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Di-And Tri-Hydroxylated Kaurane Derivatives From Microbial Transformation Of *Ent-Kaur-16-En-19-Ol* By *Cephalosporium Aphidicola* And Their Allelopathic Activity On *Lactuca Sativa* (Lettuce)

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Abstract: The use of microorganisms to induce chemical modifications in organic molecules is a very useful tool in organic synthesis, to obtain biologically active substances. The fungus *Cephalosporium aphidicola* is known by its ability to hydroxylate several skeleton positions of many classes of organic compounds. In this work, the microbial transformation of *ent-kaur-16-en-19-ol* (**1**) by *C. aphidicola*, afforded two hydroxylated compounds, *ent-kauran-16 β ,19-diol* (**2**) and *ent-kauran-16 β ,17,19-triol* (**3**). Their structures were established by 1D and 2D-NMR studies. Both compounds were tested for their action on the growth of radical and shoot of *Lactuca sativa*.

Key words: Biotransformations, *Cephalosporium aphidicola*, *ent-kaur-16-en-19-ol*, *ent-kauran-16 β ,19-diol*, *ent-kauran-16 β ,17,19-triol*.

Introduction

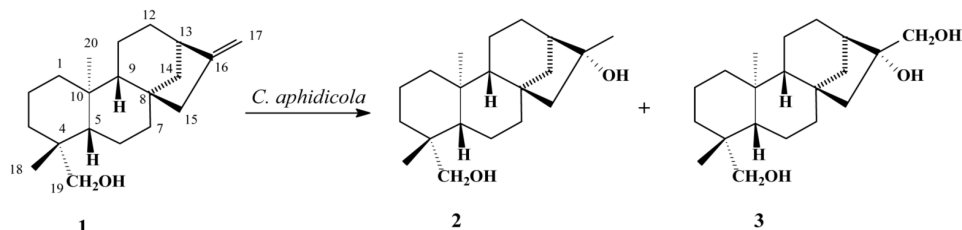
Biotransformations use living organisms or isolated enzymes to modify chemical structures and have some advantages over conventional chemical reactions [1]. This methodology have been showing a quick development along the time and represents a potential alternative for obtaining biologically active compounds of difficult access by classic synthetic methods [2,3].

Kaurane diterpenes are widely distributed in nature [4-6] and present very important biological activities, such as antitumor [7] anti-HIV [8], trypanocidal [9-10] antimicrobial [11] and allelopathic [12-13], among others. *Ent-kaur-16-en-19-oic* and *ent-kaur-9(11),16-dien-19-oic* acids and their chemical transformations derivatives are very

used substrates for biotransformations [14-17]. Kaurane diterpenes are also intermediates in the biosynthesis of a number of plant and fungal metabolites, including gibberellins, a group of diterpene lactones, as GA₃, used in agriculture to stimulate plant growth. Allelopathic activity of *ent-kaur-16-en-19-oic* and *ent-kaur-9(11),16-dien-19-oic* acids and derivatives obtained by chemical transformations of them were previously evaluated by our research group [12-13].

The fungus *C. aphidicola* promotes hydroxylations in different classes of organic compounds and has been widely used in biotransformations of kaurane diterpenes [18-20].

In this work, we describe the biotransformation of *ent-kaur-16-en-19-ol* (**1**) by *C. aphidicola*. Two hydroxylated compounds, *ent-kauran-16 β ,*



Scheme 1 – Biotransformation of *ent*-kaur-16-en-19-ol (**1**) by *C. aphidicola*

19-diol (**2**) and *ent*-kauran-16 β ,17,19-triol (**3**), were isolated (Scheme 1) and tested over their action on the growth of radical and shoot of *Lactuca sativa* (lettuce). This is the first time these compounds were obtained from this substrate using *C. aphidicola*. This is also the first report on their allelopathic activity by the best of our knowledge.

