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Article

New Terpenoids from *Croton limae* (Euphorbiaceae)

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An asymmetrical dimer of kaurane diterpene and monoterpene and four novel diterpenes were isolated from the roots of *Croton limae*, along with kaempferol-3-*O*-glucoside, ombuin-3-*O*-rutinoside and acetyl aleuritolic acid. The cytotoxic activity of the kaurane diterpene was evaluated against colorectal adenocarcinoma (HCT-116), ovarian carcinoma (OVCAR-8) and glioma (SF-295) cell lines, exhibiting IC_{50} values of 7.14, 8.19 and > 10 μg mL^{-1}, respectively.

Keywords: *Croton limae*, diterpenes, terpenoid adduct, cytotoxic activity

Introduction

Croton is the second largest genus of the Euphorbiaceae family with around 1250 species dividided in 40 sections. The *Croton* section *Argyroglossum* comprises about 15 species characterized as shrubs and trees in the neotropics. In South America, there are ten reported species, six of which occurring in the Northeast of Brazil. *Croton limae* is endemic of this region and was often confused with the most related species *C. argyrophyllus* and *C. tricolor*, however, it was recently described by Gomes *et al.*[1] as a new species belonging to this section.

Previous studies with northeastern *Croton* species have reported the occurrence of several classes of diterpenes with complex structures and interesting biological activities, including trachylobane, kaurane, crotofolane and casbane classes.[2-4] The phytochemical study of *C. limae* here presented afforded an asymmetrical dimer (**1**), in addition to four new clerodanes including a glycosyde derivative (**2**-**5**) (Figure 1), kaempferol-3-*O*-glucoside,[5] ombuin-3-*O*-rutinoside[6] and acetyl aleuritolic

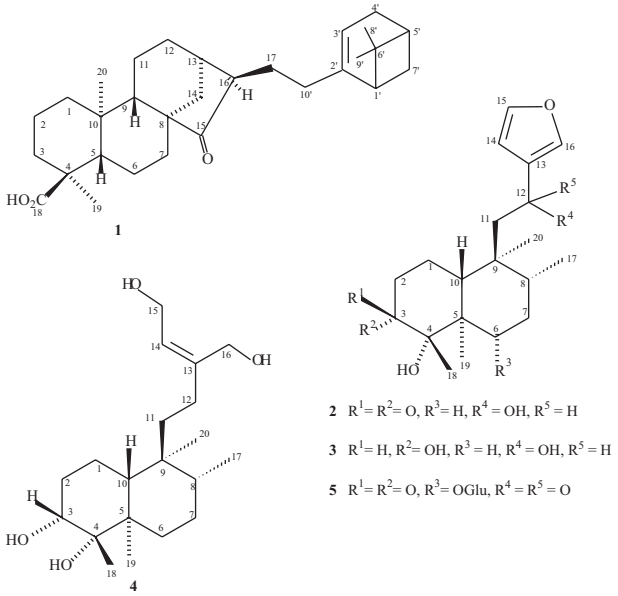

Figure 1. New terpenoids (**1**-**5**) isolated from the roots of *Croton limae.*

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acid.[7] In addition, the cytotoxicity of compound **1** was evaluated against colorectal adenocarcinoma (HCT-116), ovarian carcinoma (OVCAR-8) and glioma (SF-295) cell lines.

Experimental

General experimental procedures

Infrared (IR) spectra were recorded on a Perkin-Elmer FTIR 1000 spectrometer (Waltham, USA), using NaCl disc. The nuclear magnetic resonance (NMR) spectra were performed on Bruker Avance DRX 500 or DPX 300 instruments, equipped with an inverse detection probe head and z-gradient accessory. All pulse sequences were standard in the Bruker XWIN-NMR software, and all experiments were conducted at room temperature. The ^{1}H and ^{13}C chemical shifts are expressed in the δ scale and were referenced to tetramethylsilane (TMS) through the residual solvent. High resolution mass spectra were recorded on an UltrOTOF-Q mass spectrometer (LC-IP-TOF model 225-07100-34, Shimadzu) either by positive or negative ionization modes of the ESI source. Optical rotations were obtained on a Perkin-Elmer Q-2000 polarimeter, at 589 nm and 25 °C. Column chromatography was performed over silica gel 60 (Vetec, 70-230 and 40-63 mesh), Sephadex LH-20 (Pharmacia) and cartridge SPE C18 (Phenomenex). Thin layer chromatography (TLC) was performed on precoated silica gel aluminum sheets (Merck) and the compounds were visualized by UV detection and by spraying with vanillin/perchloric acid/EtOH solution, followed by heating. The tested compounds were analysed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) purchased by Sigma Aldrich Co. and high-throughput screening (HTS), Biomek 3000-Beckman Coulter (Inc. Fullerton, California, USA). Absorbance was measured on a DTX 880 Multimode Detector, Beckman Coulter, Inc. Fullerton, CA, USA.

