

## Antifungal Garcinia Acid Esters from the Fruits of *Garcinia atroviridis*

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*Garcinia atroviridis*, Fruits,  $\beta$ -Lactone

Two new garcinia acid derivatives, 2-(butoxycarbonylmethyl)-3-butoxycarbonyl-2-hydroxy-3-propanolide and 1',1''-dibutyl methyl hydroxycitrate, were isolated from the fruits of *Garcinia atroviridis* guided by TLC bioautography against the fungus *Cladosporium herbarum*. The structures of these compounds were established by spectral analysis. The former compound represents a unique  $\beta$ -lactone structure and the latter compound is most likely an artefact of garcinia acid (= hydroxycitric acid). Both compounds showed selective antifungal activity comparable to that of cycloheximide (MID: 0.5  $\mu$ g/spot) only against *C. herbarum* at the MIDs of 0.4 and 0.8  $\mu$ g/spot but were inactive against bacteria (*Bacillus subtilis*, methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*), other fungi (*Alternaria* sp., *Fusarium moniliforme* and *Aspergillus ochraceus*) including the yeast *Candida albicans*.

### Introduction

*Garcinia atroviridis* Griff. ex T. Anders (local name "asam gelugor"), an endemic species in Peninsular Malaysia, is a medium-sized fruit tree that is widely used for seasoning purposes. Its leaves are also locally known as a traditional vegetable ("ulam") that are either cooked or consumed raw as a salad. Ethno-botanical and -pharmacological reports have described the significant medicinal and preservative properties of *G. atroviridis* (Burkill, 1966; Fui, 1992; Grosvenor *et al.*, 1995a, b; Mackeen *et al.*, 2000). The authors have recently shown that extracts of *G. atroviridis* exhibit strong antimicrobial, antioxidant and antitumour-promoting activities (Mackeen *et al.*, 1997; Mackeen *et al.*, 2000). Until now, phytochemical investigations of *G. atroviridis* have afforded the isolation of garcinia acid (identical to (-)-hydroxycitric acid) and its  $\gamma$ -lactone, atroviridin, atroviridone and atrovirone (Lewis and Neelakantan, 1965; Kosin *et al.*, 1998; Permana *et al.*, 2001) as well as the identification of some organic acids, viz. citric,

pentadecanoic, octadecanoic, nonadecanoic and dodecanoic acids in its fruit by GC-MS (Abdullah, 1994).

Since the authors found that the butanol extract of its fruit strongly inhibited the fungus *Cladosporium herbarum* (Mackeen *et al.*, 2000), isolation of the active compounds guided by a convenient TLC bioautography assay was carried out. This fungus is ubiquitous and may be found in plants, on the surface and/or in decaying tissue. This paper deals with the isolation, structure and anti-*Cladosporium* activity of the active compounds.

### Results and Discussion

The MeOH extract (1.8 kg) of the dried fruits (4 kg) was partitioned into petroleum ether (26 g), chloroform (CHCl<sub>3</sub>, 23 g), ethyl acetate (EtOAc, 232 g), *n*-butanol (*n*-BuOH, 598 g) and H<sub>2</sub>O (207 g) soluble fractions. The minimum inhibitory dose (MID) values against *C. herbarum* of the MeOH and *n*-BuOH (most active fraction) ex-

tracts were 100 and 25  $\mu\text{g}/\text{spot}$ , respectively. An inhibitory spot against *C. herbarum*, and a yellow colour ninhydrin-active spot were detected on silica gel TLC at  $R_f$  0.75 (EtOAc-MeOH = 9:1). The *n*-BuOH extract (598 g) of the dried fruits was subjected to silica gel column chromatography. Purification guided by activity against *C. herbarum* and a characteristic yellow colour by ninhydrin-spraying on TLC afforded two new antifungal constituents, **1** (31 mg) and **2** (50 mg).

