Biocatalysis and Biotransformation, March-June 2011, 29(2–3): 83–86

informa
healthcare

ORIGINAL ARTICLE

Microbial functionalization of (–)-ambroxide by filamentous fungi

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Abstract

Biocatalysis is a very useful tool for organic chemists to functionalize organic compounds under working conditions milder than chemical ones. This methodology has special significance since it can be an easy way to introduce a functional group in a non-reactive carbon, regio- and stereoselectively. In order to look for new compounds with antioxidant activity we report the transformation of the natural substrate (–)-ambroxide using the enzyme potential of pure strains of the filamentous fungi *Alternaria alternata* and *Cunninghamella* sp., following a protocol with growing cell cultures, which produced the new compound 1β-hydroxyambroxide and the previously known compound 3β-hydroxyambroxide. After purification their structures were elucidated by spectroscopic methods. These two metabolites are the products of oxidation of ring A of the starting material, without evidence of other compounds with different functionalization. Both compounds were tested for their activity as free radical scavengers *in vitro*, using the assay of DPPH (1,1-diphenyl-2-picrylhydrazyl) radical trapping. The results demonstrated that hydroxylation of carbons C-1 and C-3 of (–)-ambroxide with β stereochemistry had no effect on biological activity as an antioxidant compared with the starting material and a reference substance.

Keywords: *Biotransformation, Cunninghamella sp., Alternaria alternata, 1β-hydroxyambroxide, 3β-hydroxyambroxide, radical scavenger activity*

Introduction

Transformation of organic natural and non-natural substrates using enzymes present in microorganisms such as bacteria and fungi is a very useful methodology for organic chemists who seek new, functionalized, enantiomerically pure compounds with potential biological activity. This biocatalysis leads to hydrolysis, reduction, oxidation, carbon–carbon bond formation, addition and elimination, glycosidic transfer, halogenation and dehalogenation reactions (Faber 1997).

The fungi used in the present study belong to the group called phytopathogens. When a fungus infects a plant, it activates the plant's defense system to generate a wide variety of secondary metabolites known as phytoalexins, which invade the fungal cells and disrupt their metabolism. Consequently, the phytopathenogenic fungi have developed enzymatic responses to the phytoalexins. The enzymes involved are similar to the p450 monooxygenases found in humans and other mammals that act by converting the phytoalexins to more water-soluble compounds, which offsets their mode of action (Rojas et al. 2001).

(–)-Ambroxide (**1**) is a natural terpenoid and is one of the components of ambergris. It has been widely used in perfumery (Teixeira et al. 2009) but has not been reported to have any biological activity. There are a few reports on the transformation of this substrate using microorganisms, particularly fungal strains (Hanson & Truneh 1996; Farooq & Tahara 2000) and plant cells (Nasib et al. 2006). However, the literature shows that *Alternaria alternata* is active in the transformation of terpenoids

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(Received 3 November 2010; revised 3 March 2011; accepted 1 April 2011)

ISSN 1024-2422 print/ISSN 1029-2446 online © 2011 Informa UK, Ltd.
DOI: 10.3109/10242422.2011.578211

(Olejniczak et al. 2001; Krishna Kumari et al. 2003) and other substrates (Ye & Guo 2005; Kurbanoglu et al. 2007), as is *Cunninghamella echinulata* (Chou et al. 2008; Xin et al. 2009).

Continuing our work on the functionalization of terpenoids (Tapia et al. 1997; Gouiric et al. 2004) we report herein the microbial oxidation of (–)-ambroxide by two filamentous fungi. When using *Cunninghamella* sp. a new compound (**2**) (Figure 1) was obtained as determined by spectroscopic methods. Moreover although compound **3** is already known, this is the first report in which it was obtained using the enzymes of the fungus *A. alternata*.

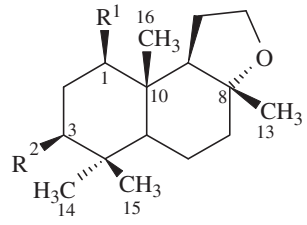
Materials and methods

Microorganisms and media

Cunninghamella sp. and *A. alternata* from the culture collection of the Laboratory of Mycology, Department of Microbiology and Immunology, National University of Río IV, Córdoba, Argentina, were used for screening experiments. Both microorganisms were stored on Sabouraud dextrose agar (Britannia Laboratories Inc.; Buenos Aires, Argentina) slants at 10°C. Liquid medium for screening and preparative-scale experiments was Sabouraud broth 2% w/w (Britannia Laboratories Inc.) sterilized at 121°C for 17 min.

