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Microbial transformation of S-(-)-Perillyl Alcohol by the filamentous fungi *Fusarium verticillioides*

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ABSTRACT

S-(-)-Perillyl alcohol (1) was transformed by *Fusarium verticilloides* over 7 days at 25°C to S-(-)-Perillyl aldehyde (2). Its structure was established by NMR spectroscopic and mass spectral studies. Total antioxidant potential and the radical-scavenging activity were determined by phosphomolybdenum and ABTS•+ assays, respectively. The results demonstrated that the oxidation of the primary alcohol of 1 had no effect on antioxidant activity when compared to starting material and a reference compound. Obtaining derivatives of 1, is an important goal of research in microbial biotechnology and medicinal chemistry.

KEYWORDS

S-(-)-perillyl alcohol;
S-(-)-perillyl aldehyde;
Fusarium verticillioides;
Biotransformation;
Antioxidant activity.

INTRODUCTION

Microbial transformation is a relevant strategy to obtain high added value compounds under controlled environmentally friendly conditions[1-3]. These reactions are important routes for introducing chemical functions into inaccessible sites of molecules and thereby produce rare structures. They can yield new drugs and existing drugs can be improved as to increased activity and decreased toxicity. Side-effects could be reduced and the stability could be increased by modification of the parent drug[4-6].

Perillyl alcohol (POH) also called *p*-mentha-1,8-diene-7-ol is a monoterpene, and thus consists of two isoprene units manufactured by the mevalonate pathway and constituent of essential oils from a number of plants; namely, perilla (*Perilla frutescens*), lavender, peppermint, ginger grass, savin, caraway, and celery seeds. It is a hydroxyl derivative of limonene and possesses chemopreventive properties. POH has cytostatic and cytotoxic effects and induces apoptosis in lung, leukocyte, prostate, and breast cancer cell lines.

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In vivo, POH has anti-metastatic effects and is a potent inhibitor of angiogenesis. Its chemotherapeutic effects are under evaluation in several clinical trials, including patients with colorectal, breast, or ovarian cancer[7-11].

Therefore, the biological derivatization of perillyl alcohol and its structural analog limonene with

the aim of producing new metabolites with a variety of biological properties, besides their use as

flavoring agents, is an important field of xenobiochemistry, pharmacology and toxicology[12-13]. The goal of this work was to obtain derivatives of perillyl alcohol through microbial transformation and evaluate their antioxidant activity. In previous work this compound was hydroxylated on methyl group of the side chain by a suspension cell culture of Catharanthus roseus[14].

Furthermore perillyl alcohol was oxidized in vivo to give the corresponding aldehyde and acid[15] and more recently microbial oxidation of endocyclic double bond was reported[4].

The goal of this work was to obtain derivatives of perillyl alcohol through microbial transformation and evaluate their antioxidant activity. For this purpose a phytopathogenic fungi like *Fusarium verticillioides* was used. This genus has an enzymatic machinery capable of transforming different structures of organic compounds[16-18].

Here perillyl alcohol transformation using a pure strain of *Fusarium verticillioides* giving as the sole product perillyl aldehyde, and the evaluation of the antioxidant activity is reported. The product structure was confirmed by spectroscopic methods and by comparison with literature data[19].

This is the first report of production of perillyl aldehyde by oxidation of perillyl alcohol with a fungus of genus *Fusarium*.

MATERIAL AND METHODS

Equipment

Gas Chromatography (GC) analyses were performed on a Shimadzu QP5050A equipped with a split/splitless injector, quadrupole mass spectrometer (EI 70 eV), Class 5000 data software, and NIST 107 mass spectral library. The injector was set at 300 ºC. For separation, a VF-5ms, 30 m x 0.25 mm x 0.25 μm column was used. Helium was the carrier gas (1.1 ml min^{-1}). 0.2 microlitres of the sample was injected in split mode at a split ratio of 20:1. The GC oven temperature was programmed as follows: initial temperature of 50 ºC held for 1 min. The temperature was increased up to 280 ºC at 18 ºC/min and held for 20 min. The detector temperature was set at 300 ºC. For the Mass Spectrometry (MS) system, the acquisitions were performed in scan mode (from 35 to 800 amu). Peak identification was carried out by analogy of mass spectra with those of the mass library and comparing the retention times and the mass spectra with those corresponding to commercial standards.

All ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 400, Ultrashield, at a frecuency for ^{1}H NMR of 400.16 MHz and for ^{13}C NMR of 100.62 Hz, with a Dual BBI Probe at 25 ºC using $CDCl_3$-d6 (99.8%, Merck) as the solvent. Chemical shift values are reported in parts per million (δ), relative to tetramethylsilane (TMS), as internal standard and coupling constants (*J*) being given in Hertz (Hz). All ^{13}C NMR spectra were proton-decoupled, and were confirmed by using the 135º DEPT technique (Distortionless Enhancement by Polarization Transfer), as well as by COSY (Correlation Spectroscopy, 1H–1H) and HSQC (Heteronuclear Single Quantum Coherence, ^{1}H–^{13}C Correlation). The relative integrals of peak areas are in agreement with those expected for the assigned structures.