Plant material

Croton limae A. P. Gomes, M. F. Sales & P. E. Berry was collected at Andaraí county, Bahia State, Northeast of Brazil, in December 2009. The specimen was authenticated by Prof Maria Lenise S. Guedes, from the Instituto de Biologia, Departamento de Botânica, Universidade Federal da Bahia (UFBA), Bahia, Brazil, and a voucher specimen (92958) was deposited at the Herbário Alexandre Leal Costa (ALCB), Universidade Federal da Bahia, Bahia, Brazil.

Extraction and isolation

Roots (1.7 kg) of *C. limae* were pulverized and extracted with hexane at room temperature. The solvent was removed under reduced pressure to yield a residue (40.2 g), which was then extracted with EtOH to yield the correspondent extract (32.1 g).

The hexane extract (40.2 g) was coarsely chromatographed over silica gel column yielding six fractions by elution with hexane (F-1), hexane:$CHCl_3$ 1:1 (F-2), $CHCl_3$ (F-3), $CHCl_3$:EtOAc 1:1 (F-4), EtOAc (F-5) and MeOH (F-6). Successive flash chromatography of fraction F-2 (12.1 g) using $CHCl_3$:EtOAc 8:2, $CHCl_3$:EtOAC 7:3, $CHCl_3$:EtOAc 1:1, EtOAc and MeOH, afforded compound **1** (48.2 mg) and acetyl aleuritolic acid (95.0 mg). Fraction F-3 (10.4 g) was purified over Sephadex LH-20 by elution with MeOH to afford three fractions. Successive chromatography over Si gel of sub-fraction F-3(2) (3.75 g) using hexane:EtOAc 7:3 as an isocratic eluting mixture, yielded compound **3** (13.8 mg). Silica gel column chromatography of the sub-fraction F-3(3) (1.54 g) using CH_2Cl_2:EtOAc 8:2, CH_2Cl_2:EtOAc 7:3, CH_2Cl_2:EtOAc 1:1, EtOAc and MeOH, yielded compound **2** (22.4 mg).

The EtOH extract (32.1 g) was suspended in a mixture of MeOH:H_2O 1:1 and submitted to partition with hexane, $CHCl_3$, and EtOAc. The EtOAc fraction (3.6 g) was further purified over Sephadex LH-20 by elution with MeOH to afford five fractions. Fraction F-3 (1.10 g) was fractionated on a SPE C18 cartridge by elution with MeOH:H_2O 9:1, MeOH:H_2O 1:1 and H_2O to afford compounds **4** (17.1 mg) and **5** (11.6 mg). Fraction F-5 (977.3 mg) was submitted to the same procedure to afford kaempferol-3-*O*-glucoside (7.2 mg) and ombuin-3-*O*-rutinoside (10.4 mg).

Cytotoxic assays

Tumor cell lines HCT-116, OVCAR-8, and SF-295 were provided by the National Cancer Institute (Bethesda, MD, USA). Cancer cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mmol L^{-1} glutamine, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin, at 37 °C with 5% CO_2. Cytotoxicity of compound **1** was evaluated using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay.[8] For all experiments, cells were plated in 96 well plates (10^5 cells *per* well for adherent cells or 3×10^5 cells *per* well for suspended cells in 100 μL of medium). The tested compounds (0.05-25 g mL^{-1}) dissolved in dimethyl sulfoxide (DMSO) were added to each well incubated for 72 h. Doxorubicin was used as the

positive control. Control groups received the same amount of DMSO. After 69 h of incubation, the supernatant was replaced by fresh medium containing MTT (0.5 mg mL^{-1}) for 3 h. The solid MTT formazan product formed was dissolved in 150 μL of DMSO, and absorbance was measured at 595 nm.

15-Oxo-17(10'-α-pinenyl)-*ent*-kauran-18-oic acid (**1**)

White solid; m.p. 122.3-123.9 °C; $[\alpha]_D^{25}$ –56.1 (*c* 0.1, CH_2Cl_2); IR (KBr) ν_{max} / cm^{-1} 2985, 2914, 2866, 1726, 1696, 1459, 1446, 1386, 1265, 1178, 1109, 1065, 1002, 950, 885, 802, 704, 665; ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) see Tables 1 and 2; HRESIMS (negative mode) *m/z* 451.3250 $[M - H]^+$ (calcd. for $C_{30}H_{43}O_3$, 451.3213).

