IJAMBR 4 (2015) 1-6 ISSN 2053-1818

International Journal of Applied Microbiology and Biotechnology Research
www.bluepenjournals.org/ijambr

Conversion of (-)-ambroxide by whole cells of *Fusarium verticillioides* (Hypocreales, Nectriaceae)

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Article History
Received 06 November, 2015
Received in revised form 09 December, 2015
Accepted 15 December, 2015

Keywords:
Biotransformation, 3β-hydroxiambroxide, 1β-hydroxiambroxide, 6β-hydroxiambroxide, Antioxidant activity, Cytotoxicity.

Article Type:
Full Length Research Article

ABSTRACT

In recent years, the growing attention on the field of biotransformations has positioned it as one of the most promising research areas. A main reason for this interest lies on the fact that organic chemists regard it a very useful tool for functionalizing organic compounds, with many potential applications in the manufacturing of useful products in various industrial processes. This methodology is highly relevant because it allows to easily introduce a functional group into a non-reactive carbon, both regio- and stereoselectively, in bio-friendly conditions, while avoiding environmental pollution. In addition, the use of whole cells of pathogenic fungi as an enzyme source is a very interesting approach because of the large number of genera and species that can therewith be formed, as well as by the ample range of enzymatic processes that can be achieved with them. This article reports on the microbial transformation of (-)-ambroxide (1) made with enzymes found in whole cells of *Fusarium verticillioides,* a phytopathogenic fungus. Besides, the bioactivity of the starting compound and those of the obtained products are analyzed and contrasted. Biotransformation of 1 with *F. verticillioides* on potato dextrose broth, gave three metabolites: 3β-hydroxiambroxide (2), 1β-hydroxiambroxide (3) and 6β-hydroxiambroxide (4). In the evaluation of antioxidant activity assay conducted with the DPPH method, compound 4 proved to be more active than the starting compound. On the other hand when cytotoxic activity was tested using *Artemia persimilis* lethality assay, all metabolites showed lower cytotoxicity than that of the starting substrate.

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INTRODUCTION

In the last decade, biotransformations have received increasing interest and currently are one of the most promising research areas on account of its potential application to the production of raw materials and products used in various industrial processes (Velasco et al., 2009).

Biotransformations are characterized by the versatility, efficiency, regioselectivity, chemoselectivity and enantioselectivity of the enzymatic processes involved. Furthermore, the metabolization of substrates undergoing a biotransformation takes place under non aggressive conditions, while reagents and solvents regularly used in these processes show low toxicity and, consequently, are considered environmentally friendly (Correa, 2009). Moreover, with biotransformation it is possible to obtain a single or multiple products, which would otherwise

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require performing many steps when using classical synthesis methods (Castellanos, 2007).

Microorganisms are capable of transforming a wide variety of organic compounds, such as terpenes, hydrocarbons, alkaloids, steroids, antibiotics and amino compounds, into their metabolites. Many compounds having therapeutic properties, along with industrial interest, were obtained through microbial transformation (Choudhary et al., 2004).

Among the great diversity of organisms used in biotransformations, phytopathogenic fungi are an interesting family because of their large number of genera and species, as well as by the wide range of enzymatic processes made possible by these fungi (Correa, 2009).

(-)-Ambroxide (**1**) or Ambrox is a sesquiterpene-like compound, synthesized from the structurally related sclareol (Barrero et al., 1993). It has a strong aroma and was isolated from the gastrointestinal tract of the sperm whale (Choudhary et al., 2004; Barton et al., 1994).

There are several reports on biotransformation of (-)-ambroxide (1) (Hanson and Truneh, 1996; Nasib et al., 2006; Musharraf et al., 2012). In 2004, Choudhary et al. (2004) conducted the biotransformation of (-)-ambroxide (1) with *Fusarium lini* to obtain four metabolites, all of which hydroxylated with α stereochemistry at different ring positions. At our laboratory, our team carried out the biotransformation of (-)-ambroxide (1) by *Alternaria alternata* and *Cunninghamella* sp., and obtained the already known 3β-hydroxiambroxide metabolite, and a new oxidation product 1β-hydroxiambroxide.

Genus *Fusarium* is active in the processing of various chemicals, as shown in the literature (Glenn et al., 2003; Krishna et al., 2003; Gliszczynska et al., 2011; Zhang et al., 2013).

