Antibacterial Activity of Naphthoquinones and Triterpenoids from *Euclea natalensis* Root Bark

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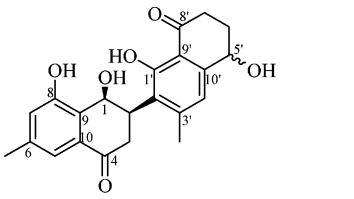
Phytochemical studies of an ethanolic extract of *Euclea natalensis* root bark afforded two new compounds, octahydroeuclein (**1**) and 20(29)-lupene-3β-isoferulate (**2**), in addition to three known compounds, shinanolone (**3**), lupeol, and betulin. The chemical structures of **1** and **2** were determined by spectroscopic means. Shinanolone (**3**) showed inhibitory activity against Gram-positive bacterial strains and a drug-sensitive strain of *Mycobacterium tuberculosis* at a concentration of 0.1 mg/mL.

The World Health Organization (WHO) has estimated that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their health care.[1] Tuberculosis (TB) is increasing worldwide due to the emergence of multidrug-resistant strains of *Mycobacterium tuberculosis*. This increase is being potentiated by the AIDS epidemic. TB accounts for 2−3 million deaths and almost one-third of AIDS-related deaths worldwide.[2]

In our continuing search for new lead compounds from higher plants for TB treatment[3−5] we have investigated the root bark of *Euclea natalensis* A. DC. (Ebenaceae). *E. natalensis* occurs in a variety of habitats including coastal and inland forests as well as bushveld. It is widely distributed in tropical and subtropical Africa and is common on the east coast of South Africa.[6,7] The roots are used for treating different forms of chest complaints such as bronchitis, pleurisy, and chronic asthma by the Zulu.[7] Several triterpenoids and naphthoquinones have been identified from *E. natalensis* root bark.[8,9] Bioassay-guided isolation of an ethanolic extract of *E. natalensis* root bark, using bioautographic TLC antibacterial assays,[10] led to the isolation of shinanolone (**3**)[11] as an active principle. Two new compounds, octahydroeuclein (**1**) and 20(29)-lupene-3β-isoferulate (**2**), in addition to two known compounds, lupeol and betulin, were isolated as inactive constituents. The structural determination of **1** and **2** and the antibacterial activity of these compounds are described herein.



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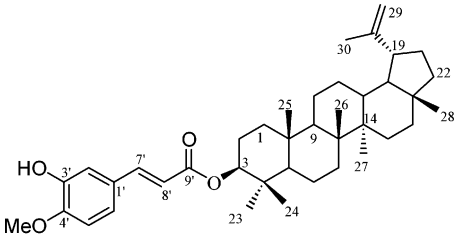

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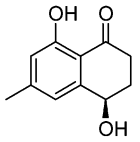

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Compound **1** was obtained as amber yellow crystals, and HREIMS showed a molecular ion peak $[M]^+$ at *m/z* 382.14217, corresponding to $C_{22}H_{22}O_6$. The ^1H and ^{13}C NMR data showed three aromatic proton singlets at δ_H 6.93, 6.69, and 6.65 (δ_C 121.1, 117.8, and 120.5; H-4′, H-7, and H-5), a proton at δ_H 3.70 (δ_C 40.3, H-2), three methylene groups at δ_H 4.10, 2.50 (δ_C 36.2, CH_2-3), 2.90, 2.60 (δ_C 34.7, CH_2-7′), and 2.25, 2.06 (δ_C 31.1, CH_2-6′), two methines attached to carbons bearing oxygen at δ_H 4.73, 4.70 (δ_C 66.5, 71.5; H-5′, H-1), and two aromatic methyl singlets at δ_H 2.28, 2.30 [δ_C 21.7 (2C); Me-11, Me-11′]. The above NMR data indicated the presence of a structure similar to euclein[12] with two units, of which one is similar to shinanolone (**3**), isolated from the same source, except for the substitution at C-6, which was indicated from the absence of an aromatic signal at 6.82 (H-7) of **3**. The second part showed signals corresponding to 3,4-dihydro-2*H*-naphthalen-1-one with two hydroxyls at positions C-1 and C-8. The reduction of the C-1 carbonyl group from the euclein molecule was evidenced by the presence of only one *peri*-hydroxyl group (δ_H 12.2, of the first part) and HMBC correlations of H-1/C-3, C-10 and H-3/C-1, C-2, C-4 (Figure 1). The points of attachment between the two units at C-2′/C-2 were evidenced from HMBC correlations of H-2/C-2′, C-1′, and C-3′. The relative configurations of the C-1 and C-2 asymmetric centers were deduced from the comparison of the coupling constants (J_{1-2} = 3.4 Hz) with that of similar compounds, which indicated a $H_{1\alpha}-H_{2\alpha}$ configuration.[13,14] In addition, NOESY spectra of **1** showed correlations between H-1α/H-2α and H-3α. The relative configuration at C-5′ could not be determined from the coupling constants of H-5′ (5.0, 6.0 Hz), although the NOESY spectra showed correlations between H-5′/H-6′a (δ_H 2.25) and H-7′b (δ_H 2.60). On the basis of the above data, compound **1** was assigned as the new binaphthoquinone, octahydroeuclein (**1**).

