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HYDROXYLATION OF GRINDELIC ACID BY FILAMENTOUS FUNGI

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ABSTRACT

The natural diterpene grindelic acid ***1****, isolated from aerial parts of* Grindelia pulchella, *was xenobiotically bioconverted by strains of* Fusarium sp. *and* Monodyctis cataneae. Monodyctis cataneae *produced a regio and stereospecific allylic hydroxilation yielding 6-α-hydroxygrindelic acid,* ***2****, (70%).* Fusarium sp. *hydroxylated the substrate in both, an allylic and on a non-activated position to afforded 6-β-hydroxygrindelic,* ***3****, and 3-β-hydroxygrindelic acid,* ***4****, respectively.*

RESUMEN

El ácido grindélico, ***1****, un diterpeno natural aislado de las partes aéreas de* Grindelia pulchella, *fue bioconvertido xenobióticamente con cepas de los hongos* Fusarium sp. *y* Monodyctis cataneae. *Esta última cepa produjo una hidroxilación alílica regio y estereo específica que condujo al derivado 6-α-hidroxilado,* ***2****, con rendimiento del 70%. Por otra parte con cultivos de Fusarium sp. se logró hidroxilar al sustrato en posiciones activadas y no activadas de manera de obtener el 6-β-hidroxiderivado,* ***3****, y el ácido 3-β-hidroxigrindélico,* ***4****, respectivamente.*

INTRODUCTION

Grindelic acid, **1,** is the main secondary metabolite isolated from aerial parts of *Grindelia pulchella* [1]. Its hydroxylated derivatives possess potential activity towards insects [2]. Even when to access to relative high quantities to the acid is possible through traditional phytochemical operations, the chemical transformation of it into its 6-hydroxyderivative involves several steps with poor overall yield [3].

Consequently, it is of substantial interest to find out into different methods in other to obtain them. In a previous work we have reported the production of 6-β-hydroxygrindelic (25%) and 3-β-hydroxygrindelic acid (55%) by bioconversion of **1** by *Alternaria alternata* [4].

This paper deals on grindelic acid microbial oxidation by the microorganisms *Fusarium sp.* and *Monodyctis cataneae* and the relationship between culture age and bioconversion rates.

RESULTS AND DISCUSSION

Extraction procedures and relative bioconversion rates calculation.

When liquid-liquid extraction was carried out at pH 3, 6,8(17)-dehydrogrindelic acid, **5,** was recovered. Compound **5** may be a dehydration artefact of 6-hydroxygrindelic acid during extraction process in the acidified medium. In fact, compounds **2** and **3** were obtained when the pH value of the extraction procedure was fixed between 5.5-5.8.

Compounds **2**, **3**, **4** and **5** were not detected by ^{1}H-NMR analysis of the extracts from fungi

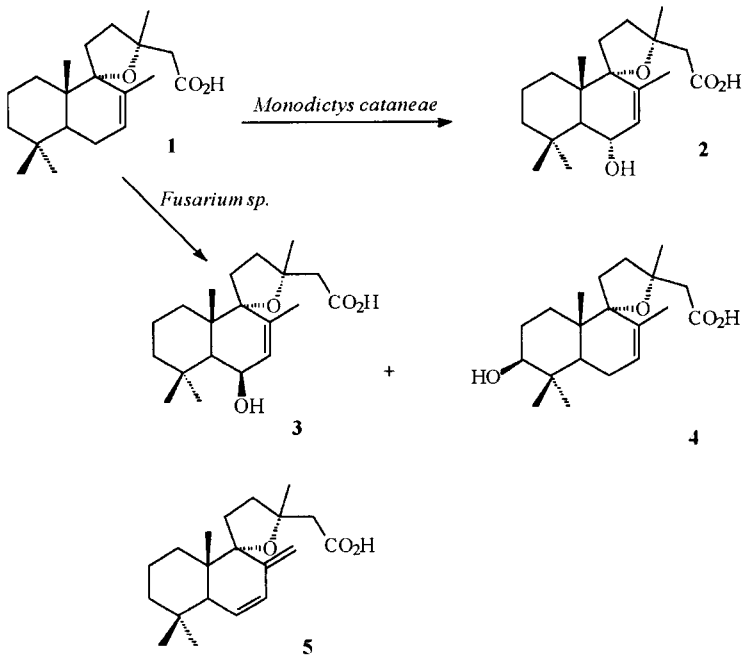

FIGURE 1. Grindelic acid and derivatives obtained by bioconversion.

