Isolation and Structure Elucidation of Ardisenone: A New, Cytotoxic Alkenylphenol from *Ardisia iwahigensis*

F. David Horgen, Hélène Guinaudeau, John M. Pezzuto, Djaja D. Soejarto, and Norman R. Farnsworth*

Program for Collaborative Research in the Pharmaceutical Sciences, Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, 833 South Wood Street, Chicago, Illinois 60612

Frederick Agcaoili, Gerlie de los Reyes, and Ru Angelie Edrada

College of Pharmacy, University of the Philippines Manila, Padre Faura Street, Ermita, Manila, Philippines

Received January 15, 1997[⊗]

A methanol extract of leaves and twigs from *Ardisia iwahigensis* demonstrated toxicity toward brine shrimp as well as LNCaP, ZR-75-1, and Lu1 human cancer cells in culture. A novel alkenylphenol, (*Z*)-1,16-bis(3-hydroxy-5-methoxyphenyl)-10-hexadecene-1,15-dione (ardisenone) (**1**), was isolated from the extract by bioassay-directed fractionation. This compound demonstrated moderate cytotoxicity against BC1, Lu1, Col2, KB, KB-V1, and LNCaP cell lines.

Ardisia iwahigensis Elmer (Myrsinaceae) is a small rain forest tree endemic to Palawan Island (Philippines). Only a few botanical specimens have been collected since the plant was first described in 1913,[1,2] and there are no records in the literature regarding the medicinal use or chemical constituents of this species.

As part of an ongoing chemical and biological evaluation of tree species occurring in study plots in the central mountain range of Palawan Island, a leaf and twig sample of *A. iwahigensis* was collected, and a MeOH extract of the air-dried, milled sample was subjected to preliminary screening against brine shrimp (*Artemia salina* Leach) nauplii to determine whether *in vitro* cytotoxicity tests should be undertaken.[3] At a concentration of 10 μg/mL, the extract immobilized 100% of the brine shrimp tested. Subsequently, the extract was tested against a panel of cultured human cancer cells (Table 1). The extract demonstrated strong activity against LNCaP (human hormone-dependent prostate cancer) and ZR-75-1 (human hormone-dependent breast cancer) cells, which was selective when compared with KB (human epidermoid carcinoma) and KB-V1 (vinblastine-resistant KB) cell toxicity. After an HPLC-MS dereplication procedure[4] failed to identify any known active compounds previously isolated from the genus, the extract was selected for bioassay-directed fractionation.

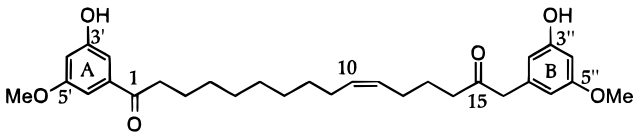
The MeOH extract was partitioned with hexane after the addition of 10% H_2O. Following the removal of MeOH from the aqueous alcohol phase, the resulting syrup was partitioned between H_2O and $CHCl_3$. The $CHCl_3$ layer yielded the most active residue, and after several purification steps guided by bioassay, ardisenone (**1**) was isolated as a transparent oil. The molecular formula of ardisenone, $C_{30}H_{40}O_6$ (HREIMS *m*/*z* 496.2844), revealed 11 degrees of unsaturation.

One-dimensional NMR experiments provided evidence of the basic skeletal structure as well as the presence of several functional groups. The ^{1}H NMR spectrum (Table 2) and homonuclear decoupling experiments suggested the presence of two 1,3,5-substituted phenyl groups, two OMe groups, a single double bond, and an aliphatic chain. The absence of a terminal methyl group and the lack of evidence of aliphatic branching suggested that two phenyl groups were connected by an unbranched, monounsaturated aliphatic chain.

Table 1. Cytotoxicity of the MeOH Extract of Leaf and Twig from *Ardisia iwahigensis* and Ardisenone (**1**)[a]

cell line[b]	MeOH extract	ardisenone (**1**)
BC1	nt	8.5
Lu1	1.8	4.9
Col2	nt	15
KB	>20	13
KB-V1(+VLB)	>20	8.0
KB-V1(−VLB)	>20	6.9
LNCaP	0.090	11
ZR-75-1	0.20	nt

[a] Data are given as estimated IC_{50} values in μg/mL; nt = not tested. [b] BC1 = human breast cancer; Lu1 = human lung cancer; Col2 = human colon cancer; KB = human epidermoid carcinoma; KB-V1(+VLB) = vinblastine (VLB)-resistant KB with 1 μg/mL VLB; KB-V1(−VLB) = VLB-resistant KB without VLB; LNCaP = human hormone-dependent prostate cancer; ZR-75-1 = human hormone-dependent breast cancer.



