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Report

16α-Hydroxy-(−)-kauranoic Acid: A Selectively Cytotoxic Diterpene from *Annona bullata*

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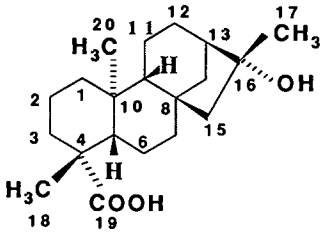
Using brine shrimp lethality to direct fractionation, extracts of the bark of *Annona bullata* Rich. (Annonaceae) have yielded 16α-hydroxy-(−)-kauranoic acid as a bioactive plant constituent. This previously known diterpene showed significant (ED_{50} 8.25×10^{-2} μg/ml) and selective cytotoxicity against A-549 (lung) cells in our panel of human tumor cells.

KEY WORDS: 16α-hydroxy-(−)-kauranoic acid; cytotoxic diterpene; *Annona bullata;* Annonaceae.

INTRODUCTION

As part of our bioassay-directed search for plants containing tumor inhibitory constituents, we previously isolated and characterized and are patenting two extremely potent antitumor acetogenins, named bullatacin and bullatacinone, from the bark of *Annona bullata* Rich. Two less-active compounds, liriodenine and (−)-kaur-16-en-19-oic acid, were also isolated (1,2). In the present report, we describe from this plant material the isolation, identification, and potent cytotoxicity of 16α-hydroxy-(−)-kauranoic acid (1), a diterpene new to *Annona* species.



(1)

Scheme 1

MATERIALS AND METHODS

Plant Material

Bark of *A. bullata* Rich. (M-06983, PL-103509) was collected at the USDA Subtropical Horticulture Research Station, Miami, Florida. The material was authenticated by Edward Garvey of the USDA. The tree originated from seeds collected in Cuba in 1933 by Robert M. Grey of Harvard University.

Instrumentation

Melting point (mp) determinations were made on a Mettler FP5 and were uncorrected. Optical rotation determinations were made on a Perkin Elmer 241 polarimeter. Infrared (ir) spectra were obtained in KBr on a Beckman IR-33. ^{1}H-NMR (proton nuclear magnetic resonance) and ^{13}C-NMR spectra were recorded on a Chemagnetics A-200. Low-resolution mass spectra (ms) were obtained on a Finnigan 400 and high-resolution ms were determined on a Kratos ms 50 through peak matching.

Bioassays

The extracts, fractions, and isolated compound were routinely evaluated for lethality to brine shrimp larvae (BST) (3). Pure compound 1 was tested in the potato disk assay for inhibition of crown gall tumors (4). Cytotoxicity tests were performed at the Purdue Cell Culture Laboratory, Purdue Cancer Center, using standard protocols for 9PS (a chemically induced murine lymphocytic leukemia), 9ASK (astrocytoma reversal), A-549 (human lung carcinoma), MCF-7 (human breast carcinoma), and HT-29 (human colon adenocarcinoma).

Isolation of Compound 1

The pulverized bark (3.9 kg) was extracted as previously described (1,2) by exhaustive percolation with 777 liters of EtOH. Vacuum evaporation left 380 g of syrupy residue (FOO1). FOO1 was partitioned between $CHCl_3/H_2O$ (1:1), and the H_2O solubles were freeze-dried and labeled FOO2 (11 g). The $CHCl_3$ solubles were vacuum evaporated to form FOO3 (181 g). The insoluble interface was air-dried (188 g) and labeled FOO4. Then FOO3 was partitioned between hexane/90% aq. MeOH (1:1). The 90% MeOH fraction

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was vacuum evaporated to a thick syrup (156 g) and labeled FOO5. The hexane residue (25 g) was labeled FOO6. The bioassay data of FOO1-FOO6, including BST, 9PS, 9KB, 9ASK, protein kinase C tests, and pesticidal activity tests, showed clearly that activities had partitioned into FOO5 (1).

