New 7,20:14,20-Diepoxy *ent*-Kauranoids from *Isodon xerophilus*

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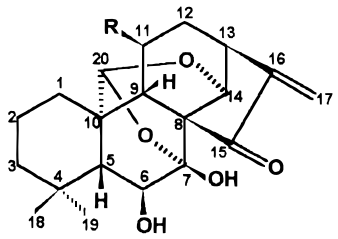
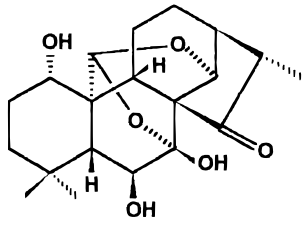
Three new 7,20:14,20-diepoxy-*ent*-kaurane diterpenoids, xerophilusins A−C (**1**−**3**), together with a known one, macrocalin B (**4**), were isolated from the leaves of *Isodon xerophilus*. Their structures were elucidated on the basis of their spectral properties and X-ray crystallographic analysis. Compounds **1**, **2**, and **4** showed significant cytotoxic activity against K562, HL-60, and MKN-28 cells.

Isodon species are rich in *ent*-kaurane diterpenoids, some of which have antitumor, antibacterial, and antiinflammatory activities.[1−5] In our continuing studies on bioactive diterpenoids from this genus,[6−10] the EtOAc extract of *Isodon xerophilus* (C. Y. Wu et H. W. Li) H. Hara (Labiatae) demonstrated significant inhibitory effect on K562, HL-60, HCT, and MKN-28 cells. Phytochemical investigation on this plant, a perennial shrub native to Yunnan province and not studied with respect to its chemical constituents to date, has led to isolation of four compounds, including three new *ent*-kauranoids (xerophilusins A−C, **1**−**3**). Compounds **1**−**3** have two unique epoxy units formed by ether bridges from C-20 to C-7 and C-14, and only two diterpenoids with similar skeletons were reported previously.[11,12] Compounds **1**, **2**, and a known compound (**4**) exhibited significant cytotoxicities against K562, HL-60, and MKN-28 cells and slightly inhibited the growth of HCT cells. We herein present the structure elucidation of **1**−**3** and the cytotoxic activity of **1**, **2**, and **4**.



1 R = OAc
2 R = H
4 R = OH



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Results and Discussion

The 70% aqueous acetone extract prepared from the leaves of *I. xerophilus* was partitioned between EtOAc and H_2O and then *n*-BuOH and H_2O. Repeated column chromatography (CC) of the EtOAc layer on silica gel and RP-18 yielded xerophilusins A−C (**1**−**3**) and macrocalin B (**4**).[12]

Xerophilusin A (**1**) was obtained as an amorphous powder and gave *m*/*z* 405.1983 $[M + H]^+$ ($C_{22}H_{29}O_7$) by positive HRFABMS. It contained an acetoxyl group as deduced from its NMR signals: δ 1.79 (3H, s) in the ^{1}H NMR spectrum, δ 170.0 (s) and 21.3 (q) in the ^{13}C NMR spectrum. The presence of a carbonyl group conjugated with an exocyclic methylene was suggested by the following spectral data: UV (MeOH) λ_{max} (log ϵ) 234.5 (3.68) nm; IR (KBr) ν_{max} 1712 and 1646 cm^{-1}; ^{1}H NMR δ 5.47 and 5.95 (each 1H, s); ^{13}C NMR δ 117.6 (t), 150.0 (s), and 199.1 (s). Also observed in the ^{13}C NMR and DEPT spectra of **1** were four quaternary carbons, including a ketalic one at δ 101.0, an acetal group at δ 98.7, six methines including three oxygenated ones at δ 72.7, 67.9, and 70.0, four methylenes, and two tertiary methyl groups. All these data were consistent with an *ent*-kaur-16-en-15-one diterpenoid having two ether linkages at C-20. In the ^{1}H NMR spectrum, absence of a C-20 methyl signal and a sharp 1H singlet at δ 5.13 and a 1H doublet at δ 4.53 provided further evidence for the proposed structure. Comparison of the above spectra with those of macrocalin (**4**)[12] confirmed the presence of two epoxy units and established the 7β-hydroxy-7α,20:14α,20-diepoxy-*ent*-kaur-16-en-15-one skeleton that was substituted by one acetoxyl and one hydroxyl.