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The IR spectrum gave a broad absorbance in the carbonyl range (1689 cm^{-1}), while the ^{13}C NMR (Table 2) and APT spectra exhibited two carbonyl carbons, at δ 201.6 and 209.8, and confirmed the presence of an aliphatic double bond and two benzene rings bearing six protonated carbons. The chemical shift of four of the aromatic carbons suggested the presence of an oxygenated substituent on each; since two OMe groups were present, it was deduced that the aromatic substitution was completed by two phenolic moieties. A bathochromic shift demonstrated in the UV spectrum with the addition of base is consistent with the presence of phenolic groups.

Carbon and proton chemical shift assignments (Table 2) and functional group positions were obtained through analysis of COSY, HMQC, HMBC, and ROESY experiments. With respect to ring A, the COSY and homonuclear decoupling experiments showed a direct cou-

* To whom correspondence should be addressed. Tel.: (312) 996-7254. Fax: (312) 996-7107. E-mail: norman@uic.edu.

[⊗] Abstract published in *Advance ACS Abstracts,* May 1, 1997.

S0163-3864(97)00049-9 CCC: $14.00

Table 2. ^{1}H and ^{13}C NMR Assignments of Ardisenone (**1**)[a]

position	^{1}H	^{13}C
1		201.6
2	2.90 (t, 7.3)	38.7
3	1.70 (quintet, 7.3)	24.4
4−8	1.20−1.35 (m)	28.9−29.4
9	1.94 (m)	27.0
10	5.35 (m)	131.0
11	5.25 (m)	128.5
12	1.98 (m)	26.4
13	1.60 (quintet, 7.3)	23.6
14	2.48 (t, 7.3)	41.2
15		209.8
16	3.59 (s)	50.3
1′		138.9
2′	7.06 (m)	107.7
3′		157.4
4′	6.64 (t, 2.0)	106.3
5′		161.0
6′	7.05 (m)	106.0
5′-OMe	3.81 (s)	55.6
1″		136.3
2″	6.36 (m)	108.9
3″		157.4
4″	6.34 (br t, 2.0)	100.2
5″		161.1
6″	6.31 (m)	107.5
5″-OMe	3.74 (s)	55.3

[a] Chemical shifts are reported in ppm ($CHCl_3$ = 7.264 ppm, $CDCl_3$ = 77.00 ppm), and proton multiplicities and coupling constants (in Hz) are in parentheses.

pling between the proton at δ 6.64 (H-4′) and the protons at δ 7.05 (H-6′) and 7.06 (H-2′). Several HMBC correlations confirmed the 1,3,5-substitution pattern. The proton assigned as H-4′ was one-bond coupled to the carbon at δ 106.3 (C-4′), which in turn was long-range coupled to H-2′ and H-6′. At the same time, H-4′ showed HMBC-correlations between the carbons at δ 106.0 (C-6′) and 107.7 (C-2′). Although H-2′ and H-6′ partially overlapped, C-2′ and C-6′ absorbances were distinguished on the basis of two-bond HMBC correlations observed between H-2′ and the carbon at δ 157.4 (C-3′) and between H-6′ and the carbon at δ 161.0 (C-5′). The OMe at δ 3.81 correlated in HMBC with the carbon at δ 161.0 (C-5′) and, in ROESY, showed a cross peak with protons at δ 6.64 (H-4′) and 7.05 (H-6′), thus placing the OMe on ring A at the 5′-position. The carbon at δ 201.6, due to a carbonyl, showed HMBC correlations with the aromatic protons H-2′ and H-6′ and the aliphatic protons H-2 (δ 2.90) and H-3 (δ 1.70), which indicates that the carbonyl is positioned α to ring A.

With respect to ring B, homonuclear decoupling showed meta-coupling between H-2″ (δ 6.36) and H-4″ (δ 6.34) and H-4″ and H-6″ (δ 6.31). Subsequently, the full assignment of ring B atoms was facilitated by HMQC and HMBC correlations. The OMe at δ 3.74 demonstrated a long-range correlation with the carbon at δ 161.1 (C-5″) and ROESY cross peaks with the protons at δ 6.34 (H-4″) and 6.31 (H-6″), establishing its position at C-5″. The sharp two-proton singlet at δ 3.59 (H-16) was long-range coupled to C-6″ (δ 107.5), while its carbon of attachment (δ 50.3) showed long-range coupling to H-2″ and H-6″. Further, the carbonyl carbon resonating at δ 209.8 (C-15) gave long-range cross peaks with the methylene protons at positions 13 (δ 1.60), 14 (δ 2.48), and 16 (δ 3.59). The only arrangement consistent with these correlations requires the carbonyl to be at the β-position to ring B.

