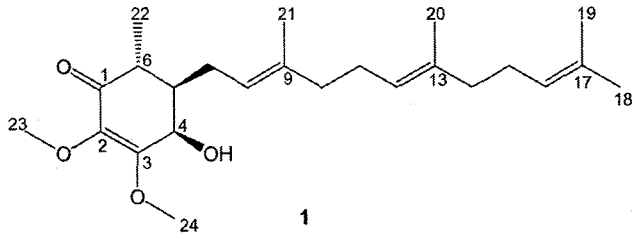
A New Cytotoxic Agent from Solid-State Fermented Mycelium of *Antrodia camphorata*

Tzong-Huei Lee[1], Ching-Kuo Lee[1], Wan-Lin Tsou[2], Sheng-Yung Liu[2], Mao-Tien Kuo[2], Wu-Che Wen[2]



1

Abstract

Antroquinonol (**1**), an ubiquinone derivative, was isolated from the solid-state fermented mycelium of *Antrodia camphorata* (Polyporaceae, Aphyllophorales), a parasitic fungus indigenous to Taiwan. The structure of compound **1** was elucidated by the analysis of their spectroscopic data. Its cytotoxic activities were evaluated against MCF-7, MDA-MB-231 (human breast carcinoma), Hep3B, HepG2 (human liver carcinoma) and DU-145, LNCaP (human prostate carcinoma) cell lines, and the IC_{50} values ranged from 0.13 ± 0.02 to 6.09 ± 0.07 μM.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Antrodia camphorata Wu, Ryvarden & Chang (Polyporaceae, Aphyllophorales) is a parasitic fungus on the inner heartwood wall of rotted trees of the endangered *Cinnamomum kanehirai* Hay (Lauraceae) in Taiwan [1]. As a folk remedy, its fruiting body has long been used for food and drug intoxication, and the treatment of diarrhea, abdominal pain, hypertension, itching of the skin, and liver cancer [2]. However, due to its slow growth rate in nature and since it cannot be mass produced in the greenhouse thus far, the fruiting bodies are very rare and expensive. In order to search for materials which would substitute for the fruiting bodies collected in nature, the cultured mycelium of *A. camphorata* has been found to exhibit anti-inflammatory activity [3], vasorelaxation [4], cytotoxic activity against several tumor cell lines [5], protection of oxidative damage in normal human erythrocytes [6], and anti-hepatitis B virus activity [7] in biological studies. Until now, the chemical study of the cultured mycelia of *A. camphorata* had only been conducted by Nakamura et al. whereby five new maleic and succinic acid derivatives were isolated [8]. In this paper we describe the identification of the new ubiquinone derivative **1** from the solid-state fermented mycelium of *A. camphorata* and its cytotoxic effects against six human carcinoma cell lines including MCF-7, MDA-MB-231, Hep3B, HepG2, DU-145 and LNCaP.

Compound **1** was isolated as light yellow oil, and its molecular formula, $C_{24}H_{38}O_4$, was established through analysis of its ^{13}C-NMR and HR-FAB-MS data. The IR spectrum of **1** confirmed the presence of a conjugated carbonyl group (1668 cm^{-1}) and a hydroxy group (3443 cm^{-1}). The ^{13}C-NMR spectrum accompanied with DEPT analysis displayed 24 signals including five methyl carbons, five methylene carbons, two methine carbons in the aliphatic region, two methoxy carbons, one carbinoyl carbon, eight olefinic carbons as well as one carbonyl carbon (Table **1**). The ^1H-

Table **1** ^1H- and ^{13}C-NMR spectroscopic data ($CDCl_3$, 500 MHz) for compound **1** [δ in ppm, mult. (*J* in Hz)][a]

