Bioactive *ent*-Kaurene Diterpenoids from *Annona senegalensis*

Majekodunmi O. Fatope and Oladimeji T. Audu

Department of Chemistry, Bayero University, PMB 3011, Kano, Nigeria

Yoshio Takeda

Faculty of Arts and Sciences, University of Tokushima, Minamijosanjimacho 1-Chome, Tokushima 770, Japan

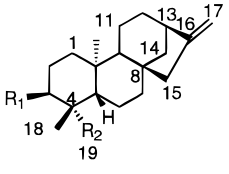
Lu Zeng, Guoen Shi, Hiroko Shimada, and Jerry L. McLaughlin*

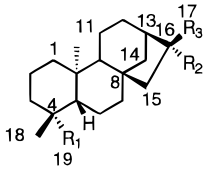
Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana 47907

Received August 10, 1995[⊗]

Activity-guided fractionation of the stem bark of *Annona senegalensis* gave four bioactive *ent*-kaurenoids (**1**−**4**). Compound **2** showed selective and significant cytotoxicity for MCF-7 (breast cancer) cells (ED_{50} 1.0 μg/mL), and **3** and **4** exhibited cytotoxic selectivity for PC-3 (prostate cancer) cells but with weaker potencies (ED_{50} 17−18 μg/mL). The structure of the new compound, **3**, was deduced from spectral evidence.

Annona senegalensis Pers. (Annonaceae), "Gwander Daaji" in Hausa, is a fruit-bearing savanna shrub.[1] It is cultivated in tropical Africa by seeds, coppicing, or root suckers. In northern Nigeria the ripe fruits are eaten. The inhabitants of the savanna belt of Nigeria use the shoots as sign posts and supports for thatched roofs because of their resistance to termites. In other cultures, the seeds are used to control lice,[2] the stem bark to kill intestinal worms,[3] and the root bark to treat cancer.[4] Various parts of *A. senegalensis* have been demonstrated to have antimalarial,[5] antitumor,[4] antitrypanosomal,[6] macrofilaricidal,[7] and insect-growth regulatory[8] properties. The reported chemical components of *A. senegalensis* include tannins and free amino acids from the stem bark,[9] *ent*-kaurenoids from the root bark,[10,11] essential oils[12] and (−)-roemerine[13] from the leaves, and acetogenins from the seeds.[14] Considering the contribution of *A. senegalensis* to herbal medicine and agricultural practices in Sub-Sahelian Africa, we have reinvestigated the stem bark of the plant for potential pesticidal and antitumor compounds. The current report describes the isolation and structural elucidation of four bioactive *ent*-kaurene diterpenoids (**1**−**4**).





1 R_1 = OH, R_2 = CH_3 **3** R_1 = CO_2H, R_2 = OAc, R_3 = CH_2OAc

2 R_1 = H, R_2 = CO_2H **4** R_1 = CO_2Me, R_2 = H, R_3 = CO_2H

The 10% aqueous MeOH-partitioned fraction of the $CHCl_3$ extract of the stem bark showed significant lethality (LC_{50} < 1.0 μg/mL) in the brine shrimp lethality test (BST).[15,16] It was also cytotoxic to several human solid tumor cell lines[17−21] at ED_{50} values of < 10^{-2} μg/mL (see Experimental Section). Repeated chromatography of the aqueous MeOH- and hexane-partitioned extracts established *ent*-kaurene diterpenoids as major chemical components and acetogenins as minor components. The structures and bioactivities of the Annonaceous acetogenins are well established,[14,22,23] but only a few reports can be found in the literature[24,25] regarding the bioactivities of the *ent*-kaurene diterpenes, a significant chemical type within the Annonaceae.[26] Bioactivity-directed fractionation of the hexane and the aqueous MeOH-soluble extracts on Si gel gave the bioactive *ent*-kaurenoids **1**−**4**. Acetogenins were detected, and are being studied, as components of the more active and more polar fractions.