In this work, we report on the capability of enzymes present in *Fusarium verticillioides* for modifying the structure of a natural compounds, (-)-ambroxide (1). This is a promising substrate because it could make functionalized precursors of natural labdane dipertenes showing biological activities, such as antimicrobial, antituberculosis, cytotoxic, antiviral and antiprotozoal (Chinou, 2005). We also report the antioxidant and cytotoxic activity of the obtained metabolites compared to that of the starting compound.

MATERIALS AND METHODS

Microorganisms and media

F. verticillioides obtained by culture collection at the Laboratory of Mycology, Department of Microbiology and Immunology, Universidad Nacional de Río IV, Córdoba, Argentina, was used for screening experiments. Microorganisms were stored in Sabouraud dextrose agar (Britannia Laboratories Inc.; Buenos Aires, Argentina) slants at 10°C. The liquid medium chosen for screening and for preparative-scale experiments was Potato Dextrose broth 2% w/w (Britannia Laboratories Inc.) sterilized at 121°C for 17 min.

Biotransformation

Preliminary screening

Screening experiments were carried out by following a standard two-stage protocol (Bustos et al., 2012), conducted 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 inoculum for a fresh stage II culture. The substrate was added to the incubation media 24 h after inoculating the stage II cultures, as a 1.5% solution in DMSO:EtOH (5:1, v/v) at a final concentration of 0.2 mg/mL of stage II medium. Substrate controls were composed of a sterile medium, to which substrate was added, and was incubated without microorganism. Culture controls consisted of fermentation blanks in which microorganisms were grown under same conditions, but without adding substrate. Fermentation was sampled during 21 days, with 48 h intervals, by taking 0.5 mL of culture medium that was extracted with EtOAc. The organic layers were chromatographed on TLC plates.

Preparative scale biotransformation

In this scale, we followed the general steps of small-scale biotransformation. An aliquot of 4 mL of culture medium of stage I was aseptically transferred to five 1000 mL Erlenmeyer flasks, each one containing 400 mL of sterile culture medium. They were placed in a shaker and incubated during four days (stage II) in the same conditions as those of the preliminary screening. (-)-Ambroxide (1) was added to a 0.2 mg/mL final concentration and the fermentation was continued for 20 days until no trace of the starting material was detected when checked through TLC.

Determination of biological activities

Antioxidant activity

This test was performed with the DPPH method. In order to determine the antioxidant capacity, a 100 ml DPPH

solution with a 20 mg/L concentration was prepared. The solution was prepared at the time of the trial and, given its instability; it was kept protected from light. From each compound; methanolic solutions were prepared, with concentrations of 0.2; 0.15; 0.1; 0.05 mg/mL. The chosen reference standard was ascorbic acid (vitamin C) and the procedure was the same as for the analyzed compounds. A volume of 0.50 mL of each sample was mixed with 1 mL of the DPPH solution. Moreover, as a blank sample, each compound without DPPH dissolved in methanol was used, and a target that consisted of the DPPH solution. All samples were allowed to react for 20 min in the dark, at room temperature.

The antioxidant activity for each compound at various concentrations was performed six times at 510 nm. The absorbance values measured at above concentrations were analyzed in pairs through T-test, with Bonferroni correction to prevent any chance of Type I error. The R Statistical Software version 3.0.2 also known as GNUS, was used.

Cytotoxic activity

This activity was determined through *A. persimilis* lethality assay. Subsequently 2 mg of each starting substrate and metabolites obtained from biotransformations were weighed, and 2 mL of a mixture of solvents (EtOH:DMSO, 5:1) was added. From this mother solution, (1000 ppm) solution volumes at 100, 75, 50, 25 and 10 ppm concentrations were prepared. Three vial flasks were used for each concentration.

Three replicates were performed by adding 10 larvae into each vial tube of different concentration, and then 2 mL being flush with seawater was added as well. Three vials tubes with only 2 ml of seawater and larvae were also used as negative control. Caffeine was chosen as a positive control with same concentrations as above. After 24 h, dead larvae were counted (Colman and Anderson, 1995).

The ED50 (effective dose that kills 50% of larvae, expressed in ppm) of all compounds was performed using the program Finney. A cytotoxicity comparison between the starting compounds and the metabolites obtained by testing Goodness of Fit (X^2), using the program STATISTICA 7, was performed. The cytotoxic activity for each compound concentration, for the various proposals in this study, was determined at three times.