^{1}H−^{1}H COSY and HMQC experiments showed signals at δ 3.71 and 5.06 (H-6 and H-11), indicating that the hydroxyl and the acetoxyl are at C-6 and C-11, respectively. The HMBC spectrum showed long-range couplings between H-20 and C-7 and H-20 and C-14, which confirmed the acetal at C-20 (Figure 1). The orientation of 6-OH and 11-OAc could be determined by the signals of H-6 and H-11. For a 7α,20:14α,20-diepoxy-*ent*-kauranoid, a distorted boat form of the B-ring results in a ca. 90° dihedral angle between H-6α and H-5β, and a chairlike conformation of the C-ring[11] causes the dihedral angles of H-11α with H-12β and H-11α with H-9β to be both ca. 90°. Thus, when the substituents at C-6 and C-11 are β-oriented, H-6α exhibits a narrow doublet, and H-11α shows a broad doublet owing to the coupling with H-12α. The signals of H-6 (1H, d, J = 1.6 Hz) and H-11 (1H, d, J = 5.7 Hz) thus confirmed the β-orientation of 6-OH and 11-OAc. The stereochemistry of H-6 and H-11 was also verified by the following ROESY correlations: H-6α with Me-18, 19; H-11α with H-1α, β; H-11α with H-12α (Figure 2). Thus, xerophilusin A (**1**) was concluded to be 6β,7β-dihydroxy-11β-acetoxy-7α,20:14α,20-diepoxy-*ent*-kaur-16-en-15-one.

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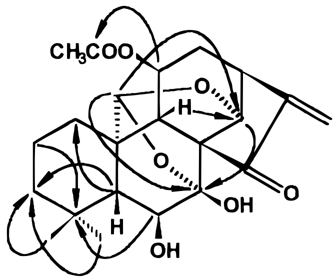

Figure 1. Selected HMBC correlations of **1**.

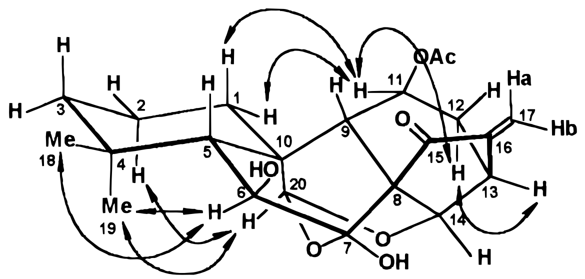

Figure 2. Key ROESY correlations of **1**.

Table 1. Cytotoxic Activity of Compounds **1**, **2**, and **4**

		IC50 (μg/mL)			
test substance	MW	K562	HL-60	HCT	MKN-28
1	404	2.22	0.45		0.12
2	346	0.73	0.29		0.17
4	362	8.07	2.05		1.68
mitoxantrone	444.5	0.0334	0.29	1.54	0.02

Xerophilusin B (**2**), $C_{20}H_{26}O_5$ (HRFABMS), differed from **1** only in the lack of one acetoxyl group. Comparison of ^{1}H and ^{13}C NMR spectra revealed that the signals at δ 5.06 (H-11α) and δ 67.9 (C-11) in **1** were replaced by the ones at δ 1.61 (H-11α), 1.51 (H-11β), and δ 17.3 (C-11) in **2**, respectively. Noticeable upfield shifts of C-9 ($\Delta\delta$ −7.2 ppm) and C-12 ($\Delta\delta$ −6.5 ppm) in **2** were also observed. These differences indicated that **2** was the 11-deacetoxylated compound of **1**. Thus, xerophilusin B (**2**) was characterized as 6β,7β-dihydroxy-7α,20:14α,20-diepoxy-*ent*-kaur-16-en-15-one.

