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Antibacterial Activity of Labdane Diterpenoids from *Stemodia foliosa*

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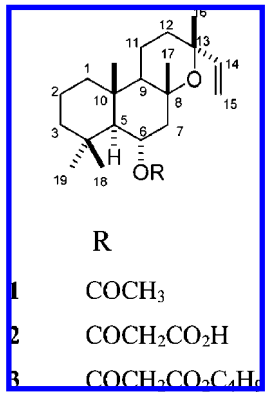
As part of a continuing interest in exploring the chemistry of Brazilian medicinal plants, three new labdane diterpenoids, 6α-acetoxymanoyl oxide (**1**), 6α-malonyloxymanoyl oxide (**2**), and 6α-malonyloxy-*n*-butyl ester manoyl oxide (**3**), together with the known betulinic acid, lupeol, sitosterol, and stigmasterol, were isolated from the aerial parts of *Stemodia foliosa.* The structures of **1**−**3** were established on the basis of interpretation of spectroscopic data, including HRESIMS, and 1D and 2D NMR techniques. All compounds were tested against a bacteria panel consisting of *Staphylococcus aureus*, *Bacillus cereus*, *B. subtilis*, *B. anthracis*, *Micrococcus luteus*, *Mycobacterium smegmatis,* and *M. phlei*. Compound **2** showed moderate activity against these strains, with MIC values in the range 7−20 μg/mL.

The genus *Stemodia* Benth. (Scrophulariaceae) is represented by 40 species distributed in Asia, Africa, Australia, and America.[1] Iridoids and diterpenes are widely distributed in this genus, with the tetracyclic stemodane diterpenoids being an unusual structural type isolated from *Stemodia maritima*,[2] a plant used medicinally in the Caribbean region to treat stomachache, edema, and swelling.[3]

Several *Stemodia* species are shrubs distributed in the tropical and subtropical areas of the world.[4] In Brazil, *Stemodia foliosa* Benth., known as "meladinha", is widespread, and in the north-eastern region, it is used popularly as a bioinsecticide and to treat respiratory infections.[5] Our previous investigation on *S. foliosa* resulted in the isolation of stearic acid 4-[(*n*-pentoxy)phenyl] ester, which showed significant activity against the Gram-positive bacteria *Bacillus cereus* and *B. subtilis* and the fast-acid bacterium *Mycobacterium fortuitum.*[6] In the course of a program aimed at identifying new bioactive compounds from Brazilian plant species, we have investigated the ethanolic extract from the aerial parts of *S. foliosa*. This has led to the isolation of three new labdane diterpenoids, 6α-acetoxymanoyl oxide (**1**), 6α-malonyloxymanoyl oxide (**2**), and 6α-malonyloxy-*n*-butyl ester manoyl oxide (**3**), together with several known compounds, betulinic acid, lupeol, stigmasterol, and sitosterol. We report herein the isolation and characterization of **1**−**3** and their antibacterial properties.