3-Oxo-15,16-epoxy-4α,12-dihydroxy-*ent-neo*-clerodan-13(16),14-diene (**2**)

Yellow solid; m.p. 115.5-117.0 °C; $[\alpha]_D^{25}$ –3.1 (*c* 0.1, MeOH); IR (KBr) ν_{max} / cm^{-1} 3447, 2935, 2869, 1707, 1453, 1375, 1161, 1083, 940; ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR data (75 MHz, $CDCl_3$) see Tables 1 and 2; HRESIMS (positive mode) *m/z* 357.2038 $[M + Na]^+$ (calcd. for $C_{20}H_{30}O_4Na$, 357.2042).

15,16-Epoxy-3α,4α,12-trihydroxy-*ent-neo*-clerodan-13(16),14-diene (**3**)

Colorless solid; m.p. 129.7-131.2 °C; $[\alpha]_D^{25}$ –2.0 (*c* 0.1, MeOH); IR (KBr) ν_{max} / cm^{-1} 3287, 2926, 2865, 1597, 1502, 1440, 1386, 1302, 1161, 1119, 1067, 1026, 1009, 971, 956, 875, 790, 718, 661; ^{1}H NMR (500 MHz, MeOD) and ^{13}C NMR (125 MHz, MeOD) see Tables 1 and 2; HRESIMS (positive mode) *m/z* 359.2168 $[M + Na]^+$ (calcd. for $C_{20}H_{32}O_4Na$, 359.2193).

3α,4α,15,16-Tetrahydroxy-*ent-neo*-cleroda-13E-ene (**4**)

White solid; m.p. 106.5-108.2 °C; $[\alpha]_D^{25}$ –9.7 (*c* 0.1, MeOH); IR (KBr) ν_{max} / cm^{-1} 3349, 2931, 1714, 1653, 1453, 1378, 1276, 1093, 1051, 1011, 978, 910, 865, 721; ^{1}H NMR (300 MHz, MeOD) and ^{13}C NMR (75 MHz, MeOD) see Tables 1 and 2; HRESIMS (positive mode) *m/z* 363.2524 $[M + Na]^+$ (calcd. for $C_{20}H_{36}O_4Na$, 363.2506).

3,12-Dioxo-15,16-epoxy-4α-hydroxy-6-(β-glucopyranosyl)-*ent-neo*-clerodan-13(16),14-diene (**5**)

White solid; m.p. 108.6-109.8 °C; $[\alpha]_D^{25}$ –82.0 (*c* 0.1, MeOH); IR (KBr) ν_{max} / cm^{-1} 3414, 2926, 1699, 1664, 1377, 1151, 1028, 870, 796; ^{1}H NMR (300 MHz, MeOD) and ^{13}C NMR (75 MHz, MeOD) see Tables 1 and 2; HRESIMS (positive mode) *m/z* 533.2357 $[M + Na]^+$ (calcd. for $C_{26}H_{38}O_{10}Na$ 533.2409).

Results and Discussion

Compound **1**, a white solid, has a molecular formula of $C_{30}H_{44}O_3$ as established by the ion at *m/z* 451.3250 $[M - H]^+$ in the high-resolution electrospray ionisation mass spectrometry (HRESIMS). The IR spectrum showed bands at 1728 and 1697 cm^{-1} relative to carbonyl groups and at 3500 cm^{-1} relative to hydroxyl. The ^{1}H NMR spectrum of **1** displayed four distinct methyl singlets at δ_H 1.26 (CH_3-8'), 1.15 (CH_3-19), 1.10 (CH_3-20) and 0.82 (CH_3-9'), and an olefinic proton at δ_H 5.21 (br s, H-3'), besides several signals attributable to hydrogens attached to sp^3 carbons (Table 1). The ^{13}C NMR spectrum with the aid of DEPT and HMQC experiments indicated thirty signals relative to two carbonyls at δ_C 184.9 (C-18) and 224.1 (C-15), two unsaturated carbons at δ_X 148.0 (C-2') and 116.6 (C-3'), and twenty six other signals for non functionalized sp^3 carbons (four methyls, twelve methylenes, six methines and four quaternaries) (Tables 1 and 2). The HMBC spectrum provided evidences for the ABC rings of a kaurane-type moiety by the long-range correlations between the hydrogens of the methyl at δ_H 1.10 (H-20) with the methylene carbon at δ_X 38.6 (C-1) and the methines at δ_X 49.5 (C-5) and 52.3 (C-9), besides the correlations of the methyl at δ_H 1.15 (H-19) with the methylene carbon at δ_C 36.9 (C-3) and the methine at δ_X 49.5 (C-5), and with the carbonyl at δ_X 184.9 (C-18). Moreover, the correlations between the hydrogen at δ_H 1.20 (H-9), with the carbons at δ_X 18.0 (C-20), 49.5 (C-5), 38.6 (C-1), 24.8 (C-12) and 37.4 (C-14), and the hydrogen at δ_H 2.48 (H-13) with the carbons at δ_X 53.2 (C-8), 17.7 (C-11) and the carbonyl at δ_X 224.1 (C-15) confirmed this suggestion. From the above data, the two carbonyl groups were undoubtedly located at C-18 and C-15, respectively. On the basis of spectroscopic comparison of the diterpene moiety with those related for the *ent*-kaur-16-en-15-oxo-18-oic acid a great similarity has been observed between both compounds, and confirmed the partial structure.[9]