Position	^{13}C[a]	^1H	HMBC (H→C)	NOESY (H⟷H)
1	197.1 s			
2	135.9 s			
3	160.5 s			
4	68.0 d	4.33 d (3.4)	2, 3, 5, 6	5, 8
5	43.4 d	1.74 m		4, 7, 8, 22
6	40.3 d	2.51 dq (11.2, 6.9)	1, 4, 5, 7, 22	7, 22
7	27.0 t	2.22 dd (7.4, 7.4)	4, 5, 6, 8, 9	5, 6, 8, 21, 22
8	121.0 d	5.14 t (7.4)	5, 7, 10, 21	4, 5, 7, 10
9	138.1 s			
10	39.8 t	2.03 m, 2.07 m	8, 9, 11, 12, 21	8, 11
11	26.4 t	2.03 m, 2.07 m	9, 10, 12, 13	10, 12, 20, 21
12	123.8 d	5.07 t (6.9)	10, 11, 14, 20	11, 14
13	135.4 s			
14	39.7 t	1.95 m, 2.03 m	12, 13, 15, 16, 20	12, 15
15	26.7 t	1.95 m, 2.03 m	13, 14, 16, 17	14, 16, 19, 20
16	124.3 d	5.07 t (6.9)	14, 15, 18, 19	15, 18
17	131.3 s			
18	25.7 q	1.65 s	16, 17, 19	16, 19
19	17.7 q	1.58 s	16, 17, 18	15, 18
20	16.0 q	1.58 s	12, 13, 14	11, 15
21	16.1 q	1.64 s	8, 9, 10	7, 11
22	12.3 q	1.15 d (6.9)	1, 5, 6	5, 6, 7
23	60.6 q	3.65 s	2	24
24	59.2 q	4.05 s	3	23

[a] Multiplicities were obtained from DEPT experiments.

Affiliation: [1] Graduate Institute of Pharmacognosy, Taipei Medical University, Taipei, Taiwan, R.O.C. · [2] Golden Biotechnology Corporation, Taipei, Taiwan, R.O.C.

Correspondence: Dr. Wu-Che Wen · 5 F. No.27–6 Sec.2 Chung Cheng East Rd · Danshuei Town · Golden Biotechnology Corporation · Taipei · Taiwan 251 · Republic of China · Phone: +886-2-2808-5538 · Fax: +886-2-7703-0103 · E-mail: wwc@goldenbiotech.com.tw

Received June 6, 2007 · **Revised** August 28, 2007 · **Accepted** September 5, 2007

Bibliography: Planta Med 2007; 73: 1–3 © Georg Thieme Verlag KG Stuttgart · New York · DOI 10.1055/s-2007-990232 · Published online ■ ■ · ISSN 0032-0943

NMR spectrum exhibited signals for four primary methyl groups [δ_H = 1.58 (3H×2, s, H_3-19, -20), 1.64 (3H, s, H_3-21), 1.65 (3H, s, H_3-18)], five methylene groups [δ_H = 1.95, 2.03 (2H×2, m, H_2-14, -15), 2.03, 2.07 (2H×2, m, H_2-10, -11), 2.22 (2H, dd, J = 7.4, 7.4 Hz, H_2-7)], two methine protons [δ_H = 1.74 (1H, m, H-5), 2.51 (1H, dq, J = 11.2, 6.9 Hz, H-6)], two methoxy groups [δ_H = 3.65 and 4.05 (3H×2, s, H_3-23, -24)], one carbinoyl proton [δ_H = 4.33 (1H, d, J = 3.4 Hz, H-4), and three olefinic protons [δ_H = 5.07 (2H, t, J = 6.9 Hz, H-12, -16), 5.14 (1H, t, J = 7.3 Hz, H-8)] (Table **1**). On account of the molecular formula $C_{24}H_{38}O_4$, the degree of unsaturation of **1** was six including one carbonyl and four olefinic functionalities. Thus, there should be one ring in **1**. Analysis of COSY and HSQC spectral data allowed the assignments of three spin systems including an aliphatic chain [(H_3-22)-(H-6)-(H-5)-(H_2-7)-(H-8)-], a hydroxy-bearing branch {-(H-5)-[H(OH)-4]-} and two three-resonance units [-(H_2-10)-(H_2-11)-(H-12)-] and -(H_2-14)-(H_2-15)-(H-16)-]. In the HMBC spectrum, key long-range proton-carbon correlations including H_3-23 (δ_H = 3.65)/C-2 (δ_C = 135.9), H_3-24 (δ_H = 4.05)/C-3 (δ_C = 160.5), H-4 (δ_H = 4.33)/C-2 (δ_C = 135.9), C-3 (δ_C = 160.5), H_2-7 (δ_H = 2.22)/C-5 (δ_C = 43.4), C-6 (δ_C = 40.3), H_3-22 (δ_H = 1,15)/C-1 (δ_C = 197.1), C-5 (δ_C = 43.4), C-6 (δ_C = 40.3), H_3-21 (δ_H = 1.64)/C-8 (δ_C = 121.0), C-9 (δ_C = 138.1), C-10 (δ_C = 39.8), H_3-20 (δ_H = 1.58)/C-12 (δ_C = 123.8), C-13 (δ_C = 135.4), C-14 (δ_C = 39.7) and H_3-18 (δ_H = 1.65), H-19 (δ_H = 1.58)/C-16 (δ_C = 124.3), C-17 (δ_C = 131.3) established the connectivities of the fragments assigned from above data. All these data suggested that **1** adopted an ubiquinone Q-3 skeleton except that its Δ^5 was reduced, and a carbonyl group at C-4 was replaced by a hydroxy group [9]. In the NOESY spectrum of **1**, some mutual correlations were listed as follows: H_2-7/H_3-21, H_3-21/H_2-11, H_2-11/H_3-20 and H_3-20/H_2-15, which indicated that the configurations of both Δ^8 and Δ^{12} were of the *E* form. The relative configuration of the remaining ring structure was deduced by the ^{1}H-NMR of **1**, especially for the J values of H-4 and H-6. Accordingly, H-4, H-5 and H-6 should be quasi-equatorial, quasi-axial and quasi-axial oriented, respectively, to fit the coupling constants of H-4/H-5 (J = 3.4 Hz) and H-5/H-6 (J = 11.2 Hz). Further evidence of the relative configuration was distinguishing features in the NOESY spectrum including mutual correlations of H_3-22/H-5, H_2-7/H-6, H-4/H-5, and a lack of correlation between H-5 and H-6, as shown in Fig. **1**. All these findings confirmed that **1** adopted a half-chair conformation, as demonstrated by molecular modeling. Thus, **1** was characterized as the shown structure, and named antroquinonol.

