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Fungal hydroxylation of (–)-α-santonin

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Abstract: Functionalization of organic compounds using enzymes present in microorganisms is a very useful tool for organic chemists, since it is a method carried out under milder conditions than chemical ones and allows the introduction of functional groups in a nonreactive carbon in a regioselective and stereoselective way. In order to look for new compounds derived from natural products with antioxidant and cytotoxic activity, the sesquiterpene lactone (–)-α-santonin was transformed using a pure strain of *Cunninghamella* spp. A two-stage standard protocol with growing cells was followed, which led to 8β-hydroxy-α-santonin. The structure of the product was unequivocally elucidated by spectroscopic methods. Both the starting material and metabolite were tested in vitro for antioxidant activity using the 1,1-diphenyl-2-picrylhydrazyl method, and cytotoxic activity was tested using the *Artemia salina* (brine shrimp) method. In both assays, the new compound was less active than substrate and reference compounds, which means that the introduction of a hydroxyl group on carbon-8 of (–)-α-santonin with β stereochemistry did not improve the tested biological activities.

Keywords: biotransformation, *Cunninghamella* spp., 8β-hydroxy-α-santonin, antioxidant activity, cytotoxic activity

Introduction

To functionalize organic compounds, not only do chemical resources exist, but there are also tools that biology gives us through the use of microorganisms that are capable of transforming an organic compound into a recoverable product by chemical reactions catalyzed by enzymes contained in cells, ie, biotransformation. Biotransformation technology has proven to be an additional useful tool in organic synthesis.[1,2] In fact, there are numerous examples of using one or several biological steps in the synthesis of organic compounds with biological activity.[3–6] The outstanding property for which the biocatalysts are favorably distinguished from ordinary chemical ones is their high specificity for the reaction they catalyze as well as for the structure and stereochemistry of the substrate they accept and the product they form. Besides its natural substrates, many enzymes accept foreign substrates, but with similar structural characteristics to the natural substrate, and thus catalyze "unnatural" reactions with compounds supplied to the medium. The reactions catalyzed by enzymes can be, in general, of different types: oxidation-reduction, transfer of groups, hydrolysis reactions, addition to double bonds, isomerization reactions, and bond formation reactions.[7] Functionalization by oxidation of nonreactive carbons, which is often a key step in organic synthesis, via the traditional method is plagued by numerous drawbacks, such as metal-based toxic oxidants, unwanted side reactions, and many difficulties in performing them

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in a regioselective and stereoselective way. Many of these disadvantages can be avoided by using biological methods such as those mentioned above, particularly in cases in which stereoselectivity is required. The enzymes that catalyze the direct incorporation of molecular oxygen in an organic molecule are called oxygenases.[8,9] Particularly, the hydroxylation of hydrocarbons is one of the most useful biotransformations; in general, the relative reactivity of carbon atoms in the microbial hydroxylation follows the order secondary > tertiary > primary.[10,11] Virtually any nonreactive carbon may be hydroxylated by a microorganism.[12–14] This functionalization can be achieved, for example, by using phytopathogenic fungi with enzymatic machinery that involves enzymes similar to P450 monooxygenases, also found in humans and other mammals.[15,16]

(–)-α-santonin (compound 1) is a sesquiterpene lactone isolated from species of the genus *Artemisia*[17–19] with antihelmintic activity.[20] This compound has been transformed by fungi, bacteria, and plant cells.[21–26] Here, the transformation of (–)-α-santonin (compound 1) – using a pure strain of *Cunninghamella* spp. – and the structure of the compound obtained, 8β-hydroxy-α-santonin (compound 2; Figure 1), and its antioxidant and cytotoxic activities are reported. The stereochemistry of compound 2 was established by spectroscopic methods. This is the first report of 8β-hydroxy-α-santonin (compound 2) obtained by microbial hydroxylation of the substrate (compound 1) by using this fungus, as in previous works the same biotransformation was achieved with a pure strain of *Aspergillus niger*.[27,28]

Materials and methods

Microorganism and media

Cunninghamella spp. from the culture collection of the Laboratory of Mycology, Department of Microbiology and Immunology, National University of Rio Cuarto, (Cordoba, Argentina) was used for screening experiments.

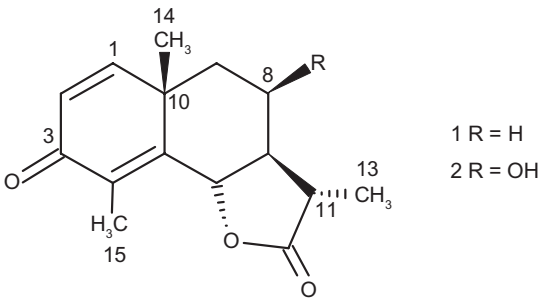

Figure 1 Structure of (–)-α-santonin (compound 1) and its biotransformation product (compound 2).