Materials And Methods

General

Solvents (hexane, dichloromethane, ethyl acetate, methanol) from VETEC (Brazil), and were of PA grade. Silica gel Merck (Darmstadt, Germany) 70-240 and 230-400 mesh were used for chromatography column and silica gel Merck 60G was used for thin layer chromatography. Reagents to prepare the culture medium were purchased from Difco (Sparks, MD, USA). Melting points were determined with a Kofler hot plate apparatus and are uncorrected. Nuclear Magnetic Resonance (NMR) spectra (1D and 2D) were recorded in CDCl₃, at room temperature, on a Bruker Avance DRX 400 MHz, from Bruker Analytic, Ettlingen, Germany. *Ent*-kaur-16-en-19-ol (**1**) was furnished by Dr. Henriete S. Vieira, Departamento de Química, ICEx, UFMG, Brazil.

Biotransformation

Stock culture of *C. aphidicola* (CCT 2163) was maintained on PDA under refrigeration and small sections of this agar were transferred to Erlenmeyer flasks containing a liquid medium (200 mL/flask) comprised of: glucose (50.0 g/L), NaH₂PO₄ (5.0 g/L), MnSO₄·6H₂O (2.0 g/L), KCl (1.0 g/L), glycine (2.0 g/L) and 2.0 mL of trace elements solution [CO(NO₃)₂,

0.10 g/L, FeSO₄, 1.0 g/L, CuSO₄, 0.15 g/L, ZnSO₄, 1.61 g/L, MnSO₄, 0.10 g/L, (NH₄)₆Mo₇O₂₄·4H₂O, 1.0 g/L] of distilled water; pH was adjusted to 5 with 10% HCl solution and the culture was incubated over magnetic stirring for 3 days. Cells in suspension (15 mL portions) were transferred to new Erlenmeyer flasks and reached suitable growth after 24 h. Then, the substrate **1** (0.69 g) was added (0.5 mL/flask, 53 mg/mL). After 13 days, the flasks contents were extracted with ethyl acetate and the solvent was taken out under vacuum. Residue (1.37 g) was chromatographed over silica gel (21.5 g), using hexane, CH₂Cl₂, AcOEt and CH₃OH, either neat or in mixtures of increasing polarity. A group of fractions (G-7, 81.5 mg), eluted from CH₂Cl₂/AcOEt 1:1, was rechromatographed over silica gel (3.2 g), using the same solvent system. G-7-2 (25.0 mg) gave an amorphous white solid [3.7 mg, *ent*-kauran-16 β ,19-diol, (**2**)], corresponding to a single spot by TLC (hexane/AcOEt 75:25), by another silica gel column chromatography. G-7-3 (17.0 mg), after new purification by silica gel column chromatography, gave a crystalline white solid [12.8 mg, *ent*-kauran-16 β ,17,19-triol (**3**)], also pure by TLC (eluents: hexane/AcOEt 70:30).

Bioassay

Lactuca sativa (cv Grand Rapids) seeds were purchased from Isla Pak, RS, Brazil. All undersized and damaged seeds were discarded. Germination and growth were conducted in 100 mm Petri dishes containing a 9.0 cm sheet of Whatman no. 1 filter paper as suport. Then, 25 lettuce seeds were placed per dish with 10 mL of a test (10⁻⁴, 10⁻⁶ and 10⁻⁸ M) or a control solution. All

solutions were prepared with deionized water and their pH values [buffered with 10 mM 2-(*N*-morpholino) ethanesulfonic acid, MES] were adjusted to 6.0 - 6.5 with NaOH solution. Concentrations lower than 10^{-4} M were obtained by dilution series. All tests were triplicated. Dishes were covered with Parafilm to reduce evaporation and incubated in the dark at 25 °C, in a controlled-environment growth chamber, for 5 days. After this time, number of germinated seeds were counted (a seed was considered to be germinated when the radicle was at least 0.2 mm long), the lengths of radical and shoots were measured (using a pachymeter). During the measurement process, the dishes were kept at 4 °C to avoid subsequent growth. The osmotic pressure values were measured on a microsmometer and ranged between 30 and 38 mOsmolar [15].

