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3-Hydroxycineole bioproduction from 1,8-cineole using *Gymnopilus spectabilis* 7423 under resting cell conditions

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Abstract: This report describes the high yield biotransformation of 1,8-cineole by the strain *Gymnopilus spectabilis* 7423, a common fungus isolated from the *Eucalyptus* tree. The biotransformation was conducted under resting cell conditions and different parameters were tested in order to achieve up to 90% bioconversion. Only two regioisomers were detected, and they were identified as 3-α-hydroxy-1,8-cineole and 2-α-hydroxy-1,8-cineole obtained in a 82:8 ratio.

Keywords: *Gymnopilus spectabilis*, 1,8-cineole, biooxidation, hydroxycineols, biocatalysis, green chemistry

1 Introduction

Between 1980 and 2012 the global cellulose demand increased from 128 to 174 million tonnes. This rise also came with a change among both consumer and producing countries. Currently, the cellulose import is led by China since its internal production is insufficient to meet the demand [1].

In this context, the forest industry in Uruguay has grown considerably for the last 17 years. After the approval of the forestry law in 1987, the forested area with exotic species increased from 25.000 to 990.030 hectares (ha) in 2012, with Eucalyptus being the most widely planted genus, covering 726.323 ha. According to the Ministry of Agriculture and Fisheries, the main planted species of Eucalyptus are *Eucalyptus globulus*, *Eucalyptus grandis*, and *Eucalyptus dunnii* [2]. The forests are exclusively dedicated to the production of wood or pulp for the paper industry, and as a result, no use has been planned for leaves and branches, which are left on the ground. Recent estimations indicated that in Uruguay the discarded material reached 48.000 kg per ha after the logging of trees [3].

Eucalyptus leaves are rich in essential oils. The essential oil can be easily obtained by steam distillation from the leaves that are considered a waste material for the forestry industry. The main component of eucalyptus essential oil in most species is the monoterpene cyclic ether, 1,3,3-trimethyl-oxabicyclo[2.2.2]octane, commonly known as eucalyptol, 1,8-cineole, or cineole (**1**) (Figure 1). This component accounts for about 70% of the total oil as determined by gas chromatography [4], and it is easily obtained from the brute essence through precipitation upon cooling [5].

cineole (**1**) (systematic numbering)
(1*S*,4*S*)-1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane

1,8-cineole (**1**)
traditional numbering

Figure 1. Two common representations of cineole. Note that the traditional numbering does not match the systematic numbering.

The molecule 1,8-cineole is chemically stable and several researchers have reported its selective oxidation by different techniques, using either chemical [6] or biocatalytic processes [3, 6b, 7]. The activity of 1,8-cineole derivatives has been tested and it was reported that, although 1,8-cineole is active as an insect deterrent, oxidized derivatives are more active as deterrents, antifeedants, or insecticides than 1,8-cineole itself [8]. In addition, the oxygenated derivatives from 1,8-cineole

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are high value-added compounds with applications in food, perfume, and pharmaceutical industries [9]. Apart from its use as an intact molecule, hydroxycineoles have been employed as precursors in the preparation of more complicated organic materials [10].

Diverse applications of these oxygenated terpenes and the wide availability of 1,8-cineole from a natural source prompted us to study the biooxidation of 1,8-cineole as a means to obtain value-added compounds and to develop new biologically active compounds intended for use as potential herbicides [11].

In this report we present the biooxidation of 1,8-cineole under resting cell conditions using the basidiomycete *Gymnopilus spectabilis* 7423 as a biocatalyst. The reaction takes place with a very high yield (90%) and excellent regioselectivity (see Figure 2).

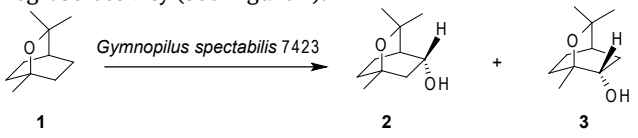

Figure 2. Biooxidation of 1,8-cineole by a fungi strain and formation of 2-α-hydroxycineole (**2**) and 3-α-hydroxycineole (**3**).