The microorganism was stored on Sabouraud dextrose agar (Britania Laboratories, Buenos Aires, Argentina) slants at 10°C. Liquid medium for screening and preparative-scale experiments was Sabouraud broth 2% weight/weight (Britania Laboratories) sterilized at 121°C for 17 minutes.

Equipment

Hydrogen-1 and carbon-13 nuclear magnetic resonance (NMR) spectra were recorded on an Avance™ II AV400 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) in deuterated chloroform with tetramethylsilane as the internal standard. Multiplicity (distortionless enhancement by polarization transfer) and two-dimensional spectra (hydrogen-1–hydrogen-1 correlation spectroscopy, heteronuclear single quantum correlation, heteronuclear multiple-bond correlation, and sensitivity rotating-frame nuclear Overhauser effect spectroscopy) were obtained using standard Bruker software. The high-resolution mass spectrum (electrospray ionization mass spectroscopy) was obtained on a micrOTOF-Q II™ spectrometer (Bruker) by electrospray ionization positive mode. Optical rotation was recorded on a Jasco P1020 spectropolarimeter (Jasco Inc, Easton, MD) in chloroform solution using a cell of 1-dm path length with the concentration (g/mL) indicated in parentheses. Thin layer chromatography was performed on Kieselgel 60 GF_{254} precoated plates (Merck and Co, Inc, Whitehouse Station, NJ) using dichloromethane:acetone (9.5:0.5 volume/volume [v/v]) as elution solvent mixture. Detection was achieved by spraying with p-anisaldehyde:ethanol:acetic acid:sulfuric acid (0.1:17:2:1 v/v/v/v) followed by heating at 150°C. Column chromatographies were run using Kieselgel 60 H (Merck) as the stationary phase. All organic solvents were distilled prior to use.

Biotransformation

Screening experiments were done following a standard two-stage protocol under the same conditions as previously described.[14] The preparative scale fermentation was performed by growing *Cunninghamella* spp. on Sabouraud liquid medium for 72 hours at 25°C on rotary shaker (Promax 2020, Weidolph, Germany) at 100 rpm. This culture was used to inoculate four 1-L Erlenmeyer flasks containing 500 mL of sterilized liquid medium. Incubation was continued for a further 24 hours. After that, (–)-α-santonin (400 mg) as a 1.5% solution in dimethyl sulfoxide:ethanol (5:1 v/v) was added to a final concentration of 0.2 mg/mL broth, and the fermentation was continued for 18 days.

Extraction and purification

After the time indicated above, the Erlenmeyer flasks were sonicated for 10 minutes, and the mycelium was filtered off and washed with distilled water (50 mL) and ethyl acetate (50 mL). Both phases were joined and the aqueous phase was extracted with ethyl acetate (4 × 100 mL). The organic layers were combined, washed with brine 20% (100 mL), dried with anhydrous sodium sulfate, and evaporated under vacuum to give an oily residue (487.4 mg). Purification by column chromatography using petroleum ether:acetone (100:0 to 93:7 v/v) as the mobile phase gave 44.6 mg of an impure compound, which was further purified with dichloromethane:acetone (100:0 to 89:11 v/v) as the mobile phase to give 8β-hydroxy-α-santonin (compund 2; Figure 1) (25.2 mg) as a colorless oil. The spectral data was $[\alpha]_D$: −0.1006 ± 0.0003 (chloroform, c = 0.005); high-resolution positive electrospray ionization mass spectroscopy, m/z: 285.10997 ($[M + Na]^+$); and calcd for $C_{15}H_{18}NaO_4$: 285.10973. Hydrogen-1 NMR (400.3 MHz, deuterated chloroform) and carbon-13 NMR (100.03 MHz, deuterated chloroform) data are listed in Table 1. The starting material was also recovered (118.1 mg), eluted with dichloromethane:acetone (95:5).