Xerophilusin C (**3**), $C_{20}H_{28}O_6$, was obtained as colorless prismatic crystals. Being a minor constituent in the leaves of *I. xerophilus* and having a qualified crystal quality, it was subjected to X-ray crystallographic analysis directly. The final atomic parameters of the non-hydrogen atoms are listed in Table 1 of the Supporting Information. The stereostructure of **3** (Figure 3) shows eight rings: A (C_1, C_2, C_3, C_4, C_5, C_{10}), B (C_5, C_6, C_7, C_8, C_9, C_{10}), C (C_8, C_9, C_{11}, C_{12}, C_{13}, C_{14}), D (C_8, C_{14}, C_{13}, C_{16}, C_{15}), E (C_5, C_6, C_7, O_4, C_{20}, C_{10}), F (C_7, C_8, C_9, C_{10}, C_{20}, O_4), G (C_7, C_8, C_{14}, O_5, C_{20}, O_4), and H (C_8, C_{14}, O_5, C_{20}, C_{10}, C_9). The conformations of the first four rings were chair, distorted boat, chairlike, and envelope forms, respectively. The E, F, G, and H rings had distorted boat conformations. In conclusion, **3** was a new *ent*-kaurane diterpenoid having the structure 16-(*S*)-methy-1α,6β,7β-trihydroxy-7α,20:14α,20-diepoxy-*ent*-kaur-15-one.

Compounds **1**, **2**, and **4** were tested for their ability to inhibit four kinds of human tumor cells, K562, HL-60, HCT, and MKN-28. IC_{50} (50% inhibitory concentration) values for these compounds are listed in Table 1, in which mitoxantrone is included as a positive reference substance. Compounds **1**, **2**, and **4** showed prominent inhibitory effects on K562, HL-60, and MKN-28 cells with IC_{50} values lower than 10 μg/mL. The IC_{50} value of **2** against HL-60 was equal to that of mitoxantrone (0.29 μg/mL). Compounds **1**, **2**, and **4** were relatively noncytotoxic against HCT cells, having IC_{50} values between 55 and 62 μg/mL.

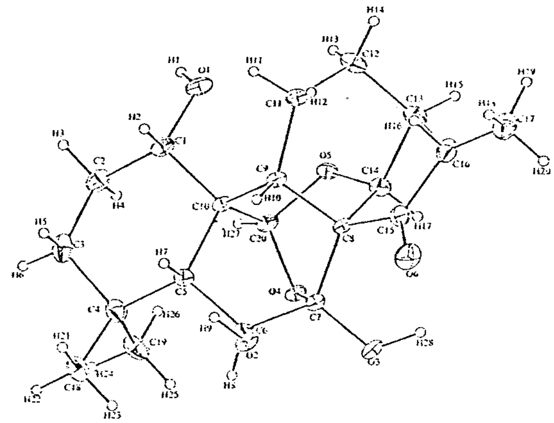

Figure 3. Stereostructure of **3**.

Experimental Section

General Experimental Procedures. All melting points were measured on an XRC-1 micro melting point apparatus and are uncorrected. Optical rotations were taken on a SEPA-300 polarimeter. IR spectra were measured on a Bio-Rad FTS-135 spectrometer with KBr pellets. UV spectra were obtained on a UV 210A spectrometer. MS were recorded on a VG Auto Spec-3000 spectrometer. 1D- and 2D-NMR spectra were run on Bruker AM-400 and DRX-500 instruments with TMS as internal standard, respectively.

Plant Material. The leaves of *I. xerophilus* were collected in Yuanyang prefecture of Yunnan Province in November, 1998, and air-dried. The identity of plant material was verified by Prof. Zhong-Wen Lin, and a voucher specimen (KIB 98-11-25 Lin) is deposited in the Herbarium of the Department of Taxonomy, Kunming Institute of Botany, Academia Sinica.

Extraction and Isolation. The dried and powdered leaves (8.3 kg) were extracted with 70% Me_2CO and filtered. The filtrate was concentrated and extracted with EtOAc and *n*-BuOH successively. A portion (440 g) of the EtOAc extract (660 g) was subjected to column chromatography on a silica gel column eluting with a $CHCl_3$−Me_2CO (1:0−0:1) gradient system to yield **4** (1.0 g) and fractions I−VII. Fraction III was further purified by repeated column chromatography over silica gel (petroleum ether−Me_2CO, 8:2) and RP-18 (MeOH−H_2O, 2:3) to afford **1** (900 mg) and **2** (340 mg). Fraction IV was subjected to column chromatography on silica gel (petroleum ether−Me_2CO, 7:3), RP-18, and MCI-gel CHP-20P (MeOH−H_2O) to give **3** (3 mg).