2 Methods

2.1 Chemicals

1,8-Cineole (99.8%) was provided by the Agroindustrial Technology Center (Cochabamba, Bolivia).

2.2 Microorganisms

The *Gymnolipus spectabilis* 7423 strain was provided by the Mycology Department of Facultad de Ciencias, UdelaR. The microorganisms were maintained on malt agar slants at 5ºC until use. For trials they were transferred onto malt agar plates, and the strain was cultivated at 25ºC.

2.3 Biotransformations assays

The experiments were carried out under sterile conditions in two stages: I and II for biomass production and biotransformation, respectively. These conditions were designed as follows.

Stage I: Biomass production
Agar discs of 1.0 cm diameter, punched from cultures growing on potato dextrose agar (PDA) over 7 days at 28°C, were used to inoculate Erlenmeyer flasks containing malt extract (ME) liquid broth. Mycelium was grown at 25°C over 10 days with shaking at 100 rpm.

Stage II: Biotransformation of 1,8-cineole
The pellets obtained in the previous stage were filtered, rinsed, and transferred to a flask with 0.05 M buffer phosphate under the same conditions of stirring rate and temperature used for Stage I. The influence of 1,8-cineole concentration, buffer pH, reaction time, as well as the influence of varying the amount of biomass, were studied. At the selected time (see Table 2) the free cells and dissolved chemical compounds were extracted with dichloromethane.

Extraction, purification and identification of bioconversion products
The liquid medium was separated from the mycelium by filtration. The mycelium free medium was extracted three times, each time using a volume of organic solvent equal to one-third of the total aqueous volume. The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude mixture containing 1,8-cineole

Table 1. ^{13}C-NMR analysis of compound **3**. Pick to pick comparison of chemical displacement values (δ, ppm) with **3**-α- and **3**-β-cineol obtained by Carman [6b].

Carbon numbering	Comp. 3	3-α (ref 6b)	Dif (ppm)	Dif (%)	3-β (ref 6b)	Dif (ppm)	Dif (%)
C-OH	73.3	73.2	0.1	0.09%	73.4	-0.1	-0.18%
C1	70.9	70.9	0.0	0.00%	70.8	0.1	0.14%
C3	65.2	65.3	-0.1	-0.10%	70.1	-4.9	-6.94%
C2	42.8	42.9	-0.1	-0.12%	43.2	-0.4	-0.82%
C4	40.3	40.4	-0.1	-0.20%	40.7	-0.4	-0.94%
C6	31.0	31.0	0.0	0.08%	30.7	0.3	1.06%
C10	29.0	28.9	0.1	0.20%	30.4	-1.4	-4.74%
C9	28.3	28.3	0.0	0.08%	30.1	-1.8	-5.90%
C7	27.1	27.1	0.0	0.04%	26.8	0.3	1.16%
C5	13.9	13.9	0.0	-0.13%	21.4	-7.5	-35.13%

(**1**), 2-α-hydroxycineole (**2**), and 3-α-hydroxycineole (**3**) was purified by flash chromatography on silica gel using a hexanes/ethyl acetate gradient as mobile phase. Each one of the compounds was identified by GC, GC-MS and/or 1H- and 13C-NMR spectroscopy.

