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Short Communication

Hydroxylation of 1,8-cineole by *Mucor ramannianus* and *Aspergillus niger*

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Abstract

The monoterpenoid 1,8-cineole is obtained from the leaves of *Eucalyptus globulus* and it has important biological activities. It is a cheap natural substrate because it is a by-product of the *Eucalyptus* cultivation for wood and pulp production. In this study, it was evaluated the potential of three filamentous fungi in the biotransformation of 1,8-cineole. The study was divided in two steps: first, reactions were carried out with 1,8-cineole at 1 g/L for 24 h; afterwards, reactions were carried out with substrate at 5 g/L for 5 days. The substrate was hydroxylated into 2-exo-hydroxy-1,8-cineole and 3-exo-hydroxy-1,8-cineole by fungi *Mucor ramannianus* and *Aspergillus niger* with high stereoselectivity. *Trichoderma harzianum* was also tested but no transformation was detected. *M. ramannianus* led to higher than 99% of conversion within 24 h with a starting high substrate concentration (1 g/L). When substrate was added at 5 g/L, only *M. ramannianus* was able to catalyze the reaction, but the conversion level was 21.7% after 5 days. Both products have defined stereochemistry and could be used as chiral synthons. Furthermore, biological activity has been described for 3-exo-hydroxy-1,8-cineol. To the best of our knowledge, this is the first report on the use of *M. ramannianus* in this reaction.

Key words: biotransformation, monoterpene, 1,8-cineole, *Mucor ramannianus*, *Aspergillus niger*.

The monoterpenoid 1,8-cineole is the main component of the essential oil extracted from leaves of *Eucalyptus globulus* L. Many biological activities have been attributed to *Eucalyptus* oil and 1,8-cineole. It is of particular interest since large areas are dedicated to *Eucalyptus* cultivation for wood and pulp production being leaves a by-product of this process. Thus, 1,8-cineole is abundant, cheap and easy available, and it has being extensively used in pharmaceutical, flavor and fragrance industries (Carvalho and Fonseca, 2006; Rodríguez *et al.*, 2006; Batish *et al.*, 2008; García *et al.*, 2009). Although 1,8-cineole has wide applicability as antimicrobial and pesticide (Knight, 2009; Barton *et al.*, 2010), some of its derivatives less frequent in nature may be more effective.

1,8-Cineole has low chemical reactivity since there are no activated C-H bonds in the molecule. The regioselective hydroxylation of such structure is considered a challenge in organic synthesis and studies on the chemistry of this monoterpenoid is mostly related to the cleavage of the ether bridge to obtain p-menthane derivatives (Boggiato *et al.*, 1987; Liu and Rosazza, 1990). Some studies involving only synthetic pathways (Boggiato *et al.*, 1987; Barton *et al.*, 2010) or synthetic and enzymatic steps have been successfully used (Luzzio and Duveau, 2002), however dangerous conditions and toxic reagents are employed and the products cannot be classified as “natural”.

Microbial transformation can be used as a main tool when such kind of selective transformation should be per-

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formed and the standard organic synthesis procedures are inefficient. Besides the high selectivity, biocatalysis has the advantages of mild reaction conditions and eco-friendly appeal (Nakamura *et al.*, 2003). However, there are some difficulties in the biotransformation of terpenoids, as low solubility in water and toxicity to microbial cells (Guardiola *et al.*, 1996). Microbial transformations of 1,8-cineole have been studied and the main reactions are oxidations, hydroxylations and hydrogenolysis of the ether bridge (Batish *et al.*, 2008; García *et al.*, 2009; Liu and Rosazza, 1990; Nishimura *et al.*, 1982; Knight 2009; Williams *et al.*, 1989). In general, biotransformations led to a mixture of compounds.

The aim of this work was to report studies about the biotransformation of 1,8-cineole by filamentous fungi. Three microorganisms were tested: *Aspergillus niger*, *Mucor ramannianus* and *Trichoderma harzianum*.

