 6.38 (2H, s, H-5, -5'). $^{13}\text{C-NMR}$ (CDCl_3 , 100 MHz): δ_{C} 31.4 (C-7, -7'), 56.4 (OMe-3, -3'), 60.7 (OMe-6, -6'), 61.0 (OMe-2, -2'), 107.2 (C-5, -5'), 125.2 (C-4, -4'), 137.3 (C-1, -1'), 140.4 (C-2, -2'), 143.2 (C-6, -6'), 145.2 (C-3, -3'). ESIMS: $m/z = 395$ $[\text{M}+\text{H}]^+$. HRESIMS: $m/z = 395.1710$ $[\text{M}+\text{H}]^+$ (calcd. for $\text{C}_{20}\text{H}_{27}\text{O}_8$, 395.1706).

3.4. Cells and Viability Assay

Murine colorectal cancer CT26 cells were cultured in RPMI1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT) and 2 mM L-glutamine at 37 °C in a humidified 5% CO_2 incubator. The viability of CT26 cells was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) colorimetric assay. Human chronic myeloid leukemia K562 cells were cultured in RPMI 1640 medium, 10% FBS and 2 mM L-glutamine at 37 °C in an incubator. The viability of K562 cells with compound treatments for one day were measured using the Trypan blue dye exclusion test. The IC_{50} values of compounds in CT26 and K562 cells were determined by using SigmaStat software (Jandel Scientific, San Rafael, CA, USA).

4. Conclusions

In this study, three $\text{C}_6\text{-C}_1$ derivatives, as well as four phytosteroids with rearranged skeletons were isolated from the industrial fermented products of *Antrodia camphorata*. Of the compounds isolated, four phytosteroids exhibited significant cytotoxicity against cancer cell lines, which may, to some extent, account for the traditional uses of this fungal product as a health food to treat cancer. More experiments should be performed to deduce the action modes of these compounds.

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Author Contributions

Hui-Fen Liao, Yueh-Hsiung Kuo, and Horng-Huey Ko designed research; Tzong-Huei Lee, Chien-Chih Chen, and Hsun-Shuo Chang performed research; Jih-Jung Chen, Ping-Jyun Sung, Mei-Hwei Tseng, and Sheng-Yang Wang contributed new analytical tools and reagents; Tzong-Huei Lee and Yueh-Hsiung Kuo wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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Sample Availability: Not available.

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