The hexane phase obtained from liquid−liquid partition of a bioactive EtOH extract from the aerial parts of *S. foliosa* gave three new compounds (**1**−**3**) after sequential column chromatographic procedures on silica gel. Compound **1** was obtained as a colorless, amorphous solid, with $[\alpha]_D^{30}$ +44.5. Electrospray-ionization MS showed a strong $[M - H]^+$ ion peak at *m/z* 347 (100), and HRESIMS gave a $[M]^+$ ion at *m/z* 348.5243, compatible with the molecular formula $C_{22}H_{36}O_3$, which is consistent with a diterpene structure. IR absorptions at 1737 cm^{-1} indicated the presence of an ester carbonyl group. The ^1H NMR spectrum of **1** (Table 1) displayed signals corresponding to five tertiary methyls at δ 1.39 (CH_3-17, s), 1.23 (CH_3-16, s), 1.00 (CH_3-20, s), 0.87(CH_3-19, s), and 0.86 (CH_3-18, s), three olefinic hydrogens at δ 5.86 (1H-14, dd, J = 17.0, 11.0 Hz), 4.83 (1H-15, dd, J = 11.0, 1.5 Hz), and 5.03 (1H-15, dd, J = 17.0, 1.5 Hz), one oxymethine hydrogen at δ 5.08 (H-6, ddd, J = 11.5, 11.9, 4.5 Hz), and one acetyl methyl group at δ 2.00 (s). From the ^{13}C NMR spectrum (Table 1) a labdane diterpene structure type was proposed for **1**.[7,8] The signals corresponding to olefinic carbons (δ 147.5 and 110.4) of a monosubstituted vinyl group were analyzed together with three oxygen-substituted carbons (δ 70.8, 74.0, 73.5) and one acetoxyl group (δ 170.0, 21.9) and suggested that **1** is a manoyl oxide derivative.[9] Also, on the basis of ^{13}C NMR data, the relative stereochemistry of C-16 was deduced, since the value attributed to this carbon is quite different for the manoyl oxide series (C-16, δ ca. 28.5) from that in the *epi*-manoyl oxide series (C-16, δ ca. 33.0).[10,11] This is consistent with the NOESY spectral data (Figure 1). Thus, taking into account these considerations, compound **1** was determined to be a manoyl oxide. The acetoxyl function at C-6 was also established on the basis of ^1H and ^{13}C NMR data. The chemical shift of H-6 in conjunction with the coupling constants observed for the signal corresponding to this hydrogen (Table 1) was diagnostic for the attachment of the acetoxyl group in an α-position.[12] Additionally, NOESY data permitted the determination of the relative configuration of C-6, C-16, and C-17 (Figure 1), thereby confirming **1** to be a new compound. Total assignments of ^1H and ^{13}C NMR signals were also confirmed by the COSY, TOCSY, HMQC, and HMBC spectra (Table S1, Supporting Information). Accordingly, diterpene **1** was characterized as 6α-acetoxymanoyl oxide.

Compound **2** was isolated as an optically active white solid ($[\alpha]_D^{30}$ +46.9) and exhibited spectroscopic data quite similar to those of compound **1**. HRESIMS afforded the molecular formula $C_{23}H_{36}O_5$ and a molecular ion at *m/z* 392.5341, 44.0098 amu higher

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Table 1. ^{1}H and ^{13}C NMR Data for Compounds **1**−**3**[a]