Compound **2** was deduced to have the molecular formula $C_{40}H_{58}O_4$ on the basis of HREIMS (*m/z* 602.4456). The ^1H NMR data of **2** showed signals for seven singlet methyls

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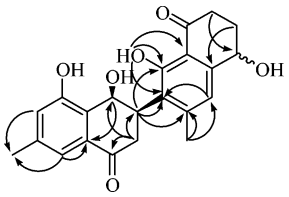

Figure 1. HMBC correlations of **1**.

(δ_H 1.00, 0.95, 0.93, 0.90, 0.88, and 0.77), including an isopropenyl group [δ_H 1.66 (Me-30), 4.52 and 4.65 (H-29)], an (*E*)-isoferulate moiety [δ 3.90 (OMe), 6.26 and 7.56 (H-8′, H-7′), three aromatic protons 6.88 (H-5′), 7.01 (H-2′), and 7.04 (H-6′)], a methine proton bearing an ester δ 4.45 (obscured by olefinic protons), and a typical lupenol H_β-19 proton signal (δ_H 2.33).[15] By comparison of the NMR data with those of lupeol, which has been isolated from the same source, compound **2** was assigned as a lupeol derivative with an extra (*E*)-isoferuloyl moiety at C-3. The position of the methoxy group of the isoferuloyl moiety at C-4′ was confirmed by a HMBC experiment, which showed correlations of MeO/C-4′, H-2′/C-4′, and H-6′/C-4′. The relative configurations of C-3 could not determined from the recorded spectra of **2**; however, hydrolysis of **2** gave lupeol with a OH-3β configuration, which was identified on the basis of comparison of its mp, $[\alpha]_D$, and ^{1}H NMR spectrum with literature values.[15]

All compounds were tested for their antibacterial and antimycobacterial (*Mycobacterium tuberculosis*) activity, but only shinanolone (**3**) exhibited inhibitory activity against Gram-positive bacteria (*Bacillus pumilus* ATCC 27142, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, and *Streptococcus faecalis*) and a drug-sensitive strain of *M. tuberculosis* (MRC strain no. H37Rv ATCC27294), at a concentration of 0.1 mg/mL. The new binaphthoquinone, octahydroeuclein (**1**), was not active, while the new triterpenoid 20(29)-lupene-3β-isoferulate (**2**) showed activity only against *B. pumilus* (0.1 mg/mL). The reference antibiotic, streptomycin sulfate, inhibited the growth of all bacterial species tested in this study at 0.01 mg/mL, except *Pseudomonas aeruginosa* and *Serratia marcescens*, which were inhibited at 0.05 and 0.1 mg/mL, respectively. *M. tuberculosis* was found to be susceptible to the drugs streptomycin and ethambutol at concentrations of 0.004 and 0.006 mg/mL, respectively.

Experimental Section

General Experimental Procedures. Melting points are uncorrected. Optical rotations were measured with a Perkin-Elmer 141 polarimeter. IR spectra were recorded on a Perkin-Elmer 1310 spectrophotometer. UV spectra were recorded using a Pharmacia LKB-ultraspec 111 UV spectrophotometer. NMR spectra were recorded using a Bruker ARX 300 or a Bruker Avance DRX 500 MHz. Mass spectra were obtained with a JEOL JMS-AX505 W mass spectrometer. Silica gel [Merck, Kieselgel 60 (0.063−0.200 mm) and (0.015−0.040 mm)] was used for column chromatography. Silica gel plates (Merck, Kieselgel 60 F_{254}) were used for TLC.

Plant Material. *E. natalensis* roots were collected from Tembe Elephant Park in KwaZulu-Natal Province of South Africa in May 2001. The plant material was identified kindly by Prof. AE van Wyk, and a voucher specimen (N.L. 22) was deposited in the Schweickerdt Herbarium (PRU), Pretoria, South Africa.

