

Two new antibacterial norabietane diterpenoids from cyanobacteria, *Microcoleous lacustris*

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Abstract Two abietane diterpenes were isolated from cyanobacteria *Microcoleous lacustris*, 20-nor-3 α -acetoxy-abieta-5,7,9,11,13-pentaene and 20-nor-3 α -acetoxy-12-hydroxy-abieta-5,7,9,11,13-pentaene. These compounds were assayed against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Salmonella typhi*, *Vibrio cholerae*, *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli*, and *Klebsiella pneumoniae*. Both compounds showed activity against *S. aureus*, *S. epidermidis*, *S. typhi*, and *V. cholerae*, but not against the other bacteria.

Keywords Cyanobacteria · *Microcoleous lacustris* · Abietane diterpenes

Introduction

Cyanobacteria are among the world's most ancient inhabitants. They are single-celled organisms that live in fresh, brackish, and marine water, and use sunlight to make their own food. In warm, nutrient-rich environments, microscopic cyanobacteria can grow quickly, creating blooms

that spread across the water's surface and may become visible. Because of the color, texture, and location of these blooms, the common name for cyanobacteria is blue-green algae. A large number of cyanobacteria are proving to be rich sources of a variety of bioactive natural products of scientific and commercial interest [1]. The filamentous mat-forming cyanobacterium *Microcoleous lacustris* (Rabenhorts) Farlow ex Gomont (Oscillatoriaceae) forms massive growths in calcareous tropical streams from central Mexico [2]. As a part of our continued phytochemical study of *M. lacustris* we previously reported its cardiovascular effect [3]. The present study reports the isolation of two diterpenes from the hexane extract of the cyanobacterium *M. lacustris* with antimicrobial activity.

Methods

Source sample collection

Microcoleous lacustris field samples were collected from Balsas basin in February and July 2006, from one spring in Los Manantiales, state of Morelos, and were taxonomically authenticated in the Laboratorio de Investigación de Ficología de la Facultad de Ciencias UNAM. A voucher specimen of the plant is stored for reference. After the collection, the samples were kept in an ice chest. Upon return to the laboratory, the samples were washed with distilled water and extraneous plant/animal material was removed before subsequent use in the test.

Preparation of extract

Microcoleous lacustris samples were dried at room temperature and ground into a fine powder. Two hundred.

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grams of powder was heated to reflux temperature (Soxhlet) with 2.5 L of hexane, chloroform, methanol and water for 3 h. The solvents were removed under reduced pressure using a rotatory evaporator to constant weight. The percentage yields obtained for the hexane, chloroform, methanol, and water extracts were 6.46, 1.89, 3.4, and 1.72%, respectively.

Antimicrobial activity determination

Isolated compounds were dissolved in 1:10 diluted ethanol and further dilutions were made with sterile distilled water. The paper disk-diffusion method was used for preliminary, qualitative evaluation of the compounds' antimicrobial effects [4]. Overnight incubations of cultures of bacteria, namely *Bacillus subtilis* NCTC 542, *Bacillus cereus* ATCC 17689, *Staphylococcus aureus* NCTC 8531, *Staphylococcus epidermidis* ATCC 55654, *Escherichia coli* ATCC 25922, *Vibrio cholerae* ATCC 17689, *Salmonella typhi* ATCC 13310, *Proteus mirabilis* ATCC 14153, and *Klebsiella pneumoniae* ATCC 14786 were adjusted to approximately 10^6 c.f.u./ml according to McFarland turbidity standards [5] and spread over the appropriate media (Mueller-Hinton agar for bacteria) in Petri dishes. Filter paper discs (\varnothing 5 mm) impregnated with the solutions (each disc containing 200 μ g of compound) were placed on the air-dried surface of the medium inoculated with the respective microorganisms. Discs containing the diluent were used as control. After overnight incubation at 37°C, the zones of inhibitions around the discs were measured. The compounds that produced zones greater than or equal to 15 mm were tested to determine the quantitative antimicrobial effects in respective broth media. The macrodilution method [6] was used, and the minimal inhibitory concentration (MIC) values (μ g/ml) were determined. Gentamicin and kanamycin were used as positive antibiotic controls, and as a negative control, sterile disks (Difco) impregnated with dimethyl sulfoxide (DMSO) were used.

General experimental procedure

IR spectra were run in KBr on a Perkin Elmer 1710 spectrophotometer. All NMR experiments were performed on a Varian 300 Hz Spectrometer.

