ANTI-HBV PRINCIPLE FROM THE CULTURE BROTH OF *ANTRODIA CAMPHOROTA* (STRAIN # CCRC-35396)

Chien-Chang Shen[1], Hui-Chuan Yang[1], Ray-Ling Huang[1], Jinn-Chu Chen[2] and Chien-Chih Chen[1]

[1]National Research Institute of Chinese Medicine
Taipei, Taiwan
[2]Biotechnology Center, Grape King Inc.
Taoyuan, Taiwan
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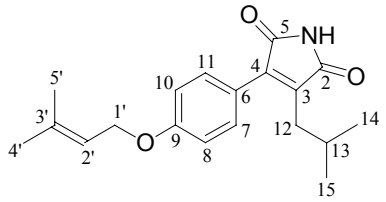
Bioassay-guided fractionation resulted in the isolation of an anti-HBV pyrroledione, 3-isobutyl-4-{4-[(3-methyl-2-butenyl)oxy]phenyl}-1*H*-pyrrole-2,5-dione (**1**), from the culture broth of fungus *Antrodia camphorota* (strain CCRC-35396). The structure of compound **1** was determined by spectral analyses. Compound **1** suppressed both HBsAg and HBeAg expression with the moderate inhibition percentages of 35.2 and 12.8%, respectively, at the non-cytotoxic concentration of 50 μM.

Key words: *Antrodia camphorota*, Pyrroledione, Anti-HBV.

INTRODUCTION

The fruiting bodies of *Antrodia camphorota* Chang & Chou, sp. nov. have been used in the treatment of diarrhea, abdominal pain, hypertension, itchy skin and liver cancer and as detoxicant in Taiwanese folk medicine[1]. Previous phytochemical investigations of the fruiting bodies of *A. cinnamonea* resulted in the isolation of a series of triterpene acids[2-4]. Recently, Nakamura et al. reported five new maleic and succinic acid derivatives from the mycelium of *A. camphorota*[5]. In the course of our preliminary microbial screening program, we found that the culture broth of the strain CCRC 35396 of *A. camphorota* exhibited anti-HBV activity. Using the bioassay-guided fractionation, an anti-HBV pyrroledione, 3-isobutyl-4-{4-[(3-methyl-2-butenyl)oxy]phenyl}-1*H*-pyrrole-2,5-dione (**1**)[5], was isolated from the culture broth of this fungus.



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Correspondence to: Chien-Chih Chen, National Research Institute of Chinese Medicine, Tel: 02-28201999 ext. 6701, Fax: 02-28264276, E-mail: ccchen@nricm.edu.tw

MATERIALS AND METHODS

General Experimental Procedures

The melting point was determined on a Yanaco MP-I3 micro melting point apparatus and was uncorrected. The IR spectrum was recorded on a Nicolet Avatar 320 FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were taken on a Varian Unity INOVA 500 spectrometer. The UV spectrum was recorded on a Hitachi U-3200 spectrophotometer. The MS spectrum was obtained on a Finnigan GCQ spectrometer.

Source of Organism

The strain of the fungus *A. camphorota* (# CCRC-35396) was purchased from Food Industry Research and Development Institute, Hsinchu, Taiwan, Republic of China.

Fermentation of Organism

The strain CCRC-35396 was inoculated into 1 L of the medium (1.0% glucose, 0.5% soybean powder, 0.5% peptone, 0.01% $MgSO_4$, 0.01% KM-72 antifoam, pH 4.0) in a 2-L Hinton flask at 28 °C on a rotary shaker for ten days. The mycelium was aseptically transferred to a 200-L fermenter containing 120 L of the above medium and was incubated at 28 °C for twelve days.

Extraction and Isolation

The cultures were concentrated under vacuum to give a residue. The residue (1 Kg) was extracted with acetone and the acetone extract was chromatographed on a silica gel column using a gradient elution of n-hexane/EtOAc to afford four fractions. The second fraction (n-hexane/EtOAc = 5/1 eluate) was rechromatographed on a silica gel column and eluted with n-hexane/EtOAc (10:1) to give ten fractions. The third and fourth fractions were combined and were chromatographed on a Sephadex LH-20 column (MeOH) to give **1** (15 mg).

Compound 1

Yellow needles, mp. 90-91 °C; UV (MeOH) λ_{max} nm (log ε): 230 (4.34), 355 (3.70); IR (KBr) 3230, 2928, 2872, 1764, 1705, 1601, 1509, 1354, 1248, 1173, 982 cm^{-1}; ^{1}H and ^{13}C NMR, in Table 1; CIMS *m/z* 331 $[M+NH_4]^+$, 263, 145.

Anti-hepatitis B Virus

Antiviral analyses were performed as previously described[6,7]. Briefly, the HBV-producing cell line MS-G2 was plated onto 24-well flat-bottomed tissue culture plates at a density of 3×10^5 cells/mL-well. Each cell was properly