Compound **1** was obtained as white crystals and formulated as  $\text{C}_{15}\text{H}_{26}\text{O}_8$  from HRFAB-MS and CI-MS data. The IR spectrum showed the presence of hydroxyl ( $3518$  and  $3433\text{ cm}^{-1}$ ) and carbonyl ( $1751$  and  $1732\text{ cm}^{-1}$ ) groups. The  $^{13}\text{C}$ -NMR spectrum with DEPT displayed signals due to two methyls ( $\delta$  13.6, 13.6), one methoxyl ( $\delta$  53.4), five methylenes ( $\delta$  19.0, 19.0, 30.5, 30.5, 39.8), two oxymethylenes ( $\delta$  64.9 and 66.4), one oxymethine ( $\delta$  77.3), one oxygen-bearing quaternary carbon ( $\delta$  77.5) and three ester carbonyls ( $\delta$  170.0, 170.6 and 172.7). The  $^1\text{H}$ -NMR spectrum showed the presence of two hydroxyls ( $\delta$  3.29, 1H, d,  $J = 8.9$  Hz, secondary hydroxyl;  $\delta$  3.92, 1H, s) and of the methoxyl ( $\delta$  3.83, 3H, s). The  $^1\text{H}$ -NMR signals ( $\delta$  0.91, 6H, *m*;  $\delta$  1.37, 4H, *m*;  $\delta$  1.57, 1.67, 2H each, *m*;  $\delta$  4.06, 2H, *m*;  $\delta$  4.25, 2H overlapped with another H) together with the  $^1\text{H}$ - $^1\text{H}$  COSY and  $^1\text{H}$ - $^{13}\text{C}$  HETCOR crosspeaks indicated the presence of two butoxyl groups. The  $^1\text{H}$ - $^1\text{H}$  COSY experiment also revealed that the secondary hydroxyl proton ( $\delta$  3.29, 1H, *d*,  $J = 8.9$  Hz) was coupled with the overlapped proton at  $\delta$  4.25. The  $^1\text{H}$ - $^{13}\text{C}$  HETCOR experiment exhibited that the methylene carbon ( $\delta$  39.8) carried two protons ( $\delta$  2.99, 1H, d,  $J = 16.5$  Hz,  $\delta$  3.02, d, 1H,  $J = 16.5$  Hz) appearing as a pair of clear doublets of the AB type. This observation indicated that no hydrogen-bearing carbons were connected to this methylene. Thus the following partial structures were deduced: two butoxycarbonyl, one methoxycarbonyl, one secondary and one tertiary alcohol groups, and one methylene group not neighbouring any hydrogen-bearing carbons. The connectivity of these partial structures was established by HMBC correlations (Fig. 1) that only led to the structure 1',1''-dibutyl methyl hydroxycitrate (**1**). This compound would be an artefact resulting from garcinia acid during the extraction of plant material with methanol and butanol

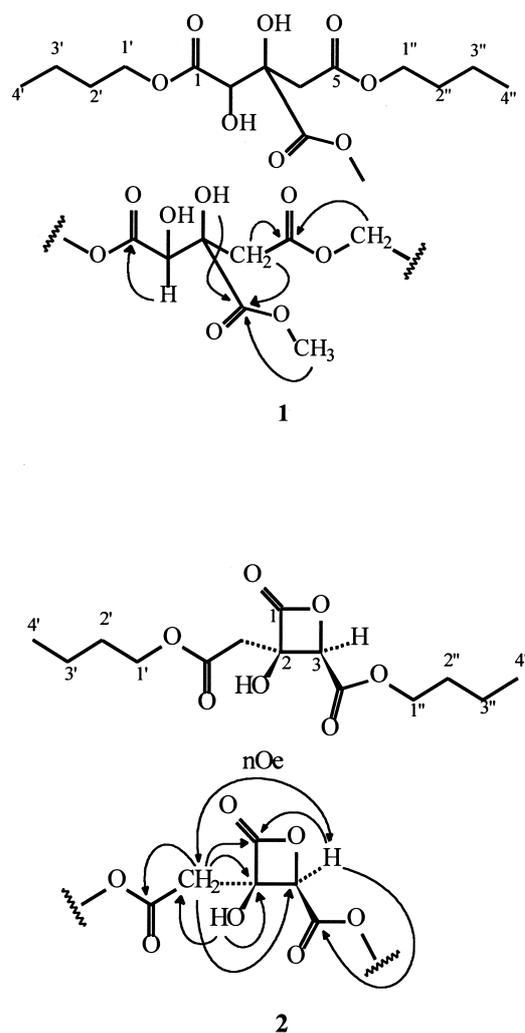


Fig. 1. Structures of **1** and **2** and their selected HMBC (arrow) correlations.