Isolation of compounds

Air-dried powdered algae (200 g) of *M. lacustris* was extracted in a Soxhlet with hexane, and the solvent was eliminated under reduced pressure at 40°C giving an extract (12.9 g) that was subjected to chromatography on silica gel (400 g; 50 \times 7 cm column) with mixtures of

hexane/acetone/ethyl ether (14:1:2). All of the eight fractions obtained were tested for antibacterial activity. Inhibition zones were located mainly in fractions 2 and 4. Fraction 2 was subject to chromatography over Sephadex LH-20 (80 g) with the same eluent as above to yield five secondary fractions. Subfraction 3 was finally purified on preparative silica gel thin layer chromatography eluting with hexane/acetone 8:1.5 to give **1** (0.003% weight of the algae). Further column chromatography on silica fraction 4 eluting with CHCl_3 /ethyl ether (3:1) yielded six fractions. Fraction 3 yielded **2** (0.0024% weight of the algae) after separation on preparative silica gel thin layer chromatography using CHCl_3 -MeOH (9:3) as eluent.

Compound analysis

Compound 1

Yellow oil; HRFABMS: m/z 326.4091 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{21}\text{H}_{26}\text{O}_3$ 326.4060); IR ν_{max} (KBr) 3,388 (OH), 2,960, 2,933, 2,871, 1,723 (C = O), 1,676, 1,590, 1,489, 1,458, 1,372, 1,267 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3): δ 1.32 (ddd, $J = 13.9, 13.3, 4.6$ Hz, H-1 α), 1.54 (m, H-1 β), 1.37 (m, 2 α), 1.84 (ddd, $J = 13.9, 13.6, 5.0, 4.8$ Hz, H-2 β), 4.30 (m, Hz H-3), 7.36 (d, $J = 8.7$ Hz, H-6), 7.14 (d, $J = 8.7$ Hz, H-7), 7.56 (s, H-11), 7.52 (s, H-14), 3.30 (1H, septet, $J = 6.8$ Hz, H-15), 1.14 (3H, d, $J = 6.8$ Hz, H-16), 1.16 (3H, d, $J = 6.8$ Hz, H-17), 0.93 (3H, s, Me-18), 0.98 (3H, s, Me-19), 2.10 (s, OAc), 7.10 (s, OH). ^{13}C NMR (300 MHz, CDCl_3): δ 30.3 (C-1), 19.5 (C-2), 68.3 (C-3), 31.6 (C-4), 124.2 (C-5), 129.0 (C-6), 119.3 (C-7), 124.6 (C-8), 138.6 (C-9), 132.6 (C-10), 131.1 (C-11), 147.9 (C-12), 147.3 (C-13), 138.6 (C-14), 29.1 (C-15), 23.9 (C-16), 23.2 (C-17), 21.5 (C-18), 29.9 (C-19), 167.9 (OCOMe), 21.5 (OCOMe).

Compound 2

Yellow oil; HRFABMS: m/z 310.3712 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{21}\text{H}_{26}\text{O}_2$ 310.3697); IR ν_{max} (KBr) 2,960, 2,930, 2,867, 1,731 (C = O), 1,694, 1,598, 1,497, 1,404, 1,361, 1,252 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3): δ 1.69 (ddd $J = 14.0, 14.0, 5.0$ Hz, H-1 α), 1.51 (m, H-1 β), 1.31 (m, H-2 α), 1.94 (ddd, $J = 14, 14, 5.0, 4.5$ Hz, H-2 β), 4.22 (m, Hz H-3), 7.10 (d, $J = 8.7$ Hz, H-6), 7.45 (d, $J = 8.7$ Hz, H-7), 7.52 (d, $J = 7.9$ Hz, H-11), 7.64 (d, $J = 7.9$ Hz, H-12), 7.34 (s, H-14), 3.36 (1H, septet, $J = 6.8$ Hz, H-15), 1.15 (3H, d, $J = 6.8$ Hz, H-16), 1.17 (3H, d, $J = 6.8$ Hz, H-17), 0.90 (3H, s, Me-18), 0.92 (3H, s, Me-19), 2.3 (s, OAc). ^{13}C NMR (300 MHz, CDCl_3): δ 30.6 (C-1), 22.9 (C-2), 68.4 (C-3), 31.7 (C-4), 124.2 (C-5), 129.7 (C-6), 124.7 (C-7), 129.0 (C-8), 132.7 (C-9), 131.1 (C-10), 132.2 (C-11), 128.7 (C-12), 147.3 (C-13), 139.0 (C-14), 30.0 (C-15), 23.2 (C-

16), 23.9 (C-17), 22.9 (C-18), 30.0 (C-19), 168.0 (OCOMe), 21.2 (OCOMe).

