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Activity-Guided Isolation of Constituents of *Cerbera manghas* with Antiproliferative and Antiestrogenic Activities

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Abstract—Two new cardenolides, (−)-14-hydroxy-3β-(3-*O*-methyl-6-deoxy-α-L-rhamnosyl)-11α,12α-epoxy-(5β,14β,17βH)-card-20(22)-enolide (**1**), (−)-14-hydroxy-3β-(3-*O*-methyl-6-deoxy-α-L-glucopyranosyl)-11α,12α-epoxy-(5β,14β,17βH)-card-20(22)-enolide (**2**), and a known cardenolide, (−)-17β-neriifolin (**3**), were isolated from the roots of *Cerbera manghas* as antiproliferative and antiestrogenic principles when evaluated against a human colon cancer cell line (Col2) and the Ishikawa cell line, respectively. Two known lignans, (−)-olivil (**4**) and (−)-cycloolivil (**5**), were also isolated but were inactive in the assay systems used.

Cancer chemoprevention can be defined as the prevention, delay, or reversal of cancer by ingestion of dietary or pharmaceutical agents capable of modulating the process of carcinogenesis.[1–3] Strategies for discovering potential cancer chemopreventive agents involve the regulation of cell proliferation and differentiation, including modulation of cell signal transduction,[4] enhancement of intercellular communication,[5] inhibition of oncogene activity,[6] as well as induction of apoptosis.[7] As part of our current work to discover novel plant-derived cancer chemopreventives, a battery of mechanism-based in vitro assays is employed for screening purposes.[8] Accordingly, we have utilized a human colon cancer cell line (Col2) to search for antiproliferative agents, as well as the Ishikawa cell line (derived from a human endometrial adenocarcinoma), to discover new natural product antiestrogens.[9]

Cerbera manghas L. (Apocynaceae) is synonymous with *Cerbera odollam* Gaertn., a small tree found along the coasts of southeast Asia, tropical Australia, and Polynesia.[10] Extracts of *C. manghas* have shown analgesic, anticonvulsant, cardiotonic, and hypotensive activities.[11,12] All parts of this species contain a latex with emetic and cathartic properties. Previous phytochemical investigations on *C. manghas* have resulted in the isolation of several cardenolides[13–15] and lignans.[16,17]

In the present investigation, *C. manghas* was selected for study, since an ethyl acetate-soluble extract of the roots was found to exhibit significant antiproliferative activity in a human colon cancer cell line (Col2) and antiestrogenic activity using the Ishikawa cell line. Bioassay-monitored fractionation of the active extract using these assays led to the isolation of two new (**1** and **2**) and three known constituents (**3** and **5**), which were then evaluated for their individual biological activities. The structure elucidation of compounds **1** and **2** and the biological evaluation of **1**–**5** are reported in this communication.

Isolation and Structure Elucidation of Compounds 1 and 2

The roots of *C. manghas* were collected in October 1976 in Fiji. These were stored at ambient temperature and milled just prior to being used for the present investigation. A voucher specimen (accession number CA-2375) has been deposited at the University of Illinois Pharmacognosy Field Station. The milled roots (5.0 kg) were extracted exhaustively by maceration with MeOH:H_2O (9:1; 3×10 L). After filtration and evaporation of the solvent, the resultant extract was diluted with H_2O to afford an aqueous MeOH solution (80%), and then partitioned with petroleum ether and EtOAc, respectively, to afford dried petroleum ether- (18.0 g) and EtOAc-soluble (65.0 g) residues. The EtOAc extract exhibited significant activities when evaluated against Col2 and Ishikawa cells (IC_{50} 0.8 and 1.8 μg/mL, respectively).

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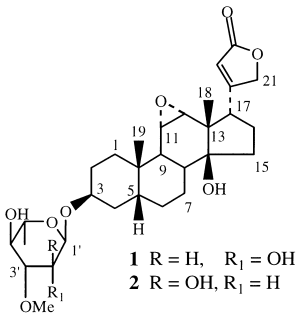