Additionally, were also observed in the HMBC spectrum the correlations of the methine at δ_H 2.10 (H-16) with carbons at δ_X 24.8 (C-12), 53.2 (C-8) and 35.5 (C-10'), besides the correlations of the olefinic methine at δ_H 5.21 (H-3') with carbons at δ_X 35.5 (C-10'), 45.8 (C-1') and 41.0 (C-5'), and those of remaining methyl groups at δ_H 0.82 (H-9') and 1.26 (H-8') with carbons δ_X 45.8 (C-1') and 41.0 (C-5'). On the basis of the foregoing evidence and comparison with the literature data, the monoterpene moiety was characterized as α-pinene.[10] Thus, the structure of **1** was fully established as an unprecedented asymmetrical dimer of a kaurane diterpene bearing a monoterpene unit at C-16. The relative stereochemistry was elucidated

Table 1. ^{1}H NMR chemical shifts of compounds **1**-**5** (δ in ppm, J in Hz)

H	**1**[a,c]	**2**[b,c]	**3**[a,d]	**4**[b,d]	**5**[b,d]
	δ_H	δ_H	δ_H	δ_H	δ_H
1	0.86 (td, 12.8, 3.30) 1.76 (m)	1.68 (m) 2.22 (m)	1.19 (m) 1.54 (m)	1.33 (m) 1.58 (qd, 13.1, 5.2)	1.81 (qd, 13.2, 4.3) 2.08 (m)
2	1.63 (m)	2.39 (ddd, 14.2, 4.9, 1.9) 2.56 (td, 14.2, 4.9)	1.18 (m) 1.58 (m)	1.63 (td, 14.2, 7.3) 2.03 (ddd, 14.2, 7.3, 1.7)	2.33 (dq, 14.2, 2.0) 2.45 (td, 14.2, 6.8)
3	1.60 (m) 1.79 (m)	–	3.48 (t, 2.5)	3.48 (brs)	–
4	–	–	–	–	–
5	1.77 (m)	–	–	–	–
6	1.25 (m) 1.76 (m)	1.50 (m) 1.64 (m)	1.43 (m)	1.34 (td, 13.0, 4.1) 1.67 (dt, 13.0, 3.1)	3.95 (dd, 11.6, 3.9)
7	1.24 (m) 1.92 (m)	1.44 (m) 1.42 (m)	1.32 (m) 1.44 (m)	1.35 (m) 1.49 (m)	1.62 (dd, 13.5, 2.4) 2.04 (m)
8	–	1.70 (m)	1.82 (m)	1.50 (m)	2.05 (m)
9	1.20 (s)	–	–	–	–
10	–	2.51 (dd, 12.4, 2.9) 1.59 (m)	1.38 (m)	1.87 (m)	2.64 (dd, 13.3, 2.6)
11	1.53 (m)	1.95 (dd, 15.7, 9.0)	1.77 (dd, 15.1, 6.8) 1.92 (dd, 15.1, 6.8)	1.40 (d, 15.9) 1.51 (d, 15.7)	2.86 (d, 16.1) 2.92 (d, 16.1)
12	1.65 (m) 1.67 (m)	4.92 (dd, 9.0, 1.5)	4.74 (t, 6.2)	2.05 (m)	–
13	2.48 (m) 1.30 (d, 13.8)	–	–	–	–
14	12.43 (d, 13.8)	6.38 (brs)	6.48 (d, 0.9)	5.47 (t, 6.8)	6.78 (br s)
15	–	7.38 (m)	7.46 (t, 1.5)	4.14 (d, 6.8)	7.59 (br s)
16	2.10 (m)	7.38 (m)	7.48 (s)	4.10 (s)	8.02 (br s)
17	1.28 (m) 1.94 (m)	0.79 (d, 6.7)	0.92 (d, 6.7)	0.80 (d, 6.0)	0.95 (d, 6.6)
18	–	1.37 (s)	1.06 (s)	1.19 (s)	1.45 (s)
19	1.15 (s)	0.81 (s)	1.13 (s)	1.12 (s)	0.97 (s)
20	1.10 (s)	0.74 (s)	0.76 (s)	0.75 (s)	0.87 (s)
1'	2.03 (m)	–	–	–	4.45 (d, 7.7)
2'	–	–	–	–	3.13 (dd, 9.2, 7.9)
3'	5.21 (br s)	–	–	–	3.33 (m)
4'	2.18 (d, 17.4) 2.24 (d, 17.4)	–	–	–	3.27 (q, 9.5)
5'	2.08 (m)	–	–	–	3.36 (m)
6'	–	–	–	–	3.66 (dd, 11.8, 5.4) 3.85 (dd, 11.8, 2.2)
7'	1.12 (m) 2.35 (m)	–	–	–	–
8'	1.26 (s)	–	–	–	–
9'	0.82 (s)	–	–	–	–
10'	1.98 (m) 2.05 (m)	–	–	–	–

