TRACHYLOBANE AND KAURANE DITERPENES FROM Croton floribundus SPRENG.

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A new trachylobane diterpene ent-trachyloban-18,19-diol (1) was isolated from root bark of Croton floribundus, along with known diterpenes ent-trachyloban-19-oic acid (2), 15β-hydroxy-ent-trachyloban-19-oic acid (3), ent-trachyloban-19-ol (4), ent-kaur-16-en-19-oic acid (5), ent-kaur-16-ene-6,19-diol (6) and ent-16β-hydroxykaur-11-en-19-oic acid (7). ent-trachyloban-18,19-diol (1) was submitted to derivatization reactions affording four new compounds (8-11). Cytotoxic activity of diterpenes 1, 3, 4, 7-11 against three human cancer cell lines was evaluated. No compounds showed cytotoxic potential with IC₅₀ values greater than 25 µg/mL. Compound 6 was evaluated against five human cancer cell lines, showing moderate effect against three cancer cell lines, MDA-MB-435, HCT-8 and HCT-116, with IC₅₀ values of 14.32, 13.47 and 12.1 µg/mL, respectively.

Keywords: diterpenes; Croton floribundus; Euphorbiaceae.

INTRODUCTION

Trachylobane and kaurane are diterpenoids biosynthetically derived from the monomeric monolactone. Kaurenes have been reported in several families of higher plants, with kaurenoic acid and its natural derivatives found particularly in the Asteraceae and Euphorbiaceae families. These compounds have been largely cited as cytotoxic, antitumoral, genotoxic, antiinflammatory, antibacterial, antifungal and moluscidal. On the other hand, trachylobane is found in some species of Euphorbiaceae, Annonaceae, Asteraceae, Lamiaceae and Leguminosae. Although the biological activities of trachylobane compounds are poorly investigated, they are cited to possess vasorelaxant and cytotoxic properties.

As part of an investigative effort to find bioactive diterpenoids in native species from Northeastern Brazil flora, this study reports the phytochemical investigation of the root bark of Croton floribundus Spreng., a tree with ethnobotanical use in the treatment of syphilis and ulcers. In this work, the new ent-trachyloban-18,19-diol is reported, along with the known ent-trachyloban-19-oic acid (2), ent-15β-hydroxytrachyloban-19-oic acid (3), ent-trachyloban-19-ol (4), ent-kaur-16-en-19-oic acid (5), ent-kaur-16-ene-6,19-diol (6) and ent-16β-hydroxykaur-11-en-19-oic acid (7). ent-trachyloban-18,19-diol 1 was derivatized to the diacetate, dimethoxyl and dialyl derivatives 8-10, respectively. In addition, an unusual oxidized product 11 was obtained by oxidation with PCC and chromic acid. The cytotoxicity of the compounds 1, 3, 4, 6-11 against a small panel of cancer cell lines was evaluated (Figure 1).

RESULTS AND DISCUSSION

Compound 1 was isolated as a colorless solid, with [α]D²⁰ -41° (c 0.1, CHCl₃), m.p. 148.2-149.3 °C. Its molecular formula C₄₆H₇₀O₈ was established based on its quasi-molecular ion at m/z 327.2349 [M + Na]+(calc for C₄₆H₇₀O₈Na 327.2295), in the HRESIMS. The IR spectrum contained absorption bands of the hydroxyl group evidenced by the characteristic absorptions at 3332 and 1029 cm⁻¹.

The ¹H NMR spectrum displayed signals for two angular methyl groups at δ 0.90 (s, 3H-20) and 1.12 (s, 3H-17), and deshielding signals at δ 3.91 (d, J = 10.5 Hz, H-19a), 3.88 (d, J = 10.5 Hz, H-18a), 3.71 (d, J = 10.5 Hz, H-19b), and 3.33 (d, J = 10.5 Hz, H-18b), that...
were attributed to two oxymethylene groups attached to a quaternary carbon. In addition, the presence of the shielded doublets at $\delta$ 0.58 (d, $J = 7.5$ Hz, H-12) and 0.83 (dd, $J = 7.5$ and 2.4 Hz, H-13) of a tetrasubstituted cyclopropane ring suggested that I belongs to the trachylobane series.

