









## Identification of the Main Specialized Metabolites of *Ceanothus caeruleus* and Cytotoxic Effects of *a-nor*-Lupane Derivatives<sup>†</sup>

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**Abstract:** The dichloromethane (DCM) and ethyl acetate (EtOAc) extracts of *C. caeruleus* yielded nine known compounds, including an *A-nor*-lupane triterpenoid identified as gouanic acid B (**1**). Additionally, its acetyl derivative, acetylgouanic acid B (**2**), is reported here for the first time as a natural product. Furthermore, we tested the bioactivity of natural products **1** and **2** and their dimethyl ester derivatives **3** and **4** against cancer cell lines MCF-7, A549, HeLa, and K562. Among these, compounds **1** (IC<sub>50</sub> = 36.4 ± 4.0 μM), **2** (IC<sub>50</sub> = 21.6 ± 4.3 μM), and **4** (IC<sub>50</sub> = 33.0 ± 2.0 μM) demonstrated moderate to good activity against the K562 cell line while maintaining a satisfactory survival rate in non-cancerous bMEC cells. Notably, the natural triterpenes **1** and **2** and derivative **4** showed remarkable outcomes in cytotoxicity tests due to their specificity against K562 leukemia cells.

**Keywords:** *Ceanothus caeruleus*; triterpenoid; bioactive compounds; cytotoxicity activity; non-cancerous cell line. © 2025 ACG Publications. All rights reserved.

### 1. Plant Source

Certain species of *Ceanothus* play a significant role in traditional medicine. For example, *Ceanothus caeruleus*, native to the State of Michoacán in Mexico and commonly referred to as

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## Secondary metabolites of *Ceanothus caeruleus*

"chaquira's flower" or "leather rod," is used to treat various ailments, including wounds, pimples, insect bites, foot inflammation, stomach issues, and diarrhea [1, 2].

Flowers, leaves, shrubs, and stems of *Ceanothus caeruleus* Lag. were collected on February 2023 near the Estribo Grande Panoramic Viewpoint (19°30'52.5''N, 101°38'36.8''W, 2348 masl) at Pátzcuaro, Michoacán, Mexico. A voucher specimen (EBUM 3658) was identified by M.Sc. Patricia Silva-Sáenz and deposited at EBUM Herbarium of the Facultad de Biología of the Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, Mexico.

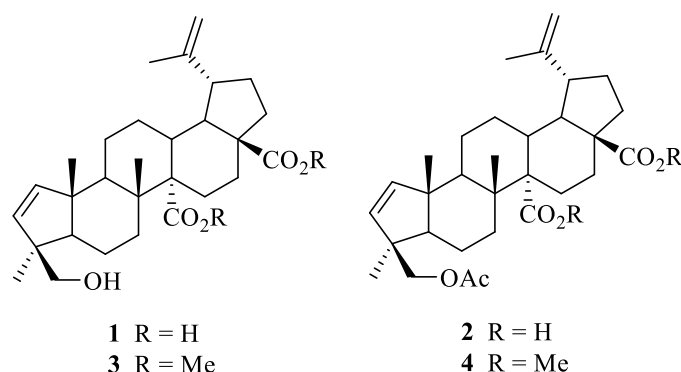
## 2. Previous Studies

There are no previous reports about the chemical composition of this plant.