Equipment

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE II AV-400 spectrometer (UNC) in $CDCl_3$ with TMS as internal standard. Multiplicity (DEPT) and ^{2}D spectra (SOCY, HSQC, HMBC and sensitivity ROESY) were obtained using standard Bruker software. The high-resolution mass spectrum (ESI-MS) was obtained on a Bruker micrOTOF-Q II spectrometer in electrospray ionization positive mode (+ESI). Optical rotation was recorded on a Jasco P1020 spectropolarimeter (UNC) in MeOH solution using a cell of 10 cm path length at the concentration (*c*, g/mL) indicated. TLC was performed on Kieselgel 60 GF_{254} pre-coated plates using petroleum ether (PE)–EtOAc (7:3, v/v). 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. Radial chromatography (preparative thin-layer chromatography, PTLC) was run on a Chromatotron model 7924 T instrument (Herrison Research, UNC) on silica gel GF_{254} (1 mm thick; Merck; Buenos Aires, Argentina). All organic solvents were distilled prior to use.



1 R^1 = H; R^2 = H
2 R^1 = OH; R^2 = H
3 R^1 = H; R^2 = OH

Figure 1. Structure of (–)-ambroxide (**1**) and its biotransformation products **2** and **3**.

Biotransformation

Screening experiments were carried out following a standard two-stage protocol (Abourashed et al. 1999) 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 the inocula of 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 sterile medium to which substrate was added and incubated without microorganisms. 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 fermentations were performed in five 1-L Erlenmeyer flasks containing 400 mL of sterile culture medium and then incubated at 25–30°C on shaker at 80 rpm. (–)-Ambroxide (**1**) 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.

Extraction and purification

After the indicated time, liquors were combined, mycelium filtered off and washed with H_2O (200 mL). The washings were combined with the filtrate and then extracted with EtOAc (4 × 200 mL). Organic layers were combined, washed with brine 20% (w/v) (2 × 100 mL), dried (anhydrous Na_2SO_4) and the solvent evaporated under vacuum, giving a brownish oily residue (142.7 mg) when *Cunninghamella* sp. was the microorganism used. After purification by PTLC using hexane (Hex)–EtOAc (8:2, v/v) as mobile phase, compound **2** (18.4 mg), 1β-hydroxyambroxide, was obtained as an oil. $[a]_D$: –0.014° (Cl_3CH, c = 0.613). EI-MS, *m/z* (rel. int.): 252 (72%). High-resolution positive ESI-MS, *m/z*: 253.21670 ($[M + H]^+$). Calcd for $C_{16}H_{29}O_2$: 253.21621. IR (KBr) ν (cm^{-1}): 3424 (–OH), 2918, 2863, 1446, 1376, 1092 (C–O), 1000.

^{1}H NMR (400.3 MHz, $CDCl_3$) and ^{13}C NMR (100.03 MHz, $CDCl_3$) data are listed in Table I.

Applying the same methodology for the biotransformation and extraction but using a pure strain of *A. alternata* yielded a pale yellow oily residue (540 mg), which was purified by PTLC using a mixture of PE–EtOAc (7:3, v/v) as elution solvent. Compound **3** (3.3 mg), 3β-hydroxyambroxide, was isolated as a pure oil. The structure of **3** was analyzed by ^{1}H and ^{13}C NMR spectroscopy and confirmed when compared with bibliographical data (Hanson and Truneh 1996; Farooq and Tahara 2000).

Antioxidant activity

The antioxidant activity of compounds **2** and **3** were tested using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical method (Potterat 1997; Molyneux 2004; Önay-Uçar et al. 2006). A solution of DPPH (5×10^{-5} M) in MeOH was prepared and protected from light prior to use. Sample solutions of compounds **1**, **2** and **3** (0.5%, w/v) and ascorbic acid (0.1%, w/v) as a reference in the same solvent were made. Aliquots of each sample and reference solutions were mixed with DPPH solution in a 1:2 (v/v) ratio and incubated at room temperature for 15 min in the dark.

A control containing MeOH and DPPH solution in the same ratio was used as a blank. Also a mixture of sample solution in MeOH was prepared to avoid inaccurate absorptions. The absorbance of all these solutions was then measured at 510 nm and the radical-scavenging activity expressed as percentage inhibition of DPPH using the formula:

$$\%\ \text{inhibition DPPH} = 1 - \frac{A_{\text{sample}}}{A_{\text{blank}}} \times 100$$

Results are shown in Table II.

Table I. ^{1}H (400.3 MHz) and ^{13}C (100.03 MHz) NMR spectral data of compound **2** ($CDCl_3$, TMS as internal standard; δ in ppm, $\mathcal{J}$ in Hz).