Chemical shifts and comparative analysis of BB and DEPT-13C NMR spectra revealed 20 lines in agreement with the suggested molecular formula. These data allowed clear deduction of the presence of two oxygen-bearing methylene carbons at $\delta$ 74.0 (C-18) and 65.0 (C-19), and 18 other sp3 non-functionalized carbon signals (two methyls, eight methylenes, four methines and four quaternaries). The unambiguous assignment of all carbons and hydrogens was possible by the HMBC spectrum analysis (Table 1). In particular, the presence of two magnetically equivalent carbons was evidenced by the correlations of the three hydrogens at $\delta$ 0.58 (H-12), 1.12 (H-17) and 1.60 (H-6) with a single signal at $\delta$ 20.7, and the correlations of hydrogens at $\delta$ 0.94 (H-5) and 1.14 (H-9) with the carbon at $\delta$ 53.5.

Table 1. $^1$H and $^{13}$C NMR (500 and 125 MHz) of I in CDCl$_3$, $J$ in Hz, $\delta$ in ppm

<table>
<thead>
<tr>
<th>C</th>
<th>$^1$H</th>
<th>HMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.52 (d, $J = 11.2$)</td>
<td>3.90 (2H-3, 3H-20)</td>
</tr>
<tr>
<td>2</td>
<td>1.55 (d, $J = 7.5$)</td>
<td>17.3</td>
</tr>
<tr>
<td>3</td>
<td>2.00 (d, $J = 11.2$)</td>
<td>30.4 (2H-1, H-18, H-19a)</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>41.7</td>
</tr>
<tr>
<td>5</td>
<td>0.94 (m)</td>
<td>53.5 (2H-1, 2H-3, H-18, H-19, 3H-20)</td>
</tr>
<tr>
<td>6</td>
<td>1.60 (m)</td>
<td>20.7</td>
</tr>
<tr>
<td>7</td>
<td>1.35 (m)</td>
<td>39.3 (2H-15)</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>40.6 (2H-6, H-11, H-13)</td>
</tr>
<tr>
<td>9</td>
<td>1.14 (m)</td>
<td>53.5 (2H-1, 2H-11, H-12, 2H-15, 3H-20)</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>38.2</td>
</tr>
<tr>
<td>11</td>
<td>1.88 (d, $J = 13.0$)</td>
<td>21.1</td>
</tr>
<tr>
<td>12</td>
<td>1.64 (d, $J = 7.5$)</td>
<td>20.7 (H-13, 2H-14, 2H-15, 3H-17)</td>
</tr>
<tr>
<td>13</td>
<td>0.56 (m)</td>
<td>24.4 (2H-15, 3H-17)</td>
</tr>
<tr>
<td>14</td>
<td>3.80 (d, $J = 7.5$)</td>
<td>33.5 (H-9, H-12, 2H-15)</td>
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<td>15</td>
<td>1.36 (d, $J = 11.2$)</td>
<td>50.5 (3H-17)</td>
</tr>
<tr>
<td>16</td>
<td>1.23 (d, $J = 11.2$)</td>
<td>22.6 (2H-14)</td>
</tr>
<tr>
<td>17</td>
<td>1.12 (s)</td>
<td>20.7 (H-12, H-13, 2H-15)</td>
</tr>
<tr>
<td>18</td>
<td>3.38 (d, $J = 10.5$)</td>
<td>74.0 (H-5, H-19)</td>
</tr>
<tr>
<td>19</td>
<td>3.33 (d, $J = 10.5$)</td>
<td>65.0 (H-3, H-5, H-18)</td>
</tr>
<tr>
<td>20</td>
<td>0.90 (s)</td>
<td>15.1 (H-1b, H-5, H-9)</td>
</tr>
</tbody>
</table>