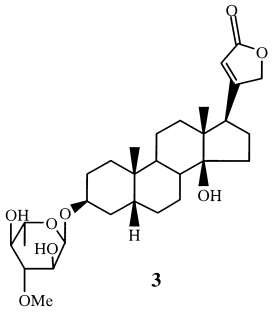

Activity-guided fractionation of the EtOAc-soluble residue using these cell lines, involving successive Si gel, reversed-phase C_{18}, and Sephadex LH-20 chromatographic steps, afforded two new cardenolides, namely, (−)-14-hydroxy-3β-(3-*O*-methyl-6-deoxy-α-L-rhamnosyl)-11α,12α-epoxy-(5β,14β,17βH)-card-20(22)-enolide (**1**),[18] (−)-14-hydroxy-3β-(3-*O*-methyl-6-deoxy-α-L-glucopyranosyl)-11α,12α-epoxy-(5β,14β,17βH)-card-20(22)-enolide (**2**),[18] a known cardenolide, (−)-17β-neriifolin (**3**),[19,20] as well as two known lignans, (−)-olivil (**4**) and (−)-cycloolivil (**5**).[16,19]

The molecular formula of compound **1** was determined as $C_{30}H_{43}O_9$ by positive-ion HRFABMS. Comparison of its ^1H and ^{13}C NMR data with deacetylcerbertin[21] and several of its analogues indicated that **1** is a cardenolide.[13–15] In addition, a significant mass spectral fragment peak was observed at *m/z* 389 $[M+H-160]^+$, suggesting that only one sugar unit was present in **1**. The anomeric carbon and proton NMR signals were observed at δ_C 97.5 and δ_H 4.94 (d, $J=1.7$ Hz), respectively. The aglycon was therefore assigned the elemental formula $C_{23}H_{31}O_5$, which is consistent with a cardenolide having two hydroxyl (IR, ν_{max} 3426 cm^{-1}) and one epoxide[21] (δ_C 55.4 and 63.7; δ_H 3.05 and 2.89, each doublet with $J=3.6$ Hz) functionalities. In the ^1H NMR spectrum, characteristic methylene protons at C-21 (δ_H 4.82 dd, $J=18.0$, 1.0 Hz) and an olefinic proton at C-22 (δ_H 5.95 s) were also observed.[13] The relative locations of these functional groups were established from HMBC NMR spectral observations, in which cross-peaks were observed between δ_H 0.96 (Me-18) and δ_C 63.7 (C-12), 45.9 (C-17), and 84.1 (C-14). Additional correlations were observed between δ_H 2.89 (H-12) and δ_C 49.1 (C-13) and 84.1 (C-14), and between δ_H 3.05 (H-11) and δ_C 35.4 (C-9) and 36.0 (C-10). In addition, the H-17 signal at δ_H 3.29 showed correlations with the signals at δ_C 28.4 (C-16), 49.1 (C-13), 117.7 (C-22), and 172.8 (C-20). In terms of the sugar moiety of **1**, the proton signals at δ_H 1.30 (3H, d, $J=6.0$ Hz, H-6′), 3.45 (1H, dd, $J=3.0$, 9.0 Hz, H-3′), 3.49 (3H, s, H-3′-OCH_3), 3.54 (1H, m, H-4′), 3.74 (1H, m, H-5′), 4.03 (1H, br s, H-2′), and 4.94 (1H, d, $J=1.7$ Hz, H-1′) in addition to their coupling constants were assignable by a ^1H–^1H COSY experiment. By comparison with L-thevetose (3-*O*-methyl-6-deoxy-α-L-glucopyranose), the sugar moiety of neriifolin,[20] the only difference was the opposite orientation at H-2′ for these monosides, thereby affecting the chemical shifts and coupling constants observed. Those signals corresponding to H-1′–H-3′ for compound **1** were observed at δ_H 4.03 (1H, br s, H-2′), 4.94 (1H, d, $J=1.7$ Hz, H-1′) and 3.45 (1H, dd, $J=3.0$, 9.0 Hz, H-3′), in contrast to δ_H 3.58 (1H, dd, $J=8.9.0$, 4.4 Hz, H-2′), 4.86 (1H, d, $J=4.4$ Hz, H-1′), and 3.25 (1H, t, $J=8.9$ Hz, H-3′) for neriifolin.[20] Thus, the component sugar of **1** was assigned as an L-acofriose (3-*O*-methyl-6-deoxy-α-L-rhamnose) unit.[13,20]