Compounds **1**, **2**, and **4** were identified by comparison of their NMR data with those in the literature.[11,18,27] The bioactive *ent*-kaurenoid (**3**) showed spectral properties similar to those of **2**, with the exception that, in the ^{13}C-NMR spectra (Table 1), the signals for the terminal double bond were replaced by those attributable to C-17 oxymethylene and C-16 carbinyl groups. As expected, the signals that are higher than δ 2.43 in the ^{1}H NMR of **3** arose from the resonance of an AB system at δ 4.41 and 4.37 (J = 12.5 Hz). These signals were assigned to the two hydrogens at C-17. The value of the chemical shifts and the presence of two acetate signals at δ 2.08 and 2.04 (each 3H, s) established C-16 and C-17 as the attachment sites for the acetate esters. Compound **3**, a diacetate ester, has a molecular formula of $C_{24}H_{36}O_6$ as supported by the NMR data. This finding was substantiated by a molecular ion peak at m/z 421 $(MH)^+$ in the CIMS and by peaks at m/z 361 and 301 corresponding to successive losses of acetic acid from the molecular ion. HR CIMS confirmed the molecular formula, M^+ 421.2594 (found), 421.2590 (calcd). Compound **3** is a new natural product.

Compound **2** was active in the brine shrimp test (BST LC_{50} 16 μg/mL), and it was significantly selective for MCF-7 (breast cancer, ED_{50} 1.0 μg/mL) cells among six human solid tumor cell lines.[17−21] Compounds **3** and **4** were not significantly cytotoxic to this variety of human tumor cells (ED_{50} > 20 μg/mL); however, for PC-3 (prostate cancer) cells, modest ED_{50} values of 17.71 μg/mL (**3**) and 17.38 μg/mL (**4**) were observed. Compared

[⊗] Abstract published in *Advance ACS Abstracts,* March 1, 1996.

0163-3864/96/3259-0301$12.00/0

Table 1. ^{13}C-NMR ($CDCl_3$, δ) Spectral Data of *ent*-Kaurene Diterpenes **3** and **4**

position	**3**	**4**
1	40.7	40.7
2	19.1	19.2
3	37.8	38.1
4	41.2	43.9
5	56.0	57.0
6	20.9	22.2
7	39.7	41.7
8	43.7	44.5
9	56.8	56.2
10	41.6	40.0
11	19.1	18.3
12	27.0	30.0
13	43.3	45.3
14	50.6	39.8
15	37.2	39.4
16	87.0	27.4
17	66.2	179.8
18	28.9	28.8
19	183.7	178.1
20	15.4	15.4
Me(Ac)	21.8	
	21.6	
C=O	170.7	
	171.0	
CO_2Me		51.4

with the biological activities of the acetogenin-containing fraction (see Experimental Section), the fractions that are rich in these *ent*-kaurene diterpenoids were significantly less bioactive. The diterpene **2**, nevertheless, demonstrated a significant cytotoxic selectivity that would serve to justify further investigations into the structure−activity relationships among this class of compounds.

Experimental Section

General experimental procedures: melting points Mel-Temp apparatus, uncorrected; column chromatography, Si gel (Merck, 60−200 mesh); TLC, Si gel (Whatman, K6F 60° A, viewed both under a UV lamp and by spraying with 5% phosphomolybdic acid in EtOH); HPLC, Rainin system equipped with Dynamax software, a Rainin UV-detector (set at 220 nm), normal phase 250 × 21-mm Si gel column, eluted with a gradient of hexane−MeOH−THF; optical rotations, Perkin-Elmer 241 polarimeter; IR spectra, Perkin-Elmer 1600 FT IR spectrometer; UV spectrra, Beckman DU-7 spectrometer; ^1H- and ^{13}C-NMR spectra, Varian VXR-500S or JEOL GSX-400 spectrometers; FABMS, JEOL D-300 spectrometer; CIMS, Finnigan 4000 spectrometer; HRCIMS, Kratos 50 spectrometer.

Plant Material. The bark of *A. senegalensis* was collected at Yelwa village in Bebeji Local Government area of Kano State, Nigeria, in January 1994. The plant was identified by Mr. Ali Garko and Dr. Y. Karatella of the Department of Biological Sciences, Bayero University, Kano. A voucher sample of the plant is deposited in the Herbarium (Herbarium No. 5) of Bayero University, Kano, Nigeria. The stem bark was air-dried and milled.

