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Notes

Constituents of *Oxandra* cf. *xylopioides* with Anti-inflammatory Activity

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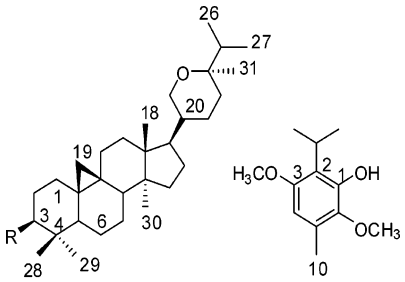
A new triterpenoid (**1**) derived from 24-methylcycloartanol was isolated from the leaves of *Oxandra* cf. *xylopioides*. An unusual structure of the new compound was assigned as **1**, for which the name berenjenol is proposed, on the basis of the spectroscopic data of the natural product and of its derivatives **2** and **3**. The leaves also afforded the known monoterpene isoespintanol (**4**). Compounds **1** and **4** significantly reduced the paw edema induced by carrageenan by 64% and 43%, at 3 h, respectively. Moreover, **4** reduced IL-1β production by 72% at 100 μM and reduced IL-1β mRNA synthesis.

The Annonaceae constitutes a primitive family of plants belonging to the order Magnoliales. This family, comprising around 130 genera and approximately 2000 species,[1] produces several highly bioactive metabolites.[2] For the genus *Oxandra*, chemical studies have been reported for only four of 26 species[3−6] and have resulted in the isolation of bis-dehydroaporphines, alkaloids, azafluorenone alkaloids,[4,7] and triterpenes.[6] According to published reports, alkaloids have been isolated only from the bark of *Oxandra xylopioides* Diels.[3,7,8] In the present work, a new and unusual cycloartane triterpene, berenjenol (**1**), and a previously known monoterpene, isoespintanol (**4**), were isolated from the CH_2Cl_2 and petroleum ether extracts of the leaves of this plant. The structure of the new compound **1** was established on the basis of the interpretation of its NMR and MS data.



1 R = OH
2 R = OAc
3 R = O

4

Inflammation is a response of animal organisms to physiological or pathological situations. Different mediators are implicated in the evolution and resolution of this process, including vasoactive amines, arachidonate metabolites, peptidic mediators, and free radicals, and its resolution could be focused by different mechanisms.[9] Plants from the Annonaceae are a potential source of anti-inflammatory compounds, because some alkaloids from this family have demonstrated capacity for inhibiting pro-inflammatory enzymes and have antioxidant properties.[10] In addition, triterpenes, and specifically cycloartenol derivatives, have demonstrated anti-inflammatory activity in different experimental models of inflammation.[11]

Compound **1** was obtained as colorless crystals, $[\alpha]_D$ +59 (*c* 0.8, $CHCl_3$). The molecular formula, $C_{31}H_{52}O_2Na$, was established on the basis of the positive-ion HRTOFMS (*m*/*z* 479.3644) and the ^{13}C NMR and DEPT NMR spectra. Its IR spectrum showed a broad absorption band attributable to a hydroxyl group (3395 cm^{-1}). The ^{13}C NMR and DEPT spectra of **1** displayed a total of 31 carbon signals, constituted by seven methyl, 12 methylene, six methine, and six quaternary carbons. The ^1H NMR spectrum of **1** revealed two doublets at δ 0.31 (1H, d, J = 4.3 Hz) and 0.51 (1H, d, J = 4.3 Hz), characteristic of a cycloartan-3β-ol derivative.[12] In addition, five tertiary methyl singlet resonances at δ 1.00, 0.96, 0.93, 0.85, and 0.77 and two secondary methyl resonances at δ 0.86 (1H, d, J = 6.8 Hz) and 0.83 (1H, d, J = 6.8 Hz) were observed. The signal at δ 3.24 (1H, dd) indicated the presence of a proton attached to a carbon bearing a hydroxyl group. This was confirmed by acetylation of **1** with acetic anhydride/pyridine, which afforded monoacetate **2**. The FABMS of **2** showed a peak at *m*/*z* 498, compatible with the molecular formula $C_{33}H_{54}O_3$. Oxidation of **1** with Jones' reagent gave **3**, which showed a new signal due to a ketone carbonyl at δ 216.2 (Table 1) and the disappearance of the signal assignable to the hydroxyl found in **1**. In addition, two signals at δ 3.20 (1H, t, J = 11.2 Hz) and 3.79 (1H, dd, J = 11.2, 1.8 Hz) were assigned to an ether function of the type $CH-CH_2-O-C$.[12] These 1D NMR data, in combination with the observed 2D $^1H-^1H$ COSY, HSQC, and HMBC correlations (Figure 1), suggested the presence of a 24-methyl-21,24-tetrahydropyran ring in the structure of **1**, closely related to the *Oxandra asbekii* triterpenes.[6,13] On the basis of its

