Journal of Molecular Catalysis B: Enzymatic 59 (2009) 173–176

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Biooxidation of 1,8-cineole by *Aspergillus terreus*

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ARTICLE INFO

Article history:
Received 14 July 2008
Received in revised form 11 February 2009
Accepted 16 February 2009
Available online 28 February 2009

Keywords:
Aspergillus terreus
1,8-cineole
Biooxidation
Hydroxycineole
Monooxigenase

ABSTRACT

Biotransformation of 1,8-cineole by a strain of *Aspergillus terreus* isolated from Eucalyptus leaves was investigated.

This strain produced four oxygenated compounds identified as 2-*exo*-hydroxy-1,8-cineole, 2-*endo*-hydroxy-1,8-cineole, 3-*exo*-hydroxy-1,8-cineole and 3-*endo*-hydroxy-1,8-cineole, with good bioconversion percentage and high stereoselectivity.

1. Introduction

The monoterpene cyclic ether, 1,3,3-trimethyl-oxabicyclo [2.2.2]octane commonly known as eucalyptol, 1,8-cineole, or cineole (**1**) (see Scheme 1) is an abundant monoterpene present in the essential oils of several species being the main component of *Eucalyptus globulus* Labill oils [1].

This monoterpene has been shown to have biological activity against plants [2–4] microorganisms [5] and insects [6,7].

It is extensively used in pharmaceutical preparations for external application and also as a nasal spray [8]. In turn, due to its pleasant and distinctive flavor, it is also used in the food and cosmetic industries [9].

The 1,8-cineole, is a cheap raw material and it is easily obtained from *Eucalyptus* essential oils. Its transformation into more valuable compounds is recognized as being of great economic potential to the food, perfume and pharmaceutical industries.

Some studies have been carried out on the oxygenation of 1,8-cineole by chemical [10,11] and biological processes [12–19]. However, none of these biotransformations represent a significant breakthrough on the transformation of this abundant and cheap substance into more valuable products.

Obtaining oxygenated derivatives of 1,8-cineole involves stereospecific introduction of molecular oxygen in non-activated carbon atoms, which is not easy to carry out by classical chemical synthesis [14]. Besides, the oxidation of this compound by traditional chemical methods usually gives a mixture of stereoisomers. Because of the correlation between structure and biological activity, it is desirable to have methodologies that yield the product in its optically pure form.

The production of new compounds via a biotechnological route offers a number of advantages. One important attribute of microbial biocatalysis is the ability to synthesize products that can be labeled as natural, if derived from natural substrates, and added to foods without being considered as additives [20].

Our group has been working in the characterization of useful biocatalysts for the synthesis of oxygenated monoterpene derivatives.

In this paper we describe a biocatalytic procedure for the transformation of 1,8-cineole using whole cells of *Aspergillus terreus* isolated from *Eucalyptus* leaves.

This fungus biotransformed 1,8-cineole (**1**) into four oxygenated species (**2**, **3**, **4**, **5**) with good bioconversion percentage and high stereoselectivity (Scheme 2).

2. Experimental

2.1. Chemical and reagents

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

α-Terpineol (90%) was purchased from Aldrich.

m-Chloroperoxybenzoic acid was purchased Sigma–Aldrich.

Ketoconazole and Metronidazole were provided by Roemmers (Montevideo, Uruguay).

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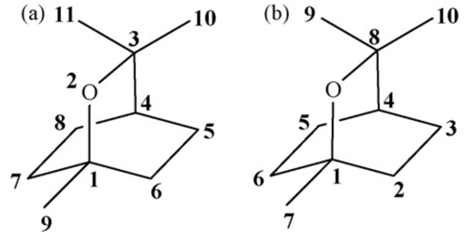
1381-1177/$ – see front matter
doi:10.1016/j.molcatb.2009.02.013



Scheme 1. (**1a**) 1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octane and (**1b**) 1,8-cineole.

2.2. Fungi isolation and identification

Fragments of *Eucalyptus* leaves were transferred to minimal medium BG-11 [21] supplemented with 1,8-cineole (0.5%) as carbon and energy source. After several rounds of serial culturing (1:10 dilutions) at 37 °C and 150 rpm (orbital shaker Sanyo IOXX400.XX2.C), the cell culture was spread onto the minimal medium plates (1.5% agar plates) with 1,8-cineole in the lid, recovering an isolated strain.

For identification of this strain, a culture was grown on Czapek yeast extract agar (CYA) at 5, 25 and 37 °C; malt extract agar (MEA) and 25% glycerol nitrate agar (G25N) at 25 °C. All plates were incubated for 7 days. Fungal identification was done according to Pitt [22]. This strain was kept at the Collection of the Microbiology Department (School of Chemistry, Montevideo, Uruguay) as BFQU 121.