Xerophilusin A (1): white amorphous powder: $[\alpha]^{23}_D$ −139.4° (*c* 0.57, MeOH); UV (MeOH) λ_{max} (log ϵ) 234.5 (3.68) nm; IR (KBr) ν_{max} 3435, 3205, 2943, 2866, 1735, 1712, 1646, 1495, 1453, 1362, 1239, 1061 cm^{-1}; ^{1}H NMR (acetone-d_6, 400 MHz) δ 1.74 (1H, m, H-1α), 1.43 (1H, m, H-1β), 1.57 (2H, m, H_2-2α, 2β), 1.44 (1H, m, H-3α), 1.24 (1H, m, H-3β), 1.34 (1H, d, J = 1.6 Hz, H-5β), 3.71 (1H, d, J = 1.6 Hz, H-6α), 2.42 (1H, s, H-9β), 5.06 (1H, d, J = 5.7 Hz, H-11α), 2.46 (1H, ddd, J = 13.5, 5.7, 3.8 Hz, H-12α), 1.73 (1H, m, H-12β), 3.21 (1H, m, H-13α), 4.53 (1H, d, J = 6.0 Hz, H-14β), 5.95 (1H, s, H-17a), 5.47 (1H, s, H-17b), 0.94 (3H, s, Me-18), 0.84 (3H, s, Me-19), 5.13 (1H, s, H-20), 1.79 (3H, s, OAc); ^{13}C NMR (acetone-d_6, 100 MHz) δ 28.1 (t, C-1), 19.2 (t, C-2), 41.7 (t, C-3), 34.1 (s, C-4), 63.2 (d, C-5), 72.7 (d, C-6), 101.0 (s, C-7), 56.0 (s, C-8), 52.7 (d, C-9), 44.0 (s, C-10), 67.9 (d, C-11), 32.7 (t, C-12), 39.4 (d, C-13), 70.0 (d, C-14), 199.1 (s, C-15), 150.0 (s, C-16), 117.6 (t, C-17), 31.5 (q, C-18), 23.4 (q, C-19), 98.7 (d, C-20), 170.0 (s, OAc), 21.3 (q, OAc); EIMS m/z 404 $[M]^+$ (45), 386 $[M - H_2O]^+$ (10), 371 $[M - H_2O - Me]^+$ (1), 358 $[M - H_2O - CO]^+$ (7), 344 $[M - AcOH]^+$ (27), 326 $[M - AcOH - H_2O]^+$ (12), 317 (20),

298 (100), 280 (37), 269 (92), 255 (26), 242 (32), 229 (36), 211 (26); positive HRFABMS *m/z* 405.1983 (calcd for $C_{22}H_{29}O_7$, 405.1913).

Xerophilusin B (2): colorless needles (Me_2CO): mp 173−174 °C; $[\alpha]^{22}_D$ −150.0° (*c* 0.46, C_5H_5N); UV (MeOH) λ_{max} (log ϵ) 231 (3.78) nm; IR (KBr) ν_{max} 3445, 3293, 2987, 2946, 2923, 2870, 1727, 1639, 1464, 1445, 1391, 1303, 1244 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) δ 1.68 (1H, d, J = 13.3 Hz, H-1α), 1.02 (1H, m, H-1*β*), 1.38 (2H, m, H_2-2α, 2*β*), 1.34 (1H, d, J = 12.4 Hz, H-3α), 1.11 (1H, dd, J = 3.8, 12.4 Hz, H-3*β*), 1.52 (1H, d, J = 1.5 Hz, H-5*β*), 4.21 (1H, d, J = 1.5 Hz, H-6α), 2.75 (1H, d, J = 7.0 Hz, H-9*β*), 1.61 (1H, dd, J = 12.7, 6.4 Hz, H-11α), 1.51 (1H, overlap, H-11*β*), 2.24 (1H, m, H-12α), 1.37 (1H, m, H-12*β*), 3.10 (1H, m, H-13α), 4.95 (1H, d, J = 6.2 Hz, H-14*β*), 6.17 (1H, s, H-17a), 5.27 (1H, s, H-17b), 1.05 (3H, s, Me-18), 0.91 (3H, s, Me-19), 5.48 (1H, s, H-20); ^{13}C NMR (C_5D_5N, 100 MHz) δ 27.7 (t, C-1), 19.0 (t, C-2), 41.4 (t, C-3), 33.8 (s, C-4), 63.4 (d, C-5), 73.0 (d, C-6), 101.6 (s, C-7), 57.7 (s, C-8), 45.5 (d, C-9), 43.1 (s, C-10), 17.3 (t, C-11), 26.2 (t, C-12), 40.3 (d, C-13), 70.1 (d, C-14), 199.9 (s, C-15), 149.1 (s, C-16), 118.3 (t, C-17), 31.7 (q, C-18), 23.4 (q, C-19), 98.1 (d, C-20); EIMS *m/z* 346 $[M]^+$ (9), 328 $[M - H_2O]^+$ (2), 300 $[M - H_2O - CO]^+$ (100), 282 $[M - H_2O - CO - H_2O]^+$ (9), 271 (68), 259 (23), 239 (2), 229 (4); positive HRFABMS *m/z* 347.1809 (calcd for $C_{20}H_{27}O_5$, 347.1858).

