Two New Components of the Aspochalasins Produced by *Aspergillus* sp.

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Aspergillus sp. FO-4282 was found to produce two new components of the aspochalasins. Their structures were determined by spectroscopic analyses.

In the course of our screening program for new antimalarial antibiotics, strain FO-4282, a fungal soil isolate and identified as *Aspergillus* sp., was found to produce two new components of the aspochalasins. They were characterized by the presence of a 17,18-epoxy moiety in the structures and were given name aspochalasins F (**1**) and G (**2**). In this paper we describe the taxonomy of the producing strain and the fermentation, isolation, structure elucidation and biological activity of **1** and **2**.

Materials and Methods

Microorganisms

Strain FO-4282, the aspochalasin-producing culture, was isolated from a soil sample. It was grown at 27°C on YpSs agar slants, and preserved under oil sealing.

Unidentified soil bacterium strain L, a soil bacterium newly isolated in our laboratory, was used as an indicator of potential antimalarial activity. This strain was selected because it was tolerant to hemin, and was sensitive to artemisinin only in the presence of hemin. It was grown on nutrient agar, and stored at room temperature for laboratory use. It was also preserved under lyophilization. The taxonomy and classification of the bacterium is now underway.

Fermentation

Spores and mycelia on an agar slant of strain FO-4282 were scraped off to inoculate into a test tube (i.d. 2 × 20 cm) containing 10 ml of a seed medium composed of glucose 2.0%, yeast extract 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, Polypepton 0.5%, KH_2PO_4 0.1%, and agar 0.1% (in tap water, pH 6.0 adjusted with 6N NaOH). After incubation with reciprocal shaking (300 rpm) at 27°C for two days, 2 ml of this seed culture was transferred into flasks (500-ml volume) containing 100 ml of a second seed medium composed of soluble starch 4.0%, solvent extracted toasted soybean meal (Nikko Oil Mills Co. Tokyo, Japan) 2.0%, 1/10N sodium thiosulfate 32 μl/liter, $FeSO_4 \cdot 7H_2O$ 0.05%, K_2HPO_4 0.05%, and KCl 0.03% (in tap water, pH 6.5 adjusted with 6N NaOH), and then the flasks incubated with rotary shaking (210 rpm) at 27°C for two days. The second seed culture (1.2 liters thus obtained was transferred into two jar fermenters (70-liter volume) containing 30 liters of a production medium. The production medium was composed of soluble starch 3.0%, soybean flour 2.0%, glycerol 1.0%, dry yeast 0.3%, KCl 0.3%, $CaCO_3$ 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, allophane 0.5% and adecanol (antifoam, Asahi Denka, Tokyo) 0.01%, (in tap water, pH 6.5 before sterilization adjusted with 6N NaOH). Allophane is an aluminosilicic clay (Shinagawa Chemicals, Tokyo) capable of trapping inorganic phosphate. When added to a fermentation medium, it often increases antibiotic yields by decreasing phosphate contents in the culture[1]. The jar fermenters were run at 27°C for 68 hours with aeration (15 liters/minute) and agitation (250 rpm).

Detection of Antibiotic Activity

Antibiotic activity in fermentation broths and purification steps were estimated by the conventional paper disc assay using a newly isolated unidentified bacterium strain L as indicator. L-seeded nutrient agar (L), and nutrient agar supplemented with hemin (100 μg/ml) (LH), and LH plus tocopherol (400 μg/ml) (LHT), were incubated at 37°C for 24~40 hours. Artemisinin was

used as positive control. This method is based on the finding of MESHNICK *et al.* that the antimalarial activity of artemisinin involves heme-dependent radical generation[2]. Artemisinin showed no growth inhibition on L-agar, but provided a big inhibition zone in the presence of hemin (LH-agar). The inhibition zone became smaller and hazy upon supplementation with α-tocopherol, a radical scavenger (LHT- agar) (see also Table 3). It was assumed that the heme-dependent growth inhibition against bacterium L, or in other words, artemisinin-like growth inhibition pattern of +/−, ++, + on L-, LH-, and LHT-agars, respectively, was an indication that the culture had an activity of heme-dependent radical generation, just as artemisinin did, in the microbiological assay system employed. The culture of FO-4282 was thus picked up as candidate because it showed an artemisinin-like growth inhibition pattern on the three agar plates with the largest inhibition zone on LH.