[a] ^{1}H NMR data were recorded at 500 MHz; [b] ^{1}H NMR data were recorded at 300 MHz; [c]solvent $CDCl_3$; [d]solvent MeOD.

from NOESY data and comparison with kaurane-type diterpenoids. In this spectrum were observed correlation between the both methyls groups at δ_H 1.15 (CH_3-19) and 1.10 (CH_3-20), as well as the correlation between the hydrogens at δ_H 1.77 (H-5) and 1.20 (H-9). The orientation of the monoterpene group at C-16 was assigned as β due the strong nOe correlations observed between the hydrogens at δ_H 2.10 (H-16) and 1.30 (H-14); and δ_H 2.43 (H-14) and 1.10 (CH_3-20) (Figure 2). This finding was confirmed by the chemical shift observed for C-12 at δ_X 24.8 that was shielded in comparison to the *ent*-kaur-16-en-15-oxo-18-oic acid at δ_X 32.4. This effect can be explained by the γ-gauche interaction of the C-12 with the methylene group at C-17, as observed for other reported kaurane dimers with

Table 2. ^{13}C NMR chemical shifts of compounds **1**-**5** (δ in ppm)

C	**1**[a,c]	**2**[b,c]	**3**[a,d]	**4**[b,d]	**5**[b,d]
	δ_C	δ_C	δ_C	δ_C	δ_C
1	38.6	23.8	18.1	17.7	24.1
2	18.4	36.4	30.6	31.2	37.7
3	36.9	216.0	77.9	77.1	214.1
4	47.6	81.8	77.3	77.5	83.9
5	49.5	45.4	43.6	42.7	51.0
6	21.9	31.7	34.1	33.7	85.4
7	33.7	26.8	27.9	28.0	35.8
8	53.2	37.3	38.6	37.5	36.7
9	52.3	39.9	40.6	39.9	43.0
10	39.2	42.2	44.4	41.9	43.9
11	17.7	45.9	46.8	38.8	47.8
12	24.8	63.5	63.9	29.5	197.4
13	33.0	131.5	132.0	144.1	130.9
14	37.4	108.4	110.0	127.3	109.4
15	224.1	143.7	144.7	59.0	146.1
16	52.9	138.4	140.4	60.5	149.9
17	23.4	16.0	16.9	16.6	16.6
18	184.9	21.9	23.4	21.3	23.0
19	16.2	15.3	16.6	18.0	11.0
20	18.0	17.9	19.0	19.0	17.9
1’	45.8	–	–	–	106.6
2’	148.0	–	–	–	75.3
3’	116.6	–	–	–	78.1
4’	31.4	–	–	–	71.7
5’	41.0	–	–	–	78.0
6’	38.1	–	–	–	62.8
7’	31.9	–	–	–	–
8’	26.5	–	–	–	–
9’	21.3	–	–	–	–
10’	35.5	–	–	–	–

[a] ^{13}C NMR data were recorded at 125 MHz; [b] ^{13}C NMR data were recorded at 75 MHz; [c]solvent $CDCl_3$; [d]solvent MeOD.

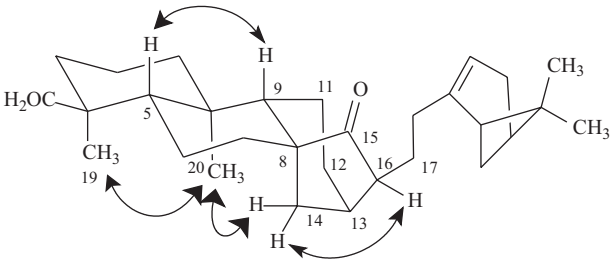

Figure 2. Important dipolar couplings observed through the NOESY for compound **1**.

β orientation.[11,12] Thus, compound **1** was determined to be the new 15-oxo-17(10’-α-pinenyl)-kauran-18-oic acid.