RESULTS AND DISCUSSION

After stopping the biotransformation an extraction was made, showing a 256 mg residue. Purification was done by column chromatography, using dichloromethane: acetone (CH_2Cl_2:Me_2CO) (100:0 to 4:1, v/v) as the mobile phase. Further purification by preparative thin-layer chromatography (PTLC) using dichloromethane: acetone (CH_2Cl_2:Me_2CO) (8:2, v/v) as mobile phase, led to obtaining three metabolites: 1β-hydroxyambroxide (3.7 mg; yield 0.9%), 3β-hydroxyambroxide (2.7 mg; yield 0.6%)(Allendes et al., 2011) and 6β-hydroxyambroxide (3.6 mg; yield 0.86%) (Figure 1). The resulting polarity order was: 6β-hydroxyambroxide (4) > 1β-hydroxyambroxide (3) > 3β-hydroxyambroxide (2).

The analysis of ^{1}H NMR and C^{13} NMR spectrum of metabolite 2 (Table 1) showed that the chemical shift values are clearly consistent with those of the same compound previously obtained in our laboratory with *A. alternata* (Allendes et al., 2011), following the procedures of Hanson and Truneh (1996), using *Cephalosporium aphidicola* fungi, and Farooq and Tahara (2000), using *Botrytis cinerea* fungi.

The data obtained from the ^{1}H NMR and C^{13} NMR spectrum of 3 (Table 1) are consistent with the proposed structure of the 1β-hydroxyambroxide compound (3). This compound was previously obtained by our research team using *Cunninghamella* sp. fungi (Allendes et al., 2011).

Compound 4 was analyzed through ^{1}H NMR and C^{13} NMR (Table 1) and the chemical shift values were correspondent with those of the compound obtained by Musharraf et al. (2012) using *Macrophomina phaseolina* fungi.

In the lethality assay with *A. persimilis*, the effective dose expressed in ppm (ED50 dose that kills 50% of the larvae), calculated with Finney software, gave the following results for both the starting substrate and the biotranformation metabolites: 1: ED50= 4.8729 ppm; 2: ED50 = 99.7222 ppm; 3: ED50= 73.9240 ppm; 4: ED50= 122.6044 ppm.

The results from this cytotoxic activity bioassay showed that the citotoxicity of metabolites is lower than that of the substrate, which demonstrates that the compounds resulting from the biotransformation of substrate (-)-ambroxide (1) are detoxification products of fungi metabolism.

All three metabolites resulting from the transformation of 1 showed sharp differences when contrasted with the starting substrate (1 and 2 X^2: 298.1602, df = 2, $p < 0.0000001$, 1 and 3 X^2: 190.3078, df = 2, $p < 0.0000001$, 1 and 4 X^2: 355.1804, df = 2, $p < 0.0000001$).

As previously mentioned, cytochrome P450 catalyse diverse reactions and are key enzymes in fungal primary and secondary metabolism, and xenobiotic detoxification (Lah et al., 2011). In the bioassay of the antioxidant capacity measuring absorbance (510 nm) by the DPPH method, the DPPH inhibition percentage of each compound at different concentration levels (Table 2); it