Further evidence for a trachylobane skeleton was obtained by the long-range correlations in the HMBC spectrum. In this experiment, correlation signals for the hydrogen of cyclopropane moiety at $\delta$ 0.88 (H-13) with the carbons at $\delta$ 20.7 (C-12), 22.6 (C-16) and 40.6 (C-8) were observed, besides the correlation between the hydrogen at $\delta$ 0.56 (H-12) and the carbons at $\delta$ 53.5 (C-9), 33.5 (C-14), 20.7 (C-17) and 33.5 (C-14). In addition, the two oxymethylene groups at $\delta$ 3.91 and 3.71 (2H-19 and 2H-18, respectively) showed the same correlations with the carbons at $\delta$ 30.4 (C-3), 53.5 (C-5) and 41.7 (C-4), and confirmed the attachment of both groups to C-4 of the trans-decalin system.

On the basis of the leporatory nature of I, along with the knowledge of the co-occurrence of ent-trachylobane diterpenoids in plants of the Croton genus, it was possible to suggest that compound I belongs to the entanio series. From the previously established evidence, compound I was determined to be the new ent-trachyloban-18,19-diol. Comparison of NMR data of compound I with other trachylobanes indicated a close structural similarity with ent-26e,18,19-traquialbontinol, previously isolated from Psiadia punctulata, and that possesses one additional hydroxyl group at C-2.

Compounds 2-7 were identified as ent-trachyloban-19-19-oic acid (2), ent-15β-hydroxytrachyloban-19-19-oic acid (3), ent-trachyloban-19-ol (4), ent-kaur-16-en-19-oic acid (5), ent-kaur-16-en-6α,19-diol (6) and ent-16α-hydroxykaur-11-en-19-oic acid (7), by comparison of their spectroscopic data with those reported in the literature.

Compound I was the major compound isolated from the roots of C. floribundus. Thus, considering the reported cytotoxic activity for kaurene and trachylobane diterpenoids, we analyzed the cytotoxic effects of the natural compounds 1, 3-7. In order to correlate cytotoxicity with chemical structure, four new semi-synthetic trachylobane derivatives 8-11 were prepared from I, and their cytotoxic activity also evaluated. ent-trachyloban-18,19-diol 1 was acetylated with acetic anhydride and pyridine to afford the diacetate derivative. Reaction with methyl iodide and KOH yielded the dimethoxyl 9, while the reaction with allyl bromide and KOH yielded the diallyl 10. In addition, oxidation with PCC yielded the unusual oxidized product 11.

The $^1$H NMR spectrum of compound 11 showed similar signals to those described for 1. The only slight difference was the presence of one additional broad singlet at $\delta$ 2.46 (s, H-4), and the disappearance of the two diastereotopic oxymethylene protons 2H-18 and 2H-19. The NMR $^{13}$C spectrum of 11 showed only 19 spectral lines. The comparative analysis of the $^{13}$C NMR data of 11 with those observed for compound 1 revealed a shielded signal attributable to one carboxyl group at $\delta$ 181.3 (C-19), in place of the signals of the oxymethylene groups at $\delta$ 65.0 and $\delta$ 74.0 (C-18 and C-19) observed in 1. These assignments suggested compound 11 is a nor-diterpene containing a carbonyl group at C-19. Although unexpected, the isolation of this compound could be explained by oxidation of the two oxymethylene groups in 1 to give a dicarboxylic acid precursor, followed by CO$_2$ loss. Indeed, the thermal instability of malonic acid derivatives toward decarboxylation is well-known, and has been proposed as being due to a possible concerted mechanism where proton transfer is concurrent with carbon-carbon bond fission.