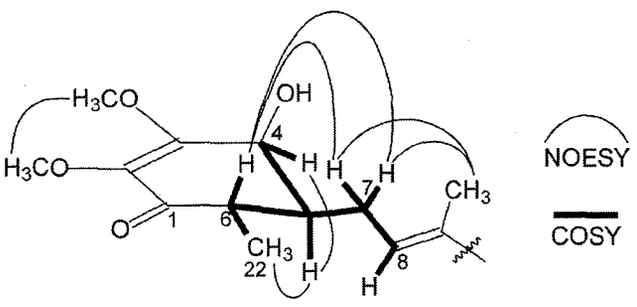


Fig. 1 Half-chair form conformation for **1**, which accommodates the observed key NOESY (arc) and ^{1}H-^{1}H COSY (bold).

Compound **1** was further evaluated for its cytotoxicity against MCF-7, MDA-MB-231 (human breast carcinoma), Hep3B, HepG2 (human liver carcinoma) and DU-145 and LNCaP (human prostate carcinoma) cell lines. The cell viabilities were assessed with the MTT assay [10]. As shown in Table **2**, **1** with IC_{50} values ranging from 2.19 ± 0.04 to 6.09 ± 0.07 μM **1** showed only moderate toxicities toward MCF-7, MDA-MB-231, HepG2, DU-145 and LNCaP cancer cells when compared with relevant clinical chemotherarpeutic drugs, while with an IC_{50} value of 0.13 ± 0.02 μM against Hep3B it seemed to be outstanding when compared with the corresponding positive control, lovastatin, with an IC_{50} value of 20 ± 2 μM.

Materials and Methods

General experimental procedures: Optical rotations were measured on a JASCO DIP-1000 digital polarimeter (Kyoto, Japan). ^{1}H- and ^{13}C-NMR were acquired on a Bruker DMX-500 SB spectrometer (Ettlingen, Germany). Mass spectra were obtained using a Finnigan Thermo Quest MAT 95XL spectrometer (Bremen, Germany). IR spectra were recorded on a Perkin Elemer Spectrum one spectrometer (Waltham, MA, USA). UV spectra were measured on a Thermo Heλios α spectrophotometer (Waltham, MA, USA). Column chromatography was carried out with silica gel (Merck; Darmstadt, Germany) and Sephadex LH-20 gel (Amersham Biosciences; Uppsala, Sweden). Pre-coated silica gel plates (Si 60 F_{254}, 0.2 mm, Merck) were used for analytical TLC.