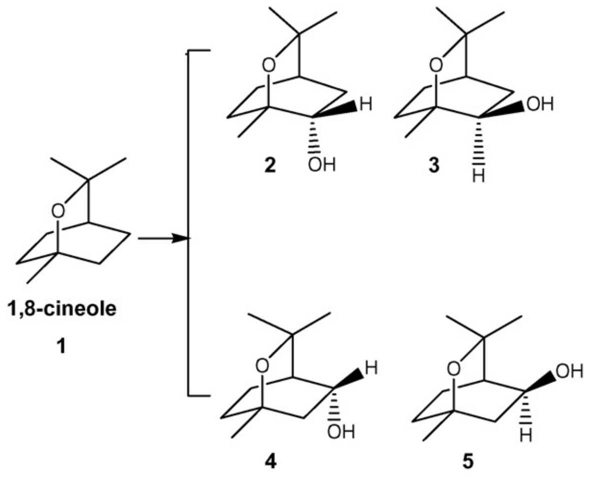
For routine procedures, the strain was grown in PDA slants (DIFCO, Detroit, USA) at 28 °C until sporulation, and then kept at 4 °C.

2.3. Biotransformation assays

YMPG (yeast extract 0.5%, malt extract 1%, bacteriological peptone 0.5%, glucose 1%) was used as the culture media. A spore suspension in sterile physiological serum was used as inoculum reaching a final concentration of 10^6 spores/mL in the culture media.

1,8-Cineole was added after 48 h of incubation such as to attain a final concentration of 0.1% in the culture media.

Biotransformations in the presence of inhibitors were carried out in the same conditions described above. The ketoconazole or metronidazole were added after 48 h of incubation to a final 50 mM [23] concentration in the culture media and 1,8-cineole was added 15 min after the addition of the inhibitor.



Scheme 2. Oxygenated products derived from 1,8-cineole: (**2**) 2-*exo*-hydroxy-1,8-cineole; (**3**) 2-*endo*-hydroxy-l,8-cineol; (**4**) 3-*exo*-hydroxy-1,8-cineole; (**5**) 3-*endo*-hydroxy-1,8-cineole.

Samples were taken for analysis at 4, 7, 10, and 14 days.

The growth of the fungus, with and without inhibitor, was monitored by measuring dry weight at 100 °C.

Three negative controls were performed, one of them using 1,8-cineole in culture media (without inoculum), a second one inoculated but containing neither substrate nor inhibitor, and a third one similar to the later but including the corresponding inhibitor.

All experiments were carried out in triplicate in 500 mL conical flasks containing 100 mL of culture media each one.

2.4. Extraction and identification of bioconversion products

In order to isolated enough amount of each product to achieve spectral experiments, we carried out the biotransformation experiments by fermentation in 6 conical flasks (2 L) containing 400 mL of culture media each one with 0.1% of 1,8-cineole (total starting material 2400 mg), placed in an orbital shaker Sanyo IOX400 XX2.C, agitation 100 rpm at temperature 28 °C.

The liquid medium was separated from the mycelia by filtration, and then was extracted with CH_2Cl_2. The mycelia were washed several times with the same solvent. Organic phases were combined and then dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to obtain 1392 mg of the crude mixture of 1,8-cineole **1** and hydroxycineoles **2**, **3**, **4**, and **5**.

Crude mixture reaction was flash-chromatographed on 150 g silica gel SAI (32-63 μm particle size, 60 Å pore size, 40 cm length × 6.5 cm diameter) using *n*-hexane/ethyl acetate gradient from 4:1 to 2:1.

Each one of these compounds was identified by GC–MS and/or ^{1}H and ^{13}C NMR.

2.5. Analysis conditions

HRGC analyses were performed in a Shimadzu GC14B equipped with FID and EZ Chrom integration software for data processing. A fused silica capillary column (30 m × 0.32 mm i.d.) with bonded SE52 (0.40–0.45 μm thickness) was used. Temperature program: 60 °C, 8 min; 60–210 °C at 3 °C/min; injector temperature: 240 °C; detector temperature: 250 °C. Carrier gas: N_2 at 0.50 kg/cm^2; injection system: split ratio 1:100.

HRGC–MS was carried out in Shimadzu QP 5500 in the conditions described above, using He as carrier gas. Ionization voltage 70 eV, temperature interface: 250 °C.