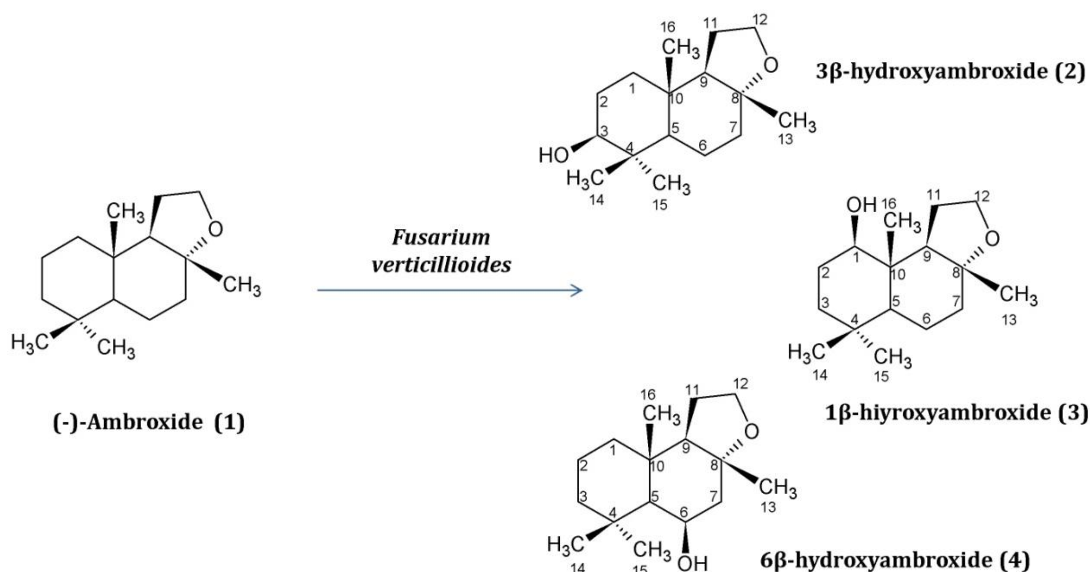

Figure 1. Biotransformation reaction of (-)-ambroxido (**1**) with *Fusarium verticillioides*.

Table 1. Nuclear magnetic resonance spectra of (-)-ambroxide (1), 3β-hydroxyambroxide (2), 1β-hydroxyambroxide (3) and 6β-hydroxyambroxide (4).

Carbon	δH ppm (J)				δC ppm			
	1	2	3	4	1	2	3	4
1	1.05 1.5 brs	1.53 t 1.56 t	3.36 dd (4.9, 10.8)		39.9	38.1	79.9	39.9
2	1.32 d 1.29 d	1.15	2.02 m	1.72 m	18.4	27.1	25.7	20.4
3	1.22 d	3.23 dd	1.45 m		42.4	79.0	40.5	39.4
4	0.83 s				33.1	38.7	32.7	40.1
5	0.98 dd	0.94	0.93 m	1.76 d (2.0)	57.2	55.9	55.9	52.8
6	0.65 t	1.75 1.40	1.15 m	3.41 t (2.2)	20.6	20.4	20.6	73.1
7	1.94 t 1.97 t	1.96 dd	1.43 m 2.00 m	1.84 m 1.87 m	39.7	39.5	39.5	25.6
8	1.09 s				79.9	79.8	80.2	80.5
9	1.41 m	1.37 t	1.33 dd (4.6, 13.4)	1.38 m	60.1	60.1	58.9	48.7
10	0.84 s				36.2	35.9	32.7	39.5
11	1.76 m	1.75	2.02 m	1.70 m	22.6	22.7	28.0	22.2
12	3.93 m 3.84 q	3.86 q 3.96 m	3.86 q (1.0, 9.0)	3.79 m 3.85 m	65.0	64.9	64.9	64.9
13	0.85 s	1.09 s	0.83 s	1.03 s	21.1	21.0	21.1	21.3
14	0.87 s	0.86 s	0.89 s	0.78 s	33.6	28.2	33.1	32.9
15	1.10 s	0.80 s	1.11 s	1.02 s	21.2	15.1	20.9	20.9
16	0.89 s	0.85 s	0.86 s	0.77 s	15.0	15.2	10.9	14.8

References: d, Doublet; dd, double doublet; dt, double triplet; td, triple doublet; t, triplet; m, multiplet; q, quartet; s, singlet. The NMR spectrum of this metabolite was performed in deuterated chloroform (Cl3CD).

Table 2. % of Inhibition of DPPH results for all compounds (-)-ambroxide (1), 3β-hydroxyambroxide (2), 1β-hydroxyambroxide (3) and 6β-hydroxyambroxide (4).