Initially, the in vitro anticancer activity of tested compounds was assessed against three human cancer cell lines (HCT-116; OVACR-8 and SF-295) by using MTT assay. Based on data collected from three independent experiments, results showed that only ent-kaur-16-en-6α,19-diol (6) exhibited a moderate effect against the HCT-116 cell line with an IC$_{50}$ value of 12.1 µg/mL (Table 2), while the other tested compounds showed IC$_{50}$ greater than 25 µg/mL (data not shown). In a second set of experiments, compound 6 was tested against another three tumor cell lines (HL-60, HCT-8 and MDA-MB-435), showing moderate cytotoxic effects on MDA-MB-435 cells with IC$_{50}$ values of 14.3 and 13.5 µg/mL, respectively (Table 2). The cytotoxicity of kaurene diterpenoids has been widely discussed in the literature. Costa-Lotufo et al. studied the effects of ent-kaur-16-en-19-oic acid (5) in developing sea urchin (Lytechinus variegatus) embryos on tumor cell growth. The results showed cytotoxic and anti-proliferative actions of ent-kaur-16-en-19-oic acid (5) against leukaemic cells of 95.3%, and against both MCF-7 breast carcinoma and HCT-8 colon cancer cells of 47.5%. Cavalcanti et al. demonstrated that kaurenic acid also had moderate cytotoxicity against cancer cell lines, with
Uchôa et al. 12.1 (9.1-16.1) Cl0.1, CHCl0.24 (0.2-0.27) 2= 3.5 Hz, H-7), 1.23 (1H, C) C-NMR values ranging from 9.1 to 14.3 µg/mL, where its mechanism of action was apparently related to DNA interaction ultimately leading to the inhibition of topoisomerase I and apoptosis induction. Therefore, the structural similarity between ent-kaur-16-en-19-oic acid (5) and ent-kaur-16-en-6α,19-diol (6), justifies the moderate effect exhibited by the latter compound in this work.

Table 2. Cytotoxic activity expressed by IC50 in µg/mL of compound 6 for cancer cell lines

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50(µg/mL)</th>
<th>Doxorubicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ent-kaur-16-en-6α, 19-diol (6)</td>
<td>&gt; 25</td>
<td>0.26 (0.17-0.3)</td>
</tr>
<tr>
<td>SF-295</td>
<td>&gt; 25</td>
<td>0.24 (0.2-0.27)</td>
</tr>
<tr>
<td>HCT-116</td>
<td>12.1 (9.1-16.1)</td>
<td>0.12 (0.09-0.17)</td>
</tr>
<tr>
<td>HL-60</td>
<td>&gt; 25</td>
<td>0.02 (0.01-0.02)</td>
</tr>
<tr>
<td>MDA-MB-435</td>
<td>14.3 (12.8-16.0)</td>
<td>0.48 (0.34-0.66)</td>
</tr>
<tr>
<td>HCT-8</td>
<td>13.5 (10.2-17.8)</td>
<td>0.01 (0.01-0.02)</td>
</tr>
</tbody>
</table>

* Data are presented as IC50 values and 95% confidence intervals obtained by nonlinear regression for all cell lines from three independent experiments.

**EXPERIMENTAL**

**General**

IR spectra were recorded on a Perkin-Elmer FT-IR 1000 spectrometer (Waltham, USA), using a NaCl disc. The NMR spectra were acquired on a Bruker Avance DRX 500 spectrometer, equipped with an inverse detection probe head and z-gradient accessory working at 499.9 (1H) and 124.97 MHz (13C), respectively. All pulse sequences were standard in the Bruker XWIN-NMR software, and all experiments conducted at room temperature. The samples, dissolved in CDCl3 (0.6 mL), were transferred to 5 mm tubes. The 1H and 13C chemical shifts were expressed in the δ scale and referenced to TMS through the residual CHDCl3 as an internal standard.

**Plant material**

*C. floribundus* was collected from Pacoti county (Ceará State, Northeast Brazil) and authenticated by E. P. Nunes of the Departamento de Biologia, Universidade Federal do Ceará. A voucher specimen (#39851) was deposited at the Herbarium Prisco Bezerra (EAC), Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, Ceará, Brasil.