The optical purity was determined in a GC–GC Shimadzu GC 17A. The first GC is equipped with a SE52 column and the second one with a modified β-ciclodextrin chiral capillary column. Temperature program: 50 °C (6 min), 50–90 °C at 2 °C/min, 90 °C (20 min); 90–180 °C at 2 °C/min, 180 °C (10 min); injector temperature 250 °C, detector temperature 280 °C, carrier gas: He; injection system:split (ratio 1:150).

NMR spectra were recorded using a Bruker DPX-400 Avance Spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C).

3. Results and discussion

A native strain was isolated from *Eucalyptus* leaves in the presence of **1** as the sole carbon and energy source. This microorganism was identified as *Aspergillus terreus* by our laboratory.

This strain produced four oxygenated derivatives from **1** (see Scheme 2) that were identified as 2-*exo*-hydroxy-1,8-cineole **2**, 2-*endo*-hydroxy-1,8-cineole **3**, 3-*exo*-hydroxy-1,8-cineole **4** and 3-*endo*-hydroxy-1,8-cineole **5**.

The major product (**2**) was identified by comparison with the literature data and co-injection in GC with the synthetic **2**, prepared from alpha-terpineol according to bibliography [24].

The isolated amount for compounds **3** and **5** was not enough to correctly identify these products by NMR, therefore we prepared

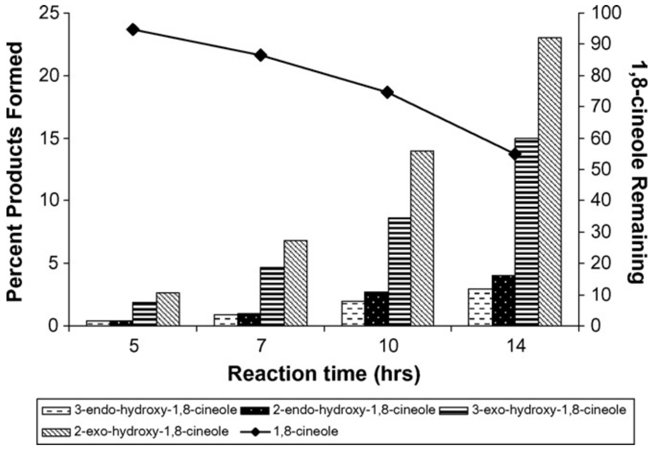


Fig. 1. Evolution of hydroxylated products.

3 by the following process: oxidation of **2** (PCC/CH_2Cl_2, r.t. 2 h) to obtain 2-oxo-1,8-hydroxycineole followed by reduction with $NaBH_4$ ($NaBH_4$, MeOH, 0 °C, 2 h). This yielded the mixture of **2** and **3**, that was co-injected with the crude product allowing the identification of compound **3**.

Compound **4** was isolated and identified by GC–MS and ^1H and ^{13}C NMR and compared to previous reports [10,12,14,16,17,25].

The bioconversion percentage of oxygenated products increased with reaction time and the best percent conversion was obtained at 14 days (Fig. 1).

This biocatalysts did not present high regioselectivity since 2- and 3-hydroxycineoles were obtained in similar percentage. However, it shows good stereoselectivity, since the *exo* position was preferred over the *endo* position by a ratio of 84:16 (Figs. 1 and 2).

In all previous reports, the biotransformation of 1,8-cineole with bacterium only produced the 2 oxygenated derivates [15–17,19,20].

However, reports on the biotransformation with other fungus showed that the hydroxylated position was more variable. Our results partially agree with previous reports. We detected 2-*endo*-hydroxycineole which was not reported previously as biotransformation product of **1** by fungus. On the other hand we did not detect the oxo derivates of **1**, as has been reported in previous literature [16,17].

The regioselectivity and stereoselectivity obtained with our *Aspergillus terreus* strain were very similar to the one obtained with the *Aspergillus niger* strain reported by Nishimura et al. [16], but the biotransformation percentage with our strain was higher than *Aspergillus niger* by 57%.

Rasmusen et al. reported the biotransformation of **1** with a *Penicillium* strain that yielded only the 3-hydroxy and oxo derivatives [17]. They did not report the biotransformation percentage.

The enantiomeric selectivity was low, for the 2 hydroxycineoles the ee was 12%, for both stereoisomers (*exo* and *endo*).

A low enantioselectivity was also observed for the 3 hydroxycineoles, the *endo* product presented an ee of 16% while the *exo* product yielded a racemic mixture ($[\alpha]_D^{25}$, 0 °C).

