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

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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 β-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 µg/spot) only against *C. herbarum* at the MID of 0.4 and 0.8 µg/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 ochraceous*) 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 -pharmaceutical 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 garcinic acid (identical to (−)-hydroxycitric acid) and its γ-lactone, atroviridin, atrovirisidone and atrovirinone (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 (Abdulah, 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 (CHCl₃, 23 g), ethyl acetate (EtOAc, 232 g), n-butanol (n-BuOH, 598 g) and H₂O (207 g) soluble fractions. The minimum inhibitory dose (MID) values against *C. herbarum* of the MeOH and n-BuOH (most active fraction) ex-
Extracts were 100 and 25 µg/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 C$_{15}$H$_{26}$O$_8$ from HRFAB-MS and CI-MS data. The IR spectrum showed the presence of hydroxyl (3518 and 3433 cm$^{-1}$) and carbonyl (1751 and 1732 cm$^{-1}$) groups. The $^{13}$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 oxy-methylenes ($\delta$ 64.9 and 66.4), one oxymethine ($\delta$ 77.3), one oxygen-bearing quartenary carbon ($\delta$ 77.5) and three ester carbonyls ($\delta$ 170.0, 170.6 and 172.7). The $^1$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 $^1$H-NMR signals ($\delta$ 0.91, 6H, m; $\delta$ 1.37, 4H, m; $\delta$ 1.57, 1H, 1.67, 2H each, m; $\delta$ 4.06, 2H, m; $\delta$ 4.25, 2H overlapped with another H) together with the $^1$H-$^1$H COSY and $^1$H-$^1$C HETCOR crosspeaks indicated the presence of two butoxyl groups. The $^1$H-$^1$C 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 $^1$H-$^1$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 butoxy carbonyl, one methoxy carbonyl, 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.

Compound 2 was obtained as a yellow oil and formulated as C$_{14}$H$_{22}$O$_7$ based on its HRFAB-MS and Cl-MS data. This formula was CH$_4$O less than that of compound 1, suggesting that 2 lacked CH$_3$OH. A close similarity of the $^{13}$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 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 (δ 4.25) of 1 changed to a singlet and shifted downfield (δ 4.85) in 2. There was only one structure in which the secondary hydroxyl oxygen was involved in the β-lactone ring among the three possible β-lactones of hydroxycitric acid. Additionally, HMBC correlations were also consistent with proposed structure (Fig. 1). The NOESY experiment showed correlation between the signal at δ 2.77 (one of the methylene protons) and the methine proton signal at δ 4.89 to suggest a cis configuration (Fig. 1). Therefore, it was elucidated that 2 was 2-(butoxy-carbonylmethyl)-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 µg and 0.8 µg/spot, respectively and was comparable to the antifungal standard, cycloheximide (MID: 0.5 µg/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 µg/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

1H- and 13C-NMR spectra were recorded in CDCl₃ 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 (uncorr.) was determined on a Kofler 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-positive), 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 µl of inoculum (adjusted to 10⁵-10⁶ CFU/ml) with 20 ml of agar at 45 °C and allowed to set (NA for bacteria and PDA for fungi) in a petri dish (∆90 cm is most likely an artefact of garcinia acid. Compounds 1 and 2 were loaded onto each Whatman No.1 filter paper discs (∆6 mm) and placed on the previously inoculated agar. The plates were inverted and incubated for 24 h at 30 °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 (∆90 cm) 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 5000xg for 20 min. The supernatant (0.5 ml) was well-mixed with 20 ml of PDA at 45 °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 (1μl/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) and successively extracted three times each with petrol, CHCl3, EtOAc and n-BuOH to give the respective residues of the extracts (26 g, 23 g, 22 g, 2.2 g) were then rechromatographed on silica gel.

**Co-chromatography**

Subsequently, the most active fraction (4 kg) was chromatographed on silica gel (670 g) to show an inhibition zone was recorded as the first five fractions. Antifungal activity appeared as clear inhibition zones against the dark background of *C. herbarum* spores. The most active fraction (294 mg) 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 × 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 of 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 while crystals (23 mg). Co-chromatography showed it to be identical to the previously isolated compound (1).

**′,′′-DIBUTYL METHYL HYDROXYCITRATE (1)**

White crystals (hexane); *Rf* = 0.45, ninhydrin-yellow, silica gel TLC, C15H27O8 (7:3); mp 64 °C (uncorr.); [α]D + 150° (MeOH; c 0.01); UV λmax (MeOH) nm (log ε): 271 (2.5); IR λmax (KBr): 3343 (OH), 3518 (OH), 1751 (C=O), 1732 (C=O) 335.1706; found, 335.1709; CI-MS (NH3) m/z: 352 [M + NH4]+; 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); 1H NMR (500 MHz, CDCl3): 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); 13C NMR (125 MHz, CDCl3): 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; [α]D -60° (MeOH, c 0.02); Rf = 0.5, ninhydrin-yellow, silica gel TLC, C14H23O7

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