In the first step of the study, reactions were carried out with 1,8-cineole at 1 g/L for 24 h. Filamentous fungi were grown using a standard protocol (Ribeiro *et al.*, 2009). *Aspergillus niger*, *Mucor ramannianus* and *Trichoderma harzianum* belong to the collection of the 'Departamento de Engenharia Bioquímica, Escola de Química, UFRJ'. Spores were allowed to grow for 48 h, under 150 rpm and 30 °C in a medium containing 1% glucose, 0.5% yeast extract, 0.5% peptone, 0.1% $(NH_4)_2SO_4$, and 0.1% $MgSO_4.7H_2O$. After that period, biomass was separated by filtration through filter paper (Whatman™ grade 4, pore size: 20-25 μm), washed with distilled water and directly added to 50 mL of a solution containing only 0.5 g of glucose, 50 mg of 1,8-cineole and distilled water. Biomass was used at concentration of 10 g/L (dried weight). The substrate 1,8-cineole was dissolved in 0.5 mL of ethanol before the addition in reaction medium. The initial reaction conditions were based on the conditions described by Ribeiro *et al.* (2009) for the biotransformation of ethyl 4-chloro acetoacetate. Reactions were carried out in 500 mL cotton plugged Erlenmeyer flasks at 30 °C and 150 rpm, in duplicates. After 24 h of incubation, the biomass was separated by filtration through filter paper (Whatman™ grade 4, pore size: 20-25 μm) and washed with distilled water. The liquid phase was extracted twice, with 20 mL of ethyl acetate in each extraction. The organic phase was dried over anhydrous Na_2SO_4, filtered through filter paper (Whatman™ grade 4, pore size: 20-25 μm) and concentrated under vacuum.

To evaluate whether substrate concentration could influence on the catalytic activity, it was tested a new reaction condition with higher substrate concentration. After growing, filamentous fungi were added to 100 mL of a solution containing only 1 g of glucose, 500 mg of 1,8-cineole and distilled water. The reaction was carried out in 500 mL cotton plugged Erlenmeyer flasks for 5 days at 30 °C and 150 rpm, in duplicates. The biomass was separated by filtration through filter paper and the liquid phase was extracted as described above.

Substrate and products were analyzed by gas chromatograph (Agilent 6890N) coupled to a quadripolar mass spectrometer (Agilent 5973N), with ionization by electronic impact (70 eV). The apparatus was fitted with a HP-5MS column (30 m x 0.25 mm x 0.25 μm). Carrier gas was helium at flow of 0.5 mL/min. The injector port temperature was 250 °C and the scan-range was 40-700 *m/z*. The oven program was as follows: 40 °C held for 5 min, then ramped at 4 °C /min to 300 °C, with a final hold time of 10 min. Interpretation and identification of the fragmentation mass spectrum was carried out by comparison with the Wiley NBS mass spectrum data base. Purified products by preparative thin layer chromatography(performed in aluminum sheets recovered with silica gel 60 and eluted with cyclohexane/ ethyl acetate, 1:1), were characterized by spectral MS and NMR analyses and comparison with literature data.

Figure 1 and Table 1 show products obtained and conversion levels obtained in the first step of this study. The retention times were: 1,8-cineole, 16.4 min; 2-exo-hydroxy-1,8-cineole, 24.2 min; 3-exo-hydroxy-1,8-cineole, 24.8 min.

Highly stereoselective hydroxylation of 1,8-cineole was observed when *A. niger* and *M. ramannianus* were used as biocatalysts, since the *exo* position was preferred over the *endo* position. Two products were obtained: 2-exo-hydroxy-1,8-cineole and 3-exo-hydroxy-1,8-cineole (Figure 1), which have not usually been formed as chemical reaction products (Parshikov *et al.*, 2000). In the presence of *M. ramannianus* other two substances that were not identified, represented 3.6% of the total peak area of the products. No reaction was detected when *T. harzianum* was tested. These biocatalysts also showed regioselectivity, especially *A. niger*, since the 2-position was preferred over the 3-position by a ratio of 1.9:1.

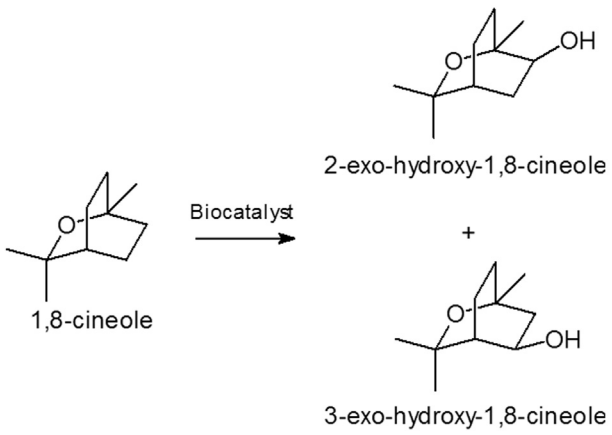

Figure 1 - Biotransformation of 1,8-cineole to 2-exo-hydroxy-1,8-cineole and 3-exo-hydroxy-1,8-cineole.

Table 1 - Biotransformation of 1,8-cineole (1 g/L) by filamentous fungi. Incubation: 30 °C, 150 rpm, 24 h.