The position of the double bond was unambiguously assigned by following successive COSY and HMBC correlations in the aliphatic chain from C-14 through C-9. The configuration of the double bond was determined by measuring the coupling constants between the olefinic protons while decoupling the vinylic protons; the value measured, J = 10.8 Hz, indicates *Z* geometry.[5] The *Z* configuration was confirmed by the chemical shift of the vinylic carbons, δ 27.0 (C-9) and δ 26.4 (C-12); in the case of the *Z* configuration, the chemical shift expected for vinylic carbons in environments similar to those of **1** would be near δ 27−30, while the *E* configuration would result in chemical shifts near δ 33.[6] Therefore, the structure of the compound was fully elucidated as (*Z*)-1,16-bis(3-hydroxy-5-methoxyphenyl)-10-hexadecene-1,15-dione (ardisenone) (**1**).

Table 1 lists the cytotoxicity results for ardisenone. The compound demonstrated moderate cytotoxicity against BC1 (human breast cancer), Lu1 (human lung cancer), Col2 (human colon cancer), KB, KB-V1, and LNCaP cell lines. Further work is continuing with the aim of isolating additional cytotoxic principles from the plant extract.

Experimental Section

General Experimental Procedures. Proton-detected NMR experiments, with the exception of COSY, were performed on a General Electric GE Omega 500 spectrometer operating at 500.1 MHz utilizing a Nalorac IDT 500-5GE inverse detection probe, whereas ^{13}C NMR, APT, and COSY experiments were performed on a Varian 300XL operating at 75.4 and 299.9 MHz for carbon and proton, respectively. ^{1}H, HMQC, HMBC, and ROESY experiments were obtained using standard sequences from the GE library. HMQC and HMBC were optimized for J_{CH} = 140 Hz and $^nJ_{CH}$ = 7 Hz, respectively. ^{13}C, APT, and COSY spectra were obtained using standard sequences from the Varian library. $CDCl_3$ was used as solvent for all experiments, and the chemical shift offset was accomplished by setting the $CHCl_3$ singlet to 7.264 ppm for ^{1}H spectra and the $CDCl_3$ triplet to 77.00 ppm for ^{13}C spectra. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. The UV and IR spectra were recorded on Beckman DU-7 UV and Midac FTIR spectrophotometers, respectively. EIMS and HREIMS were measured on a Finnigan MAT-90 mass spectrometer. VLC and CC were carried out on Merck Si gel 60 (40−63 μm particle size). Preparative TLC was performed on precoated Merck Si gel 60 F_{254} (0.25 mm-thick layer) plates, and fractions were monitored using vanillin/sulfuric acid spray (0.5% vanillin in 20% concd H_2SO_4/EtOH). Semipreparative HPLC was carried out on a Waters 600 HPLC system with a Waters 996 photodiode array detector.

Plant Material. A leaf and twig sample of *A. iwahigensis* was collected December 2, 1992, from a study plot on Palawan Island, Philippines. The plot is located on the lower northeastern slope of Thumb Peak in Barangay Simpocan, Municipality Puerto Princesa (elevation, 620−680 m; latitude, 9° 48′ north, 118° 36′ east). The fresh plant material was coarsely chopped and air-dried. Voucher specimens (Horgen *et al.* 65) were deposited at the John G. Searle Herbarium of the Field Museum of Natural History, Chicago, IL, and at the Philippine National Herbarium, Manila.

Extraction and Isolation. In preparation for the brine shrimp toxicity assay, 20 g of the air-dried plant

material was further dried at 60 °C for 30 min, ground, and extracted with 50 mL of MeOH. The solvent was evaporated from the extract in an oven at 60 °C, and the residue was assayed for brine shrimp toxicity.

For cytotoxicity testing, a 3 g portion of the milled, air-dried plant material was extracted with MeOH three times. After the extracts were combined and the solvent was removed under reduced pressure (at ≤40 °C), the residue was tested for cancer cell toxicity.

For the purpose of bioassay-directed fractionation, 700 g of the air-dried plant material was milled and extracted exhaustively with MeOH. The resulting extract was concentrated under reduced pressure (at ≤40 °C) and partitioned between MeOH/H_2O (9:1) and hexane. The aqueous MeOH phase was concentrated under reduced pressure (at ≤40 °C) and further partitioned between $CHCl_3$ and H_2O. The organic phase was dried with anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure.