## 3. Present Study

Organic extracts from *Ceanothus caeruleus* were prepared using maceration and reflux extraction with dichloromethane (DCM) and ethyl acetate (EtOAc) (see Supplementary Information). DCM maceration of the flowers produced a mixture of  $\beta$ -sitosterol and stigmaterol (0.8%), ceanothenic acid (3.7%), alphitolic acid (5.2%), veratric acid (4.5%), and  $\beta$ -sitosterol  $\beta$ -D-glucopyranoside (2.2%), with structures confirmed by  $^1\text{H}$  NMR data comparison [3, 4-14]. Notably, this is the first report of alphitolic acid in the *Ceanothus* genus. Subsequent EtOAc extraction yielded lower amounts of ceanothenic acid (0.5%),  $\beta$ -sitosterol  $\beta$ -D-glucopyranoside (0.8%), and kaempferol (0.9%) [15]. For the leaves, DCM extraction isolated ceanothenic acid (2.7%), alphitolic acid (5.5%),  $\beta$ -sitosterol  $\beta$ -D-glucopyranoside (2.0%), and gouanic acid **1** (2.0%) similar to flower yields, along with betulinic acid as a minor product (0.7%) [16]. EtOAc extraction gave  $\beta$ -sitosterol  $\beta$ -D-glucopyranoside (0.8%) and acetylgouanic acid **2** (1.4%), whose structure was elucidated through 1D and 2D NMR, as it has not been previously reported as a natural product (see Supplementary Information). From the stems, DCM extraction yielded ceanothenic acid (2.1%) and  $\beta$ -sitosterol  $\beta$ -D-glucopyranoside (4.2%). DCM extraction of the roots provided high yields of dehydroabiatic acid (22.8%) [17], while EtOAc extraction identified *A-nor*-lupane ceanothic acid (21%) [18-21] (see Tables S1 and S2). The presence of component **2** in the plant has been confirmed through methanolic extraction by macerating the leaves without using any other solvent beforehand. The  $^1\text{H}$  NMR spectrum of the methanol extract showed a distinctive singlet signal attributed to the acetyl group, which verifies the presence of acetyl *A-nor*-lupane (See Figure S21 and S22). Additionally, the same methanolic extract of the leaves underwent HPLC analysis, using compound **2** as a standard. Data were recorded at a wavelength of 220 nm, with characteristic peaks for compound **2** observed at a retention time of 3.298 minutes for the pure compound and 3.402 minutes for the crude leaves extract, falling within a normal range variation [22], providing compelling evidence of its presence in *C. caeruleus* (see Figures S24-S28).

*Acetylgouanic acid B (2)*: colorless amorphous solid (decomposes above 100 °C),  $[\alpha]_{589} = +60$ ,  $[\alpha]_{578} = +62$ ,  $[\alpha]_{546} = +72$ ,  $[\alpha]_{436} = +129$ ,  $[\alpha]_{365} = +220$  (*c* 0.26, acetone at 25 °C, see supporting information for details of measurement); IR (KBr)  $\nu_{\text{max}}$  3450-2650 (-COOH), 2942 and 2869 (C-H), 1739 and 1687  $\text{cm}^{-1}$  (C=O).  $^1\text{H}$  NMR (300 MHz, pyridine-*d*<sub>5</sub>)  $\delta$  6.19 (1H, d, *J* = 5.9 Hz, H-2), 5.48 (1H, d, *J* = 5.9 Hz, H-3), 5.08 (1H, brs, H-29), 4.85 (1H, brs, H-29'), 4.23 (1H, d, *J* = 11.1 Hz, H-24), 4.11 (1H, d, *J* = 11.1 Hz, H-24'), 3.68 (1H, m, H-19), 2.04 (3H, s, AcO), 1.94 (3H, s, CH<sub>3</sub>-30), 1.18 (3H, s, CH<sub>3</sub>-26), 1.04 (3H, s, CH<sub>3</sub>-25), 1.02 (3H, s, CH<sub>3</sub>-23);  $^{13}\text{C}$  NMR (75.4 MHz, pyridine-*d*<sub>5</sub>)  $\delta$  179.6 (C-28), 178.9 (C-27), 171.2 (AcO), 151.6 (C-20), 144.3 (C-2), 135.1 (C-3), 110.7 (C-29), 68.8 (C-24), 63.2 (C-5), 60.7 (C-14), 56.9 (C-17), 52.6 (C-18), 51.2 (C-10), 49.2 (C-4), 48.9 (C-9), 48.3 (C-19), 42.0 (C-8), 40.5 (C-13), 39.0 (C-7), 38.1 (C-22), 35.7 (C-16), 31.5 (C-21), 29.2 (C-15), 26.9 (C-12), 24.6 (C-23), 24.0 (C-11), 21.1 (AcO), 20.8 (C-25), 19.6 (C-30), 18.7 (C-26), 18.5 (C-6); EIMS *m/z* (rel. int.): 512 [M]<sup>+</sup> (0.4), 452 (4), 439 (17), 393 (16), 371 (54), 325 (12), 173 (100), 119 (82), 107 (84), 105 (81), 91(59). HRESIMS *m/z* 513.3213 (calcd. for C<sub>31</sub>H<sub>45</sub>O<sub>6</sub> + H, 513.3211).