Fermentation of Antrodia camphorata: *A. camphorata* (strain No. 438bp), isolated and identified by Prof. Dr. Shean-Shong Tzean in National Taiwan University, was inoculated into 1-L Erlenmeyer flasks containing 0.1 g NaCl, 10.0 g peptone (Merck), 2.0 g yeast extract (Merck), 10.0 g agar (Merck), 10.0 g grain (a mixture of rice, corn and glutinous rice), and deionized water was added to a total volume of 1000 mL. The medium was adjusted to pH 6.5–7.5 by using 1 N NaOH and 1 N HCl. The fermentation was conducted under sterile conditions at 25–30 °C for 12–14 weeks.

Extraction and isolation: The mycelium together with the medium from the above fermentation were dried and ground into powder (500 g), and extracted three times with 2500 mL *n*-hexane by stirring (4800 rpm) at room temperature for 6 h. The crude extract was then filtered and concentrated in vacuum to dryness (45 g). Subsequently, this residue was re-dissolved in 25 mL of *n*-hexane, and applied onto a silica gel gravity column (230–400 mesh, 5×45 cm) which was eluted sequentially with mixtures of *n*-hexane and EtOAc [10:1 (v/v) to 10:4 (v/v)] in a stepwise gradient mode. Fractions of 20 mL eluent were collected and each was analyzed by thin layer chromatography using a solution of *n*-hexane/EtOAc [10:4 (v/v)] for development. UV 254 nm illumination to exhibit yellow fluorescence was used to group the compounds with similar skeletons. The fractions eluted by *n*-hexane and EtOAc [10:3 (v/v) to 10:4 (v/v)] were combined and evaporated under reduced pressure to yield 5 g of the antroquinonol mixture. The antroquinonol mixture was further purified by a open column of Sephadex LH-20 (5×70 cm) with 95% ethanol as eluent to give pure antroquinonol (**1**) (25 mg).

Antroquinonol (**1**): Light yellow oil; $[\alpha]_D^{18}$: +72.7 (*c* 0.28, $CHCl_3$); UV (MeOH): λ_{max} (log ε) = 267 (4.0) nm; IR (KBr): λ_{max} = 3443

Table 2 IC_{50} values of compound **1** against six human cancer cell lines

Compounds	IC_{50} (μM)[a]					
	MCF-7[b]	MDA-MB-231[b]	Hep3B[c]	HepG2[c]	DU-145[d]	LANCaP[d]
1	2.19 ± 0.04	2.64 ± 0.05	0.13 ± 0.02	4.30 ± 0.03	4.64 ± 0.06	6.09 ± 0.07
Taxol[e]	0.0020 ± 0.0001	0.10 ± 0.02	–	–	0.010 ± 0.001	0.0020 ± 0.0002
Lovastatin[e]	–	–	20 ± 2	–	–	–
Acacetin[e]	–	–	–	0.070 ± 0.003	–	–

[a] Cells were treated with various concentrations of test compounds for 3 days. Cell growth was determined by MTT assay. The IC_{50} value resulting form 50% inhibition of cell growth was calculated. Each value represents the mean of three independent experiments.

[b] MCF-7, MDA-MB-231 as human breast carcinoma cell lines.

[c] Hep3B, HepG2 as human liver carcinoma cell lines.

[d] DU-145, LANCaP as human prostate carcinoma cell lines.

[e] Taxol, lovastatin and acacetin, chemotherarpeutic drugs, were used as reference compounds in this study.

(–OH), 2927, 1668 (C = O), 1650, 1621, 1454, 1355, 1239, 1141, 1016 cm^{-1}; ^{1}H-NMR data: see Table **1**; ^{13}C-NMR data: see Table **1**; FAB-MS (NBA): m/z = 391 $[M + H]^+$; HR-FAB-MS (NBA): m/z = 413.2666 $[M + Na]^+$; calcd. for $[C_{24}H_{38}O_6 + Na^+]$: 413.2668.