Analytical Procedures

HPLC analyses were carried out using a Senshu HPLC system (model SSC-6530) equipped with a variable wave length UV detector (model SSC-6500), with Pegasil ODS (i.d. 20×250 mm) and CN-5251 (i.d. 20×250 mm) columns. An open column chromatography was performed using reverse phase silica gel 60 (Senshu Scientific, Tokyo) with mixtures of acetone and *n*-hexane as eluant (0:100~100:0). UV spectra were measured with a Shimadzu spectro-photometer, model UV-240. FAB-MS were recorded by a JEOL spectrometer, model JSM-A-505 HA. All NMR spectra were recorded on a JEOL JNM-EX270 spectrometer.

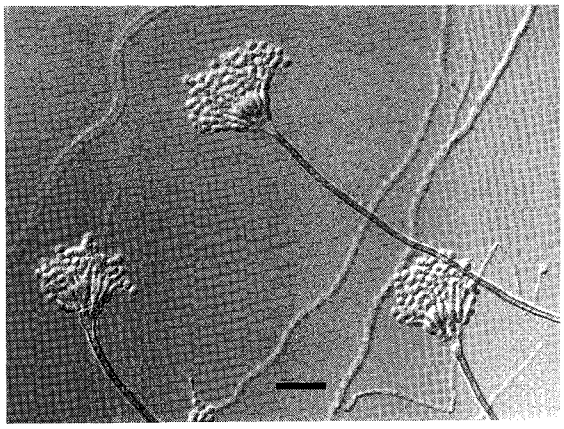
Results and Discussion

Taxonomy of the Producing Strain FO-4282

The fungal strain FO-4282 was isolated from a soil sample collected at the Arisugawa Park, Minato-ku, Tokyo. CYA (Czapek Yeast Extract Agar: yeast extract 0.5%, sucrose 3.0%, K_2HPO_4 0.1%, $NaNO_3$ 0.3%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, $CuSO_4 \cdot 5H_2O$ 0.0005%, and agar 1.5%), MEA (Malt Extract Agar: malt extract 2.0%, peptone 0.1%, glucose 2.0%, agar 2.0%), and CY20S (Czapek Yeast Extract Agar with 20% Sucrose) media were used for identification of the fungal strain FO-4282. Colonies on CYA were 35 to 37 mm in diameter after culturing at 25°C for 7 days. The surface of a colony was flat with a velvety texture and pale yellow. The reverse of colonies were pale yellowish brown. Colonies on CY20S were 39 to 41 mm in diameter after culturing at 25°C for 7 days. The surface of a colony was flat with a velvety texture and yellowish gray. The marginal area of a colony was floccose. The reverse of colonies were bamboo in color. No soluble pigment was produced in either medium. On CYA at 37°C, growth was nil. Morphological observation was carried out under a microscope (Olympus Vanox-S AH-2). When grown on CYA at 25°C for 7 days, the conidiophores were born from substrate hyphae, and smooth and colorless hyphae were 100 to 380 μm in length and 2.5 to 4.5 μm in width. A vehicle was produced at the top of the conidiophores and was spherical or subspherical and 6.3 to 10 μm in diameter. The upper three quarter of the vehicle beared flask-shaped phialides which are 7.5 to 10 μm in length and 2.5 to 3.8 μm in width as shown in Fig. 1. The conidial ontogy was enteroblastic. The phialidic conidia were globes to subglobes, 2.5 to 3.5 μm in diameter and yellowish gray in color. No tereomorphs were observed. From the above characteristics, strain FO-4282 was identified as the genus *Aspergillus*, and named *Aspergillus* sp. FO-4282.

Fig. 1. Photograph of conidiophores and conidia of *Aspergillus* sp. FO-4282 grown on CYA at 25°C for 7 days.

Bar represents 20 μm.



Fermentation and Isolation

A preliminary optimization of fermentation conditions indicated that the addition of allophane to the production medium resulted in an increased production of active substances by FO-4282. Therefore the fermentation was carried out in allophane-supplemented medium (see Materials and Methods). The effects of metal salts, which often affect fermentation yields in fungi, were not studied here. A typical time course of aspochalasins production

by *Aspergillus* sp. FO-4282 in a jar fermenter is shown in Fig. 2. The anti-LH activity detected by a paper disc method (see Materials and Methods) appeared after incubation for 24 hours. The fermentation was stopped after incubation for 68 hours and the cultured broth (55 liters) was used for isolation of active compounds.