Bioassays. The brine shrimp lethality test (BST) was performed according to standard protocols,[15,16] and LC_{50} values, in μg/mL, were determined for partitioned fractions, pooled chromatographic fractions, and the isolated compounds. Certain fractions and pure compounds were also tested for cytotoxicity in a panel of six human solid tumor cell lines at the Purdue Cancer Center, using a 7-day MTT assay and standard protocols for A-549 (lung carcinoma),[17] MCF-7 (breast carcinoma),[18] HT-29 colon (adenocarcinoma),[19] A-498 (kidney carcinoma),[17] PC-3 (prostate adenocarcinoma),[20] and PACA-2 (pancreatic carcinoma)[21] with Adriamycin as a positive control.

Extraction and Isolation. The pulverized plant material (824 g) was extracted by percolation with $CHCl_3$ (4 × 2.0 L) and concentrated on a rotavapor at 35 °C to give a residue (67.2 g, BST LC_{50} 8.3 μg/mL). This residue was dissolved in 10% aqueous MeOH (500 mL), partitioned with hexane (500 mL), and separated into fractions that were individually evaporated to obtain the hexane (18.5 g), the 10% aqueous MeOH (34.53 g), and the MeOH−hexane interphase (11.7 g) extracts. The MeOH-soluble extract gave the following biological activities: BST, LC_{50} < 1.0 μg/mL; A-549, ED_{50} < 10^{-2} μg/mL; MCF-7, ED_{50} < 10^{-2} μg/mL; HT-29, ED_{50} 1.0 μg/mL; A-498, ED_{50} < 10^{-2} μg/mL; PC-3, ED_{50} < 10^{-2} μg/mL, and PACA-2, ED_{50} < 10^{-2} μg/mL; Adriamycin gave the following ED_{50} values (μg/mL) in the same run (A-498 1×10^{-2}, MCF-7 1.85×10^{-1}, HT-29 4.37×10^{-2}, PC-3 2.23×10^{-2}, and PACA-2 2.05×10^{-2}). A portion of the hexane-soluble fraction (5.1 g) was subjected to column chromatography on Si gel (150 g) and eluted with hexane and a gradient of hexane−Et_2O. Fractions were combined on the basis of their TLC patterns and bioactivity in the BST assay. A BST-active fraction (150 mg, BST LC_{50} 101 μg/mL) eluted with hexane was identified as **1**. A portion of the MeOH-soluble fraction (30.0 g) was fractionated on an open column of Si gel (250 g), eluting with hexane and a gradient of hexane-EtOAc, hexane−EtOAc−MeOH, and CH_2Cl_2−EtOAc−MeOH. Eluents were pooled into six fractions based on TLC and BST analyses. Fraction 1 (2.23 g, BST LC_{50} < 30 μg/mL), eluted with hexane−EtOAc (9.5:0.5), fraction 2 (1.69 g, BST LC_{50} 48 μg/mL), eluted with hexane−EtOAc (9.1), and fraction 4 (1.06 g, BST LC_{50} < 1.0 μg/mL), eluted with hexane−EtOAc−MeOH (1:0.95:0.05), were separately rechromatographed on Si gel columns. The bioactive components of fraction 1 were eluted with hexane−$CHCl_3$ (1.95:0.05) and further purified on HPLC to give **2** (31.0 mg), A-549 (lung), ED_{50} 13.59 μg/mL; MCF-7 (breast), ED_{50} 1.0 μg/mL; HT-29 (colon) ED_{50} 31.39 μg/mL; A-498 (kidney) ED_{50} 47.97 μg/mL; PC-3 (prostate) ED_{50} 15.19 μg/mL, and PACA-2 (pancreas) ED_{50} 22.13 μg/mL; Adriamycin, as a positive control, gave ED_{50} (μg/mL) values of 5.06×10^{-3} (lung), 1.52×10^{-1} (breast), 1.11×10^{-2} (colon), 3.67×10^{-2} (kidney), 2.25×10^{-2} (prostate), and 3.79×10^{-3} (pancreas) in the same run. Hexane−$CHCl_3$ (1.6:0.4) similarly eluted with bioactive components of fraction 2 from which **4** (14.3 mg) and **3** (18.8 mg) were isolated after purification on HPLC. Fraction 4 (BST LC_{50} 0.35 μg/mL) was a mixture of acetogenins that coeluted with a brownish material on column chromatography, TLC, and HPLC in neutral or acidic solvent systems.