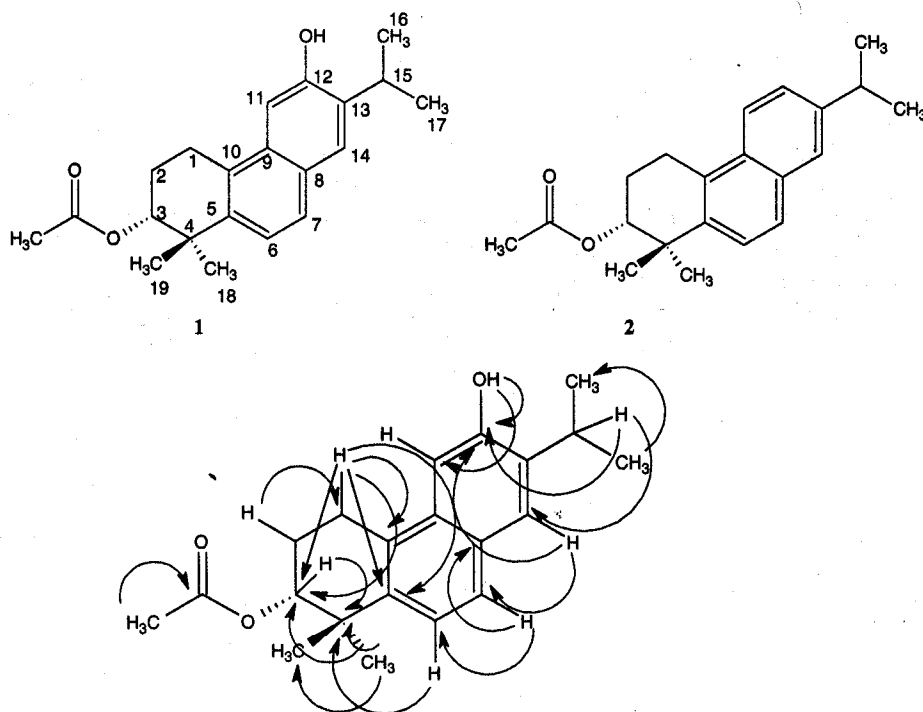
Results

Compound **1** was obtained as an oil, and its molecular formula was assigned as $C_{21}H_{26}O_3$ by HRFABMS. The IR spectrum exhibited absorbancies at 1,605, 1,580, and 1,520 cm^{-1} for the aromatic ring and at 1,723 cm^{-1} for an ester carbonyl. The latter absorption was correlated with the signal at δ_c 167.94. The presence of a hydroxyl group at 3,388 cm^{-1} was also indicated. Analysis of the ^{13}C and DEPT spectra revealed that the compound possesses five methyls, two methylenes, six methines, and eight quaternary carbons. The presence of the three rings in the structure was deduced from the molecular formula and the observation in the ^{13}C NMR spectrum of the ten unsaturated carbons at δ_c 132.62, 124.17, 128.99, 119.30, 124.64, 138.59, 131.08, 147.88, 147.26, and 138.59. In addition, an ester carbonyl group at δ_c 167.94 was attributed to the tricyclic skeleton, and five double bonds for two aromatic rings and the signals remaining indicated the presence of another ring formed by saturated carbons. However, a significant downfield shift for C-12 indicated substitution of a hydroxyl group at this position at δ_c 147.88 and H-12 at δ_H 7.30 (d, $J = 1.6$ Hz) [7]. Analysis of 1H NMR spectroscopic data revealed resonances for two methyl groups of the isopropyl side chain (δ_H 1.14, d, $J = 6.8$ Hz and 1.16,

d, $J = 6.8$ Hz); two other methyl groups were observed at δ_H 0.93 (Me-18) and 0.98 (Me-19). 1H NMR spectroscopic data also indicated ten degrees of unsaturation as double-bond equivalents, all of which were attributed to the tricyclic ring system. One of the oxygen atoms in the molecule was accounted for in the hydroxyl group, the other two in the acetyl group [8]. In the 1H NMR spectrum of **1**, the multiple resonances of C-3 methine proton at δ 4.30 indicated an α -axial orientation of the acetyl group at C-3. The heteronuclear multiple bond correlation (HMBC) spectrum showed 1H - ^{13}C long-range correlations between methyl protons at δ 0.93 (H-18) and δ 68.3 (C-3), 31.6 (C-4), and 21.5 (C-19) (Fig. 1).