2.4 Analytical conditions

High-resolution gas chromatography (HRGC) analyses were performed on a Hewlett-Packard HP 5890 Series GC equipped with FID and EZChrom integration software for data processing. A fused silica capillary column (30 m, 0.32 mm i.d.) with bonded CARBOWAX™ (0.25 μm thickness) was used. The following parameters were employed: temperature program, 60°C (8 min) and 60–210°C at 3 °C/min; injector temperature, 240°C; detector temperature, 250°C; N_2 carrier gas pressure, 0.50 kg/cm^2; and injection system split ratio, 1:100. HRGC-MS was carried out on a Shimadzu QP 2010 instrument using these conditions and He as a carrier gas; an ionization voltage of 70 eV and a temperature interface of 250ºC were applied. The optical purity was determined with a GC-GC Shimadzu GC 17A; the first GC was equipped with a SE52 column and the second one with a modified β-cyclodextrin chiral capillary column. The temperature program proceeded as follows: 50°C (6 min), 50–90°C at 2°C/min, 90°C (20 min), 90–180°C at 2°C/min, and 180ºC (10 min). The following parameters were used: injector temperature, 250°C; detector temperature, 280°C; carrier gas, He; and injection system split ratio, 1:150. Optical rotations were measured using a Kruss Optronic GmbH P8000 polarimeter with a 0.5 mL cell (concentration *c* given as g/100 mL).

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance DPX-400 instrument. Chemical shifts (δ) are given in parts per million (ppm) and coupling constants (*J*) are reported in hertz (Hz).

3 Results and discussion

3.1 Identification of metabolites

Oxygenated terpenes **2** and **3** were identified by GC, GC-MS, Chiral GC, polarimetry, and ^{1}H- and ^{13}C-NMR spectroscopies. The more easily accessible 2-α-hydroxycineol (**2**) was unequivocally identified by comparison with an authentic standard synthesized in our laboratory. This minor metabolite was coinjected with the standard and coeluted under the GC conditions optimized for separation of this group of compounds. In addition, racemic compound **2**, obtained by chemical synthesis, was analyzed by chiral chromatography, but the enantiomers were inseparable under all conditions tested. Therefore, no conclusion can be drawn regarding the enantioselectivity for this compound.

The main product of the biotransformation, 3-α-hydroxycineol (**3**), demanded a more extensive analysis. Since an authentic standard of **3** was not available, we relied on NMR analysis for its unequivocal identification as the α-isomer (See Table 1). As shown in the table, the chemical shifts of the isolated compound **3** and the 3-α-hydroxycineol reported by Carman [6b] are identical within the experimental conditions, while the 3-β isomer exhibits significant differences. The enantiomers of compound **3** were also inseparable in our chiral column, but since we obtained a significant amount, we measured the optical rotation of a chemically pure sample of the metabolite and determined it to be a racemic mixture, as the specific optical rotation value was 0 within experimental error: $[\alpha]_D^{20} = 0.15$ (c = 1.04 g/100 mL, CH_2Cl_2).

Several experiments were performed in order to find a set of conditions that would allow for a high degree of 1,8-cineole bioconversion into oxygenated products. The experiments were carried out under sterile conditions in stages I and II, as explained previously. A summary of the chosen conditions tested in the Stage II experiments and the percent conversion observed is shown in Table 2.

As can be noted in Table 2, the pH value was a key factor in these experiments, and a value of pH = 6 appeared to be adequate to maximize the reaction yield. When the reaction was run at pH = 7, the highest bioconversion percentage achieved was only 6% (entries 1 to 3), while at pH = 6 the minimum degree of conversion was 15% and the highest 90%. Moreover, the added amount of 1,8-cineole was an important parameter in the final bioconversion percentage. For instance, the bioconversion was higher with an addition of 100 μL of 1,8-cineole compared to the bioconversion percentage with 50 μL of the terpene (entries 5 and 6).

The selectivity of the reaction is also noteworthy. Figure 3 shows the graph of the relative composition of the mixture at the end of each of the experiments. The highest bioconversion percentage was obtained under the conditions given in entry 6 (Table 2). For **STAGE I**, the conditions were as follows: temperature, 25°C; stirring speed, 100 rpm; broth, 2% malt extract; and growing time, 10 days. The following conditions were used in **STAGE II**: temperature, 25°C; stirring speed, 100 rpm; phosphate buffer, 0.05 M (pH = 6); and reaction time, 7 days. Only two regioisomers were obtained and one of them accounted for 82% of the final composition. The mixture was analyzed

Table 2. Tested conditions for biooxidation of 1,8-cineole trials.