***ent*-3β-Hydroxykaur-16-ene (1) and *ent*-Kaur-16-en-19-oic Acid (2).** The spectral properties of **1** and **2** were identical with those previously reported.[27−29]

***ent*-16,17-Diacetoxykauran-16-oic acid (3):** mp 185−186 °C; $[\alpha]^{25}_D$ −60.5° (*c* 1.5, $CHCl_3$); UV λ (MeOH) max (log ϵ) 225 (2.53) nm; IR (film) ν max 1733, 1701,

1470, 1369, 1258, 1035 cm^{-1}; CIMS (isobutane) m/z $(MH)^+$ 421 (3), 362 (22), 361 (100), $(MH - HOAc)^+$, 315 (5), 301 (40), $(MH - 2HOAc)^+$; HRCIMS m/z 421.2594 for $C_{24}H_{36}O_6$ (calcd 421.2590); ^1H NMR ($CDCl_3$, 500 MHz), δ 4.41 (1H, d, $J = 12.5$ Hz, 17a), 4.37 (1H, d, $J =$ 12.5 Hz, 17b), 2.43 (1H, m, H-13), 2.08 (3H, s, OAc), 2.04 (3H, s, OAc), 1.23 (3H, s, H-18), 0.94 (3H, s, H-20), 2.20−0.80 (the rest of the protons). ^{13}C NMR, see Table 1.

***ent*-19-Carbomethoxykauran-19-oic acid (4):** $[\alpha]^{25}_D$ −74.4° (*c* 0.86, $CHCl_3$); UV λ (MeOH) max (log ϵ) 225 (2.53) nm; IR (film) ν max 1723, 1699, 1233 cm^{-1}; CIMS (isobutane) m/z $(MH)^+$ 349 (100), 335 (12), 298 (33), 123 (13); HRCIMS m/z 349.2372 for $C_{21}H_{32}O_4$ (calcd 349.2379); ^1H NMR ($CDCl_3$, 500 MHz), δ 9.75 (1H, s, $-CO_2H$), 3.65 (3H, s $-CO_2Me$), 2.95 (1H, dt, $J = 6.0$ Hz, H-16), 2.57 (1H, m, H-13), 1.16 (3H, s, H-18), 0.81 (3H, s, H-20) 2.20−0.76 (the rest of the protons). ^{13}C-NMR, see Table 1. **4** was previously reported but without complete spectral data.[11]

Acknowledgments. M. O. F. is grateful to the International Foundation for Science, Stockholm, for a grant (F/2018-1) and to USIS for a Fulbright Research Fellowship. This investigation was supported in part by RO1 grant CA 30909 from the National Cancer Institute, National Institutes of Health. The authors are grateful to Mr. Ali Garko and Dr. Y. Karatela of the Biological Sciences Department, Bayero University, Kano, for the identification of the plant material and to the Cell Culture Laboratory, Purdue Cancer Center, for the cytotoxicity data.