cultures without the addition of substrate (Control assays). No other grindelane derivatives were detected in the ethereal extracts when **1** was added to the culture media without microorganisms (control assays).

Bioconversion rates were calculated using the relative area of characteristic peaks from the ^{1}H-NMR spectra [5]. The H-14 signals appeared as a double doublet overlapped for grindelic acid and its derivatives at δ 2.66, so this signal was used as a parameter for all the area comparisons. To analyze relative occurrence of 6-α-hydroxygrindelic acid the area ratios of the new signal of the geminal proton H-6, at δ 4.20 and of H-14 at δ 2.66, were compared. In a parallel way, the isomer 6-β was analyzed by comparison of H-6 signal at δ 4.4 vs. H-14 signal area. On the other hand, 3-β-hydroxygrindelic acid was analyzed by measuring the new signal at δ 3.27 which correspond to the alcoholic based proton at position H-3.

Bioconversion of grindelic acid with *Monodyctis cataneae.*

The bioconversion process carried out by *Monodyctis cataneae* yielded 6-α-hydroxygrindelic acid as a unique product. This regio and stereospecific allylic hydroxylation allows us to obtain a metabolite that is not easy to get by chemical transformation (Figure 1). Moreover, the important fact of this process was the unusually high rate of bioconversion, which reached the 70 % of substrate transformed into an only one final product (Figure 2).

By analyzing bioconversion rates according to the culture age it was possible to see that, when the substrate was added to cultures in stationary phase (96 h) the bioconversion begun approximately 70 h later than when it was incorporated at the end of log phase (48 h). In addition, the final rates, at 220 h of incubation, in this experience were considerably lower (40 %) than the ones observed by inoculating grindelic acid to 48h-old cultures in which transformed product recovering was nearly the 70 %.

Bioconversion of grindelic acid with *Fusarium sp.*

When grindelic acid biotransformations were carried out by cultures of *Fusarium sp.* and the substrate were added to 48 hours-old fungal cultures two different hydroxylated derivatives were

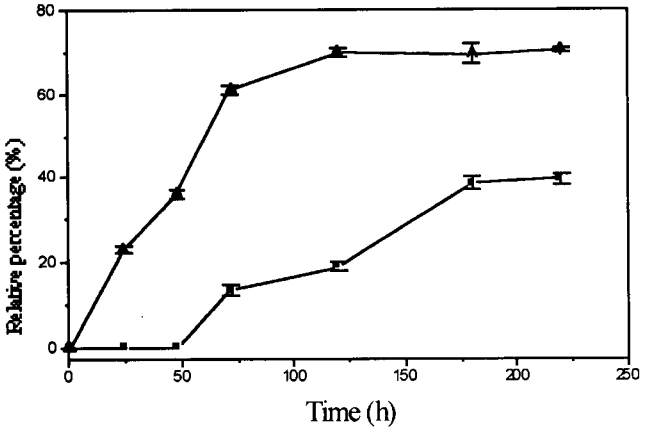

FIGURE 2. Time course of production of 6-α-hydroxygrindelic acid (▲) in 48 hours-old cultures, (■) in 96 hours-old cultures of *Monodyctis cataneae*.

obtained: the allylic hydroxylated derivative 6-β-hydroxygrindelic acid, **3,** and 3-β-hydroxygrindelic acid, **4**, where the hydroxylation took place on a non activated position (Figure 1). Although, the yields of this process were considerable lower (7 % and 8 % for the allylic and non allylic hydroxylated derivatives respectively) than the ones described above it is a very interesting reaction under a chemical point of view due to the difficulty to access to non activated positions by other ways.