	1		**2**		**3**	
position	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	38.7 CH_2	1.57, m 1.45, m	38.7 CH_3	1.66, br d (12) 0.92, br t (12)	38.7 CH_3	1.66, br d (12) 0.92, br t (12)
2	18.1 CH_2	1.67, m 1.49, dt (16, 6)	18.0 CH_3	2.08, m 1.43, m	18.3 CH_3	2.08, m 1.43, m
3	43.3 CH_2	1.16, dt (16) 1.37, dt (16)	43.3 CH_3	2.60, br d (12.5) 1.03, m	43.7 CH_3	2.6, br d (12.5) 1.03, m
4	33.6 C		33.2 C		33.0 C	
5	58.8 CH	1.32, d (11.0)	58.6 CH	1.32, d (11)	58.6 CH	1.32, d (11)
6	70.8 CH	5.08, ddd (11.5, 11, 4.5)	72.4 CH	5.08, ddd (10.5, 11, 4.8)	72.4 CH	5.08, ddd (10, 11, 4.8)
7	49.8 CH_2	2.11, dd (11.5, 12.0) 1.87, dd (4.5, 12.0)	49.2 CH	2.20, dd (10.5, 10.8) 1.46, dd (4.8, 10.8)	49.5 CH	2.20, dd (10, 10.8) 1.46, dd (4.8, 10.8)
8	74.0 C		74.3 C		73.9 C	
9	54.1 CH	1.78, m	54.0 CH	1.44, br d (12.5)	54.7 CH	1.44, br d (12.5)
10	37.8 C		37.9 C		37.8 C	
11	15.4 CH_2	1.55, m 1.42, m	15.4 CH_2	1.58, m 1.39, m	15.6 CH_2	1.58, m 1.39, m
12	34.6 CH_2	1.52, m	34.6 CH_2	1.50, m 2.07, m	34.6 CH_2	1.50, m 2.07, m
13	73.5 C		73,5 C		73.4 C	
14	147.5 CH	5.86, dd (17.5, 11.0)	147.5 CH_2	5.86, dd (17.4, 10.7,)	147.4 CH_2	5.86, dd (10.7, 17.4)
15	110.4 CH_2	4.83, dd (11.0, 1.50) 5.03, dd (17.0, 150)	110.9 CH_2	4.92, dd (10.7, 1.6) 5.14, dd (17.4, 1.6)	110.3 CH_2	5.14, dd (17.4, 1.6) 4.92, dd (10.7, 1.6)
16	29.1 CH_3	1.23, s	29.3 CH_3	1.21, s	29.4 CH_3	1.21, s
17	26.9 CH_3	1.39, s	25.9 CH_3	1.37, s	27.0 CH_3	1.37, s
18	36.0 CH_3	0.86, s	36.2 CH_3	0.84, s	36.1 CH_3	0.84, s
19	21.8 CH_3	0.87, s	22.7 CH_3	0.86, s	22.1 CH_3	0.86, s
20	16.4 CH_3	1.00. s	16.5 CH_3	0.99, s	16.6 CH_3	0.99, s
−OAc	170.0 C					
	21.9 CH_3	2.00, s				
1′			173.9 C		166.9 C	
2′			43.6 CH_2	3.31, s	42.5 CH_2	3.20, s
3′			171.1 C		165.6 C	
1″					65.6 CH_2	3.84, m
2″					36.1 CH_2	1.47, m
3″					19.9 CH_2	1.37, m
4″					13.8 CH_3	0.93, t (7.0)

[a] 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR; $CDCl_3$.

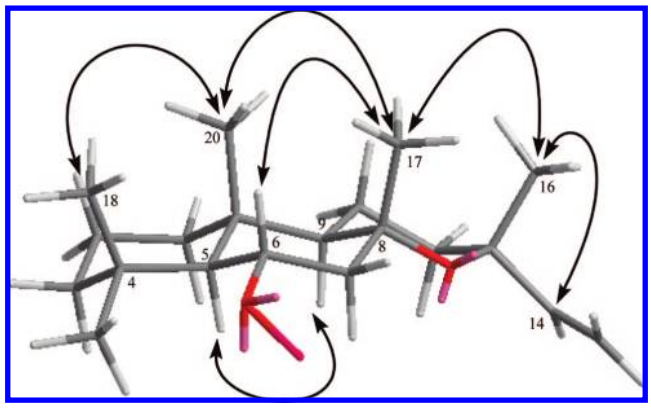

Figure 1. Some NOESY correlations observed for diterpenes **1**−**3**.

than that of **1**, suggesting the presence of an additional carboxyl group. Accurate analysis of spectroscopic data including the ^{1}H and ^{13}C NMR spectra (Table 1) demonstrated that compounds **1** and **2** are closely related, indicating the same diterpene skeleton for **2**. The only difference observed in the ^{1}H NMR spectrum of **2** was the singlet signal at δ 3.31, instead of the signal for the acetyl group observed for compound **1**. The ^{13}C NMR and DEPT spectra revealed the presence of three additional carbons for **2** at δ 173.9 (s), 171.1 (s), and 43.6 (t). These observations together with $^{1}H-^{1}H$ COSY and HMBC data (Table S2, Supporting Information) indicated that diterpene **2** has a malonyloxy moiety at C-6. These findings were confirmed by the HMBC spectrum, due to a ^{3}J cross-peak between H-6 (δ 5.08) and C-1′ (δ 173.9) and a ^{2}J cross-peak between H-2′ (δ 3.31) and C-1′(δ 173.9) and C-3′ (δ 171.1). In addition, like diterpene **1**, the coupling constants between H-6 and H-5/H-7 (J = 10.5, 11.0, 4.8 Hz) indicated the same relative configuration for the substituent at C-6. The relative configuration of the chiral centers C-8 and C-13 in **2** were assigned by NOE correlations between CH_3-16 and CH_3-17 (Figure 1), in agreement with a manoyl oxide diterpene skeleton.[7,8] On the basis of the above evidence, compound **2** was characterized as 6α-malonyloxymanoyl oxide.