Concentration (mg/ml)	Percentage of Inhibition of DPPH				
	Ascorbic acid	1	2	3	4
0.005	96	36	40	43	39
0.01	97	36.1	34	43	40
0.015	98	38	31	51	41
0.02	99	40	25	55	42

was calculated using the following formula:

Percentage Inhibition DPPH = 1- (A_{sample} - A_{blank}) × 100

The statistical analysis made for (-)-ambroxide (1) and metabolites have shown significant differences: for 1β-hydroxyambroxide (3) at 200 ppm (T-test: t = 17.1616, df = 4, p = 6.763e-05), 150 ppm (T-test: t = 21.0785, df = 7, p = 1.361e-07), 100 ppm (T-test: t = 31.2852, df = 12, p = 7.159e-13) and 50 ppm (T-test: t = 16.8449, df = 11, p = 3.342e-09) and for 6β-hydroxyambroxide (4) to 200 ppm (T-t test: t = -46.5449, df = 4, p = 1.274e-06), 150 ppm (T-test: t = -22.2196, df = 9, p = 3.582e-09), 100 ppm (T-test: t = -14.8634, df = 11, p = 1.255e-08) and 50 ppm (T-test: t = -5.6146, df = 11; p = 0.0001569). There are no significant differences between values for the substrate and the 3β-hydroxyambroxide metabolite (2) at 200 ppm (T-test: t = 0.9954, df = 5, p = 0.3652), 150 ppm (T-test: t = 0.2714; df = 9, p = 0.7922). There are small differences at 100 ppm (T-test showed t = 2.2713, df = 11, p = 0.04421) and 50 ppm (T-test: t = 4.6904, df = 11; p = 0.0006604).

It was also noted that differences were significantly lower ($p<0.05$) for all compounds with respect to ascorbic acid. The 1β-hydroxyambroxide (3) compound was shown to have higher antioxidant capacity than the starting substrate; while for 3β-hydroxyambroxide (2) difference was observed at lower concentrations of 100 and 50 ppm. Equally to compound 6β-hydroxyambroxide (4), the activity was lower than that of the starting compound.

Biotransformation using whole cells of microorganisms, such as phytopathogenic fungi, is a simple, economical and clean methodology for modifying structurally organic compounds. It could be verified that all hydroxylations were obtained on a same face, which lets us assume that these compounds have been anchored by the enzyme, leaving the α face hidden and, consequently, the β face exposed, on which the nonreactive carbon atoms C-1, C-3 and C-6 of (-)-ambroxide were oxidized in a regioselectively way.

These experimental results let assume that the enzymes responsible for the oxidation of unreactive carbon atoms of the chosen substrate might be cytochrome P450. For example, in recent years, the involvement of cytochrome P450 enzymes in many fungal complexes bioconversion processes has been described (van den Brink and van Gorcom, 1998). These enzymes functionalized unreactive carbons C-1, C-3 and C-6 of (-)-ambroxide, which suggests that the mono-oxygenase of this fungus is not specific; that is, it has multiple active sites where it can biotransform the substrate 1.

Cytochrome P450 enzymes are described as flexible, moving upon binding of substrate so as to favor catalytic reaction (Werk-Reichhart and Fayereisen, 2000). Although there are other enzymes capable of performing oxidative transformations similar to that of P450, such as flavin-dependent monooxygenases and non-heme iron oxygenases, it is worth noting that, what makes P450s special is its versatility shown by the range of substrates and type of reaction they catalyze and its exquisite capability to perform regio and stereoselective oxidation reactions in numerous molecules that are physiologically and biotechnologically important (Munro et al., 2013). Many naturally occurring antioxidants are found in foods, among which are, catechins in green tea, red wine resveratrol, curcumin curry seasoning, ascorbic acid (vitamin C) in citrus, as well as synthetic butylhydroxytoluene and butylhydroxyanisole. A shared feature found in them all is that they have, among other, one or more hydroxyl groups linked to aliphatic or aromatic carbons. Consequently, it is reasonable to think that the metabolites obtained in this work, which were oxidized nonreactive carbons, could present some free-radical scavenging capability, which made us subject them to the already described test.

The results show that the antioxidant activity of the obtained compound 1β-hydroxyambroxide (3) was higher than that of the starting substrate, and this may be explained by the position and stereochemistry of the hydroxyl group in this compound.

As regards the cytotoxicity bioassay with *A. persimilis*, all hydroxylated metabolites showed lower cytotoxicity than that of the starting substrate. This shows that the formation of hydroxy compounds have proved to be detoxification products, because these compound types are generally more water-soluble and, therefore, more susceptible to be eliminated by organisms (Pothuluri et al., 2000).

ACKNOWLEDGEMENTS

The authors express their appreciation to Universidad Nacional de San Juan, CONICET, Agencia Córdoba Ciencia, and SECyT-UNC for the financial support.

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