When substrate was added to 96h-old cultures only 3-β-hydroxygrindelic acid, **4,** was obtained with a final rate of 9 % of conversion (Figure 3).

These results, together with the one reported previously, showed us the possibility to choose among different fungi in order to obtain different derivatives.

Particularly, to produce regio and stereospecific hydroxylation like the one performed by *Monodyctis cataneae*. In addition, these procedures allow us to explore into different fermentation variations, to obtain, alternatively, different bioactive metabolites with a unique fungal strain.

MATERIALS AND METHODS

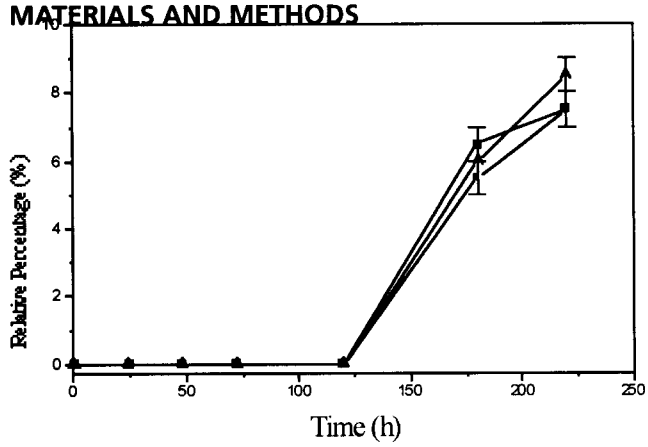

FIGURE 3. 6-β-hydroxygrindelic acid (■) and 3-β-hydroxygrindelic acid (●) in 48-h-old cultures and 3-β-hydroxygrindelic (▲) acid in 96-h-old cultures of *Fusarium sp.*

Microorganism and culture conditions.

The fungal strains were isolated from natural sources [6] and maintained in solid Czapek media. Growth curves were performed in Czapek liquid media incubated at 28 ± 2 °C and shaken at 180 rpm, dried weight evaluation was performed each 12 h.

Isolation of substrate.

Grindelic acid was isolated from aerial parts of *Grindelia pulchella* by the method described elsewhere [1].

Biotransformation assay.

Spore suspensions were inoculated to Czapek liquid medium, incubated at 28±2°C and shaken at 180 rpm for 2 days. Mycelium was then transferred to the same fresh culture medium (100 ml in 500 ml conical flask) and grown under the conditions above described.

Grindelic acid was solubilized in ethanol and added to 48 and 96 hours-old cultures at a final concentration in culture media of 1.0 mg/ml. TLC and 200 MHz 1H-NMR analyses were employed to monitor the bioconversion processes. Blank assays without substrate and without fungi were carried out. Samples were harvested each 24 h after substrate addition. Each experience was performed three times with two replicates each.

Extraction, purification and quantification.

Culture medium and mycelia were separated by vacuum filtration. The filtrate was acidified to pH 5.0 -5.8 with 0.5M HCl before extraction with Et_2O four times. The combined ethereal layers were evaporated in vacuum to dryness. Cultures were subjected to liquid-liquid extraction with Et_2O and purified by CC in silica-gel 70-230 mesh 60 Å eluted with an increasing polarity mixture of n-hexane: EtOAc. Identification was performed by comparison of their ^{1}H-NMR data with values reported by Bohlmann *et al*, [7] for compound **4**, by Rose *et al*, [2] for metabolite **2,** Gonzales Sierra *et al*, [8] for both, **2** and **3** and by Guerreiro *et al*, [1] for the substrate **1**. Relative bioconversion rates were calculated by ^{1}H-NMR using the relative area of characteristic peaks according to Miyazawa *et al*, [5].

Statistical methods:

All experiments were repeated three times and statistical analyses were carried out using the ANOVA test.

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