The relative stereochemistry of compound **1** at the H-11/H-12 and H-17 positions was established by analysis of ^1H NMR coupling constants and a 2-D ROESY experiment. The H-11/H-12 epoxy unit was assigned with α-stereochemistry, since ROESY cross-peaks were observed between δ_H 3.05 (H-11) and δ_H 1.03 (Me-19), and between δ_H 2.89 (H-12) and δ_H 0.96 (Me-18), respectively. In addition, the observed resonances for compound **1** at δ_C 15.8 (C-18), 38.6 (C-8), and 45.9 (C-17) are compatible and in agreement with an α- instead of β-configuration for the epoxide.[21] The stereochemistry of H-17 was assigned as β based on the coupling constant at δ_H 3.29 (t, $J=9$ Hz), consistent with published data for 17α-cardenolides.[13,22] Higuchi et al. have reported differences between the chemical shifts of the C-17 cardenolide isomers, in which the 17β-cardenolide H-17 resonance was observed at δ_H 2.76 (1H, m), in contrast to the 17α-cardenolide H-17 signal at δ_H 3.20 (1H, t-like).[22] Accordingly, the structure of **1** was determined as (−)-14-hydroxy-3β-(3-*O*-methyl-6-deoxy-α-L-rhamnosyl)-11α,12α-epoxy-(5β,14β,17βH)-card-20(22)-enolide.

Compound **2** was shown to possess a molecular formula of $C_{30}H_{43}O_9$. Comparison of its ^1H and ^{13}C NMR spectra with the known deacetylcerbertin[21] indicated that they were very closely related isomers, with the only difference being the relative stereochemistry at H-17 for these cardenolides, thereby affecting the chemical shifts and coupling constants observed. The sugar moiety of **2** was assigned as L-thevetose on the basis of direct comparison of its ^1H NMR spectral data with those of the sugar of neriifolin (**3**).[20] Significant ^1H NMR signals for the 17α-cardenolide were observed at δ_H 3.32 (1H, t, $J=9.0$ Hz, H-17), and δ_H 0.96 (3H, s, H-18) in contrast to δ_H 2.78 (1H, dd, $J=8.8$, 5.0 Hz, H-17) and δ_H 0.88 (3H, s, H-18) for a 17β-cardenolide.[20] Thus, the structure of **2** was determined as (−)-14-hydroxy-3β-(3-*O*-methyl-6-deoxy-α-L-glucopyranosyl)-11α,12α-epoxy-(5β,14β,17βH)-card-20(22)-enolide, and only differs from **1** in its sugar unit.

Compounds **1**–**5** were evaluated for their potential as antiproliferative agents using Col2 human colon cancer cells[23,30] and antiestrogenic agents using Ishikawa cells,[9] respectively, according to established protocols. Compounds with IC_{50} (half maximal inhibitory concentration) values of ≤ 5 μg/mL are considered active.[9,23] Compounds **4** and **5** were inactive (IC_{50} values >10 μg/mL) in both assays, whereas compounds **1**–**3** exhibited significant antiproliferative and antiestrogenic activities. The IC_{50} values for growth inhibition of **1**–**3** were 0.015, 0.02, and 0.10 μg/mL, respectively, using the Col2 cell line. The presence of a C-11/C-12 epoxide group and the differential stereochemistry of the C-17-attached lactone

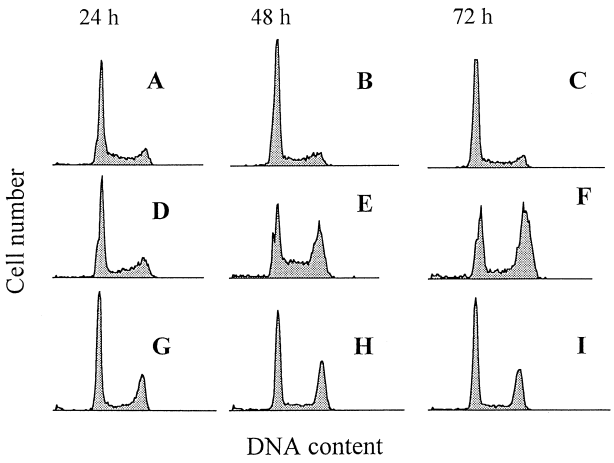

Figure 1. Flow cytometric analysis of propidium iodide stained Col2 cells treated with DMSO (0.1%) (panels A–C), compound **1** (0.03 μg/mL) (panels D–F), or compound **2** (0.04 μg/mL) (panels G–I) for the indicated time period.

ring in compounds **1** and **2** as compared with **3**, may have contributed to their more potent biological activities demonstrated in this assay. In addition, when the DNA content of cells treated with either compound **1** or **2** was measured, the fraction of cells in the G_0/G_1 phase of the cell-cycle was observed to decrease although the fraction of cells in the G_2/M phase increased (Fig. 1). While the mechanism of this block is unknown, it has been established that the cardiotonic action of cardiac glycosides is related to their ability to inhibit Na^+,K^+-dependent ATPase.[25] It is supposed that cardenolide glycosides inhibit this ATPase by means of binding to the 'digitalis'-receptor site of this membrane enzyme.[26] Since it has been suggested that the proliferation of normal and cancer cells is coupled with enhanced activity of the Na^+/K^+ pump,[27–29] inhibition is of relevance.