References and Notes

(1) Hutchison, J.; Dalziel, J. M. In *Flora of West Tropical Africa*; Keay, R. M. J., Ed.; Crown Agents: London, 1954; Vol. 1, p 51.
(2) Hirschmann, G. S.; De-Arias, A. R. *J. Ethnopharmacol.* **1990**, *29*, 159−172.
(3) Weiss, E. A. *Econ. Bot.* **1979**, *33*, 35−51.
(4) Durodola, J. T. *Planta Med.* **1975**, *28*, 32−36.
(5) Balansard, G.; Timan-David, P. Patent-France Demande, 2 550 093, Feb 8, 1985; *Chem. Abstr.* **1985**, *103*, 42666x.
(6) Igwe, A. C.; Onabanjo, A. O. *Ann. Trop. Med. Parasitol.* **1989**, *83*, 527−534.
(7) Comley, J. C. W. *Trop. Med. Parasitol.* **1990**, *41*, 1−9.
(8) Jacobson, M.; Redfern, R. E.; Mills, G. D., Jr. *Lloydia* **1975**, *38*, 455−472.
(9) Bamba, D.; Balansard, G.; Maillard, C.; Dumenil, G.; Gayte-Sorbier, A. *Plantes Med. Phytother.* **1984**, *18*, 36−45.
(10) Eshiet, I. T. U.; Akisanya, A.; Taylor, D. A. H. *Phytochemistry* **1971**, *10*, 3294−3295.
(11) Adesogan, E. K.; Durodola, J. I. *Phytochemistry* **1976**, *15*, 1311−1312.
(12) Ekundayo, E.; Oguntimein, B. *Planta Med.* **1986**, *52*, 202−204.
(13) You, M.; Wickramaratne, M. D. B.; Silva, G. L.; Chai, H.; Chagwedera, T. E.; Farnsworth, N. R.; Cordell, G. A.; Kinghorn, A. D.; Pezzuto, J. M. *J. Nat. Prod.* **1995**, *58*, 598−604.
(14) Sahpaz, S.; Laurens, A.; Hocquemiller, R.; Cave, A.; Cortes, D. *Can. J. Chem.* **1994**, *72*, 1533−1536.
(15) Meyer, B. N.; Ferrigni, N. R.; Putnam, J. E.; Jacobson, L. B.; Nichols, D. E.; McLaughlin, J. L. *Planta Med.* **1982**, *45*, 31−34.
(16) McLaughlin, J. L. In *Methods in Plant Biochemistry*; Hostettmann, K., Ed.; Academic Press: London, 1991; Vol. 6, pp 1−33.
(17) Giard, D. J.; Aaronson, S. A.; Todaro, G. J.; Arnstein, P.; Kersey, J. H.; Dosik, H.; Parks, W. P. *J. Natl. Cancer. Inst.* **1973**, *51*, 1417−1423.
(18) Soule, H. D.; Vazquez, J.; Long, A.; Albert, S.; Brennan, M. *J. Natl. Cancer Inst.* **1973**, *51*, 1409−1416.
(19) Fogh, J.; Trempe, G. In *Human Tumor Cells in Vitro*; Fogh, J. Ed.; Plenum Press: New York, 1975, p 115.
(20) Kaighn, M. E.; Narayan, K. S.; Ohnuki, Y.; Lechner, J. F.; Jones, L. W. *Invest. Urol.* **1979**, *17*, 16−23.
(21) Yunis, A. A.; Arimura, G. K.; Russin, G. K. *Int. J. Cancer* **1977**, *19*, 128−135.
(22) Rupprecht, J. K.; Hui, Y.-H.; McLaughlin, J. L. *J. Nat. Prod.* **1990**, *53*, 237−278.
(23) Fang, X.-P.; Reiser, M.; Gu, Z.-M.; Zhao, G.-X.; McLaughlin, J. L. *Phytochem. Anal.* **1993**, *4*, 27−48 and 49−67.
(24) Hui, Y.; Chang, C.; Smith, D. L.; McLaughlin, J. L. *Pharm. Res.* **1990**, *7*, 376−378.
(25) Sung, H.-D.; Lin, Z.-W.; Niu, F.-D.; Chai, H.-B.; Pezzuto, J. M.; Cordell, G. A. *Phytochemistry* **1995**, *38*, 437−442.
(26) Leboeuf, M.; Cave, A.; Bhaumik, P. K.; Mukherjee, B.; Mukherjee, R. *Phytochemistry* **1982**, *21*, 2783−2813.
(27) Gonalez, A. G.; Fraga, B. M.; Hernadez, M. G.; Hanson, J. R. *Phytochemistry* **1981**; *20*, 846−847.
(28) Hutchison, M.; Lewer, P.; MacMillan, J. *J. Chem. Soc., Perkin Trans. 1* **1984**, 2363−2366.
(29) Yamasaki, K.; Kohda, H.; Kobayashi, T.; Kasai, R.; Tanaka, O. *Tetrahedron Lett.* **1976**, 1005−1008.

NP9601566