Data Analysis

The effects on germination and growth are given as percent differences from control, and consist of the differences (in cm) between mean values of seeds with tested compounds and mean values for control (seeds grown without addition of tested compounds)/ mean values for control $\times 100$. Thus, zero represents the control, positive values represent stimulation of the studied parameter and negative values represent inhibition.

The data were evaluated using Student's *t*-tests and the differences between the experiment and control were significant at a value of $P \leq 0.05$.

The results obtained for compounds **2** and **3** are shown in Figure 2.

Results and Discussion

Compounds **2** and **3** were identified by 1D and 2D ^1H and ^{13}C -NMR as products of mono and di-hydroxylation, respectively, of the original C16-C17 double bond of substrate **1**, since in the ^1H -NMR spectrum of both products, the unsaturated exocyclic methylene signals, present on starting material at δ_{H} 4.73 and 4.79 were not observed. Also, this spectrum showed the signals for C-19 hydroxymethyl AB system for both products, those of **2** at δ_{H} 3.44 (1H, d, $J=11.2$ Hz) and 3.72 (1H, d, $J=11.2$ Hz). The ^1H NMR of **2** exhibited signals for two methyl groups at δ_{H} 0.95 (3H,

s, H-18) and 1.01 (3H, s, H-20) and for a new methyl group at δ_{H} 1.35. ^{13}C -NMR spectrum and DEPT experiment showed a chemical shift at δ_{C} 65.6, of the C-19 oxygenated methylene carbon, from the starting material. Additionally, signals for nine methylene carbons, three methine carbons, and four quaternary carbons (including a new oxygenated carbon at δ_{C} 79.3) were observed. In order to carry on a more detailed spectroscopic analysis of the products, bidimensional spectra (HSQC, HMBC and ROESY) were obtained for **2**, since they are of special help for complete attribution of terpene compounds [21]. HSQC spectrum showed the correlations between the signal at δ_{H} 1.82 with δ_{C} 49.0, attributed to C-13, by comparison to the spectroscopic data of the starting material; the signals at δ_{H} 1.43 and 1.48 were correlated to a signal at δ_{C} 18.0 (C-11); the signals at δ_{C} 1.57 and 1.88 to a signal at δ_{C} 37.5 (C-14). A correlation of the signal at δ_{H} 0.99 with the signal at δ_{C} 57.0 (C-9) was also observed. The signals corresponding to methine positions 5, 9 and 13 appeared at δ_{H} 0.91 and δ_{C} 56.8; at δ_{H} 0.99 and δ_{C} 57.0; and at δ_{H} 1.82 and δ_{C} 49.0, respectively. Also, the signal at δ_{H} 1.35 (3H) was correlated, in the HSQC spectrum, to a signal at δ_{C} 24.4, and was associated with C-17, since this was the only option for the introduction of an additional methyl group in the molecule, and consequently the new oxygenated quaternary carbon signal observed at δ_{C} 79.3 can be associated to C-16.

In the HMBC spectrum, 3J correlations between H19 β (δ_{H} 3.72) with C-18 (δ_{C} 27.0) and between H19 β (δ_{H} 3.44) with C-3 (δ_{C} 35.7) were observed. Besides, correlations between hydrogens of the methyl group at C17 (δ_{H} 1.35) and C13 (δ_{C} 49.0), C15 (δ_{C} 57.9) and C16 (δ_{C} 78.3) were further evidence of the presence of a methyl group at C-17. Therefore, **2** was identified as the product of monohydroxylation of the original exocyclic double bond from starting material (Table 1).