Antioxidant activity

The antioxidant activity of the substrate (compound 1) and 8-hydroxy derivative (compound 2) were tested using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical method; the results are shown in Table 2.[14,29] A solution of DPPH (5×10^{-5} M) in methanol was prepared and protected from light until use. Sample solutions of compounds 1 and 2, with ascorbic acid as reference (0.02%, 0.015%, 0.01%, and 0.005%), 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 for 20 minutes in the dark at room temperature. A control containing methanol and DPPH solution in the same ratio was used as a blank. A mixture of sample solutions in methanol were also prepared to avoid inaccurate absorptions. The absorbance (A) of all solutions was then measured at 510 nm and the radical-scavenging activity expressed as percentage inhibition of DPPH using the formula:

$$\text{Inhibition of DPPH (\%)} = 1 - A_{sample}/A_{blank} \times 100$$

Cytotoxic activity

The cytotoxic activity of compounds 1 and 2 was tested using the *Artemia salina* method.[30] Brine shrimp eggs of *A. salina* were hatched in artificial sea water prepared from commercial sea salt (40 g/L). A plastic chamber with two compartments (one dark, one light) separated by a partition plate with small holes for the hatching was used. The eggs were sprinkled in the dark compartment, while the other was illuminated. The eggs were incubated at 25°C–27°C. After 48 hours, the nauplii (larvae) were collected by pipette from the light side. α-(–)-santonin (compound 1) and 8β-hydroxy-α-santonin (compound 2) dissolved in dimethyl sulfoxide (0.2% weight/volume) were tested at 100, 75, 50, 25, and 10 ppm in 2 mL sea water. Three replications were done for

Table 1 Hydrogen-1 (400.3 MHz) and carbon-13 (100.03 MHz) nuclear magnetic resonance spectral data of compound 2

Carbon	δ_C (ppm)	δ_H (J)	HSQC-DEPT	HMBC
1	154.7	6.69, d (10.0)	CH	C-14, C-10, C-5, C-9, C-3
2	125.1	6.29, d (10.0)	CH	
3	185.9		C	
4	128.7		C	
5	152.1		C	
6	76.10	5.35, d (12.0)	CH	C-11, C-13, C-4, C-5
7	57.3	1.98, td (12.0, 2.6)	CH	C-11, C-4
8	64.3	4.36, brs	CH	C-10
9	44.6	1.67, dd (3.0, 14.5)	CH_2	C-14, C-10, C-1
		2.09, dd (3.0, 14.5)		C-14, C-10, C-7, C-1, C-8
10	41.5		C	
11	36.0	2.84, dq (12.0, 7.7)	CH	C-7, C-8, C-12
12	179.4		C	
13	12.3	1.28, d (7.0)	CH_3	
14	28.3	1.53, s	CH_3	
15	10.9	2.16, s	CH_3	

Note: J values in Hz; spectra in deuterated chloroform with tetramethylsilane as the internal standard.

Abbreviations: δ_C, chemical shift; δ_H, change in enthalpy; HMBC, heteronuclear multiple-bond correlation; HSQC-DEPT, heteronuclear single quantum correlation-distortionless enhancement by polarization transfer.

Table 2 Antioxidant activity of compounds 1 and 2 by the 1,1-diphenyl-2-picrylhydrazyl method[a]

Compound	Concentration (%)	A at 510 nm	Scavenging activity (% inhibition DPPH)	EC_{50}[b] (mg/mL)	AE = $1/EC_{50}$
1	0.02	0.495	40		
	0.015	0.505	39	0.225	4.44
	0.01	0.520	37		
	0.005	0.540	33		
2	0.02	0.342	36		
	0.015	0.345	35	0.30	3.33
	0.01	0.369	32		
	0.005	0.393	29		
Ascorbic acid	0.02	0.012	99		
	0.015	0.017	98	0.02	50
	0.01	0.025	97		
	0.005	0.035	96		
Control	–	0.537	–	–	–

Notes: [a]Values expressed are mean ± standard deviation of three parallel measurements; [b]concentration of substrate that causes 50% loss of 1,1-diphenyl-2-picrylhydrazyl activity.
Abbreviations: A, absorbance; AE, antiradical efficiency; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EC_{50}, half maximal effective concentration.

each concentration. Ten nauplii were used for each vial maintained at 25°C under light, and the survivors were counted after 24 hours. Caffeine was used as a positive control at the same concentrations as described above. A solvent control was also done. The average effective dose was calculated using the Finney program. Results shown in Table 3 are expressed as mortality percentage compared to caffeine, and are given by the formula:

$$\text{Mortality (\%)} = ([\%\ \text{mortality of test} - \%\ \text{mortality of control}]/[100 - \%\ \text{mortality of control}]) \times 100$$