X-ray Crystallographic Analysis of Xerophilusin C (3).[13] A colorless prismatic crystal of $C_{20}H_{28}O_6$ having approximate dimensions 0.50 × 0.40 × 0.20 mm was mounted on a glass fiber. All measurements were made on a Rigaku RAXIS-IV imaging plate area detector with graphite-monochromated Mo Ka radiation (λ = 0.71070 Å).

Cell constants and an orientation matrix for data collection corresponded to a primitive orthorhombic cell with the following dimensions: a = 13.090(2) Å, b = 20.431(3) Å, c = 6.809(4) Å, V = 1820.9600 $Å^3$. For Z = 4 and fw = 364.44, the calculated density is 1.33 g/cm^3. The space group was determined to be $P2_12_12_1$. A total of 1852 reflections were collected. The liner absorption coefficient, μ, for Mo Ka radiation is 1.0 cm^{-1}. The data were corrected for Lorentz and polarization effects. A correction for secondary extinction was applied (coefficient = 4.98550 × 10^{-7}).

The structure was solved by direct methods (SIR92) and expanded using Fourier techniques (DIRDIF94). The nonhydrogen atoms were refined anisotropically. Hydrogen atoms were included but not refined. The final cycle of full-matrix least-squares refinement was based on 1455 observed reflections ($I > 2.00\sigma(I)$) and 236 variable parameters and converged (largest parameter shift was 0.00 times its esd) with unweighted and weighted agreement factors of R = 0.072, R_w = 0.099. The standard deviation of an observation of unit weight was 1.20. The weighting scheme was based on counting statistics and included a factor (p = 0.130) to downweight the intense reflections. Plots of $\sum w(|F_o| - |F_c|)^2$ versus $|F_o|$, reflection order in data collection, sin θ/λ, and various classes of indices showed no unusual trends. The maximum and minimum peaks on the final difference Fourier map corresponded to 0.30 and −0.46 e/$Å^3$, respectively. All calculations were performed using the teXsan crystallographic software package of Molecular Structure Corporation.

Cytotoxicity Against Four Kinds of Human Tumor Cells. An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay was performed in 96-well plates. The assay is based on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to give a blue formazan product that can be measured spectrophotometrically. K562 cells at a log phase of their growth cycle (4 × 10^4 cell/mL) were added to each well (90 μL/well), then treated in four replicates at various concentrations of the drugs (10^{-4}−10^{-8} mol/L), and incubated for 48 h at 37 °C in a humidified atmosphere of 5% CO_2. After 48 h, 10 μL of MTT solution (5 mg/mL) per well was added to each cultured medium, which were incubated for a further 4 h. Then, 10% SDS−5% isobutanol−0.012 mL/L HCl was added to each well (100 μL/well). After 12 h at room temperature, the OD of each well was measured on an ELISA reader (Bioteck EL-340) at two wavelengths (570 and 630 nm). In these experiments, the negative reference agents were isochoric normal saline, 1% DMSO, or 0.1% DMSO, and mitoxantrone was used as the positive reference substance with concentrations of 10^{-5}−10^{-8} mol/L. The same method was used in cytotoxic testing against HL-60, HCT, and MKN-28.

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Supporting Information Available: Atomic coordinates for **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

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