Compound **3** was obtained as a white, amorphous solid with $[\alpha]_D^{30}$ +57.0 and exhibited a molecular ion peak at $[M^+]$ m/z 448.6454 in the HRESIMS, corresponding to $C_{27}H_{44}O_5$. The ^{1}H NMR spectrum of **3** was very similar to that of **2** (Table 1), suggesting that this compound also belongs to the manoyl oxide diterpene series. The main chemical shift differences observed in the ^{1}H NMR spectrum were due to the additional signals at δ 3.84

Table 2. Antibacterial Activity of Diterpenes **1**−**3**[a]

	minimum inhibitory concentration (μg/mL)[a]	
	compound **2**	clarithromycin[b]
S. aureus	15	0.8
B. cereus	15	
B. subtillis	15	1.7
B. anthracis	20	1.3
M. luteus	17	2.0
M. smegmatis	7	0.5
M. phlei	9	0.5

[a] Compounds **1** and **3** were inactive at 50 μg/mL. [b] Clarithromycin at 1.28 μg/mL was used as positive control.

(m), 1.47 (m), 1.37 (m), and 0.93 (t, J = 7.0 Hz). These data analyzed together with the signals at δ 65.6 (t), 36.1 (t), 19.9 (t), and 13.8 (q), observed in the ^{13}C NMR spectrum (Table 1), suggested the presence of a side-chain *n*-butyl in the structure of **3**. This structural feature was further corroborated by ^{1}H−^{1}H COSY analysis, which indicated one spin system (C-1″−C-2″−C-3″−C-4″), in agreement with a *n*-alkyl unity. The HMBC correlations of H-6 (5.08) to C-1′ (166.9), H-1″ (3.84) to C-3′ (165.6), and H-4″ to C-2″ revealed that the *n*-butyl unit is connected to the ester unit at C-6. This assumption was supported from the analysis of all spectroscopic data, including the NOESY, TOCSY, and HMBC spectra (Table S3, Supporting Information), which were consistent with the proposed structure, 6α-malonyloxy-*n*-butyl ester manoyl oxide (**3**).

Diterpenoids **1**−**3** were examined for their antibiotic activity toward the bacteria *S. aureus*, *B. cereus*, *B. subtilis*, *B. anthracis*, *M. luteus, M. smegmatis*, and *M. phlei* (Table 2). As shown in Table 2, diterpene **2** exhibited moderate antibiotic activity, and **1** and **3** were inactive. It was also interesting to note that the malonyloxy function at C-6 seems to be an important feature for the antibacterial activity. Additionally, the antibacterial activity of **2** serves to corroborate the popular uses attributed for this plant to treat infectious respiratory diseases.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer polarimeter model 341 using a sodium lamp (589 nm) at 20 °C. IR spectra were recorded on a Perkin-Elmer 1725X FT spectrometer with KBr pellets. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unit 500 spectrometer at 500 and 125.67 MHz, respectively, with $CDCl_3$ as solvent and TMS as internal standard; *g*COSY, *g*HMQC, *g*HMBC, NOESY, and DEPT NMR experiments were performed in the same spectrometer, using standard Varian pulse sequences. High-resolution mass spectra were measured on a Q-TOF Bruker spectrometer, using ESI+ mode. Column chromatography was carried out on silica gel 230−400 mesh (Merck), XAD-2 (Sigma-Aldrich), or Sephadex LH-20 (Pharmacia). TLC was carried out using silica gel 60 (>230 mesh, Merck) and precoated silica gel 60 PF_{254} plates. Spots on TLC were visualized under UV light and/or by spraying with anisaldehyde−H_2SO_4 reagent followed by heating. Preparative HPLC was performed on a preparative LC 4000 system (Waters) using C_{18} (250 mm × 21.20 mm, Phenomenex) columns.