Perillyl alcohol (purity 99.5%) was purchased from Sigma Aldrich. All chemicals were of analytical reagent grade and used as delivered.

Thin layer chromatography (TLC) was performed on Merck Sil G/UV254 silica gel plates with a fluorescent indicator, and the spots were visualized under 254 nm illumination, using petroleum ether (PE) – EtOAc (7:3, v/v) as eluting mixture, and detection was achieved by spraying with p-anisaldehyde – EtOH – AcOH – H_2SO_4 acid (0.1:17:2:1, v/v/v/v) followed by heating at 150°C. All organic solvents were distilled prior to use.

Microorganism and media

Fusarium verticillioides from the culture collection of the Laboratory of Mycology,

Department of Microbiology and Immunology, National University of Río IV, Córdoba, Argentina, was used for screening experiments. The microorganism was stored on Sabouraud dextrose agar (Britannia Laboratories Inc.; Buenos Aires, Argentina) slants at 10 °C. Liquid medium for screening and preparative-scale experiments was potato dextrose agar (PDA), sterilized at 121°C for 17 min.

Biotransformation, extraction and purification

Screening experiments were carried out following a two-stage standard protocol (I and II) in Erlenmeyer flasks (250 ml) containing 30 mL of culture medium, placed on a rotary shaker at 120 rpm and incubated at 25-30 °C for 72 h.

One milliliter of a stage I culture was used as the inoculum for a fresh stage II culture. The substrate was added to the incubation media 24 h after the inoculation of the stage II cultures as a 1.5% solution in dimethyl sulfoxide (DMSO) – EtOH (5:1, v/v) at a final concentration of 0.2 mg/ml of medium. Substrate controls were composed of sterile medium, to which substrate was added and incubated without microorganisms. The culture controls consisted of fermentation blanks in which microorganisms were grown under identical conditions, but without adding substrate. The fermentation was sampled at intervals of 48 h, taking 0.5 ml of culture medium, which was extracted with EtOAc. The organic layers were chromatographed on TLC plates.

The preparative-scale fermentation was performed in five 1L Erlenmeyer flasks containing 400 ml of sterile culture medium and these were then incubated at 25 – 30°C on a shaker at 80 rpm. POH was added to a final concentration of 0.2 mg/mL and the fermentation was continued until no starting material was detected when checked by TLC.

After the indicated time, liquors were combined, the mycelium filtered off and washing with H_2O (200 ml) was carried out. These washings and the filtrate were combined and extracted with EtOAc (4 x 200 ml). Organic layers were combined, washed with brine, dried with anhydrous Na_2SO_4, and the solvent evaporated under vacuum, giving a brownish oily residue.

Antioxidant activity

1- Total antioxidant potential by phosphomolybdenum assay. The antioxidant power of the samples was assessed with the phosphomolybdenum reduction assay according to Prieto et al.[20]. The reagent solution, protected from the light, contained ammonium molybdate (4 mM), sodium phosohate (28 mM) and sulfuric acid (600 mM). A 1 ml aliquot of this solution was mixed in Eppendorf tubes with 0.1 mL of sample diluted in MeOH at the concentrations of 10, 50 and 100 µg/mL. The tubes were capped and incubated in a thermal block at 37°C for 90 min and the absorbance of the green phosphomolybdenum complex was measured at 695 nm on a Cary 50 Conc Varian® spectrophotometer. The reducing capacity of the samples was expressed as ascorbic acid equivalents (µg/mL) used for calibration curve. Measurements were performed in triplicate. Results are shown in TABLE 1.

TABLE 1: Antioxidant capacity of POH and PALD using phosphomolybdenum method.

Concentration (µg/mL)	Perillyl Alcohol	Perillyl Aldehyde
	µg/ml eq. Ascorbic acid[b]	µg/ml eq. Ascorbic acid[b]
100	6.49 ± 0.25	7.15 ± 0.04
50	5.41 ± 0.26	6.15 ± 0.22
10	4.18 ± 0.19	4.39 ± 0.15

[a]Values expressed are mean ± S.D. of three experiments; [b]Ascorbic acid equivalent (µg/ml) through the formation of phosphomolybdenum complex.