Table 1. ^{13}C (126 MHz) and ^{1}H (500 MHz) NMR Spectral Data of Compound 1 in $CDCl_3$[a]

position	δ_C	DEPT	δ_H [mult., *J* (Hz)]	HMBC
2	171.80	C		
3	139.14	C		H-12, H-13
4	138.73[b]	C		H-7, H-11, H-12
5	171.15	C		
6	121.13	C		H-8, H-10
7	130.92	CH	7.49 (d, 8.5)	
8	114.82	CH	6.97 (d, 8.5)	H-7
9	160.02	C		H-7, H-8, H-10, H-11, H-1′
10	114.82	CH	6.97 (d, 8.5)	H-11
11	130.92	CH	7.49 (d, 8.5)	
12	32.78	CH_2	2.49 (d, 7.5)	H-13, H-14, H-15
13	28.07	CH	2.03 (m)	H-12, H-14, H-15
14	22.70	CH_3	0.88 (d, 7.0)	H-12, H-13
15	22.70	CH_3	0.88 (d, 7.0)	H-12, H-13
1′	64.86	CH_2	4.57 (d, 6.5)	
2′	119.19	CH	5.48 (t, 6.5)	H-1′, H-4′, H-5′
3′	138.71[b]	C		H-1′, H-4′, H-5′
4′	25.81	CH_3	1.79 (s)	H-2′, H-5′
5′	18.20	CH_3	1.74 (s)	H-2′, H-4′

[a] Assignments were based on DEPT, COSY, HMQC, and HMBC experiments.
[b] Assignments may be interchangeable.

attached after overnight; the cells were challenged by the test samples. DMSO was added to each culture as solvent control. All test samples were dissolved in DMSO at concentrations of 5, 25, and 50 μM, respectively. The concentration of DMSO in the media was maintained at no more than 2.5 μL/mL to ensure that it did not affect the growth of the MS-G2 cells. Subsequently, the culture media were collected on day 3 for each anti-viral assay. The antiviral activities, analyzed by an Enzyme-Linked Immunosorbent Assay (ELISA) (EverNew, Co., Taipei, Taiwan), were determined by the changes in HBsAg and HBeAg levels in the presence or absence of the test sample. The results were recorded at wavelength 492 nm with a DYNATECH MR7000 enzyme-linked immunosorbent assay reader (Guernsey, Channel Islands, United Kingdom). The percentage inhibition (%) was calculated by comparing with the DMSO solvent control group, i.e., % inhibition = [1 – OD (492 nm) of sample well/OD (492 nm) of DMSO well] × 100.

RESULTS AND DISCUSSION

Compound **1** was obtained as yellow needles from MeOH. The CIMS spectrum with ammonia as the reagent gas showed $[M+NH_4]^+$ ion at *m/z* 331 ($C_{19}H_{23}NO_3 + NH_4$). Its IR spectrum exhibited absorption bands for a N-H or O-H group at 3230 cm^{-1} and carbonyl groups at 1764 and 1705 cm^{-1}. The ^{1}H NMR spectrum (Table 1) showed two

ortho-coupled (J = 8.5 Hz) doublets at δ_H 7.49 and 6.97 (each 2H) in the aromatic region, indicated that compound **1** possessed a 1,4-disubstituted benzene ring. The signals at δ_H 5.48 (1H, t, J = 6.5 Hz), 4.57 (2H, d, J = 6.5 Hz), 1.79 (3H, s), and 1.74 (3H, s) suggested the presence of a prenyloxy moiety. Besides, the presence of an isobutyl group was indicated by the signals at δ_H 2.49 (2H, d, J = 7.5 Hz), 2.03 (1H, m), and 0.88 (6H, d, J = 7.0 Hz). The ^{13}C NMR and DEPT spectra (Table 1) showed four methyl, two methylene, six methine, and seven quaternary carbons. Among these quaternary carbons, the signals at δ_C 171.80 and 171.15 were assigned to two carbonyl groups and the signals at δ_C 139.14 and 138.73 (or 138.71) were attributed to substituted double bonded carbons. In the HMBC spectrum, cross peaks of H-12 with C-2, C-3, and C-4, and of H-7 and H-11 with C-4 indicated that the isobutyl group and the aromatic ring were attached to the conjugated double bond. The carbonyl signal at δ 171.15 was very close to that of C-2, which suggested that C-5 was also in conjugation with double bond. Furthermore, the cross peak observed between H-1′ and C-9 revealed that the prenyloxy group was linked to the aromatic ring. Thus, the structure of compound **1** was established as 3-isobutyl-4-{4-[(3-methyl-2-butenyl)oxy]phenyl}-1*H*-pyrrole-2,5-dione[5].

The antiviral activity of compound **1** was evaluated in MS-G2 cells against hepatitis B virus *in vitro*. It suppressed effectively both HBsAg and HBeAg expression with the inhibition percentages of 76.5 and 58.2% at the non-cytotoxic concentration of 100 μM and of 35.2 and 12.8% at 50 μM, respectively.

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樟芝（*Antrodia camphorota*, strain CCRC-35396）培養液中具抗 B 型肝炎病毒活性成分

沈建昌[1]　楊惠娟[1]　黃瑞齡[1]　陳勁初[2]　陳建志[1]

[1]國立中國醫藥研究所

台北，台灣

[2]葡萄王股份有限公司，生技中心

桃園，台灣

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利用活性追蹤的分離，自樟芝（*Antrodia camphorota*, strain CCRC-35396）的培養液中得到一個具抗 B 型肝炎病毒活性之 pyrroledione 類化合物，利用光譜分析確認其結構為 3-isobutyl-4-{4-[(3-methyl-2-butenyl)oxy]phenyl}-2,5-dihydro-1*H*-2,5-pyrroledione **(1)**。化合物 **1** 在 50 μM 時不產生細胞毒活性，但對 B 型肝炎病毒之 HBsAg 及 HBeAg 的表現分別有 35.2 及 12.8% 的抑制作用。

關鍵詞：樟芝，pyrroledione，抗 B 型肝炎病毒。

聯絡人：陳建志，國立中國醫藥研究所，電話：02-28201999 ext. 6701，傳眞：02-28264276，E-mail：ccchen@nricm.edu.tw