The isolation procedures for **1** and **2** from the FO-4282 fermentation broth are outlined in Fig. 3. Fifty-five liters of the broth was centrifuged and separated into a supernatant fluid (45 liters) and a mycelial cake (10 liters). The mycelial cake was extracted with acetone and concentrated *in vacuo*. The mycelial extract and culture filtrate were extracted with ethyl acetate, and concentrated *in vacuo*. The resulting yellow resinous residues were combined and extracted with *n*-hexane to obtain a crude material (67.5 g). It was applied on a column of silica gel 60 (2.6 liters) and eluted stepwise with *n*-hexane-acetone. The active fractions eluted with *n*-

Fig. 2. A typical time course of fermentation by *Aspergillus* sp. FO-4282 in a jar fermenter.

Diameter of LH inhibition zone (mm), ●; pH, △; and packed cell volume (ml/10 ml of culture broth), ○; are shown.

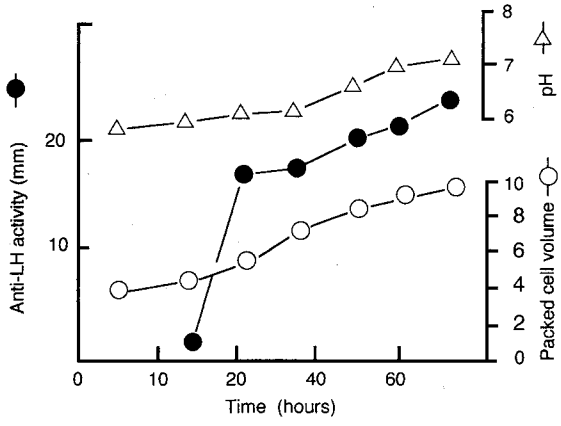
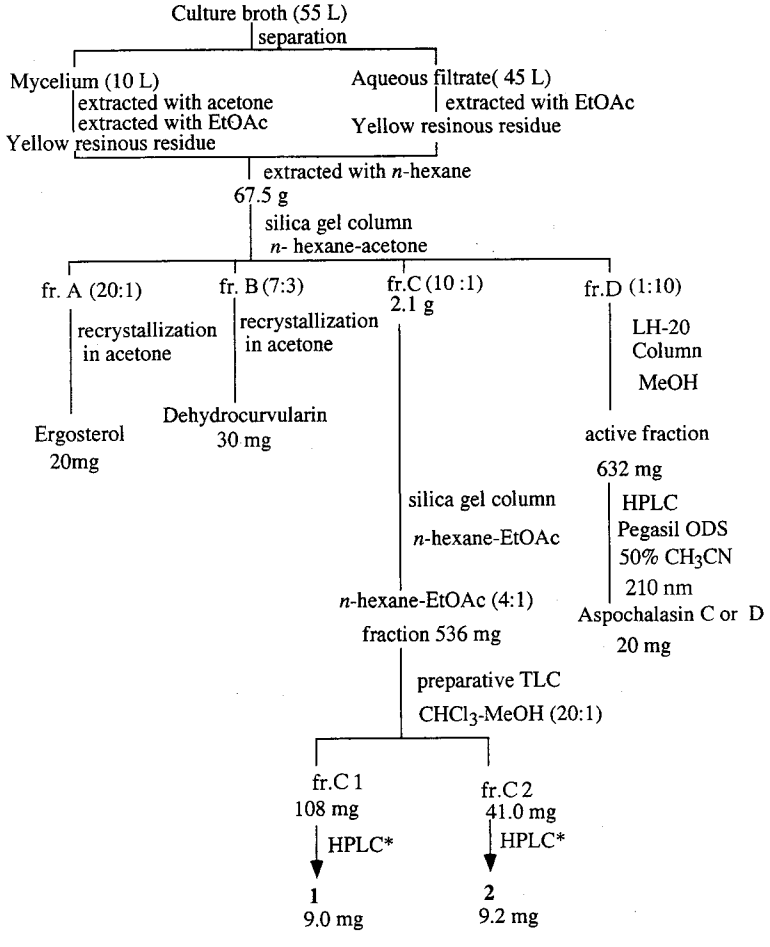


Fig. 3. Isolation procedures for aspochalasins F (**1**) and G (**2**).