The $CHCl_3$ extract (11 g) was subjected to vacuum liquid chromatography over Si gel (220 g) in a fritted disk funnel (9.5 cm diameter). Fractions were eluted with hexane/$CHCl_3$ (1:1, 1:4), $CHCl_3$, $CHCl_3$/MeOH (99:1, 98:2, 95:5, 90:10, 80:20, 60:40), and MeOH. The 36 collected fractions (400 mL each) were combined into 19 final fractions based on TLC.

A fraction (887 mg) eluting with $CHCl_3$/MeOH (99:1) showed the highest activity against the LNCaP cell line (IC_{50} = 400 ng/mL). A portion of this fraction (800 mg) was chromatographed over Si gel (40 g) with $CHCl_3$/EtOAc (10:2, 9:2, 8:2) and $CHCl_3$/MeOH (8:2). Eluted fractions (5 mL each) were combined into 13 final fractions after comparison of TLC patterns. Compound **1** (11.5 mg) was isolated from two of the resulting fractions by preparative TLC using $CHCl_3$/MeOH (95:5) as a mobile phase.

Final purification of compound **1**, prior to bioassay, was achieved with C_{18} semipreparative HPLC (YMC C_{18} ODS, 250 × 20 mm i.d.). The compound was eluted with MeOH/H_2O (8:2) at 67 min with a flow rate of 7 mL/min.

Ardisenone (1): transparent oil; $[\alpha]^{25}_D$ 0° (*c* 0.18, MeOH); IR ν max (film on AgCl plate) 3385 br, 3004, 2929 str, 2854, 1689 br, 1598 str, 1505, 1459, 1347, 1301, 1196, 1158 str, 1061, 996, 844, 681 cm^{-1}; UV (MeOH) λ max (log ϵ) 210 (4.55), 268 (3.85), 320 (3.27) nm; UV (MeOH + NaOH) λ max (log ϵ) 212 (4.71), 234 (4.44), 287 (3.91), 354 (3.33) nm; ^1H and ^{13}C NMR data, see Table 2; EIMS *m/z* (rel int) $[M]^+$ 496 (21), 478 (10), 360 (15), 359 (56), 215 (11), 191 (17), 180 (100), 166 (22), 163 (11), 151 (61), 138 (85), 137 (33), 135 (21), 123 (30), 108 (12); HREIMS *m/z* $[M]^+$ 496.2844 ($C_{30}H_{40}O_6$ requires 496.2825, ΔM −1.9 mmu).

Brine Shrimp Toxicity Assay Procedure. The procedure used was that of Meyer and co-workers[3] with the following modifications: (a) tests were performed in triplicate in test tubes, each with a final test mixture volume of 4 mL; (b) the plant extracts were dissolved in 100% DMSO prior to dilution, and dilutions were added directly to test tubes to give a final concentration of 0.5% DMSO for tubes receiving 10 and 100 μg/mL of extract and 5% DMSO for 1000 μg/mL, (c) negative controls using 0.5% DMSO and 5% DMSO were used, (d) brine shrimp nauplii were hatched in a one-compartment glass beaker that was partially exposed to incandescent light, (e) the assay was performed on nauplii after 24 h, (f) no food was added during the hatching or testing procedures, and (g) the outcome measured was "immobility," results being expressed as "percent immobilized".

Cytotoxicity Assay Procedure. The protocol, based on a colorimetric assay that employs the measurement of sulforhodamine B binding to quantitate cell growth,[7] has been described previously.[8] Extracts, fractions, and isolates were dissolved in 100% DMSO, and aqueous 10% DMSO dilutions were prepared. For each cell line, four concentrations were tested in triplicate, and the control-corrected, averaged values were subjected to nonlinear regression analysis to estimate IC_{50} values.

Acknowledgments. The dereplication procedure was carried out by Mr. James Graham, Dr. Howard Constant, and Dr. Christopher Beecher. The plant collection activities were funded by the University of Illinois at Chicago (UIC) Senior University Scholar Award to N.R.F. We thank Dr. Domingo Madulid as consultant to the plant collection and Dr. Jack Regalado for his assistance in the identification of the plant material. We are also grateful for the fellowship support to F.D.H. from the American Foundation for Pharmaceutical Education and the UIC Graduate College. Finally, we thank the Department of Environment and Natural Resources of the Republic of the Philippines and the Philippine National Museum for permission to collect plant samples on the island of Palawan.

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NP970049R