Compound **2** was obtained as a yellow oil and formulated as  $\text{C}_{14}\text{H}_{22}\text{O}_7$  based on its HRFAB-MS and CI-MS data. This formula was  $\text{CH}_4\text{O}$  less than that of compound **1**, suggesting that **2** lacked  $\text{CH}_3\text{OH}$ . A close similarity of the  $^{13}\text{C}$ -NMR of **2** to that of **1** except for the absence of a methoxyl carbon signal ( $\delta$  53.4) suggested that **2** was a dibutyl ester of a hydroxycitric acid lactone, which was proved to be a  $\beta$ -lactone from the carbonyl frequency ( $1807\text{ cm}^{-1}$ ) in the IR of **2**. The absence of the secondary hydroxyl proton signal in **2** and a downfield shift of the secondary carbinol carbon signal from **1** to **2** ( $\delta$  74.7  $\rightarrow$   $\delta$  84.2) suggested that

the secondary hydroxyl group was lactonized. This suggestion was confirmed by the observation that the secondary carbinol proton doublet ( $\delta$  4.25) of **1** changed to a singlet and shifted downfield ( $\delta$  4.85) in **2**. There was only one structure in which the secondary hydroxyl oxygen was involved in the  $\beta$ -lactone ring among the three possible  $\beta$ -lactones of hydroxycitric acid. Additionally, HMBC correlations were also consistent with proposed structure (Fig. 1). The NOESY experiment showed correlation between the signal at  $\delta$  2.77 (one of the methylene protons) and the methine proton signal at  $\delta$  4.89 to suggest a *cis* configuration (Fig. 1). Therefore, it was elucidated that **2** was 2-(butoxycarbonylmethyl)-3-butoxycarbonyl-2-hydroxy-3-propanolide.

The antimicrobial activity of compounds **1** and **2** was confined to the fungus, *C. herbarum*, with MID values of 0.4  $\mu$ g and 0.8  $\mu$ g/spot, respectively and was comparable to the antifungal standard, cycloheximide (MID: 0.5  $\mu$ g/spot). Both **1** and **2** failed to inhibit the growth of other fungi, viz. *Alternaria* sp., *F. moniliforme*, *A. ochraceous* and *C. albicans*. Interestingly, **1** and **2** were inactive ( $> 40$   $\mu$ g/spot) towards the panel of test bacteria comprising *B. subtilis*, methicillin-resistant *S. aureus*, *P. aeruginosa*, and *E. coli*. The failure of these compounds to exhibit antibacterial activity meant that the metabolites responsible for the initial antibacterial activity of the fruit extract had not been isolated (Mackeen *et al.*, 2000). Hence, TLC-bioautography based isolation had only yielded the anti-*Cladosporium* components of the fruit extract.

## Experimental

### General experimental procedures

$^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra were recorded in  $\text{CDCl}_3$  on a Varian VXR-500 instrument using TMS as the internal reference, and mass spectra were measured on a JEOL JMS-SX102A spectrometer. IR and UV spectra were obtained by Perkin-Elmer 1725X FT-IR and Shimadzu UV-3000 instruments, respectively. Specific rotation was measured with a Jasco DIP-360 polarimeter. Melting point (uncorrec.) was determined on a Kofhler hot-stage microscope. Silica gel was used for MPLC (Merck 15111), TLC (Merck 5554) and column chromatography (Merck 7734 unless specified otherwise).

### Plant material

The fruits of *G. atroviridis* were collected in Serdang, Selangor. A herbarium voucher specimen (MM-1) was prepared and deposited at the Herbarium of the Department of Biology, Universiti Putra Malaysia.

### Antimicrobial assays

The bacterial and fungal stock cultures were maintained on nutrient agar (NA) and potato dextrose agar (PDA) slants, respectively, as described before (Mackeen *et al.*, 2000). The microorganisms used for the antimicrobial assays were *Bacillus subtilis* (Gram-positive), methicillin-resistant *Staphylococcus aureus* (Gram-positive), *Escherichia coli* (Gram-negative), *Pseudomonas aeruginosa* (Gram-negative), *Candida albicans* (yeast) and *Aspergillus ochraceous* (fungus), and three phytopathogenic fungi, i.e. *Cladosporium herbarum*, *Fusarium moniliforme* and *Alternaria* sp.

### Disc diffusion method

An even spread of microorganisms was prepared by mixing 200  $\mu$ l of inoculum (adjusted to  $10^5$ - $10^6$  CFU/ml) with 20 ml of agar at 45  $^\circ\text{C}$  and allowed to set (NA for bacteria and PDA for fungi) in a petri dish ( $\varnothing$  90 mm is most likely an artefact of garcinia acid). Compounds **1** and **2** were loaded onto each Whatman No.1 filter paper discs ( $\varnothing$  6 mm) and placed on the previously inoculated agar. The plates were inverted and incubated for 24 h at 30  $^\circ\text{C}$ . Antimicrobial activity was indicated by the presence of clear inhibition zones around the discs and was recorded as MID values.