Extraction and Isolation. *E. natalensis* fresh root bark was separated mechanically, homogenized with ethanol, and extracted for 72 h (twice). The total ethanolic extracts were combined and concentrated under reduced pressure, then partitioned with *n*-hexane. The ethanolic layer was diluted to 70% with H_2O and partitioned with EtOAc. Both fractions were subjected to chromatographic separations to isolate the individual components. The hexane fraction (25 g) was chromatographed on a silica gel column using hexane/EtOAc mixtures of increasing polarity (0 to 100% EtOAc) to yield five secondary fractions (H1−H5). Fraction H4 was chromatographed on a silica gel column eluted with hexane/EtOAc (8:2) to yield pure **3** (201 mg, 0.01%). Fraction H2 (1.950 g) was chromatographed under the same conditions given above, which yielded three fractions, H21−H23. Fraction H22 (1.370 g) was crystallized from EtOAc/hexane mixtures and yielded lupeol (1.250 g, 0.062%). Fraction H3 (140 mg) was purified on a silica gel preparaive-TLC plate using toluene (100%), which yielded pure **2** (36 mg, 0.0018%). The EtOAc partition (16 g) was chromatographed on a silica gel column eluted with hexane/EtOAc mixtures of increasing polarity, which yielded six fractions (E1−E6). Fraction E6 was chromatographed on a silica gel column using $CHCl_3$/MeOH mixtures as eluents, which resulted in three fractions, E61−E63. Fraction E62 (56 mg) crystallized from MeOH and yielded **1** (30 mg, 0.0015%). Fraction E2 (789 mg) was chromatographed on a silica gel column using hexane/EtOAc (9:1) and yielded betulin (564 mg, 0.028%).

Octahydroeuclein (1,8,1′,5′-tetrahydroxy-6,3′-dimethyl-2,3,6′,7′-tetrahydro-1*H*,5′*H*-[2,2′]binaphthalenyl-4,8′-dione) (1): amber yellow crystals; mp 238−242 °C; $[\alpha]_D^{25}$ +33.0° (*c* 0.03; $CHCl_3$); IR (KBr) ν_{max} 3500, 3310, 2930, 2860, 1680, 1595, 1265, 1180 cm^{-1}; UV (MeOH) λ_{max} 267 nm (log ϵ 3.43), 336 nm (log ϵ 1.88); ^{1}H NMR (500 MHz, $CDCl_3$) δ 12.2 (1H, s, OH-1′), 6.93 (1H, s, H-4′), 6.69 (1H, s, H-7), 6.65 (1H, s, H-5), 4.73 (1H, dd, J = 5.0, 6.0 Hz, H-5′), 4.70 (1H, d, J = 3.4 Hz, H-1), 4.10 (1H, dd, J = 17.5, 13.5 Hz, H-3β), 3.70 (1H, ddd, J = 13.5, 3.6, 3.4 Hz, H-2α), 2.90 (1H, ddd, 17.8, 7.3, 4.9 Hz, H-7′a), 2.60 (1H, ddd, 17.8, 8.0, 4.9 Hz, H-7′b), 2.50 (1H, dd, J = 17.5, 3.6 Hz, H-3α), 2.30 (3H, s, H-11′), 2.28 (3H, s, H-11), 2.25 (1H, m, H-6′a), 2.06 (1H, m, H-6′b); ^{13}C NMR (75 MHz, $CDCl_3$) δ 204.7 (s, C-8′), 204.3 (s, C-4), 160.7 (s, C-1′, C-8), 148.5 (s, C-6), 147.4 (s, C-3′), 144.7 (s, C-10′), 144.1 (s, C-9), 125.3 (s, C-2′), 121.1 (d, C-4′), 120.5 (d, C-5), 117.8 (d, C-7), 113.8 (s, C-9′), 112.9 (s, C-10), 71.5 (d, C-1), 66.5 (d, C-5′), 40.3 (d, C-2), 36.2 (t, C-3), 34.7 (t, C-7′), 31.1 (t, C-6′), 21.7 (q, C-11, C-11′); EIMS *m/z* 382 [M]$^+$ (9), 364 (100), 346 (31), 329 (4), 219 (5), 201 (12), 188 (9), 177 (11), 164 (9), 135 (21), 107 (5); HREIMS *m/z* 382.14217 [M]$^+$ (calcd for $C_{22}H_{22}O_6$, 382.14164).