Cytotoxicity assay: Human breast carcinoma cell lines MCF-7 (BCRC-60436; Bioresource Collection and Research Center, Food Industry Research and Development Institute; Hsinchu, Taiwan) and MDA-MB-231 (CCRC-60425; The NHRI Cell Bank, National Health Research Institute; Miaoli County, Taiwan) were maintained in minimum essential medium (MEM; Invitrogen-Gibco; Carlsbad, CA, USA) and Dulbecco's modified Eagle's medium (DMEM), respectively. Both human liver carcinoma cell lines Hep3B (BCRC-60434; Bioresource Collection and Research Center, Food Industry Research and Development Institute; Hsinchu, Taiwan) and HepG2 (BCRC-60025, Bioresource Collection and Research Center, Food Industry Research and Development Institute) were maintained in MEM. Human prostate carcinoma cell lines DU-145 (CCRC-60348, The NHRI Cell Bank, National Health Research Institute) and LNCaP (CCRC-60088, The NHRI Cell Bank, National Health Research Institute) were maintained in MEM and Roswell Park Memorial Institute-1640 medium (RPMI-1640; Invitrogen-Gibco), respectively. Each medium was supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin. Cell growth in the presence or absence of experimental agents was determined using the MTT-microculture tetrazolium assay [10]. Briefly, 100 μL of cell suspension in logarithmic growth phase were seeded into 96-well plateS. After 24 h, the cells were exposed to various concentrations of the test compound in a volume of 50 μL for 72 h. Two hours prior to the end of incubation, 15 μL MTT solution (5 mg/mL) was added into the culture medium. Cells were lysed with 75 μL of MTT lysis buffer (20% SDS-50% DMF, Merck) and cell lysis solution were incubated at 37 °C for another 12 h to dissolve the dark blue crystals. The absorption of formazan solution at 570 nm was measured using a microplate reader. Taxol (Sigma; Seelze, Germany; purity ≥ 97%), lovastatin (Merck; purity ≥ 97.7%) and acacetin (Fluka; Buchs, Switzerland; purity ≥ 97%), the chemotherarpeutic drugs, were used as positive controls in this study.

Supporting information

^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, NOESY, HSQC, and HMBC NMR spectra for **1** are available as Supporting Information.

Acknowledgements

This research was supported by the grant from National Science Council, Taipei, Taiwan. We are grateful to Ms. Shwu-Huey Wang and Ms. Shou-Ling Huang for the NMR data acquisition in the Instrumentation Center of Taipei Medical University and Instrumentation Center of the College of Science, National Taiwan University, respectively.

References

[1] Wu SH, Ryvarden L, Chang TT. *Antrodia camphorata* ("niu-chang-chih"), new combination of a medicinal fungus in Taiwan. Bot Bull Acad Sin 1997; 38: 273–5.

[2] Chen CC, Shiao YJ, Lin RD, Shao YY, Lai MN, Lin CC et al. Neuroprotective diterpenes from the fruiting body of *Antrodia camphorata*. 2006; 69: 689–91.

[3] Shen YC, Chou CJ, Wang YH, Chen CF, Chou YC, Lu MK. Anti-inflammatory activity of the extracts from mycelia of *Antrodia camphorata* cultured with water-soluble fractions from five different *Cinnamomum* species. FEMS Microbiol Lett 2004; 231: 137–43.

[4] Wang GJ, Tseng HW, Chou CJ, Tsai TH, Chen CT, Lu MK. The vasorelaxation of *Antrodia camphorata* mycelia: involvement of endothelial Ca(2+)-NO-cGMP pathway. 2003; 73: 2769–83.

[5] Liu JJ, Huang TS, Hsu ML, Chen CC, Lin WS, Lu FJ et al. Antitumor effects of the partially purified polysaccharides from *Antrodia camphorata* and the mechanism of its action. Toxicol Appl Pharmacol 2004; 201: 186–93.

[6] Hseu YC, Chang WC, Hseu YT, Lee CY, Yech YJ, Chen PC et al. Protection of oxidative damage by aqueous extract from *Antrodia camphorata* mycelia in normal human erythrocytes. Life Sci 2002; 71: 469–82.

[7] Lee IH, Huang RL, Chen CT, Chen HC, Hsu WC, Lu MK. *Antrodia camphorata* polysaccharides exhibit anti-hepatitis B virus effects. FEMS Microbiol Lett 2002; 209: 63–7.

[8] Nakamura N, Hirakawa A, Gao JJ, Kakuda H, Shiro M, Komatsu Y et al. Five new maleic and succinic acid derivatives from the mycelium of *Antrodia camphorata* and their cytotoxic effects on LLC tumor cell line. J Nat Prod 2004; 67: 46–8.

[9] Joela H, Kasa S, Lehtovuori P, Bech M. EPR, ENDOR and TRIPLE resonance and MO studies on ubiquinones (Q-n): comparison of radical anions and cations of coenzymes Q-10 and Q-6 with the model compounds Q-2 and Q-0. Acta Chem Scand 1997; 51: 233–41.

[10] Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 1989; 119: 203–10.