The configuration of C-16 was established by analysis of ROESY spectrum, where the correlation of CH₃-20 (δ_{H} 1.01) with H-14 α , at δ_{H} 1.57, was observed. Thus, by using HSQC spectrum, the signal of H-14 β was located at δ_{H} 1.88. No ROESY correlation was observed between H-14 β and H-17 (δ_{H} 1.35). Also by ROESY spectrum, the signal related to H-11 α

was found to be δ_{H} 1.43, due to its correlation with H-9 (δ_{H} 0.99) therefore by HSQC spectrum H-11 β (δ_{H} 1.48) was located and, as this signal correlates with CH₃-17 (δ_{H} 1.35) in the ROESY spectrum, the later is unequivocally at the α -position (Figure 1). Thus, **2** was proposed to be *ent*-kauran-16 β ,19-diol. This compound was already isolated as natural product from stems of *Aristolochia rodriguesii* [22] and leaf and stems of *Ozothamnus hookeri* (Asteraceae) [23]; **2** was also obtained by chemical modification of *ent*-kaur-16-en-19-oic acid [2].

NMR spectra of product **3** were not consistent with the presence of a methyl group at C-17, since only a new signal in ¹H- NMR spectrum at δ_{H} 4.03 (2H) was observed. Two new oxygenated carbon signals were detected in ¹³C NMR spectrum, at δ_{C} 78.7 and 71.8 (quaternary and methylene, respectively, according with DEPT experiment). HSQC spectrum presented correlations between the signal at δ_{H} 0.95 with δ_{C} 57.1, associ-

ated with C-9; at δ_{H} 1.36 and 1.48 with δ_{C} 18.1 (C-11); at δ_{H} 1.57 and 1.84 with δ_{C} 37.5 (C-14). This spectrum also showed the correlation between the signal at δ_{H} 4.03 with the signal at δ_{C} 71.8. Since, the signals related to the exocyclic double bond of starting material are not present in the NMR spectra of **3**, these signals can be only associated to a hydroxymethyl group at 17 position. Therefore, the signal related to a quaternary carbon, at δ_{C} 78.7, was attributed to C-16. The β -hydroxy stereochemistry of C-16 was defined, as for **2**, based on a ROESY experiment (Figure 1), mainly due to the correlation observed between H-11 β (δ_{H} 1.48) and 17-CH₂OH (δ_{H} 4.03). Compound **3** was, then, proposed to be the *ent*-kauran-16 β ,17,19-triol. This compound was already isolated as natural product from leaf and stems of *Ozothamnus hookeri* (Asteraceae) [23], from the fern *Lindsaea javanensis*, as 19-*O*- β -D-glucopyranoside [24] and from aerial parts of *Bahia glandulosa*, as the 19-acyl derivative [25].

Table 1 – ¹H and ¹³C NMR data (δ) of *ent*-kaur-16en-19-ol (**1**), *ent*-kauran-16 β ,19-diol (**2**) and *ent*-kauran-16 β ,17,19-triol (**3**), based in HSQC correlations