### Spore germination method

Ten ml of sterile 0.85% (w/v) saline solution was poured onto the agar plate ( $\varnothing$  90 mm) of a confluent culture of *F. moniliforme* and *Alternaria* sp., respectively. The surface of the culture was gently rubbed with an inoculating loop to harvest the spores. The resulting spore suspension was transferred to a sterile centrifuge tube. The spore suspension was centrifuged at  $500\times g$  for 20 min. The supernatant (0.5 ml) was well-mixed with 20 ml of PDA at 45  $^\circ\text{C}$  and allowed to set in a petri dish. Filter paper discs containing samples were placed on the surface of the previously inoculated agar.

The petri dishes were inverted and incubated at 27 °C for 48 h. Antifungal activity was indicated by the presence of clear inhibition zone around the discs and the MID values were recorded.

#### Bioautography using *C. herbarum* on TLC

PDA medium (0.1% agar) was added to well-sporulating agar slant cultures of *C. herbarum* to harvest the spores (Homans and Fuchs, 1970). Serial dilutions of samples of known concentrations were spotted (1 µl/spot) on a TLC sheet for MID determination were sprayed adequately with the spore suspension. Sprayed TLC sheets were incubated in a wet chamber at 27 °C for 24 h in the dark. Antifungal activity appeared as clear inhibition zones against the dark background of *C. herbarum* spores. The lowest dose (µg/spot) of sample to show an inhibition zone was recorded as the MID.

#### Extraction, and isolation of **1** and **2**

The fresh fruits (31 kg) of *G. atroviridis* were sliced and dried at room temp. for two weeks based on the traditional method of processing the fresh fruits for culinary uses. The dried-sliced fruits (4 kg) were extracted with MeOH (20 l × 3) at room temp. for one week. After removal of the solvent, the residue (1.8 kg) was suspended in H<sub>2</sub>O and successively extracted three times each with petrol, CHCl<sub>3</sub>, EtOAc and *n*-BuOH to give the respective residues of the extracts (26 g, 23 g, 232 g, 598 g).

Subsequently, the most active *n*-BuOH extract (33 g) was chromatographed on silica gel (670 g) and was eluted with EtOAc to give seven fractions. The most active two fractions (**C1-A** & **C1-B**) with MIDs of 3.125 µg/spot and 6.25 µg/spot were further separated. The most active fraction (**C1-A**, 291 mg) was subjected to medium-pressure liquid chromatography (MPLC) over silica gel (100 × Ø 0.8 cm) and eluted with hexane-MeCOEt (85:15 v/v) at a flow rate of 1 ml/min. A total of 90 fractions were collected which were pooled to give five fractions based on TLC analysis (**C2-A** to **C2-E**). The fourth fraction (**C2-D**), which was the most active fraction (MID: 0.4 µg/spot), gave white crystals upon drying and was subsequently recrystallised from hexane to give **1** (8 mg). Separately, the next most active fraction (**C1-B**, 11 g) from the first step

of column chromatography mentioned above, was eluted with EtOAc on silica gel (670 g). The TLC bioautography profiles of the eight fractions (**C3-A** to **-H**) indicated that only the first six fractions (**C3-A** to **-F**) inhibited *C. herbarum* of which the first five fractions showed yellow colour against ninhydrin. The major ninhydrin-yellow fraction (2.2 g) was then rechromatographed on silica gel (Merck 9385, 110 g) with hexane-MeCOEt (85:15 v/v) and five fractions were collected. The only fraction containing the ninhydrin-yellow spot (259 mg) was rechromatographed on silica gel MPLC (100 × Ø 0.8 cm) with hexane-MeCOEt (85:15 v/v) at a flow rate of 1 ml per min. Ninety fractions were collected and combined based on TLC analysis to give three combined fractions. The second combined fraction (100 mg) was eluted with 90% hexane-MeCOEt at a flow rate at 1 ml per min on silica gel using MPLC to afford **2** (50 mg) that was obtained as a yellow oil (MID: 0.8 µg/spot). The third combined fraction was obtained as a white solid after drying and recrystallised from hexane to give white crystals (23 mg). Co-chromatography showed it to be identical to the previously isolated compound (**1**).