*HPLC condition:
Mobile phase : *n*-hexane-EtOAc (4:1)
Column : CN-5251 20×250 mm
Flow rate :7 ml/min
Detection :260 nm

hexane-acetone (10:1) were concentrated *in vacuo* to afford a brown material (2.1 g). The crude material dissolved in a small amount of $CHCl_3$ was further purified by chromatography on a silica gel column (800 ml). Elution was conducted stepwise with *n*-hexane-ethyl acetate. Active fraction (536 mg) eluted by *n*-hexane-ethyl actate (4:1) was applied on preparative TLC, and developed with $CHCl_3$-methanol (20:1). The bands extracted with methanol-$CHCl_3$ (1:1) were further purified by HPLC (mobile phase, *n*-hexane-ethyl acetate (4:1); column, CN-5251 20×250 mm; flow rate, 7 ml/minute; detection at 260 nm), to give pure **1** (9.0 mg) and **2** (9.2 mg).

Meanwhile, we have also obtained aspochalasin C or D[3,4] (8 mg), ergosterol (20 mg), and dehydrocurvularin (30 mg)[5,6] as anti-LH compounds (Fig. 3).

Structure Elucidation

The physico-chemical properties of **1** and **2** are summarized in Table 1. The molecular formulae of **1** and **2** were revealed by HR-FAB-MS as $C_{24}H_{33}NO_4$ and $C_{24}H_{33}NO_3$, respectively. Chemical shifts in the ^1H and ^{13}C NMR of **1** and **2** are shown in Table 2. The ^1H-^{13}C COSY experiments indicated the connectivity of each

Table 1. Physico-chemical properties of aspochalasins F (**1**) and G (**2**).

		1	2
Appearance		Pale Yellow	Pale Yellow
$[\alpha]_D^{25}$ (EtOH)		9.8° (*c* 1.0)	−77.9° (*c* 1.0)
mp (°C)		96~97	95
Molecular formula		$C_{24}H_{33}NO_4$	$C_{24}H_{33}NO_3$
Molecular weight		399	383
HR FAB-MS (*m/z*):	Calcd	400.2488 $(M+H)^+$	384.2539 $(M+H)^+$
	Found	400.2480 $(M+H)^+$	384.2540 $(M+H)^+$
UV λ_{max}^{MeOH} (ε)		203 (10200)	205 (9800), 240 sh, 280 sh
IR ν_{max} (KBr) cm^{-1}		3413, 2957, 2935, 1780	3434, 2960, 2927, 1686, 1626
Solubility:	Soluble	$CHCl_3$, EtOH, MeOH	$CHCl_3$, EtOH, MeOH
	Insoluble	H_2O, EtOAc	H_2O, EtOAc

Table 2. ^1H (270 MHz) and ^{13}C (67.8 MHz) NMR data of aspochalasins F (**1**) and G (**2**) in $CDCl_3$.

Position	1		2	
	δ_C	δ_H	δ_C	δ_H
1	173.2		174.9	
2NH		6.38 br. s		5.95 br. s
3	51.9	3.13 t (3.3), 3.17 t (3.3)	50.8	3.19 dd (4.9, 9.9)
4	52.0	2.74 dd (3.3, 5.3)	49.4	2.97 dd (2.3, 4.9)
5	34.4	2.96 br. s	35.0	2.51 br. s
6	140.2		140.3	
7	124.0	5.32 br. s	125.4	5.39 br. s
8	40.7	3.68 br. d (9.6)	44.9	2.93 br. d (9.9)
9	88.1		66.1	
10	47.9	1.28 m	48.3	1.23 m
11	13.8	1.21 d (7.3)	13.4	1.24 d (7.2)
12	19.8	1.73 dd (1.0, 1.0)	19.8	1.76 s
13	123.6	6.20 br. d (8.9)	125.2	6.13 br. d (9.9)
14	139.7		137.0	
15	33.3	2.13 m	34.4	2.18 m, 2.31 m
16	25.3	2.28 m, 1.41 m	26.4	1.33 m
17	59.1	3.36 dd (4.3, 4.6)	59.3	3.22 dd (4.6, 9.5)
18	54.2	3.56 ddd (1.0, 4.6, 5.0)	56.4	3.63 dd (4.6, 7.7)
19	149.8	7.33 dd (5.0, 15.5)	141.3	6.19 dd (7.7, 16.4)
20	122.8	5.88 dd (1.0, 15.5)	134.1	7.42 d (16.4)
21	166.4		198.3	
23	25.1	1.64 m	25.0	1.55 m
24	23.6	0.93 d (6.2)	23.5	0.90 d (6.5)
25	21.3	0.92 d (6.6)	21.5	0.90 d (6.5)
26	16.0	1.52 d (1.3)	15.7	1.40 s

proton and carbon.