Carbon	δ_C	δ_H ($\mathcal{J}$)	HSQC–DEPT	HMBC
1	79.9	3.36, dd (10.8, 4.9)	CH	
2	28.1	1.76, m	CH_2	
3	40.5	1.45, m	CH_2	
4	32.7		C	
5	55.9	0.93, m	CH	
6	20.6	1.15, m	CH_2	
7	39.5	1.43, m 2.00 m	CH_2	
8	80.2		C	
9	58.9	1.33, dd (13.4, 4.6)	CH	
10	41.8		C	
11	25.7	2.02, m	CH_2	
12	64.9	3.86, q (9.0, 1.0) 3.93, td (9.1, 3.3)	CH_2	C-8, C-11
13	21.1	0.83, s	CH_3	C-3, C-4, C-5
14	20.9	0.89, s	CH_3	C-3, C-4, C-5, C-14
15	33.1	1.11, s	CH_3	C-7, C-8, C-9
16	10.9	0.86, s	CH_3	C-1, C-5, C-9, C-10

Results and discussion

After checking extracts from the fermentation of compound **1** with *Cunninghamella* sp. and *A. alternata* by TLC, biotransformations were done on a preparative scale. In each case only one metabolite more polar than the starting material was obtained. After incubation for 5 days at 25–30°C *Cunninghamella* sp. had converted **1** into metabolite **2** which was recovered from the culture medium by solvent extraction and then isolated and purified by PTLC. The high-resolution ESI-MS spectrum of this new compound as an oil showed the quasi-molecular ion $[M + H]^+$ at $m/z = 253.21670$ in accordance with the formula $C_{16}H_{28}O_2$. The IR spectrum exhibited a broad absorption band at 3424 cm^{-1} attributable to the presence of a hydroxyl group in the molecule. The ^{1}H NMR spectrum of **2** showed a double doublet at $\delta = 3.36$ ppm (1H, $\mathcal{J} = 10.8$, 4.9 Hz) attributable to H-1, while the value of the coupling constants indicated that the hydroxyl group was in an equatorial β orientation (Tapia et al. 1997). The attachment of the hydroxyl group was corroborated by HMBC correlation of H_3-16 ($\delta = 0.86$ ppm, s) with C-1 ($\delta = 79.9$ ppm), C-5 ($\delta = 55.9$ ppm), C-9 ($\delta = 58.9$ ppm) and C-10 ($\delta = 41.8$ ppm). The data obtained from mono- and bidimensional experiments (Table I and Figure 2) are in full agreement with the proposed structure for compound **2** as 1β-hydroxyambroxide. On the other hand, when *A. alternata* was incubated for 9 days at the same

Table II. Antioxidant activity of compounds **1**, **2** and **3** by DPPH method[a].

Compound	*A* at 510 nm	Scavenging activity (% inhibition DPPH)	EC_{50} (mg/mL)	AE = $1/EC_{50}$
Control	0.537	–	–	–
1	0.520	3.1	8.12 ± 0.08	0.123
2	0.495	7.8	3.45 ± 0.36	0.289
3	0.525	2.2	11.56 ± 0.30	0.086
Ascorbic acid	0.051	92.1	0.058 ± 0.04	17.241

[a]Values expressed are mean ± standard deviation of three parallel measurements. EC50: concentration of substrate that causes 50% loss of the DPPH activity AE=1/EC50

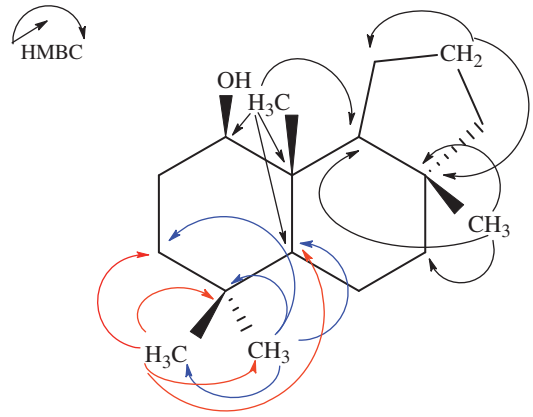


Figure 2. HMBC correlations for compound **2**.

temperature and purification conditions as described above, this led to the pure metabolite **3** as an oil; its structure was determined by comparing its ^{1}H and ^{13}C NMR spectra with literature data (Hanson and Truneh 1996; Farooq and Tahara 2000) and identified as 3β-hydroxyambroxide.

Analysis showed that both metabolites **2** and **3** thus obtained were almost inactive as radical scavengers (Table II).

Conclusions

It has been found that the enzymes, presumably monooxygenases, produced by two phytopathogenic fungi were able to functionalize the non-reactive carbons C-1 and C-3 of the A ring of (–)-ambroxide (**1**). No other products were detected, which means that only a portion of the substrate structure can fit into the enzymes' active site and be hydroxylated, generating the hydroxyl groups with the stereochemistry shown. However, alcohols **2** and **3** did not show any significant radical-scavenging activity, demonstrating that the presence of a hydroxyl group bonded to C-1 or C-3 in the β orientation does not induce such a property compared with the starting material **1**, which has very weak radical-scavenging activity itself.

Declaration of interest: The authors wish to thank Universidad Nacional de San Juan, CONICET, Agencia Córdoba Ciencia, Agencia Nacional de Promoción Científica y Técnica and SECyT – UNC for financial support. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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