FOO5 (80 g) was adsorbed onto 100 g of Celite and applied to a column of Si gel (3 kg) packed in a slurry of hexane. A gradient of hexane–$CHCl_3$–MeOH was used to elute the column, collecting 82 fractions of 100–200 ml each. Fractions were combined into pools according to their similar tlc (thin-layer chromatography) patterns [$CHCl_3$–MeOH (9:1) on Si gel, phosphomolybdic acid spray], weighed, and bioassayed by the BST.

The second most active pool, 51–60 (15 g) (BST LC_{50} = 0.0258 ppm, 0.04/0.02 ppm), was subjected to another Si gel (230–400 mesh, 600 g) column chromatograph, eluting by a gradient of $CHCl_3$–EtOAc–MeOH. An active pool of fractions 30–60, which was separated from the active acetogenins, yielded a white powder (150 mg) which was recrystallized twice (EtOAc) and characterized as the diterpene, 16α-hydroxy-(−)-kauranoic acid (1).

Acetylation

Five milligrams of 1 was mixed with 0.5 ml of anhydrous pyridine and 1 ml of acetic anhydride at 60°C for 3 days. Ice water was added, and the mixture was partitioned with $CHCl_3$. The $CHCl_3$ layer was dried using anhydrous sodium carbonate and vacuum evaporated to give a white oil which was one single spot on tlc and identified as 1 acetate.

Characterization of Compound 1

Mp 275–280°C, reported mp 275–279°C (7). $[a]_D$ = −110.4 (c, 0.0045 g/ml, abs. EtOH), reported $[a]_D$ = −92° (5). Cims (isobutane) m/z 321 (MH^+), 303 ($MH^+ - H_2O$), 257 ($MH^+ - H_2O$ − HCOOH); Cims (NH_3) m/z 338 (M + NH_4^+), 320 ($MNH_4^+ - H_2O$); exact mass (eims) 320.2357 for $C_{20}H_{32}O_3$ (cal. 320.2343). Ir (KBr) cm^{-1}: 3460, 3600–2600 (br), 1701. ^{1}H-NMR (pyridine-d_5, 200 MHz) δ ppm: 1.19 (s, CH_3–C10), 1.33 (s, CH_3–C4), 1.54 (s, CH_3–C16), 5.30 (br. OH), 0.7–2.5 (the rest of the protons). ^{13}C-NMR (pyridine-d_5, 50 MHz) δ ppm: 42.77 (t, C1), 19.90 (t, C2), 38.10 (t, C3), 43.96 (s, C4), 57.10 (d, C5), 22.96 (t, C6), 41.12 (t, C7), 45.64 (s, C8), 56.40 (d, C9), 40.09 (s, C10), 18.71 (t, C11), 27.32 (t, C12), 49.24 (d, C13), 38.80 (t, C14), 58.60 (t, C15), 77.94 (s, C16), 25.09 (q, C17), 29.40 (q, C18), 180.16 (s, C19), 16.09 (q, C20).

Characterization of 1 Acetate

Cims (isobutane): 363 (MH^+), 303 (MH^+ − HAc), 257 (303 − HCOOH). ^{1}H-NMR (200 MHz, pyridine-d_5): δ ppm: 1.19 (s, CH_3–C10), 1.33 (s, CH_3–C4), 1.70 (s, CH_3–C16), 1.93 (s, CH_3CO–).

Biological Activities of 1 and 1 Acetate

Compound 1 was active in the brine shrimp lethality test (BST) (LC_{50} = 25.69 ppm, 95% confidence interval 59.7/10.2 ppm), showed 46.73% inhibition of crown gall tumors on potato disks and gave cytotoxicities in 9PS (ED_{50} = 8.25 × 10^{-2} μg/ml), A-549 (ED_{50} = 8.60 × 10^{-2} μg/ml), MCF-7 (ED_{50} = 10.41 μg/ml), HT-29 (ED_{50} = 4.16 × 10^{-1} μg/ml), and 9ASK (cytotoxic with slight reversal at 100 μg/ml).