Compound **2** was obtained as a yellow solid. Its molecular formula $C_{20}H_{30}O_4$ was determined by HRESIMS by the ion at *m/z* 357.2038 $[M + Na]^+$. The IR spectrum displayed diagnostic absorption bands referring to hydroxyl and carbonyl groups at 3447 and 1707 cm^{-1}, respectively. The ^{13}C NMR spectrum displayed twenty signals associated with four methyl groups (δ_X 15.3, 16.0, 17.9 and 21.9), five methylenes and six methines (one of which oxygenated at δ_X 63.4 and three olefinic at δ_X 138.4, 131.5 and 108.4). In addition, five non-hydrogenated carbons including a carbonyl (δ_X 216.0) were observed (Table 2). The ^1H NMR spectrum revealed signals consistent with the presence of four methyls, three angular at δ_H 0.74 (s, CH_3-20), 0.81 (s, CH_3-19) and 1.37 (s, CH_3-18), and a tertiary at δ_H 0.79 (d, *J* 6.7 Hz, CH_3-17), an oxymethine at δ_H 4.92 (dd, *J* 5.0, 1.5 Hz, H-12), and three olefinic methines, one at δ_H 6.38 (br s, H-14) and two overlapped at δ_H 7.38 (m, H-15 and H-16) (Table 1). Signals relative to a subsystem constituted by five hydrogens belonging two diastereotopic methylenes at δ_H 2.56 (dt, *J* 14.2, 4.9 Hz, H-2β), 2.39 (ddd, *J* 14.2, 4.9, 1.9 Hz, H-2α), 2.22 (m, H-1β), and 1.68 (m, H-1α), and a methine at δ_H 2.51 (dd, *J* 12.4, 2.9 Hz, H-10), were observed by analysis of the ^1H-^1H COSY experiment. The deshielded diastereotopic protons of a methylene at δ_H 2.56 and 2.39 (2H-2) were attributed to the methylene “α” to a ketone group, due to the constant of 14.2 Hz. Furthermore, the coupling between both methylenes at δ_H 1.64 (m, H-6_{eq}) and 1.50 (m, H-6_{ax}), 1.42 (H-7_{eq}) and 1.44 (and H-7_{ax}), the latter showing correlation with the methine at δ_H 1.70 (m, H-8), revealed another subsystem of five hydrogens, which were placed between two quaternary sp^3 carbon atoms. In addition, the remaining methylene at δ_H 1.95 (dd, *J* 15.7, 9.0 Hz, H-11_{eq}) and 1.59 (m, H-11_{ax}) showed correlations with the oxymethine at δ_H 4.92 (dd, *J* 9.0, 1.5 Hz, H-12). The proposed assignment for the clerodane skeleton contained a β-monosubstituted furan moiety[9,13] was supported by analyzing the HMBC spectrum, through the long-range correlations of the methyl groups at δ_H 0.74 (H-20) with carbons at δ_X 45.9 (C-11), 42.2 (C-10) and 37.3 (C-8), and those of the methyl at δ_H 0.79 (H-17) with carbons at δ_X 39.9 (C-9) and 26.8 (C-7). The allocation of the hydroxyl group at C-12 was deduced on the basis of the correlations of the oxymethine at δ_H 4.92 (H-12) with the carbon at δ_X 39.9 (C-9), and with the carbons of the furan moiety at δ_X 108.4 (C-14) and 138.4 (C-16). On the other hand, the correlations of the methyl at δ_H 1.37 (CH_3-18) and hydrogens of the methylene at δ_H 1.68 and 2.22 (2H-1) with the carbonyl at δ_X 216.0 (C-3), besides the concomitant correlations of the hydrogens at δ_H 0.81 (CH_3-19), 2.51 (H-10), 2.39 and 2.56 (2H-2) with the non-hydrogenated oxygenated carbon at δ_X 81.8 (C-4), are consistent with the presence of a carbonyl at C-3 and a hydroxyl group at C-4. The configuration of the hydroxyl at C-4 α-oriented was definitively determined on the basis of diagnostic nOe correlations observed between the methyl at δ_H 1.37 (H-18) and the methines at δ_H 2.51 (H-10) and 4.92 (H-12) in the

NOESY experiment (Figure 3). In this spectrum were also observed important correlations between the hydrogen at δ_H 2.51 (H-10) with the hydrogens at δ_H 1.37 (H-18) and 1.67 (H-12), as well as correlations between the hydrogens at δ_H 0.74 (H-20) with the hydrogens at δ_H 0.81 (H-19) and 1.68 (H-1a) (Figure **3**) charactering a *trans*-decalin system. These data suggested that the lateral chain at C-19 was β-oriented (equatorial), confirming the final structure of compound **2** as the new 3-oxo-15,16-epoxy-4α,12-dihydroxy-*ent-neo*-clerodan-13(16),14,diene.[14]

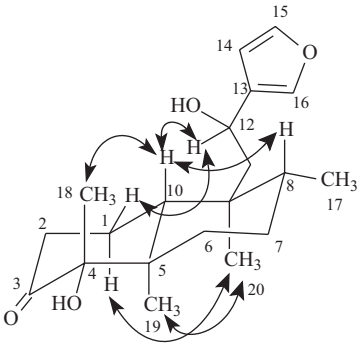

Figure 3. Dipolar coupling observed through the NOESY experiment for compound **2**.