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Table 1. NMR Spectroscopic Data (^{1}H 400 Hz, ^{13}C 100 Hz) of **1** in $CDCl_3$ and ^{13}C NMR Data of **2** and **3** in $CDCl_3$ (^{13}C 100 Hz)

position	δ_C **1**	δ_H (J in Hz) **1**	δ_C **2**	δ_C **3**
1	31.9	H_α 1.20	31.5	33.3
		H_β 1.51		
2	30.3	H_α 1.72	29.6	37.3
		H_β 1.52		
3	78.8	3.24, dd (10.9, 4.2)	80.6	216.2
4	40.0		39.4	50.1
5	47.1	1.25	47.1	47.5
6	21.1	1.55	20.8	21.1
7	26.6	H_α 1.28	26.7	25.8
		H_β 1.04		
8	47.9	1.47	47.7	48.3
9	19.9		20.0	21.0
10	26.0		26.0	25.9
11	26.3	H_α 1.08	26.3	26.6
		H_β 1.93		
12	32.2	1.46	32.1	32.0
13	45.1		45.1	45.1
14	48.7		48.7	48.6
15	35.4	1.28	35.4	35.4
16	26.8	H_α 1.28	26.7	26.8
		H_β 1.82		
17	49.4	1.51	49.4	49.4
18	18.2	0.96 s	18.2	18.3
19	29.8	H_α 0.51, d (4.3)	26.8	29.4
		H_β 0.31, d (4.3)		
20	39.2	1.42	39.3	39.2
21	65.5	H_α 3.79, dd (11.2, 1.8)	65.5	65.5
		H_β 3.20, dd (11.2, 1.8)		
22	26.1	H_α1.65	25.7	26.0
		H_β 1.22		
23	33.0	H_α 1.47	33.0	22.9
		H_β 1.34		
24	74.7		74.7	74.7
25	39.3	1.43	39.4	39.4
26	16.7	0.86, d (6.8)	16.7	16.7
27	17.1	0.83, d (6.8)	17.0	17.0
28	25.4	0.93 s	25.4	20.0
29	14.0	0.77 s	14.7	22.1
30	19.3	0.85 s	19.3	19.3
31	14.7	1.00 s	15.1	14.7
MeC=O			170.8	
MeCO			21.2	

^{1}H−^{1}H, COSY, HSQC, NOESY, HOHAHA, and HMBC NMR spectra, all ^{1}H and ^{13}C NMR signals of **1** could be assigned.

The HMBC spectrum showed correlations (Figure 1) between the methyl signals at δ_C 25.4 (C-28) and 14.0 (C-29) and the carbon signal at δ_C 78.8 (C-3), the proton signal at δ_H 3.20 (C-21), and carbon signals at δ_C 39.2 (C-20) and 49.4 (C-17). There were also correlations between signals resonating at δ_H 0.86 (Me-26) and 0.83 (Me-27) and the quaternary carbon signal at δ_C 74.7 (C-24). This was also confirmed by HMBC correlations observed from Me-18 (δ_H 0.96) to C-17 and Me-30 (δ_H 0.85). The relative stereochemistry of **1** was determined on the basis of NOESY experiments (Figure 2). In the NOESY spectrum, strong correlations were observed between CH_3-18/H-20, CH_3-18/H-21, CH_3-31/H-21, CH_3-18/H-19, CH_3-28/CH_3-29, and CH_3-28/H-3. The configuration of the hydroxyl group at C-3 was concluded to be β on the basis of coupling constants[13] of H-3 (dd, J = 10.9, 4.2 Hz), and the H-20 β-configuration of cycloartane **1** was shown by the correlations between H18/H20 and H18/H21 (Figure 2). Therefore, the structure and relative stereochemistry of **1** were confirmed as 21:24-epoxy-24-methylcycloartane, and this compound was given the trivial name berenjenol.