Compound **1** showed five methyl, three methylene, eleven methine, and five quaternary carbons in the DEPT spectra. Three partial structures of **1** were elucidated from the ^{1}H-^{1}H COSY experiment and their arrangement was analyzed by the HMBC experiment as shown in Fig. 4. The presence of epoxide at C-17/C-18 was suggested by their chemical shifts (δ 59.1 and 54.2, respectively), and was confirmed by their large $^1J_{C\text{-}H}$ values (172.0 and 173.5 Hz, respectively) in the *J*-resolved spectrum, as reported for other compounds[7]. Taking into account the molecular formula and the chemical shifts of C-9 (δ 88.1) and C-21 (δ 166.4), one remaining oxygen should be arranged between C-9 and C-21. Thus, C-8 and C-9 were connected. The chemical shifts of C-9 and C-21 were similar to those of C-9 and C-23 in cytochalasin B that corresponded to the ester moiety[8]. The coupling constant between 19-H and 20-H (J=15.5 Hz) indicated the configuration of 19*E*, and the chemical shift of C-26 (δ 16.0) revealed 13*E*-configuration as reported for other compounds[9]. So the structure of **1** was the same as that of aspochalasin C except for a 17,18-anhydro moiety and an ester bond between C-9 and C-21. Thus the structure of **1** was elucidated as (13*E*,19*E*)-10-isopropyl-14-methyl-22-oxa-17-epoxy[12]cytochalasa-6,13,19-triene-1,21-dione.

Physico-chemical properties of **2** were similar to those of **1** and HR-FAB-MS experiment indicated the lack of one oxygen atom from **1**. The chemical shifts of **2** were similar to those of **1** except for those of C-9 and C-21, in which **2** were similar to aspochalasin C. So the structure of **2** was suggested to be 17,18-anhydroaspochalasin C. The ^{1}H-^{1}H COSY and HMBC experiments also supported the structure (Fig. 5). Thus the structure of **2** was elucidated as (13*E*,19*E*)-10-isopropyl-14-methyl-17-epoxy[11]cytochalasa-6,13,19-triene-1,21-dione.

Compound **1** and **2** were named aspochalasins F and G, respectively, because aspochalasins A~E have been reported[4,10].

Biological Activity

Compound **1** showed no antimicrobial activity against bacteria, fungi and yeasts tested. Compound **2** showed moderate *in vitro* antifungal activity against *Pyricularia oryzae* and antibacterial activity (Table 3). Compounds **1** and **2** were weakly cytotoxic (Table 4). In its inhibition pattern on L, LH, and LHT, **2** was similar to artemisinin in showing the largest inhibition zone on LH-agar. It is likely that **2** is a heme-dependent radical generator at least as detected bacteriologically. This suggested the

Fig. 4. ^{1}H-^{1}H COSY and HMBC correlations of aspochalasin F (**1**).

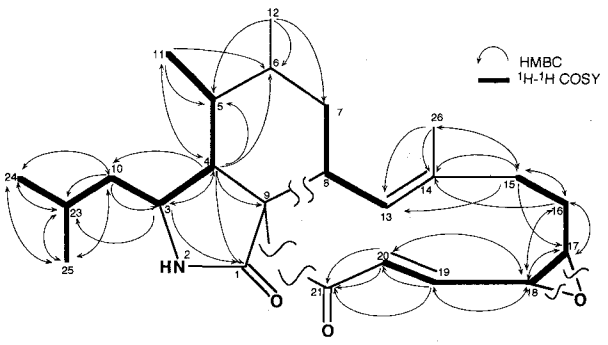


Fig. 5. ^{1}H-^{1}H COSY and HMBC correlations of aspochalasin G (**2**).