#### *1',1''-Dibutyl methyl hydroxycitrate (1)*

White crystals (hexane);  $R_f = 0.45$ , ninhydrin-yellow, silica gel TLC, C<sub>6</sub>H<sub>6</sub>-EtOAc (7:3); mp 64 °C (uncorr.);  $[\alpha]_D^{+150}$  (MeOH; *c* 0.01); UV  $\lambda_{max}$  (MeOH) nm (log  $\epsilon$ ): 217 (2.5); IR  $\lambda_{max}$  (KBr): 3343 (OH), 3518 (OH), 1751 (C=O), 1732 (C=O) HRFAB-MS *m/z* [M+H]<sup>+</sup>: calcd. for C<sub>15</sub>H<sub>27</sub>O<sub>8</sub>, 335.1706; found, 335.1709; CI-MS (NH<sub>3</sub>) *m/z*: 352 [M + NH<sub>4</sub>]<sup>+</sup>; EI-MS 70 eV, *m/z* (rel. int.): 205 (21) 171 (15) 159 (40) 145 (46) 131 (39) 99 (53) 76 (31) 57 (100) 41 (59) 29 (33); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): 0.91 (6H, *m*, H-4' & H-4''), 1.37 (4H, *m*, H-3' & H-3''), 1.57, 1.67 (each 2H, *m*, H-2' & H-2''), 2.99 (1H, *d*, *J* = 16.5 Hz, H-4), 3.02 (1H, *d*, *J* = 16.5 Hz, H-4), 3.29 (1H, *d*, *J* = 8.9 Hz, 2-OH), 3.83 (1H, *s*, MeO), 3.92 (1H, *s*, 3-OH), 4.06 (2H, *m*, H-1''), 4.25 (3H, *m*, H-1' & H-2); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): 13.6 (C-4' or C-4''), 13.6 (C-4'' or C-4'), 53.4 (MeO), 19.0 (C-3' or C-3''), 19.0 (C-3'' or C-3'), 30.5 (C-2' or C-2''), 30.5 (C-2'' or C-2'), 39.8 (C-4), 64.9 (C-1''), 66.4 (C-1'), 77.3 (C-2), 77.5 (C-3), 170.0 (C-5), 170.6 (C-1), 172.7 (MeOC=O).

2-(butoxycarbonylmethyl)-3-butoxycarbonyl-2-hydroxy-3-propanolide (2)

Yellow oil;  $[\alpha]_D^{-60}$  (MeOH, *c* 0.02);  $R_f = 0.5$ , ninhydrin-yellow, silica gel TLC,  $C_6H_6$ -EtOAc (7:3); UV  $\lambda_{max}$  (MeOH) nm (log  $\epsilon$ ): 220 (2.5); IR  $\lambda_{max}$  (NaCl): 3480 (OH), 1807 (C=O), 1750 (C=O); HRFAB-MS)  $m/z$   $[M+H]^+$ : calcd. for  $C_{14}H_{23}O_7$  303.1444; found, 303.1443; CI-MS ( $NH_3$ )  $m/z$ : 320  $[M + NH_4]^+$ ; EI-MS  $m/z$  (rel. int.): 201 (21) 184 (17) 172 (13) 145 (75) 128 (32) 99 (20) 57 (100) 41 (45) 29 (30);  $^1H$  NMR (500 MHz,  $CDCl_3$ ): 0.92 (6H, *t*,  $J = 7.3$  Hz, H-4' & H-4''), 1.35 (4H, *m*, H-3' & H-3''), 1.62 (4H, *m*, H-2' & H-2''), 2.77 (1H,

*d*,  $J = 17.7$  Hz, 2- $CH_2CO_2$ ), 3.08 (1H, *d*,  $J = 17.7$  Hz, 2- $CH_2CO_2$ ), 3.96 (1H, *s*, 2-OH), 4.10 (2H, *m*, H-1''), 4.17, 4.25 (each 1H, *m*, H-1'), 4.85 (1H, *s*, H-3);  $^{13}C$  NMR (125 MHz,  $CDCl_3$ ): 13.5 (C-4''), 13.6 (C-4'), 18.9 (C-3''), 19.0 (C-3'), 30.2 (C-2''), 30.3 (C-2'), 39.6 (2- $CH_2CO_2$ ), 66.3 (C-1''), 67.5 (C-1'), 84.2 (C-3), 78.7 (C-2), 166.3 (2- $CH_2CO_2$ ), 170.4 (3- $CO_2$ ), 171.6 (C-1).

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