Isoespintanol (**4**) was also obtained from a petroleum ether extract of the leaves of *Oxandra* cf. *xylopioides* as colorless prisms and in significant amounts. The occurrence of isoespintanol (**4**) was reported in the ethereal extract from aerial parts of *Eupatorium saltense*.[14] Compound **4** has also been synthesized as part of the structure elucidation of the natural product espintanol.[15]

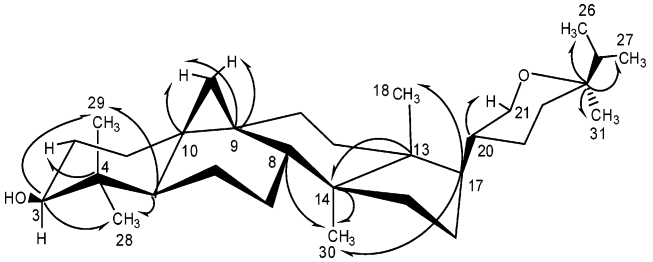

Figure 1. Selected HMBC interactions of berenjenol (**1**).

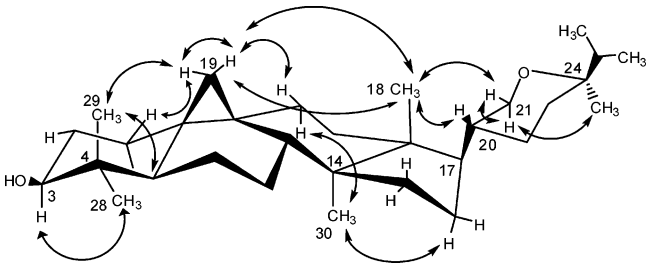

Figure 2. Selected NOESY interactions of berenjenol (**1**).

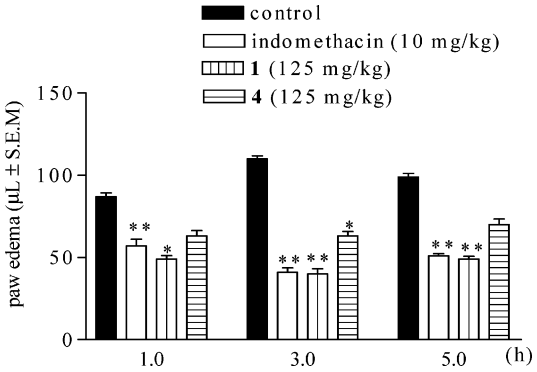

Figure 3. Effects of **1** and **4** (125 mg/kg, po) on carrageenan-induced mouse paw edema. Footpad edema was induced 1 h after the injection of carrageenan (3% w/v in saline). Footpad volume was measured 1, 3, and 5 h after irritant injection. Each point represents the mean from six to eight increases in foot paw volume, and the vertical lines indicate the SEM. Statistically significant difference with respect to the control is expressed as $^{**}p < 0.01$ or $^{*}p < 0.005$ (Dunnet's t-test).

Compounds **1** and **4** at 125 mg/kg reduced paw edema induced by carrageenan (Figure 3). Whereas **1** showed a clear effect at 1, 3, and 5 h, reducing the paw edema by 44%, 64%, and 51%, respectively, **4** had a significant effect only at 3 h, reducing the edema by 43%. Moreover, **1** and **4** did not show toxicity against RAW 264.7 macrophages at 100 μM, and no effect on NO or TNF-α production.