Results and discussion

After checking the small-scale conversion of the substrate (compound 1) using a pure strain of the fungus *Cunninghamella* spp. with thin layer chromatography, the biotransformation was carried out on a preparative scale in Sabouraud liquid medium. After a period of 18 days at 25°C, a single metabolite was isolated from the medium by liquid–liquid extraction. The purification of the impure residue by column chromatography yielded compound 2 as a transparent oily liquid more polar than the substrate (compound 1). High-resolution mass spectrum electrospray ionization mass spectroscopy showed an ion ($[M + Na]^+$) at m/z 285.10997 according to the formula $C_{15}H_{18}NaO_4$, suggesting that a hydroxyl group had been introduced. Hydrogen-1 NMR spectrum of compound 2 exhibited a signal at $\delta = 4.36$ ppm as a broad singlet attributable to hydrogen-8, a double quartet at $\delta = 2.84$ ppm ($J_{11-13} = 7.7$ Hz, $J_{11-7} = 12$ Hz) assigned to hydrogen-11, a signal as a triple doublet at δ 1.98 ppm ($J_{7-11} = 12$ Hz, $J_{7-8a} = 2.6$ Hz) assigned to hydrogen-7, then a doublet at δ 5.35 ppm ($J_{6-7} = 12$ Hz) assigned to hydrogen-6, and a signal at δ 1.53 ppm as a singlet assigned to hydrogen-14. The carbon-13 NMR spectrum showed a methyne resonance at $\delta = 64.3$ ppm and a methylene resonance at $\delta = 44.6$ ppm for carbon-8 and carbon-9, respectively. Two quaternary olefinic carbons gave signals, resonating at $\delta = 152.1$ ppm for carbon-5 and $\delta = 128.7$ ppm for carbon-4. Heteronuclear multiple-bond correlation spectrum corroborated the attachment of a hydroxyl group to carbon-8 since it showed important correlations between hydrogen-8 ($\delta = 4.36$ ppm) and carbon-10 ($\delta = 41.5$ ppm); hydrogen-11 ($\delta = 2.84$ ppm) and carbon-7 ($\delta = 57.3$ ppm), carbon-8 ($\delta = 64.3$ ppm), and carbon-12 ($\delta = 179.4$ ppm), and hydrogen-6 ($\delta = 5.35$ ppm) and carbon-11 ($\delta = 36.0$ ppm), carbon-13 ($\delta = 12.3$ ppm), carbon-4 ($\delta = 128.7$ ppm), and carbon-5 ($\delta = 152.1$ ppm) (Table 1 and Figure 2). The correlation spectroscopy spectrum displayed diagnostic interactions between hydrogen-$9_{a,b}$ ($\delta_a = 1.67$ ppm, $\delta_b = 2.09$ ppm) and hydrogen-8 ($\delta = 4.36$ ppm). The multiplicity of the hydrogen-8

Table 3 *Artemia salina* test of compounds 1 and 2

Compound	Concentration (ppm)	% mortality	LD_{50} (ppm)[a]
1	100	75.18	47,7580
	75	61.46	(64,3549–33,0042)
	50	14.99	
	25	3.7	
	10	0.68	
2	100	49.84	52,9676
	75	7.68	(88,3325–34,1548)
	50	4.99	
	25	2.5	
	10	1.11	

Note: [a]Concentration of compound that causes 50% death of *Artemia salina* nauplii.
Abbreviation: LD_{50}, median lethal dose.

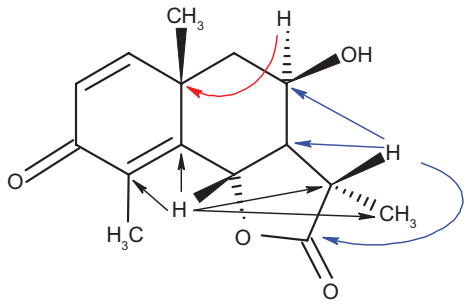

Figure 2 Heteronuclear multiple-bond correlations for compound 2.

signal, the heteronuclear multiple-bond correlation and correlation spectroscopy data, and comparison with previous studies[27,28] showed that the β stereochemistry for the hydroxyl group was unequivocally assigned to carbon-8 of compound 2, ie, 8β-hydroxy-α-santonin.

From these results, it can be seen that the enzymes present in the strain of the fungus *Cunninghamella* spp. under the experimental conditions described are monooxygenases, which allowed the substrate (compound 1) to be hydroxylyated in a regioselective and stereoselective way resulting in compound 2.

The results of the antioxidant activity test showed that compound 2 has slightly lower scavenging activity than the substrate (compound 1), suggesting that a hydroxyl group attached to carbon-8 with β stereochemistry would be responsible for this effect.

On the other hand, the *A. salina* test exhibited similar results since the activity of the metabolite (compound 2) was lower than the original compound (compound 1), also presumably due to the effect of the substituent hydroxyl group on carbon-8.

In conclusion, the introduction of a hydroxyl group at carbon-8 of α-santonin with β stereochemistry did not improve the biological activities tested here, but instead slightly diminished both.

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Disclosure

The authors report no conflicts of interest in this work.

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