Compound **3** was obtained as a colorless solid. The molecular formula $C_{20}H_{32}O_4$ was established by HRESIMS from the ion at *m/z* 359.2168 $[M + Na]^+$. The IR spectrum showed the presence of a hydroxyl band at 3287 cm^{-1}. Analysis of the ^{13}C NMR spectrum of **3** revealed that the chemical shifts were similar to those observed for compound **2** (Table 2), except for the absence of the signals relative to the carbonyl and the presence of an extra oxymethine at δ_X 77.9 (C-3) (Table 2). The ^1H NMR spectrum corroborated these observations, through the presence of an additional signal for an oxymethine at δ_H 3.48 (t, *J* 2.5 Hz, H-3). The suggestion that the carbonyl group at C-3 for compound **2** has been reduced in **3**, was confirmed by the diagnostic long-range correlations in the HMBC spectrum observed between the oxymethine proton at δ_H 3.48 (H-3) and the carbons at δ_X 18.1(C-1), 43.6 (C-5) and 23.4 (C-18). The relative stereochemistry of the hydroxyl group at C-3 was defined as α-oriented (equatorial) by the small value of the coupling constant (*J* 2.5 Hz) of the triplet observed for H-3. As for compound **2**, the NOESY experiment showed the same correlations between the hydrogens at δ_H 1.38 (H-10) with the hydrogens at δ_H 1.06 (H-18) and 4.74 (H-12), as well as correlations between the hydrogens at δ_H 0.76 (CH_3-20) with the hydrogens at δ_H 1.13 (CH_3-19) and 1.54 (H-1a), and suggested that the lateral chain at C-19 was also β-oriented (equatorial). Thus, the final structure of compound **3** was elucidated as the new 15,16-epoxy-3α,4α,12-trihydroxy-*ent-neo*-clerodan-13(16)14-diene.

Compound **4** was obtained as a white solid. The molecular formula $C_{20}H_{36}O_4$ was established by HRESIMS by the ion at *m/z* 363.2524 $[M + Na]^+$. The IR spectrum showed hydroxyl and C=C stretching bands at 3349 and 1653 cm^{-1}, respectively. The ^1H NMR spectrum signals indicated a close relationship with those observed for compound **3**, mainly relative to the decalin system (Table 1). The differences found were related to the presence of just one olefinic proton at δ_H 5.47 (t, *J* 6.8 Hz, H-14), and two oxymethylenes at δ_H 4.14 (d, *J* 6.8 Hz, H-15) and 4.10 (s, 2H-16), besides the disappearance of the signals relative to the furan moiety and the oxymethine at C-12. These data suggested that the furan moiety of compound **3** was opened in **4**. This was confirmed by comparison of their ^{13}C NMR data, since the typical furan values of compound **2** were here replaced by two oxymethylene carbons at δ_X 59.0 (C-15) and 60.5 (C-16), and a double-bond at δ_X 144.1 (C-13) and 127.3 (C-14) on **3** (Table 2). The NOESY experiment showed correlations between the hydrogen at δ_H 1.87 (H-10) with the hydrogens at δ_H 1.50 (H-8), 2.03 (H-2a), 1.67 (H-6b) and 1.19 (CH_3-18). In addition, it was observed the correlations between the hydrogens at δ_H 0.75 (CH_3-20) with hydrogens at δ_H 1.12 (CH_3-19), 1.40 (H-11b), 1.49 (H-7a) and 1.58 (H-1a), and the correlation of the hydrogen at δ_H 3.48 (H-3) with hydrogens at δ_H 1.19 (CH_3-18). From the above data we can assume that the hydroxyls groups at C-3 and C-4, and the lateral chain at C-19 have the same configuration as in compound **3**, so compound **4** was thus characterized as the new 3α,4α,15,16-tetrahydroxy-*ent-neo*-(*E*)-cleroda-13-ene.