Subcutaneous injection of carrageenan in the mouse paw induces edema, hyperalgesia, and erythema, resulting from action of pro-inflammatory agents such as bradykinin, histamine, prostaglandins, tachykinins, complement, and reactive oxygen and nitrogen species. First, there is an increase of vascular permeability due to amines, followed by an increase of arachidonic acid metabolism with production of prostaglandins (2−4 h), and finally, the infiltration of cells to inflamed tissues. Neutrophils that migrate to sites of inflammation can generate pro-inflammatory reactive oxygen and peptide mediators such as TNF-α and IL-1β, which increase the edema and tissue inflammation.[16,17] Compound **4** modified IL-1β production by 72% at 100 μM and reduced IL-1β mRNA synthesis (Figure 4). This could be a mechanism implicated in the anti-inflammatory action of **4**, but further experiments should be carried out to determine the mechanism of action of this compound.

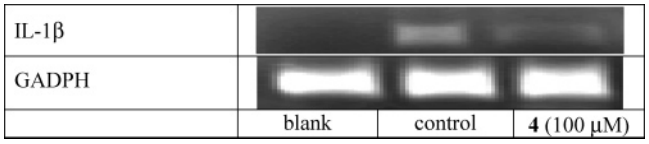

Figure 4. mRNA expression of IL-1β by RAW 264.7 macrophage cells in response to LPS. Total RNA was isolated and analyzed by RT-PCR as described in the Experimental Section.

Experimental Section

General Experimental Procedures. Melting points were measured without correction on a Büchi apparatus. Optical rotations were obtained using a cell (1.5 mL) with a 1 dm path length, on a Polartronic E (Schmidt-Haensch) polarimeter. IR spectra were recorded on a Perkin-Elmer Spectrum RX I FT-IR system in a KBr disk. UV spectra were obtained in MeOH, using a Jenway 6405 spectrophotometer. ^{1}H NMR (400 and 600 MHz) and ^{13}C NMR (75 MHz) spectra (all in $CDCl_3$) were recorded with Bruker AMX 400 and Bruker AM 600 NMR spectrometers, using TMS as internal standard. EIMS were obtained with a Nermag-Sidar R10-10C mass spectrometer. Silica gel 60 (Merck 0.063−0.200 mesh) was used for column chromatography, and precoated silica gel plates (Merck 60 F_{254} 0.2 mm) were used for TLC. TLC spots were sprayed with a mixture of sulfuric acid in acetic acid (10:90) and heating to 100−105 °C.

Plant Material. The plant material was collected in Monteria, Colombia, in May 2002 and identified by Prof. Francisco Javier Roldan Palacio, University of Antioquia. A voucher specimen is deposited at Botanical Garden Joaquin Antonio Uribe, Medellin, Colombia (voucher number 037852).

Extraction and Isolation. The powered leaves (1.0 kg) of *Oxandra* cf. *xylopioides* were extracted with petroleum ether (15 L) at room temperature and concentrated in vacuo to give a crude extract (150 g). The petroleum ether extract (30 g) was subjected to passage over a silica gel column (5 × 80 cm) eluting with a step gradient of hexane−CH_2Cl_2 (95:5, 80:20, 70:30, 100 CH_2Cl_2 each 1.0 L), to obtain four fractions. Fraction II was dissolved in CH_2Cl_2 and compound **4** recrystallized in hexane (3.0 g) (1.5%).

The residue was extracted with CH_2Cl_2 at room temperature, and the solvent was evaporated. The CH_2Cl_2 extract (65.0 g) was then fractionated by column chromatography on a silica gel column (350 g, 8 × 80 cm), eluting with a step gradient of hexane in CH_2Cl_2, starting with 100% hexane. Fractions were collected on the basis of their TLC profiles to yield eight fractions (F1−F8). Further flash column chromatography of fraction 3 (15.0 g) on silica gel (100 g), eluting with a step gradient of hexane−CH_2Cl_2 (50:50, 40:60, 30:70) to 100% CH_2Cl_2, afforded six fractions (F31−F36). Fraction 33 was dissolved in CH_2Cl_2, and berenjenol (**1**) was recrystallized in methanol (3.0 g) (0.3%).