2- Radical-scavenging activity by ABTS•+ assay. The method used was as described by Re et al.[21], based on the capacity of a sample to scavenge the blue-green coloured 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) radical cation (ABTS•+) compared with a reference antioxidant standard vitamin E analogue (Trolox®). The ABTS•+ was produced by oxidation of 7mM ABTS solution with 2.45 mM potassium persulphate solution, stored in the dark at room temperature for 12 h (time required to radical formation) since the solution is stable for 48 hours. Once formed the colored radical, the ABTS•+ solution was diluted to get an absorbance

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of 0.700 ± 0.020 at 754 nm with ethanol to obtain the working solution. After addition of 1 ml of working solution of ABTS•+ to 25 µl of different concentration of samples (10, 50 and 100 µg/ml), the absorbance was read at 754 nm after exactly 4 min on a Cary 50 Conc Varian® spectrophotometer. Measurements were performed in triplicate. The percentage inhibition was calculated against standard Trolox® calibration curve prepared for a concentration range of 0.31–0.61 µg/ml Trolox® and the results were expressed as Trolox equivalent (TE, µg/ml). Results are shown in TABLE 2.

^{13}C NMR spectroscopy and confirmed when compared with bibliographical data.

Biotransformation by F. verticilloides

The product, (S)-(-)-Perillaldehyde (2) (50 mg; 63% yield).

^{1}H NMR ($CDCl_3$): 1.39 (m, 1H, H_{5b}), 1.65 (s, 3H, $H_{3'}$), 1.74 (m, 1H, H_{5a}), 1.85 (m, 1H, H_{3b}), 2.04 (m, 2H, H_6), 2.05 (m, 1H, H_4), 2.07 (m, 1H, H_{3a}), 4.63 (s, 2H, $H_{1'}$), 7.20 (m, 1H, H_2), 9.38 (s, 1H, H_7).

^{13}C NMR ($CDCl_3$): C_7 (193.7), C_8 (149.3), C_9 (109.6), C_1 (41.3), C_2 (150.5), C_4 (40.8), C_3 (31.8), C_5 (26.4), C_6 (21.6), C_{10} (20.7).

TABLE 2 : Radical-scavenging activity of POH and PALD by ABTS•+ assay.

Concentration (µg/mL)	Perillyl Alcohol		Perillyl Aldehyde	
	TE value[b]	Percentage inhibition[c] (%)	TE value[b]	Percentage inhibition[c] (%)
100	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0,00	1.20 ± 0.11
50	0.07 ± 0.04	3.56 ± 0.54	0.08 ± 0.03	3.47 ± 0.33
10	0.24 ± 0.01	5.87 ± 0.27	0.07 ± 0,03	3.34 ± 0.28

[a]Values expressed are mean ± S.D. of three experiments; [b] Trolox equivalent (µg/mL); [c] Percentage inhibition relative to the concentration of Trolox equivalents obtained from the calibration curve.

RESULTS AND DISCUSSION

The microbial transformation samples and the control samples were extracted and analyzed as described above. After checking extracts from the fermentation of compound 1 with *Fusarium verticilloides* by TLC, biotransformations were done on a preparative scale. The resulted was a pure metabolite, (S)-(-)-perillyl aldehyde (PALD, 2) (Figure 1).

1 2

Figure 1: Structure of perillyl alcohol (compound 1) and its biotransformation product (compound 2).

The MS of the metabolite showed a molecular ion m/z 150 corresponding to the molecular formula of $C_{10}H_{14}O$.

The structure of 1 and 2 was analyzed by ^{1}H and

MS m/z (%): 150.00 (20.83); 135.10 (42.75); 132.10 (6.20); 122.10 (46.07); 121.05 (30.70); 119.10 (6.64); 117.00 (18.16); 109.10 (13.20); 108.05 (27.03); 107.05 (63.32); 106.05 (24.52); 105.05 (22.60); 95.05 (23.91); 94.05 (19.31); 93.05 (47.25); 92.05 (14.43); 91.00 (46.54); 81.05 (20.32); 80.05 (21.11); 79.05 (93.04); 78.05 (13.68); 77.00 (50.57); 69.05 (10.09); 68.10 (100.00); 67.10 (96.34); 66.05 (13.45); 65.05 (16.60); 63.05 (5.14); 63.05 (5.14); 55.05 (18.01); 54.05 (6.87); 53.05 (53.60); 52.00 (10.43); 51.00 (20.83); 50.00 (7.38); 43.05 (11.51); 42.05 (6.55); 41.05 (46.70).

CONCLUSION

A few reports are available about the biotransformation of POH (1) by microorganisms, plant cell cultures and mammals.

These study indicated that POH could be transformed to perillyl aldehyde using *F. verticillioides* in an ecofriendly way in a good yield.

On the other hand the free radical scavenging activity tested showed that perillyl aldehyde has almost the same activity as perillyl alcohol, indicating that oxidation of the primary alcohol function of substrate

1 to aldehyde is not a good way to get a compound with antioxidant properties.

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