**Figure 1.** *A-nor-lupane*-type triterpenes isolated from *C. caeruleus* (**1** and **2**) and their dimethyl ester derivatives (**3** and **4**).

Triterpene compounds with a carboxylic group at position C-28 are challenging to modify due to steric hindrance. A carbonyl group moiety is reportedly essential for better antitumor activity, and small-chain esters have been shown to enhance cytotoxicity. Consequently, even “simple” modifications, such as methylation and acetylation, can significantly improve the biological activity of natural products [23]. Thus, the dimethyl ester derivative **3** was prepared from **1** through methylation with diazomethane (see Supplementary Information), and the compound **4** was obtained by acetylation of **3** (see Supplementary Information).

*Dimethyl gouanate B (3)*: colorless amorphous solid (decomposes above 100 °C),  $[\alpha]_{589} = +38$ ,  $[\alpha]_{578} = +40$ ,  $[\alpha]_{546} = +46$ ,  $[\alpha]_{436} = +83$ ,  $[\alpha]_{365} = +142$  ( $c$  0.82, chloroform at 25 °C, see supporting information for details of measurement); IR (CHCl<sub>3</sub>)  $\nu_{max}$  3612 (O-H), 2946, and 2864 (C-H), 1709 cm<sup>-1</sup> (C=O). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz)  $\delta$  6.08 (1H, d,  $J$  = 5.9 Hz, H-2), 5.44 (1H, d,  $J$  = 5.9 Hz, H-3), 4.75 (1H, brs, H-29), 4.64 (1H, brs, H-29'), 3.69 (3H, s, OMe), 3.67 (3H, s, OMe), 3.62 (1H, d,  $J$  = 10.8 Hz, H-24), 3.53 (1H, d,  $J$  = 10.8 Hz, H-24'), 3.03 (1H, m, H-19), 2.33 (1H, m, H-15), 2.32 (1H, m, H-13), 2.07 (1H, m, H-16), 2.05 (1H, m, H-12), 1.89 (1H, m, H-22), 1.89 (1H, m, H-21), 1.72 (1H, m, H-16'), 1.69 (3H, s, CH<sub>3</sub>-30), 1.67 (1H, m, H-9), 1.60 (1H, m, H-7), 1.58 (1H, m, H-6), 1.56 (1H, m, H-18), 1.52 (2H, m, CH<sub>2</sub>-11), 1.43 (1H, m, H-7'), 1.40 (1H, m, H-6'), 1.35 (1H, m, H-21'), 1.34 (1H, m, H-22'), 1.27 (1H, m, H-12'), 1.21 (1H, m, H-5), 1.19 (1H, m, H-15'), 1.11 (3H, s, CH<sub>3</sub>-23), 1.01 (3H, s, CH<sub>3</sub>-26), 0.99 (3H, s, CH<sub>3</sub>-25). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75.4 MHz)  $\delta$  176.6 (C-28), 176.0 (C-27), 149.9 (C-20), 143.5 (C-2), 134.6 (C-3), 110.0 (C-29), 67.3 (C-24), 62.1 (C-5), 60.2 (C-14), 56.2 (C-17), 51.7 (C-18), 51.4 (OMe), 50.6 (OMe), 50.6 (C-4), 50.4 (C-10), 48.3 (C-9), 46.9 (C-19), 41.4 (C-8), 39.5 (C-13), 38.1 (C-7), 36.8 (C-22), 34.1 (C-15), 30.3 (C-21), 27.9 (C-12), 25.6 (C-16), 23.8 (C-23), 23.0 (C-11), 20.6 (C-25), 18.9 (C-30), 18.0 (C-26), 17.9 (C-6). EIMS  $m/z$  (rel. int.): 498 [M]<sup>+</sup> (0.2), 483 (0.2), 467 (7), 407 (10), 399 (24), 279 (10), 247 (8), 173 (38), 79 (100), 77 (52). HRESIMS  $m/z$  499.3427 (calcd. for C<sub>31</sub>H<sub>46</sub>O<sub>5</sub> + H, 499.3418).