Berenjenol (1): colorless crystals, mp 175−177 °C; $[\alpha]_D$ +59.0 (*c* 0.8, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 205 (3.37), 230 (2.89) nm; IR (KBr) ν_{max} 3395, 2935, 1465, 1375 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 1; EIMS *m*/*z* 456 (12), 441 (16), 438 (39), 423 (37), 413 (18), 395 (8), 274 (20), 273 (100), 95 (83); HRTOFMS *m*/*z* 479.3644 (calcd for $C_{31}H_{52}O_2Na$, 479.3644).

Berenjenol Acetate (2). Berenjenol (**1**) (50 mg) was treated with 2.0 mL of acetic anhydride−pyridine (1:1) at room temperature overnight. The mixture was poured into 5% HCl (25 mL) and then extracted three times with CH_2Cl_2. The CH_2Cl_2 extract was concentrated under reduced pressure and chromatographed on a silica gel column, eluting with petroleum ether−CH_2Cl_2 (8:2) to give the acetate **2** (47 mg, 90% yield), as a pale white, amorphous powder: mp 169−170 °C; UV (MeOH) λ_{max} (log ϵ) 205 (3.64), 225 (3.33) nm; IR (KBr) ν_{max} 2945, 1745, 1375, 1245 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.60 (1H, m, H-1), 1.74 (2H, m, H-2), 4.55 (1H, m, H-3), 0.98 (3H, s, H-18), 0.79 (1H, d, J = 4.0 Hz, H-19α), 0.76 (1H, d, J = 4.0 Hz, H-19β), 3.82 (1H, t, J = 11.2 Hz, H-21α), 3.23 (1H, dd, J = 11.2, 1.8 Hz, H-21β), 0.88 (3H, d, J = 6.8 Hz, H-26), 0.86 (3H, d, J = 6.8 Hz, H-27), 0.87 (3H, s, H-28), 0.83 (3H, s, H-29), 0.87 (3H, s, H-30), 1.02 (3H, s, H-31); ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 1; EIMS *m*/*z* 498 (27), 497 (31), 455 (72), 439 (85), 438 (60), 423 (18), 395 21), 297 (52), 273 (53), 95 (100).

Oxidation of Berenjenol (1). To a solution of berenjenol (**1**) (100 mg) in acetone (15 mL) was added Jones' reagent dropwise with stirring at 0 °C until the orange color persisted, and the mixture was allowed to stand overnight at room temperature and in darkness. The reaction mixture was dissolved in ice/water (20 mL), and the mixture was extracted with CH_2Cl_2. The extract was concentrated to dryness, and the residue was purified by column chromatography using mixtures of CH_2Cl_2−hexane as eluent to obtain 90 mg of pure compound **3** (88% yield).

3-Oxoberenjenol (3): white, amorphous powder; $[\alpha]_D$ +25.1 (c 0.39, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 205 (3.70), 225 (3.31) nm; IR (KBr) ν_{max} 2960, 1710, 1465, 1370 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.50 (1H, m, H-1α), 1.80 (1H, m, H-1α), 2.67 (1H, m, H-2α), 2.25 (1H, m, H-2β), 1.00 (3H, s, H-18), 0.76 (1H, d, J = 4.0 Hz, H-19α), 0.57 (1H, d, J = 4.0 Hz, H-19β), 3.81 (1H, t, J = 11.2 Hz, H-21α), 3.23 (1H, dd, J = 11.2, 1.8 Hz, H-21β), 0.88 (3H, d, J = 6.8 Hz, H-26), 0.84 (3H, d, J = 6.8 Hz, H-27), 1.02 (3H, s, H-28), 1.07 (3H, s, H-29), 0.87 (3H, s, H-30), 1.01 (3H, s, H-31); ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 1; ESIMS$^+$ *m*/*z* 510. 477.