In the Ishikawa cell line, the IC_{50} values of **1**–**3** were 0.0042, 0.008, and 0.09 μg/mL, respectively, when tested for an antiestrogenic response, in a manner described previously.[9] Cardiac glycosides are reported to have utility in the treatment of breast cancer,[31] possibly as a result of their structural similarity to estradiol and interaction with the estrogen receptor.[32] However, this is the first report of the antiestrogenic properties of cardenolides with a human endometrial adenocarcinoma. Further characterization of their mechanism is in progress.

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18. **Characterization data: (−)-14-Hydroxy-3β-(3-*O*-methyl-6-deoxy-α-L-rhamnosyl)-11α,12α-epoxy-(5β,14β,17βH)-card-20(22)-enolide (1)**: Colorless needles (9.5 mg, 0.0002% w/w); mp 237 °C; $[\alpha]_D^{20}$ −18.4° (*c* 0.05, $CHCl_3$); HRFABMS (positive-ion mode) *m/z* $[M+H]^+$ 549.3061 (calcd for $C_{30}H_{44}O_9$, 549.3051); FABMS *m/z* (rel. int.%): $[M+H]^+$ 549 (100), 389 (34), 353 (24), 241 (23), 187 (30), 161 (81), 149 (43); UV (MeOH) λ_{max} (log ε) 242 (3.39) nm; IR ν_{max} (film) 3426 (br), 2934, 2875, 2783, 1750, 1647, 1450, 1367, 1328, 1259 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.96 (3H, s, H-18), 1.03 (3H, s, H-19), 1.30 (3H, d, *J* = 6.0 Hz, H-6′), 2.89 (1H, d, *J* = 3.6 Hz, H-12), 3.05 (1H, d, *J* = 3.6 Hz, H-11), 3.29 (1H, t, *J* = 9.0 Hz, H-17), 3.45 (1H, dd, *J* = 3.0, 9.0 Hz, H-3′), 3.49 (3H, s, H-3′-OCH_3), 3.54 (1H, m, H-4′), 3.74 (1H, m, H-5′), 4.03 (1H, br s, H-2′), 4.82 (2H, dd, *J* = 18.0, 1.0 Hz, H-21), 4.94 (1H, d, *J* = 1.7 Hz, H-1′), 5.95 (1H, s, H-22); ^{13}C NMR ($CDCl_3$) δ 30.3 (C-1), 26.6 (C-2), 71.3 (C-3), 29.8 (C-4), 35.7 (C-5), 26.9 (C-6), 21.3 (C-7), 38.6 (C-8), 35.4 (C-9), 36.0 (C-10), 55.4 (C-11), 63.7 (C-12), 49.1 (C-13), 84.1 (C-14), 33.6 (C-15), 28.4 (C-16), 45.9 (C-17), 15.8 (C-18), 24.0 (C-19), 172.8 (C-20), 73.5 (C-21), 117.7 (C-22), 174.2 (C-23), 97.5 (C-1′), 67.5 (C-2′), 81.4 (C-3′), 71.4 (C-4′), 67.8 (C-5′), 57.0 (C-3′-OCH_3), 17.6 (C-6′-CH_3).
(−)-14-Hydroxy-3β-(3-*O*-methyl-6-deoxy-α-L-glucopyranosyl)-11α,12α-epoxy-(5β,14β,17βH)-card-20(22)-enolide (2): Colorless needles (70.0 mg, 0.0014% w/w); mp 242 °C; $[\alpha]_D^{20}$ −23.0° (*c* 0.08, $CHCl_3$); HRFABMS (positive-ion mode) *m/z* $[M+H]^+$ 549.3064 (calcd for $C_{30}H_{44}O_9$, 549.3051); FABMS *m/z* (rel. int.%): $[M+H]^+$ 549 (100), 389 (54), 371 (43), 353 (32), 193 (22), 161 (35); UV (MeOH) λ_{max} (log ε) 242 (3.20) nm; IR ν_{max} (film) 3424 (br), 2930, 2780, 1749, 1650, 1450, 1367, 1329 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.96 (3H, s, H-18), 1.05 (3H, s, H-19), 1.28 (3H, d, *J* = 6.0 Hz, H-6′), 2.89 (1H, d, *J* = 3.6 Hz, H-12), 3.05 (1H, d, *J* = 3.6 Hz, H-11), 3.15 (1H, t, *J* = 9.0 Hz, H-4′), 3.27 (1H, t, *J* = 9.0 Hz, H-3′), 3.32 (1H, t,