C	Multiplicity	1			2			3		
		¹³ C	¹ H		¹³ C	¹ H		¹³ C	¹ H	
			H α	H β		H α	H β		H α	H β
1	CH ₂	40.5	1.77	0.80	40.4	1.79	0.80	40.5	1.76	0.70
2	CH ₂	18.3	1.42	1.56	18.3	1.41	1.58	18.3	1.35	1.54
3	CH ₂	35.6	1.7	0.92	35.7	1.75	0.90	35.7	1.78	0.84
4	C	38.7	-	-	39.3	-	-	38.8	-	-
5	CH	56.2	0.8		56.8	0.91		56.9	0.88	
6	CH ₂	20.5	1.6	1.28	20.7	1.65	1.31	20.6	1.60	1.26
7	CH ₂	41.6	1.5	1.4	42.5	1.62	1.45	42.5	1.55	1.38
8	C	44.2	-	-	45.3	-	-	45.3	-	-
9	CH	56.8	0.99		57.0	0.99		57.1	0.95	
10	C	39.2	-	-	38.6	-	-	39.3	-	-
11	CH ₂	18.2	1.4	1.48	18.0	1.43	1.48	18.1	1.36	1.48
12	CH ₂	33.2	1.3	1.54	26.8	1.27	1.54	26.8	1.51	
13	CH	43.9	2.63		49.0	1.82		48.9	1.81	
14	CH ₂	39.6	1.57	1.84	37.5	1.57	1.88	37.5	1.57	1.84
15	CH ₂	49.1	1.55		57.9	1.55		58.0	1.52	
16	C	155.9	-	-	79.3	-	-	78.7	-	-
17	CH ₂ or CH ₃	102.9	4.73	4.79	24.4	1.35		71.8	4.03	
18	CH ₃	27.1	0.96		27.0	0.95		27.3	0.93	
19	CH ₂	65.5	3.44	3.75	65.6	3.72	3.44	64.8	3.37	3.70
20	CH ₃	18.1	1.01		18.2	1.01		18.3	1.31	

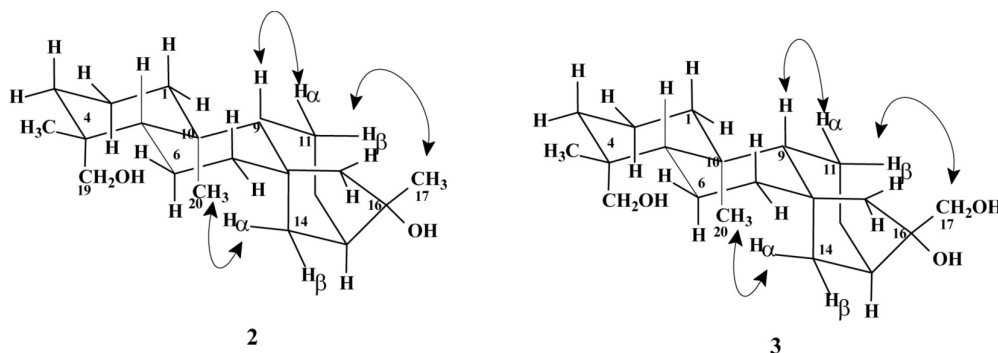


Figure 1 – Correlations observed in the ROESY spectra of compounds **2** and **3**.

The effect of compounds **2** and **3** on radical and shoot growth of *L. sativa*, in three different concentrations, was tested and the results are shown in Figure 3. Both diol and triol inhibited radical growth in all three concentrations, and the best result was observed for compound **2** at the concentration of 10^{-4} M. On shoot growth, the effect of the tested compounds was stimulatory at the concentrations of 10^{-4} and 10^{-6} M. At 10^{-8} M, both biotransformation products **2** and **3** inhibited shoot growth. The best growth stimulatory concentration was 10^{-6} M. With kaurenol (**1**), the compound used to provide derivatives **2** and **3**, radical

and shoot growth were also inhibited at the higher concentration, while stimulation of both occurred at middle concentration [14].

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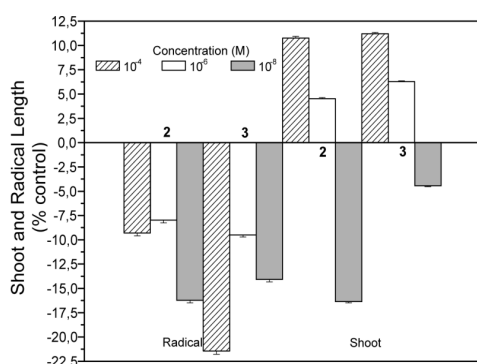


Figure 2 - Effect of diol (**2**) and triol (**3**) on radical and shoot length of *L. sativa*. Values are presented as percentage differences from the control, zero representing an observed value identical to the control, a positive value representing stimulation and a negative value representing inhibition.

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