The 1H NMR spectrum exhibited two ortho-coupled protons present in ring B. The chemical shifts δ_H 7.36 (d, $J = 8.7$) and 7.14 (d, $J = 8.7$) for H-6 and H-7 were characteristic of aromatic protons with a hydroxyl ring system as observed in 12-hydroxyabiet-1,3,5(10),6,8,11,13-heptaene [9] and 3-*O*-benzoylhosloquene [10]. Proton singlets at δ_H 7.56 and 7.52 for H-11 and H-14 indicate a tricyclic structure with two aromatic rings of abietane-type diterpenoids [11, 12]. There were two methylene groups in **1**. The acetyl group was attached at C-3, based on the correlations between H-3 and H-2 β and Me-19 in the 1H - 1H COSY spectrum. In the HMBC spectrum, C-3 correlated with H-1 α , H-1 β , H-2 α , H-2 β , H-18A, and H-18B; C-4 correlated with H-2 α , H-3 β , H-18A, H-18B, and Me-19; C-18 correlated with H-3 β ; and C-19 correlated with H-2 α ,

Fig. 1 Important long-range correlations observed in the heteronuclear multiple bond correlation (HMBC) spectrum of **1**



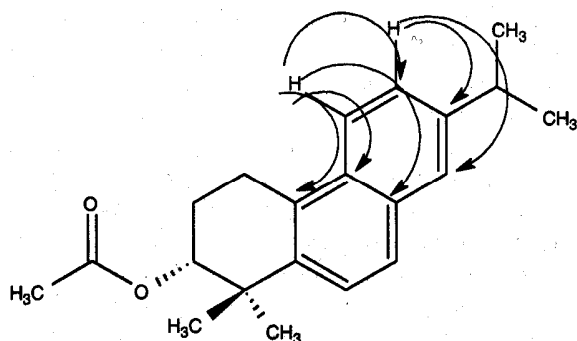


Fig. 2 Principal HMBC correlations in compound 2

H-2 β , and H-3 β . Hence, the structure of **1** was assigned as 20-nor-3 α -acetoxy-12-hydroxy-abieta-5,7,9,11,13-pentaene.

Compound **2** was also obtained as yellow oil. Mass spectral analysis suggested a molecular formula of C₂₁H₂₆O₂. The IR spectrum gave absorbances at 1,731 cm⁻¹ for an ester carbonyl, and at 1,694 and 1,598 cm⁻¹ for aromatic protons. The ¹³C NMR spectral data including DEPT revealed the presence of five methyls, two methylenes, seven methines, and seven quaternary carbons ascribable to the abietane skeleton. The ¹³C NMR spectrum of **2** showed signals for 21 carbons, only 10 of which could be attributed to aliphatic carbons (δ 30.6, 22.9, 68.4, 168.0, 21.2, 30.0, 23.2, 23.9, 22.9, and 30.0). The five signals appearing between δ 124.7 and 139.0 represented the protonated aromatic carbons. The remaining signals were attributed to six quaternary carbons and confirmed by a DEPT experiment. Therefore, compound **2** consisted of two aromatic rings with one aliphatic ring. Comparing the ¹H NMR spectrum of **2** with that of **1**, the only difference was in the chemical shifts due to the carbon around C-12, although their multiplicities were the same as those of **1**. In addition, the ¹H signals ascribable to H-11 (δ 7.52) and H-12 (δ 7.64) in **2** showed d, J = 7.9 Hz, and d, J = 7.9 Hz, respectively. The structure was completed by analysis of HMBC data, as illustrated in Fig. 2. According to these observations, **2** was elucidated as 20-nor-3 α -acetoxyabieta-5,7,9,11,13-pentaene.

The antimicrobial properties and the minimum inhibitory concentration (MIC) of compounds **1** and **2** isolated

from *M. lacustris* were determined. Compound **1** showed more activity against *S. aureus* (MIC 13.9 μ g/ml) and *S. epidermidis* (MIC 17.1 μ g/ml) than compound **2**, which exhibited MIC values of 19.1 and 21.4 μ g/ml, respectively. The MIC values of **1** and **2** were also determined against *S. typhi* (46.2 and 61.4 μ g/ml) and *V. cholerae* (261 and 286 μ g/ml). Both diterpenoids showed no activity against *B. subtilis*, *B. cereus*, *E. coli*, and *K. pneumoniae*.

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