**Extraction and isolation**

Root bark (1.58 kg) from *C. floribundus* was dried, pulverized and extracted with hexane at room temperature. The solvent was removed under reduced pressure to give the crude hexane extract (97.06 g).

The crude hexane extract was coarsely chromatographed on a silica gel column by elution with hexane, CHCl3, EtOAc and MeOH to give 4 fractions. Chromatography of the EtOAc fraction (5.5 g) using hexane:EtOAc (70:30) as an isocratic eluting mixture afforded ten subfractions. Recrystallization of subfraction F-6 (940.5 mg) with hexane provided the compound ent-trachyloban-18,19-diol (1) (350.3 mg). The sub-F-2 (97.2 mg) was rechromatographed on a silica gel and eluted with a mixture of hexane/CHCl3 (90:10) to afford ent-trachyloban-19-oic acid (2) (20.2 mg). The compound ent-15β-hydroxytrachyloban-19-oic acid (3) (13.5 mg) was isolated from subfraction F-3 (335.2 mg) by repeated chromatography over silica gel (hexane-EtOAc 85:15). Chromatography of subfraction F-4 (427.0 mg) using hexane:EtOAc (90:10) as an isocratic eluting mixture afforded ent-kaur-16α-en-6α,19-diol (6) (32.0 mg). The subfraction F-5 (325.0 mg) was subjected to chromatography using hexane:EtOAc (90:10) as an isocratic eluting mixture to yield ent-kaur-16α-hydroxykaur-11-en-19-oic acid (7) (15.0 mg).

The dichloromethane fraction (5.0 g) was chromatographed over silica gel using a gradient system of hexane/CHCl3 affording 7 subfractions. The sub fraction F-3 (982.1 mg) was subjected to flash chromatography and eluted with hexane/CHCl3 (90:10) to give the compound ent-kaur-16-en-19-oic acid (5) (323.5 mg). The subfraction F-5 (923.3 mg) was subjected to CC and eluted using a mixture of hexane/CHCl3 with increasing polarity, yielding six subfractions. Fraction 5 (923.3 mg) was submitted to semi-preparative HPLC using an isocratic hexane-isopropanol (99:1) system as the eluent, to obtain ent-trachyloban-19-ol (4) (510.0 mg).

**General procedure for preparation of derivative 8**

Compound I (30.4 mg, 0.1 mmol) was submitted to acetylation reaction using acetic anhydride (0.2 mmol) and pyridine (1.0 mL) with stirring for 3 h at room temperature. The reaction mixture was washed with CuSO4 5% solution and extracted with CH2Cl2 (3 x 20 mL). The CH2Cl2 phase was washed with water and dried with Na2SO4. Removal of the solvent yielded 28.3 mg (72.9 %) of compound 8.