Microorganism	Conversion (%)	*A** (%)	*B** (%)	*A/B* ratio
Aspergillus niger	46.1 ± 0.9	30.4 ± 1.0	15.7 ± 0.2	1.9/1
Mucor ramannianus	> 99	54.5 ± 0.2	41.9 ± 0.2	1.3/1
Trichoderma harzianum	0	0	0	-

* *A*: 2-exo-hydroxy-1,8-cineole; *B*: 3-exo-hydroxy-1,8-cineole.

One of these 1,8-cineole derivatives, 2-exo-hydroxy-1,8-cineole, is a natural product previously isolated from the juice of grape berries var. Sauvignon, contributing for the aroma note of black currant berries (Bitteur *et al.*, 1990). The other derivative, 3-exo-hydroxy-1,8-cineole, has antifungal and herbicidal activities (Barton *et al.*, 2010; Knight, 2009). Furthermore, both substances have defined stereochemistry and could be used as chiral building blocks.

In the culture broth of *A. niger* incubated with 1,8-cineole (1.43 g/L) for 7 days (when total conversion was achieved), Nishimura *et al.* (1982) found the same two products as the major metabolites. The reaction was carried out in 12 L of medium which allowed the recovered of small amounts of other three metabolites: 3-oxo-1,8-cineole, 2-oxo-1,8-cineole and 3-endo-hydroxy-1,8-cineole. García *et al.* (2009) reported the transformation of 1,8-cineole (1 g/ L) by the fungus *Aspergillus terreus* into four hydroxylated products: 2-exo-hydroxy-1,8-cineole (the major product), 2-endo-hydroxy-1,8-cineole, 3-exo-hydroxy-1,8-cineole and 3-endo-hydroxy-1,8-cineole. The transformation was conducted until 14 days and the same four products were obtained even if the inhibitors such as ketoconazol and metronidazole were added (although lower conversion levels were achieved). The bacterium *Rhodococcus* sp. (strain C1) accumulated 2-endo-hydroxy-1,8-cineole and 2-oxo-1,8-cineole in a 40 L medium containing 1,8-cineole at 1 g/L (Williams *et al.*, 1989). Rodríguez *et al.* (2006) used other *Rhodococcus* sp. strain and the same substrate concentration. They achieved 98% of conversion within 24 h and three products were formed: 2-oxo-1,8-cineole, 2-endo-hydroxy-1,8-cineole and 2-exo-hydroxy-1,8-cineole. Liu and Rosazza (1990) used *Bacillus cereus* as biocatalyst in the hydroxylation of the same substrate into 2-(*R*)-exo-hydroxy-1,8-cineole with 74% of conversion after 24 h. 1,8-Cineole also was reported by Knight (2009) to give 2-endo-hydroxy-1,8-cineole when incubated for 7 days at 0.5 g/L with the bacteria *Sphingomonas* sp. in a 20 L culturing. Differences in conversion levels and products probably have close relationship with the biocatalyst employed in the transformation o 1,8-cineole. It seems that bacteria enzymes attack preferentially the position 2 of 1,8-cineole, while fungal enzymes attack both positions 2 and 3.

Essential oils containing 1,8-cineole are extensively used in food industries, thus its metabolism in mammals must be known. In the studies on cytochrome P450 (CYP) enzymes, Miyazawa *et al.* (2001) concluded that 2-exo-hydroxy-1,8-cineole is the main human metabolite of 1,8-cineole, based on its high formation rate by cytochome P450 3A4, the major enzyme involved in the oxidation of 1,8-cineole by human liver microsomes. This found corroborates with metabolism studies using filamentous fungi, which can be considered models for the study of drug metabolism in mammals. Biotransformation processes could be a way for the production of this substance in large amounts for further toxicology studies.

High substrate concentration is important for a commercially viable biotransformation process but the cytotoxicity of monoterpenes can decrease conversion level in reactions catalyzed by whole cells (Adams *et al.*, 2003). Thus, in the second step of this study, it was tested a new reaction condition with the addition of 1,8-cineole at 5 g/L. As substrate concentration was higher, the incubation time was extended to 5 days.

As expected, there was no conversion in the presence of *T. harzianum* and it seems that this fungus does not have active enzyme to catalyze the reaction under studied conditions. The fungus *A. niger* is sensitive to high concentration of substrate and there was no conversion even after 5 days of incubation with 1,8-cineole at 5 g/L. Only *M. ramannianus* was able to catalyze the reaction in this condition, but the conversion level was 21.7% after 5 days. The same two products (12.4% of 2-exo-hydroxy-1,8-cineole and 9.3% of 3-exo-hydroxy-1,8-cineole) were detected and the ratio was kept (1.3:1), suggesting that kinetics resolution is not possible with this fungus, since the incubation times tested (24 h and 5 days) showed no influence on the ratio of products.