Entry	Biomass (g/100mL)	Buffer pH	1,8-cineole (µL/100mL)	Temp. (°C)	Reaction time (days)	conversion (%)
1	11.6	7	100	28	4	3
2	10.0	7	50	25	4	5
3	10.0	7	50	25	6	6
4	11.0	6	50	25	4	15
5	10.9	6	50	25	7	55
6	9.7	6	100	25	7	90

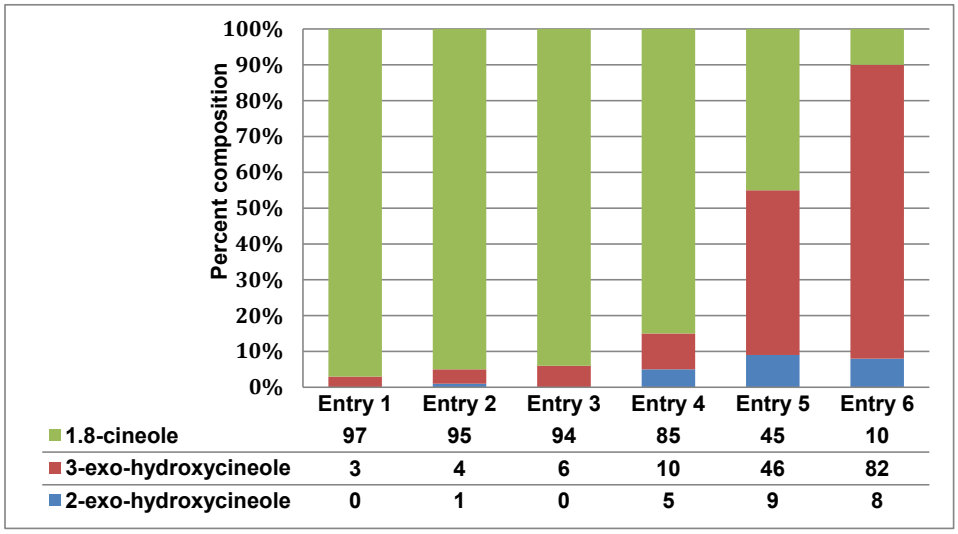

Figure 3. Relative composition of the products and the remaining substrate in the broth.

by NMR spectroscopy and GC-MS and the products were identified as 3-α–hydroxy-1,8-cineole (**3**) and 2-α-hydroxy-1,8-cineole (**2**) by comparison with literature data [6b, 12].

These results indicate that the basidiomycete *Gymnopilus spectabilis* 7423 could be an excellent biocatalyst for the practical preparation of 3-α-hydroxycineol from 1,8-cineol. Literature precedence for the biocatalytic preparation of this oxygenated terpene indicates that it is obtained in most cases by eukaryotic cells [7h, 7i, 13], but to date, the compound has only been obtained along with other oxygenated terpene derivatives. In this work compound **3** was the major product (82%) with a high bioconversion (90%). This indicates that both the strain utilized and the biotransformation conditions developed constitute an interesting method for the preparation of 3-α-hydroxycineol.

4 Conclusions

Different methods have been described for the synthesis of the oxygenated compounds from 1,8-cineole, including several attempts for their preparation using fungi [7h, 7i] and bacteria as biocatalysts [3, 7g, 7j, 7k]. The compound 3-α-hydroxycineol has also been prepared by chemical means from α-terpineol through a four step sequence: bromination with *N*-bromosuccinimide, potassium tert-butoxide elimination, peracid epoxidation, and lithium aluminum hydride reduction [6b]. The biocatalytic reactions described so far are less environmentally hazardous but produced lower conversion yields. In order to use microbial oxidations as a real tool for the transformation of 1,8-cineole into valuable oxidized compounds, it is necessary to obtain a bioconversion that is at least as good as the one obtained by traditional organic chemistry reactions. Our procedure has rendered oxygenated derivatives in very high yield and regioselectivity. This result is encouraging and suggests the possibility of using bioconversions, safer solvents, and soft reaction conditions in the fine chemicals industry.

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