Plant Material. The aerial parts of *Stemodia foliosa* were collected in July 2000 in Várzea-PE, Brazil, and identified by Dr. Alda de Andrade Chiapeta. A voucher specimen has been deposited in the herbarium of the Department of Botany, UFPE (UFP/19810).

Extraction and Isolation. The powdered, sun-dried aerial parts (1.0 kg) of *S. foliosa* were extracted with EtOH (5 × 1 L). The EtOH extract was filtered and evaporated in vacuo to obtain a dark green, gummy residue. Then, the EtOH extract was partitioned with *n*-BuOH−H_2O (1:1, 3 × 1L). The *n*-BuOH extract (41.76 g) was partitioned with MeOH−H_2O/hexane. The hexane extract (9.47 g) was fractionated by silica gel column chromatography and eluted with hexane−EtOAc into five fractions, F-1 (6% hexane), F-2 (2.8%), F-3 (9.08%), F-4 (2.44%), and F-5 (28.68%) with hexane−AcOEt (9:1). Compounds **1** (50 mg) and **2** (80 mg) were obtained by column chromatography of fraction F-2 on silica gel, eluting with hexane−EtOAc (9:1). Compound **3** (40 mg) was obtained by column chromatography of fraction F-5 on silica gel, eluting with hexane−EtOAc (1:1).

6α-Acetoxymanoyl oxide (1): white, amorphous crystals; R_f 0.4 (hexane−EtOAc, 9:1); $[\alpha]_D^{30}$ +44.5 (*c* 1.50, $CHCl_3$); IR (KBr) ν_{max} 3452, 2933, 1737 1461 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESMS *m/z* 348 [M$^+$] (18), 347 (100), 305 (30), 255 (15), 138 (5), 85 (10); HRESIMS (*m/z*) 348.5243 [M]$^+$ (calcd for $C_{22}H_{36}O_3$, 348.5205).

6α-Malonoyloxymanoyl oxide (2): white, amorphous crystals; R_f 0.28 (hexane−EtOAc, 9:1); $[\alpha]_D^{30}$ +46.95 (*c* 1.51, $CHCl_3$); IR (KBr) ν_{max} 3466, 2934, 1735, 1460 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; EIMS *m/z* 448.3 [M$^+$] (45), 274 (21.2), 273.4 (100), 190 (35.8), 95 (11.4), 81 (17.8); HRESIMS (*m/z*) 392.5341 (calcd for $C_{23}H_{36}O_5$, 392.5404).

6α-Malonyloxy-*n*-butyl ester manoyl oxide (3): white, amorphous solid; R_f 0.28 (hexane−EtOAc, 1:1); $[\alpha]_D^{30}$ +57.0 (*c* 1.51, $CHCl_3$); IR (KBr) ν_{max} 3432, 3086, 2927, 1731, 1714, 1578; ^{1}H and ^{13}C NMR, see Table 1; ESMS *m/z* 392 [M$^+$] (4.1), 347 (100), 305 (14); HRESIMS (*m/z*) 348.6454 (calcd for 348.6483).

Antibacterial Assay. Compounds **1**−**3** were tested for antibacterial activity against Gram-positive bacteria *Staphylococcus aureus* (DAUFPE 01), *Bacillus cereus* (DAUFPE 11), *Bacillus subtilis* (DAUFPE 16), *Bacillus anthracis* (DAUFPE 09), and *Micrococcus luteus* (DAUFPE 06) and fast-acid bacteria *Mycobacterium smegmatis* (DAUFPE 71) and *Mycobacterium phlei* (DAUFPE 70). Procedures for antibacterial assays have been described previously.[6]

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Supporting Information Available: 2D NMR data for compounds **1**−**3** (Tables S1−S3).This material is available free of charge via the Internet at http://pubs.acs.org.

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