*Acetylgouanic acid B dimethyl ester (4)*: colorless amorphous solid (decomposes above 100 °C).  $[\alpha]_{589} = +40$ ,  $[\alpha]_{578} = +41$ ,  $[\alpha]_{546} = +47$ ,  $[\alpha]_{436} = +86$ ,  $[\alpha]_{365} = +145$  ( $c$  0.17, chloroform at 25 °C, see supporting information for details of measurement); IR (CHCl<sub>3</sub>)  $\nu_{max}$  2944 and 2864 (C-H), 1717 cm<sup>-1</sup> (C=O). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  6.08 (1H, d,  $J$  = 5.9 Hz, H-2), 5.40 (1H, d,  $J$  = 5.9 Hz, H-3), 4.75 (1H, brs, H-29), 4.64 (1H, brs, H-29'), 4.03 (1H, d,  $J$  = 10.8 Hz, H-24), 3.97 (1H, d,  $J$  = 10.8 Hz, H-24'), 3.69 (3H, s, OMe), 3.67 (3H, s, OMe), 3.05 (1H, m, H-19), 2.36 (1H, m, H-16), 2.31 (1H, m, H-16'), 2.08 (1H, m, H-15), 2.04 (2H, m, CH<sub>2</sub>-12), 2.04 (3H, s, OAc), 2.02 (1H, m, H-15'), 1.90 (1H, m, H-22), 1.88 (1H, m, H-21), 1.69 (3H, s, CH<sub>3</sub>-30), 1.68 (1H, m, H-9), 1.63 (1H, m, H-13), 1.62 (1H, m, H-7), 1.60 (1H, m, H-6), 1.57 (1H, m, H-18), 1.51 (2H, m, CH<sub>2</sub>-11), 1.43 (1H, m, H-6'), 1.40 (1H, m, H-7'), 1.37 (1H, m, H-21'), 1.35 (1H, m, H-22'), 1.25 (1H, m, H-5), 1.08 (3H, s, CH<sub>3</sub>-23), 1.01 (3H, s, CH<sub>3</sub>-26), 1.00 (3H, s, CH<sub>3</sub>-25). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75.4 MHz)  $\delta$  176.7 (C-28), 176.1 (C-27), 171.3 (OAc), 150.0 (C-20), 143.7 (C-2), 134.2 (C-3), 110.1 (C-29), 68.4 (C-24), 62.3 (C-5), 60.2 (C-14), 56.2 (C-17), 51.8

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(C-18), 51.4 (OMe), 50.7 (OMe), 50.4 (C-10), 48.4 (C-4), 48.2 (C-9), 47.0 (C-19), 41.4 (C-8), 39.6 (C-13), 38.1 (C-7), 36.9 (C-22), 34.1 (C-16), 30.4 (C-21), 27.9 (C-15), 25.7 (C-12), 24.3 (C-23), 23.0 (C-11), 20.9 (OAc), 20.4 (C-25), 18.9 (C-30), 18.0 (C-26), 17.8 (C-6). EIMS  $m/z$  (rel. int.): 540 [M]<sup>+</sup> (0.17), 525 (0.24), 467 (4), 399 (35), 339 (15), 279 (26), 247 (11), 173 (75), 105 (100), 91 (89), 79 (58). HRESIMS  $m/z$  541.3527 (calcd for C<sub>33</sub>H<sub>48</sub>O<sub>6</sub> + H, 541.3524).