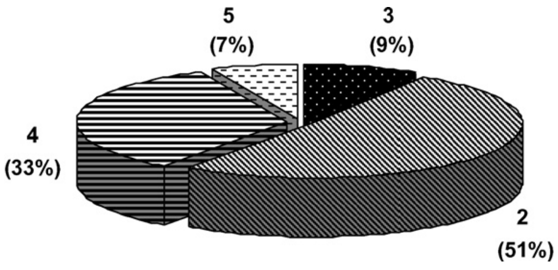


Fig. 2. Distribution of biotransformation products (%) (day 14th).

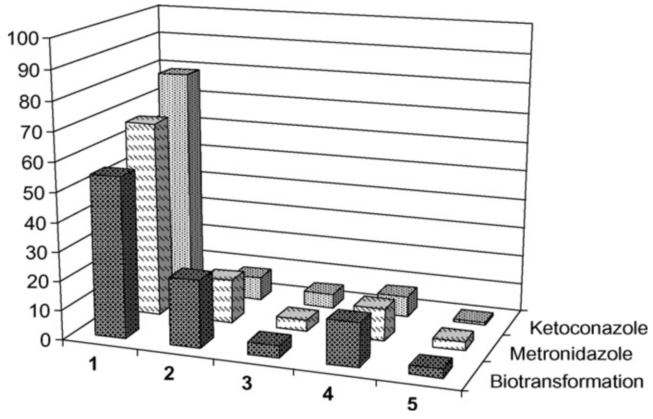


Fig. 3. Distribution of biotransformation products with and without inhibitors metronidazole and ketoconazole: (**1**) 1,8-cineole; (**2**) 2-*exo*-hydroxy-1,8-cineole; (**3**) 2-*endo*-hydroxy-1,8-cineol; (**4**) 3-*exo*-hydroxy-1,8-cineole; (**5**) 3-*endo*-hydroxy-1,8-cineole.

The production of these hydroxylated compounds implies the introduction of oxygen in non-activated carbon atoms. These types of reactions are usually catalyzed by members of the superfamily of cytochromes P450 which has been originally proposed to be responsible for the hydroxylation of **1** by a strain of *Bacillus cereus* [14].

Later, Haweck et al. reported the isolation of Cytochrome P450cin (CYP176A1) from *Citrobacter braakii* that is thought to initiate the biodegradative pathway that allows the bacterium to live on cineole as its sole carbon and energy source [13].

Based on these reports, we found it interesting to analyze if these types of enzymatic systems were involved in the production of these products by the *Aspergillus terreus* BFQU 121.

For this purpose, we included ketoconazole and metronidazole, which are well-known specific inhibitors of cytochrome P-450 systems in the biotransformation reaction.

Our results showed that addition of ketoconazole or metronidazole inhibited the biotransformation of 1,8-cineole. Although all four products were detected in the biotransformation with both inhibitors, the conversion percentage was lower than in the biotransformation without the inhibitor (Fig. 3). The percentage of total inhibition was 53% with ketoconazole and 27% with metronidazole.

The inhibition was not the same for all positions (see Fig. 3). Position *exo* was inhibited but *endo* position was not affected by the inhibitors. Given the results obtained, we conceive the existence of a Cyt P450 enzymatic system, responsible for the oxidation of **1** by *Aspergillus terreus*.

The oxidation of position *endo* could proceed by means of other type of oxidase or it could be generated from the *exo* hydroxylated product as it has been proposed previously [15,17].

We are currently analyzing the incidence of different variables on the regioselectivity, stereoselectivity and biotransformation yield in order to obtain the best conversion percentage and regioselectivity in the shortest time.

4. Conclusion

Aspergillus terreus BFQU 121 isolated from *Eucalyptus* leaves represents one type of biocatalysts that can oxygenate **1** and produces only hydroxylated products with a good yield. The bioreaction occurred with a very high stereoselectivity obtaining mainly *exo* hydroxycineoles.

The enzymes responsible for some of these oxidations could belong to the Cyt P450 group, since the main products are obtained in lower percentages when using reported inhibitors for these enzymes.

The fungi used here can perform hydroxylation reactions on **1** with a starting high concentration 1 g/L and represents a good option at the time of choosing a biocatalysts for the production of hydroxylated compounds, which could provide interesting building blocks for the synthesis of fine chemicals and biologically active compounds from a starting-point plentiful material such as 1,8-cineole.

Acknowledgements

This work was partly supported by a grant from CONICYT Clemente Estable 5071 and financially supported by PEDECIBA Química.

The authors also would like to thank BSc. Daniel Lorenzo and Dr. Eduardo Dellacassa for GC–MS analysis.

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