$J=9.0$ Hz, H-17), 3.57 (1H, dd, $J=9.0$, 4.0 Hz, H-2′), 3.69 (3H, s, H-3′-OCH_3), 3.74 (1H, m, H-5′), 3.99 (1H, m, H-3), 4.82 (2H, dd, $J=18.0$, 1.0 Hz, H-21), 4.83 (1H, d, $J=3.8$ Hz, H-1′), 5.95 (1H, s, H-22); ^{13}C NMR ($CDCl_3$) δ 30.3 (C-1), 26.6 (C-2), 73.2 (C-3), 29.8 (C-4), 36.5 (C-5), 26.9 (C-6), 21.3 (C-7), 38.9 (C-8), 35.9 (C-9), 35.6 (C-10), 55.5 (C-11), 63.8 (C-12), 49.2 (C-13), 84.3 (C-14), 33.6 (C-15), 28.4 (C-16), 46.0 (C-17), 15.8 (C-18), 24.0 (C-19), 172.8 (C-20), 73.5 (C-21), 117.7 (C-22), 174.3 (C-23), 97.5 (C-1′), 67.5 (C-2′), 81.4 (C-3′), 71.4 (C-4′), 67.8 (C-5′), 57.0 (C-3′-OCH_3), 17.6 (C-6′-CH_3).

19. **(−)-17β-Neriifolin (3)**. Colorless needles (7.0 mg, 0.00014% w/w); mp 232 °C; $[\alpha]_D^{20}$ −22.0° (*c* 0.06, $CHCl_3$); closely comparable to neriifolin by comparison with reported data (IR, ^{1}H and ^{13}C NMR, MS).[20]

(−)-Olivil (4). Colorless needles (28.0 mg, 0.00056% w/w); mp 106 °C; $[\alpha]_D^{20}$ −49.0° (*c* 0.05, $CHCl_3$); closely comparable to olivil by comparison with reported data (IR, ^{1}H and ^{13}C NMR, MS).[16]

(−)-Cycloolivil (5). Colorless needles (18.0 mg, 0.00036% w/w); mp 110 °C; $[\alpha]_D^{20}$ −35.0° (*c* 0.07, $CHCl_3$); closely comparable to cycloolivil by comparison with reported data (IR, ^{1}H and ^{13}C NMR, MS).[16]

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23. **Biological Assays for Antiproliferative Activity**. A human colon cancer cell line (Col2) was cultured in MEME medium containing 10% non-essential amino acid solution (NAA), 1×antibiotic-antimycotic (Gibco BRL, Grand Island, NY), and 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 atm. To perform the sulforhodamine B (SRB) assay, exponentially growing cells were added to 96-well microtiter plates containing test compounds dissolved in DMSO. Cells were allowed to grow at 37 °C, and, after 3 days they were fixed with trichloroacetic acid and stained with 0.4% sulforhodamine B in 1% acetic acid. The bound dye was solubilized in 0.1 M Tris and absorbance at A_{515} was measured. Percent growth was calculated from the formula: % growth = (Absorbance$_{sample}$ − Absorbance$_{day\,0}$)/(Absorbance$_{DMSO\,control}$ − Absorbance$_{day\,0}$)× 100. For additional details, see ref 30.

Cell-cycle analysis. Col2 cells were plated in T25 flasks in the presence of DMSO or compound and incubated at 37 °C. At various times, cells were trypsinized, washed with PBS and fixed with 70% ethanol. Cells were resuspended in a buffer containing trypsin.[24] RNA was degraded with RNAse A and nuclei were stained with propidium iodide. Fluorescence was measured using an Epics Elite flow cytometer (Coulter Corporation), with excitation from an Argon Ion laser at 488 nm. Histograms were analyzed using Multicycle AV software (Phoenix Flow Systems, San Diego, CA).

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