20(29)-Lupene-3β-isoferulate (2): colorless crystals; mp 115−118 °C; $[\alpha]_D^{25}$ +110.2° (*c* 0.05; $CHCl_3$); IR (KBr) ν_{max} 3500, 3310, 2930, 2860, 1680, 1595, 1265, 1180 cm^{-1}; UV (MeOH) λ_{max} 294 nm (log ϵ 1.8); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.56 (1H, d, J = 16.0 Hz, H-7′), 7.04 (1H, dd, J = 8.1, 1.5 Hz, H-6′), 7.01 (1H, d, J = 1.5 Hz, H-2′), 6.88 (1H, d, J = 8.1 Hz, H-5′), 6.26 (1H, d, J = 16 Hz, H-8′), 4.65 (1H, br s, H-29a), 4.52 (1H, br s, H-29b), 4.45 (1H, m, H-3), 3.90 (1H, s, OMe), 2.33 (1H, td, 5.6, 5.6 Hz, H-19), 1.66 (3H, s, Me-30), 1.00 (3H, s, Me-24), 0.95 (3H, s, Me-23), 0.93 (3H, s, Me-26), 0.90 (3H, s, Me-27), 0.88 (3H, s, Me-25), 0.77 (3H, s, Me-28); ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.0 (s, C-9′), 150.8 (s, C-20), 146.8 (s, C-4′), 145.7 (s, C-3′, C-7′), 126.5 (s, C-1′), 126.1 (d, C-6′), 115.7 (d, C-8′), 113.6 (d, C-5′), 108.3 (d, C-2′), 108.2 (t, C-29), 79.1 (d, C-3), 54.9 (d, C-5), 54.8 (q, OMe), 49.4 (d, C-9), 47.3 (d, C-18, C-19), 42.0 (s, C-14), 41.8 (s, C-17), 40.7 (s, C-8), 39.9 (t, C-22), 38.4 (t, C-1), 37.4 (s, C-4, −13), 37.0 (s, C-10), 35.6 (t, C-16), 34.6 (t, C-7), 30.0 (t, C-21), 28.3 (q, C-23), 27.3 (t, C-15), 26.4 (t, C-12), 23.9 (t, C-2), 22.5 (t, C-11), 19.9 (q, C-30), 18.3 (t, C-6), 17.2 (q, C-28), 15.8 (q, C-25), 15.6 (q, C-26), 15.0 (q, C-24), 14.5 (q, C-27); EIMS *m/z* 602 [M]$^+$ (20), 379 (23), 203 (65), 177 (100), 149 (30), 135 (70); HREIMS *m/z* 602.44562 [M]$^+$ (calcd for $C_{30}H_{58}O_4$, 602.43351).

Shinanolone (3):[16] $[\alpha]_D^{25}$ −8.6° (*c* 0.02; $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.37 (1H, s, OH-4), 6.82 (1H, s, H-5), 6.71 (1H, s, H-7), 4.85 (1H, dd, J = 3.6, 7.4 Hz, H-4), 2.94 (1H, ddd, 13.0, 8.2, 4.8 Hz, H-2β), 2.60 (1H, ddd, 13.0, 8.3, 3.8 Hz, H-2α), 2.33 (3H, s, Me-11), 2.30 (1H, m, H-3a), 2.06 (1H, m, H-3b); ^{13}C NMR (75 MHz, $CDCl_3$) δ 203.5 (s, C-1), 162.8 (s, C-8), 148.7 (s, C-4a), 145.0 (s, C-6), 118.5 (d, C-5), 117.3 (d,

C-7), 113.5 (s, C-8a), 67.7 (d, C-4), 34.5 (d, C-2), 31.3 (t, C-3), 22.2 (q, C-11).

Hydrolysis of 2. A 5 mg portion of **2** was added to 5 mL of aqueous KOH and left under nitrogen overnight at room temperature. The reaction mixture was neutralized with 10% HCl. Lupeol was extracted with $CHCl_3$, then purified by silica gel column chromatography using 30% EtOAc in hexane.

Antibacterial Bioassays. The antibacterial activity of the pure compounds was evaluated against *Bacillus cereus*, *B. pumilus*, *B. subtilis, Staphylococcus aureus, Streptococcus faecalis*, *Enterobacter cloacae* ATCC 13047*, Escherichia coli* ATCC 25922*, Klebsiella pneumoniae* ATCC 10031*, Pantoea agglomerans* ATCC 27155*, Pseudomonas aeruginosa* ATCC 27853*,* and *Serratia marcescens* ATCC 13880, using the agar plate method.[17] Susceptibility testing of these bacteria was done for the reference antibiotic, streptomycin sulfate, at concentrations 0.005, 0.01, 0.05, and 0.1 mg/mL. A drug-sensitive strain of *M. tuberculosis* (strain no H37Rv ATCC27294) was utilized to determine the antimycobacterial activity using a rapid radiometric method.[18] Susceptibility testing of *M. tuberculosis* was also performed for the two primary TB drugs, streptomycin and ethambutol, at concentrations of 0.004 and 0.006 mg/mL, respectively, against the H37Rv strain.

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