Carrageenan-Induced Hind-Paw Mouse Edema. Edema was induced in the right hind-paw by subplantar injection of a suspension of λ-carrageenan (3% w/v in saline, 25 μL). Test compounds (125 mg/kg) and indomethacin (10 mg/kg), in saline buffer (NaCl 0.9%, $NaHCO_3$ 0.1%, pH 7.4), were orally administered 1 h before carrageenan. The edema was measured by means of a plethysmometer (Ugo Basile) 1, 3, and 5 h after challenge and was expressed as the difference between the right and left paw volume. The control group received orally only a saline solution. The edema inhibition is expressed as the percentage of volume reduction relative to the control. Details of this method have been described earlier.[18]

Determination of Cell Viability. The cytotoxicity of compounds on cells was performed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[19] Murine RAW 264.7 macrophages were exposed to test compounds at a concentration of 100 μM in a microplate for the specified time, and then 100 μL per well of a 0.5 mg/mL solution of MTT was added and incubated at 37 °C until blue deposits were visible. The colored metabolites were dissolved in dimethyl sulfoxide. Absorbance was measured at 490 nm using a Labsystems Multiskan EX plate reader. Results were expressed in absolute absorbance readings; a decrease indicated a reduction in cell viability.

Determination of Nitric Oxide (NO) Tumor Necrosis Factor-α (TNF-α) and Interleukin-1β (IL-1β) Production in RAW 264.7 Macrophages. RAW 264.7 macrophages were cultured in DMEM medium containing 2 mM L-glutamine (Gibco), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin, and 10% fetal bovine serum (Gibco). Cells were removed from the tissue culture flask using a cell scraper and resuspended until a final relation of 1 × 106 cells/mL.[20]

RAW 264.7 macrophages (1 × 106 cells/mL) were co-incubated in 96-well culture plates (200 μL) with 1 μg/mL lipopolysaccharide at 37 °C for 24 h in the presence of test compound or vehicle. Nitrites were determined in culture supernatant by Griess reagent, and TNF-α was determined by a specific enzyme immunoassay kit from eBioscience (San Diego, CA).[20]

Semiquantitative RT-PCR. Extraction of Total Cellular RNA. RAW 264.7 macrophages (1 × 106 cells/mL) were treated with (1 μg/mL) or without lipopolysaccharide at 37 °C for 8 h in the presence of test compound or vehicle. The total cellular RNA was extracted by RNAeasy mini spin columns according to the manufacturer's protocol. The isolated RNA was precipitated with 100% cold ethanol, pelleted by centrifugation, and solved again in diethyl pyrocarbonate (DEPC)-treated H_2O. The concentration of the extracted RNA was calculated by measuring the optical density at 260 nm. The ratio of the optical density at 260 nm to that at 280 nm was always higher than 1.8.

Synthesis of First-Strand Complementary Deoxyribonucleic Acid. Briefly, 1 μg of RNA in 12.5 μL of DEPC-treated H_2O was mixed with 20 mM oligodeoxythymidine (oligo dT) 15 and heated at 65 °C for 5 min, then quick-chilled on ice. The following reagents were added to the tube: 6.5 μL of concentrated synthesis buffer (50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$; 0.5 mM dNTPs; and 0.5 U RNase inhibitor) and 200 U of the Moloney murine leukemia virus (MMLV) reverse transcriptase. The reaction was initially incubated at 42 °C for 20 min.

PCR. PCR was performed in an air thermocycler according to the manufacturer's instructions, as described previously.[21] Briefly, 10 mL of the first-strand complementary deoxyribonucleic acid (cDNA) was mixed with 0.75 mM primers, 4 U of Taq polymerase, 10 μL of reaction

buffer (2 μM Tris-HCl, pH 8.0; 0.01 μM ethylenediamine tetraacetate (EDTA); 0.1 μM dithiothreitol (DDT); 0.1% Triton X-100; 5% glycerol; and 1.5 μM $MgCl_2$), and 25 μL of water, in a total volume of 50 μL. The IL-1β primer pair was designed on the basis of published human cDNA sequence data. A temperature of 94 °C for 1 min, an annealing temperature of 60 °C for 1 min, an elongation temperature of 72 °C for 1 min for the first 30 cycles, and, finally, an elongation temperature of 72 °C for 10 min were used. Following the reaction, the amplified product was taken out of the tubes and run on 2% agarose gel.

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