1 acetate is active in the BST (LC_{50} = 61 ppm, 95% confidence interval 358/25 ppm) and less cytotoxic in A549 (ED_{50} = 6.42 μg/ml), MCF-7 (ED_{50} = 9.36 μg/ml), and HT-29 (ED_{50} = 8.72 μg/ml).

As a positive control in the human tumor-cell cytotoxicity assay, adriamycin gave values in A-549 (ED_{50} = 1.56 × 10^{-2} μg/ml), MCF-7 (ED_{50} = 7.22 × 10^{-2} μg/ml), and HT-29 (ED_{50} = 3.95 × 10^{-2} μg/ml).

RESULTS AND DISCUSSION

16α-Hydroxy-(−)-kauranoic acid (1) was isolated from the EtOH extract of the *A. bullata* bark by consecutive partitions of the EtOH extract followed by repeated BST-directed column chromatography and recrystallization to yield a white powder with mp 275–280°C. Its molecular weight of 320 was obtained by cims (isobutane) at m/z 321 (MH^+) and cims (NH_3) at m/z 320 (M + NH_4^+ − H_2O) and 338 (M + NH_4^+). High-resolution eims at 320.2357 provided the molecular formula of $C_{20}H_{32}O_3$ (cal. 320.2343). The loss of 18 mu from 321 to 303 in the cims (NH_3) suggested the presence of an OH group. The consecutive loss of 46 mu from 303 to 257 suggested the presence of –COOH. The cims (isobutane) of the acetate derivative showed a peak at m/z 363, indicating acetylation of the OH group and confirming the molecular weight of 320. Peaks in the ir spectrum at 3460 cm^{-1}, 3600–2600 cm^{-1} (broad), and 1701 cm^{-1} confirmed the above functionalities. Three singlets in the ^{1}H-NMR spectra at 1.19, 1.33, and 1.54 ppm suggested the presence of three CH_3 groups. Analysis of the fully decoupled ^{13}C-NMR and the fully coupled ^{13}C-NMR in pyridine led to the identification of the structure as 16α-hydroxy-(−)-kauranoic acid (1) (5).

16α-Hydroxy-(−)-kauranoic acid (1) was first isolated in 1968 from *Fusarium moniliforme* as a fermentation product (6). The only plant in the Annonaceae from which this compound has been previously isolated is *Xylopia acutiflora* (7). To our knowledge, there is no bioactivity previously reported in the literature for this compound. In our in-house bioassays, however, 1 was significantly active in the brine shrimp lethality test (BST) (LC_{50} = 25.69 ppm, 95% confidence interval 59.7/10.2 ppm) and showed cytotoxicities in 9PS, A-549, MCF-7, HT-29, and 9ASK (cytotoxic with slight reversal at 100 μg/ml). The results indicate that compound 1 is not generally cytotoxic but selectively cytotoxic to some cell lines such as the chemically induced murine lymphocytic leukemia (9PS, ED_{50} = 8.25 × 10^{-2} μg/ml), human lung carcinoma (A-549, ED_{50} = 8.60 × 10^{-2} μg/ml), and human colon adenocarcinoma (HT-29, ED_{50} = 4.16 × 10^{-1} μg/ml); it is much less active against the human breast carcinoma (MCF-7, ED_{50} = 10.41 μg/ml). Interestingly, the acetylation of 1 reduced the activity for BST and cytotoxicities for all three cell lines, e.g., from 8.6 × 10^{-2} to 6.42 μg/ml for A-549. This indicates that the hydroxyl group in 1 is very important for its cytotoxicity.

In addition, compound 1 showed strong inhibition (46.73%) of crown gall tumors on potato disks, which is predictive of 3PS (P388) *in vivo* murine antileukemic activity (4).

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