In summary, the filamentous fungi *M. ramannianus* and *A. niger* were able to catalyze the transformation of 1,8-cineole into two hydroxylated products: 2-exo-hydroxy-1,8-cineole and 3-exo-hydroxy-1,8-cineole. Besides the high stereoselectivity (only exo-hydroxy-1,8-cineoles were obtained), *M. ramannianus* led to almost 100% of conversion within 24 h with a starting high substrate concentration (1 g/L). To the best of our knowledge, this is the first report on the use of *M. ramannianus* in this reaction.

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References

Adams A, Demyttenaere JCR, Kimpe ND (2003) Biotransformation of (*R*)-(+)- and (*S*)-(-)-limonene to α-terpineol by *Penicillium digitatum* - investigation of the culture conditions. Food Chem 80:525-534.

Barton AFM, Dell B, Knight AR (2010) Herbicidal activity of cineole derivatives. J Agr Food Chem 58:10147-10155.

Batish DR, Singh HP, Kohli RK, Kaur S (2008) Eucalyptus essential oil as a natural pesticide. Forest Ecol Manag 256:2166-2174.

Bitteur SM, Baumes RL, Bayonove CL, Versini G, Martin CA, Serra AD (1990) 2-*exo*-Hydroxy-1,8-cineole: A new component from Grape var. Sauvignon. J Agr Food Chem 38:1210-1213.

Boggiato MV, Heluani CS, Fenik IJS, Catalán CAN (1987) Regiospecific functionalization of the monoterpene ether 1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane (1,8-cineole). Synthesis of the useful bridged g-lactone 1,3-dimethyl-2-oxabicyclo[2.2.2]octan-3→5-olide. J Org Chem 52:1505-1511.

Carvalho CCCR, Fonseca MMR (2006) Biotransformation of terpenes. Biotechnol Adv 24:134-142.

García C, Rodríguez P, Días E, Heinzen H, Menéndez P (2009) Biooxidation of 1,8-cineole by *Aspergillus terreus*. J Mol Catal B-Enzym 59:173-176.

Guardiola J, Cánovas M, Iborra JL (1996) Modelling of the biotransformation from geraniol to nerol by freely suspended and immobilised grape (*Vitis vinifera*) cells. In: R.H. Wijffels, R.M. Buitelaar, C. Bucke, J. Tramper (eds) Immobilized Cells: Basics and Applications. Elsevier Science, Noordwijkerhout, pp 349-352.

Knight AR (2009) Preparation and Bioactivity of 1,8-cineole Derivatives. Ph.D. Thesis, Murdoch University, 184 p.

Liu WG, Rosazza JPN (1990) Stereospecific hydroxylation of 1,8-cineole using a microbial biocatalyst. Tetrahedron Lett 31:2833-2836.

Luzzio FA, Duveau DY (2002) Enzymatic resolution of the 1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane (1,8-cineole) system. Tetrahedron-Asymmetr 13:1173-1180.

Miyazawa M, Shindo M, Shimada T (2001) Oxidation of 1,8-cineole, the monoterpene cyclic ether originated from *Eucalyptus polybractea*, by cytochrome P450 3A enzymes in rat and human liver microsomes. Drug Metab Dispos 29:200-205.

Nakamura K, Yamanaka R, Matsuda T, Harada T (2003) Recent developments in asymmetric reduction of ketones with biocatalysts. Tetrahedron-Asymmetr 14:2659-2681.

Nishimura H, Noma Y, Mizutani J (1982) *Eucalyptus* as biomass. Novel compounds from microbial conversion of 1,8-cineole. Agric Biol Chem 46:2601-2604.

Parshikov IA, Freeman JP, Lay Jr JO, Beger RD, Williams AJ, Sutherland JB (2000) Microbiological transformation of enrofloxacin by the fungus *Mucor ramannianus*. Appl Environ Microbiol 66:2664-2667.

Ribeiro JB, Ramos AS, Fiaux SB, Leite SGF, Ramos MCKV, Aquino Neto FR, Antunes OAC (2009) Immobilized microorganisms in the reduction of ethyl 4-chloro acetoacetate. Tetrahedron-Asymmetr 20:2263-2266.

Rodríguez P, Sierra W, S. Menéndez RP (2006) Biotransformation of 1,8-cineole, the main product of Eucalyptus oils. Electron J Biotechn 9:232-236.

Williams DR, Trudgill PW, Taylor DG (1989) Metabolism of 1,8-cineole by a *Rhodococcus* species: ring cleavage reactions. J Gen Microbiol 135:1957-1967.

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