Compound **5** was obtained as a white solid with a molecular formula $C_{26}H_{38}O_{10}$ as determined by the peak at *m/z* 533.2409 $[M + Na]^+$.The IR spectrum implied the presence of hydroxyl and carbonyl groups at 3414 and 1699 cm^{-1}, respectively. From the ^1H NMR spectrum, a monossubstituted furan system was defined by the typical signals at δ_H 8.02 (br s, H-16), 7.59 (br s, H-15) and 6.78 (br s, H-14). The magnitude of geminal coupling constants observed for the deshielded methylene groups at δ_H 2.45 (td, *J* 14.2, 6.8 Hz, H-2_{eq}), 2.33 (dq, *J* 14.2, 2.0 Hz, H-2_{ax}), 2.92 (d, *J* 16.1 Hz, H-11_{eq}), and 2.86 (d, *J* 16.1 Hz, H-11_{ax}) were characteristic of "α" methylene ketone groups (Table 1). In addition, an β-anomeric proton at δ_H 4.45 (d, *J* 7.7 Hz, H-1'), and the signals in the region of δ_H 4.45-3.13, suggested the presence of a sugar unit, that was determined to be the glucose based on the chemical shifts of the ^1H and ^{13}C NMR spectrum in comparison with literature.[15] The β-anomeric configuration was judged by the larger value of the coupling constant (*J* 7.7 Hz) of the doublet related to the anomeric hydrogen.

The location of the hydroxyl group at C-4 was established by the concomitant long-range correlations of hydrogens at δ_H 0.97 (CH_3-19), 2.64 (H-10) and 3.95 (H-6) with the carbon at δ_X 83.9 (C-4), in the HMBC spectrum. Moreover, the correlations between the hydrogen at δ_H 1.45 (CH_3-18) with the carbons at δ_X 51.0 (C-5) and 214.1 (C-3), besides the correlation of the hydrogens at δ_H 0.97 (CH_3-19) and the anomeric proton at δ_H 4.45 (H-1') with the carbon at δ_X 85.4 (C-6) confirmed the locations of carbonyl and glucosyl at C-3 and C-6, respectively. On the other hand, the correlations observed between the hydrogens at δ_H 0.87 (CH_3-20) and 2.64 (H-10) with the carbon at δ_X 47.8 (C-11), determined the location of the other carbonyl group at C-12. The equatorial orientations of the glucosyl group and hydroxyl groups at C-6 and C-3, respectively, were determined based on the value of the coupling constant observed for the oxymethine H-6 (*J* 11.6, 3.9 Hz), and by nOe correlations observed between the hydrogens at δ_H 2.64 (H-10) with those at δ_H 1.45 (CH_3-18), 2.05 (H-8) and 3.95 (H-6). Thus, compound **5** was characterized as the new 3,12-dioxo-15,16-epoxy-4α-hydroxy-6-(β-glucopyranosyl)-*ent*-*neo*-clerodan-13(16),14-diene.

The isolation of diterpene dimers is reported to several species of different genera, as symmetrical compounds with icexetane, labdane and kaurane skeletons.[16-19] The occurrence of asymmetrical dimers are more restricted, and generally involve the junction of two diterpene monomers.[20,21] In *Croton* genus, this feature is limited to the species *C. tonkinensis* and *C. micans*, as symmetrical structures of *ent*-kaurene.[22,23] The structure of an asymmetrical dimer formed by the junction between diterpene and monoterpene moities, as it is the case for compound **1**, is a feature never reported in the literature before.

The cytotoxic activity of compound **1** was evaluated against colorectal adenocarcinoma (HCT-116), ovarian carcinoma (OVCAR-8) and glioma (SF-295) cell lines, exhibiting IC_{50} values of 7.14, 8.19 and > 10 μg mL^{-1}, respectively. Previous studies showed the cytotoxicity of *ent*-kaur-16-en-15-oxo-18-oic acid against promyelocytic leukemia (L-60), glyoblastoma (SF-295), colon cancer (HCT-8) and melanoma (MDAMB-435) cell lines.[24] Since then the *ent*-kaur-16-en-15-oxo-18-oic acid is one monomer of compound **1** our results are in agreement with these findings, and with the cytotoxicity associated to kaurenoic acid derivatives.[25-28]

Conclusions

Chemical investigation of *C. limae* yielded an unusual asymmetrical dimer of a kaurane derivative with the monoterpene α-pinene (**1**), besides four new clerodanes (**2**-**5**). Based on the data collected from three independent experiments, the results showed that compound **1** exhibited a moderate cytotoxic effect against HCT-116 and OVOCAR-8 cancer cell lines with IC_{50} values of 7.14, 8.19 and > 10 μg mL^{-1}, respectively. These facts are in agreement with the knowledge that the *Croton* genus is an abundant source of several classes of structurally complex and bioactive diterpenes, which justify the efforts in pursuing the phytochemical study of other *Croton* species from the northeastern Brazil.

Supplementary Information

The NMR and mass spectra of compounds **1**-**5** are available at http://jbcs.sbq.org.br as a free-access PDF file.

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