**General procedure for preparation of derivative 9**

A total of 30.4 mg of compound 1 (0.1 mmol) was added to a stirred solution of KOH (44.8 mg, 0.8 mmol) in DMSO (3 mL), followed immediately by addition of methyl iodide (0.025 mL, 0.4 mmol). The mixture was stirred for 4 h at room temperature, after which the mixture was poured into water (10 mL) and extracted with CH2Cl2 (3 x 20 mL). The organic layer was dried with Na2SO4 and the solvent was removed under reduced pressure, to give 19.5 mg (58.7%) of compound 9.
compound 11. Colorless solid. [α]D20 -25 (c 0.1, CHCl3), IR (cm–1, NaCl disc) νmax/cm–1: 3400-2700 (O-H) and 1687 (C=O). HR-ESIMS m/z: 287.2089 ([M+H]+), 287.2011, calc. for C7H12O3. 3-H-NNMR δ6 (500 MHz, CDCl3): 2.46 (1H, s, H-4), 2.11 (1H, d, J = 12.7 Hz, H-3a), 2.05 (1H, d, J = 11.7 Hz, H-14a), 1.91 (1H, m, H-11a), 1.90 (1H, m, H-6a), 1.86 (1H, m, H-2a), 1.66 (1H, ddd, J = 14.5; 7.6, 2.0 Hz, H-11b).1.56 (1H, d, J = 12.9 Hz, H-1a), 1.48 (1H, d, J = 11.7 Hz, H-14b), 1.44 (1H, m, H-6b), 1.40 (1H, m, H-2b), 1.40 (2H, m, H-7), 1.40 (1H, m, H-15a), 1.31 (1H, m, H-3b), 1.28 (1H, m, H-15b), 1.23 (1H, s, H-5), 1.18 (1H, d, J = 11.6, 7.9 Hz, H-9), 1.14 (1H, m, H-14b), 1.12 (3H, s, H-17), 0.95 (3H, s, H-20), 0.80 (1H, ddd, J = 7.7, 3.1, H-13), 0.77 (1H, td, J = 19.2, 13.0, 3.5 Hz, H-11b), 0.57 (1H, d, J = 7.7 Hz, H-12). 13C-NMR δ6 (150 MHz, CDCl3): 78.5 (C-18), 74.2 (C-19), 59.3 (19-OCH3), 59.3 (18-OCH3), 53.2 (C-9), 50.2 (C-15), 49.5 (C-35), 41.5 (C-4), 40.7 (C-8), 39.1 (C-1), 38.9 (C-10), 33.3 (C-14), 30.9 (C-3), 24.2 (C-13), 22.4 (C-16), 20.6 (C-12), 20.6 (C-6), 20.5 (C-17), 19.9 (C-11), 17.6 (C-2), 15.0 (C-20).

CYTOTOXIC ACTIVITY AGAINST TUMOR CELL LINES

Cell line and cell culture

The tumor cell lines used in this work were HCT-8 (colorectal adenocarcinoma), HCT-116 (colorectal adenocarcinoma), MDA-MB 435 (melanoma); OVCAR-8 (ovarian carcinoma); HL-60 (leukemia) and SF-295 (glioma), kindly provided by the National Cancer Institute (Bethesda, MD, USA). All cancer cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C with 5% CO2.

MTT assay

The cytotoxicity of compounds 1, 3, 4, 7-11 was tested against three tumor cell lines, while compound 6 was tested against six tumor cell lines using the 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma Aldrich Co., St. Louis, MO/USA) reduction assay. For all experiments, cells were plated in 96-well plates (105 cells/well for adherent cells or 3×105 cells/well for suspended cells in 100 µL of medium). The tested compounds (0.05-25 µg/mL) dissolved in DMSO were added to each well (using the HTS – high-throughput screening - biomek 3000 device - Beckman Coulter Inc., Fullerton, California, USA) and incubated for 72 h. Doxorubicin was used as the positive control (0.009-5 µg/mL). Control groups received the same amount of DMSO. After 69 h of incubation, the supernatant was replaced by fresh medium containing MTT (0.1% MTT) and incubated for 3-4 h. Cell viability was determined with a plate reader (SpectraMax M2, Molecular Devices, CA, USA). The optical density (OD) was absorbed at 570 nm (blank control) and at 595 nm (OD of compound). The percentage of viability was calculated as [(OD of compound / OD of blank control) × 100%].

CONCLUSIONS

C. floribundus proved a prolific source of diterpenes, corroborating previously reported results for the Croton species. In this work, the cytotoxic activity of kaurene and trachylobane diterpenes isolated from the roots, besides the semi-synthetic trachylobane derivatives of new ent-trachyloban-18,19-diol (1), was evaluated. However, based on data collected from three independent experiments, results showed that the only ent-kaur-16-en-6,19-diol (6) exhibited a moderate effect against the three cancer cell lines, MDA-MB-435, HCT-8 and HCT-116, with IC50 value of 14.32, 13.47 and 12.1 µg/mL, respectively. The other tested compounds showed IC50 > 25 µg/mL.
SUPPLEMENTARY MATERIAL

Available at http://quimicanova.sbq.org.br, in pdf file with free access.

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