**Cytotoxic Activity Tes.:** The most common types of cancer are breast cancer (MCF-7), lung cancer (A549), cervical cancer (HeLa), and leukemia (K562). The two natural products, **1** and **2**, and their respective dimethyl ester derivatives, **3** and **4**, were tested against the four cancer cell lines (Table 1). These results showed that the four compounds have IC<sub>50</sub> values greater than 50 μM for the MCF-7 and A549 cell lines. In the HeLa cell line, the same trend was observed for three compounds, except for the dimethyl ester of gouanic acid B (**3**) with an IC<sub>50</sub> value of 18.3 μM. Regarding the K562 cell line, gouanic acid B (**1**) and the acetyl dimethyl ester derivative **4** showed an IC<sub>50</sub> with 36.4 and 33.0 μM values, respectively. In this cell line, the natural product acetylgouanic acid B (**2**) showed a good IC<sub>50</sub> value of 21.6 μM, while gouanic acid dimethyl ester **3** showed a better IC<sub>50</sub> value of 7.1 μM. The survival rate in the percentage of bovine mammary epithelial cells (bMECs) treated with **3** was low (35%), but cells treated with **1** showed an acceptable survival rate (89%) (Table 1).

**Table 1.** IC<sub>50</sub> of compounds (μM) **1**, **2**, **3**, and **4** against MCF-7, A549, HeLa, and K562 cancer cell lines and survival percentage of bMEC non-cancerous cells .

Compound	MCF-7 <sup>a</sup>	A549 <sup>a</sup>	HeLa <sup>a</sup>	K562 <sup>a</sup>	bMEC <sup>a</sup>	
					CT <sup>b</sup>	survival (%)
<b>1</b>	>50	>50	>50	36.4 ± 4.0	40	88
<b>2</b>	>50	>50	>50	21.6 ± 4.3	26	89
<b>3</b>	>50	>50	18.3 ± 2.4	7.1 ± 1.8	21	35
<b>4</b>	>50	>50	>50	33.0 ± 2.0	35	92
Actinomycin D	63 <sup>c</sup>	73 <sup>c</sup>	9 <sup>c</sup>	71 <sup>c</sup>	—	73 <sup>c</sup>

<sup>a</sup> Experiments were performed in triplicate.

<sup>b</sup> Concentrations assessed (CT) for survival experiment on bMEC were obtained by adding IC<sub>50</sub> + SE for each cell line, CT = IC<sub>50</sub> + SE.

<sup>c</sup> Survival (%) of each cell line at 10 μM of actinomycin D as control.

It is worth mentioning that in all assays, the concentration tested (CT) in normal cells had higher concentrations than the corresponding IC<sub>50</sub>. CT values were obtained by adding the corresponding maximum standard error (SE) value to each IC<sub>50</sub> value. Thus, for compounds **1**, **2**, **3**, and **4**, the CT was 40, 26, 21, and 35 μM, respectively. Noteworthy, the survival percentage of non-cancerous cells (bMEC) treated with **1**, **2**, and **4** was 88, 89 and 92%, respectively. Also, these three triterpenes showed selective cytotoxicity on K562 myelogenous leukemia cells since they showed no activity against breast (MCF-7), lung (A549), and cervical carcinoma (HeLa) cancer cells.

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## Supporting Information

Supporting Information accompanies this paper on <http://www.acgpubs.org/journal/records-of-natural-products>

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