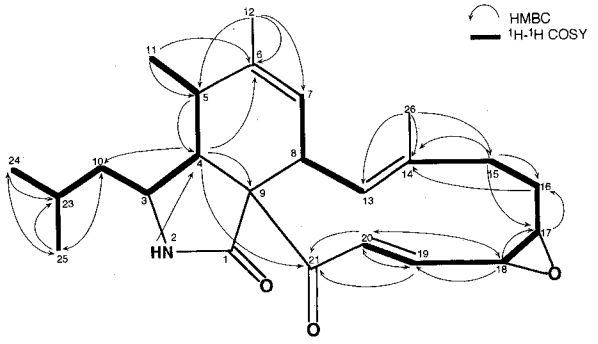
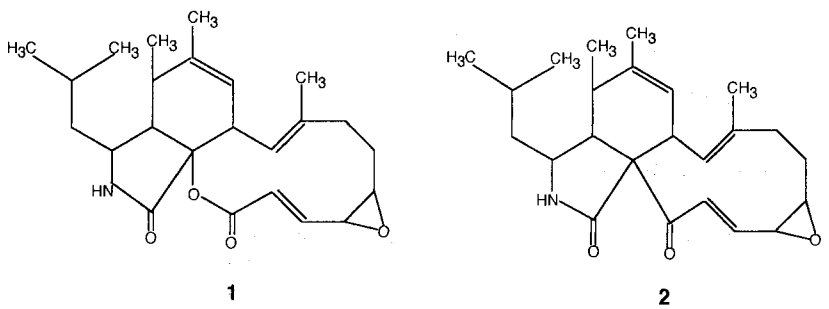


Fig. 6. Structures of aspochalasins F (**1**) and G (**2**).



1 **2**

Table 3. Antimicrobial spectra of aspochalasins F (**1**), G (**2**) and artemisinin.

Test micro-organism	Medium	Diameter of inhibition zone[a] (mm)		
		1	**2**	Artemisinin
Bacillus subtilis KB 27	A	—	19.5	
Bacillus subtilis KB 27	D	—	13.5	
Staphylococcus aureus KB 210	A	—	11.5	
Micrococcus luteus KB 40	A	—	15.0	
Mycobacterium smegmatis KB 42	A	—	—	
Escherichia coli KB 213	A	—	—	
Escherichia coli KB 176	A	—	—	
Pseudomonas aeruginosa KB 105	A	—	—	
Xanthomonas oryzae KB 88	C	—	—	
Bacteroides fragilis KB 169	B	—	—	
Acholeplasma laidlawii KB 174	K	—	15.0	
Pyricularia oryzae KF 180	C	—	14.0	
Aspergillus niger KF 103	C	—	—	
Mucor racemosus KF 223	C	—	—	
Candida albicans KF 1	C	—	—	
Saccharomyces cerevisiae KF 26	C	—	—	
Bacterium L	L	—	14	—
Bacterium L	LH	—	18	26
Bacterium L	LHT	—	13 (Hazy)	20 (Hazy)

[a] Paper discs (8 mm diameter) with 30 μg of aspochalasin F (**1**), G (**2**) and artemisinin.
A: Nutrient agar (Nissui, Tokyo). 37°C, 20 hours.
B: GAM agar (Nissui, Tokyo). Anaerobic chamber 37°C, 24 hours.
C: Potato-glucose agar. 27°C, 72 hours.
D: DAVIS' defined medium.
K: Mycoplasma medium (Kitasato, Tokyo).
L: L-seeded nutrient agar.
LH: L + hemin.
LHT: LH + Tocopherol.

Table 4. Cytotoxic activities of aspochalasins F (**1**) and G (**2**).

Cell line	IC_{50} (μg/ml)	
	1	**2**
Melanoma B16	>25	>25
KB/VP-2	>25	>25
KB/VJ-300	>25	>25
PC-3/CDP-5	>25	>25
CPAE	>25	>25
HL-60	25	25
MH-60	18	14

KB: Epidermoid carcinoma.
KB/VP-2: Etoposide-resistant KB cell line.
KB/VJ-300: Vincristine-resistant KB cell line.
PC-3/CDP-5: Human prostate/cisplatin-resistant cell line.
CPAE: Calf pulmonary arterial endotherial cells.
HL-60 Cell: Human leukemia cell.
MH-60 Cell: Mouse IL-6 dependent cell.

possibility that this compounds was an active antimalarial agent just as artemisinin, a heme-dependent radecal generator[2]. Very recently, however, **1** and **2** were shown to be inacive in an *in vitro* antimalarial assay, although other non-peroxide radical generators were potently active[11].

Acknowledgments

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