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HYDROXYLATION OF DEHYDROABIETIC ACID BY *FUSARIUM* SPECIES

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Key Word Index—*Fusarium oxysporum*; *F. moniliforme*; biotransformation; dehydroabietic acid; 1β-hydroxydehydroabietic acid; antibacterial activity.

Abstract—A novel compound, 1β-hydroxydehydroabietic acid has been obtained by the microbial transformation of dehydroabietic acid, using cultures of *Fusarium oxysporum* and *F. moniliforme*. Its antibacterial activity was also tested. © 1997 Elsevier Science Ltd. All rights reserved

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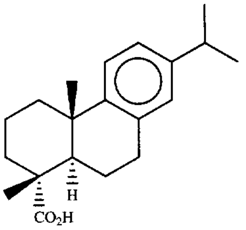
INTRODUCTION

Biotransformations are useful methods for producing medicinal and agricultural chemicals from both active or inactive natural products. Dehydroabietic acid (**1**) is an example of the former. It is well known that this diterpene has a broad range of antimicrobial activity being active against bacteria and fungi [1–4]. As part of our research programme to investigate new antimicrobial compounds we have examined **1** and its metabolites. It has been shown that **1** and its methyl ester can be transformed by different microorganisms. Bacteria and fungi have provided hydroxylation, decarboxylation, oxidation and hydrolysis products [5–10]. For these microbial modifications, *Flavobacterium resinovorum*, *Arthrobacter* sp., *Corticium sasakii*, *Alcaligenes euthrophus* and *Pseudomonas* sp. were used. However, there are no reports using other microorganisms. In the present work, we report the microbial hydroxylation of **1** by *Fusarium oxysporum* and *F. moniliforme* and the structural determination of the new metabolite obtained and its antimicrobial activity.

RESULTS AND DISCUSSION

Preliminary screening studies using different microorganisms cultures were carried out in order to determine those capable of transforming dehydroabietic acid (**1**). Several strains of *Fusarium*, *Trichoderma*, *Rhyzopus* and *Cunninghamella* were cultivated and tested using TLC monitoring. These studies indicated that *Fusarium moniliforme* and *F. oxysporum* could biotransform compound **1** to form, in both cases, only one more polar metabolite than the starting material.



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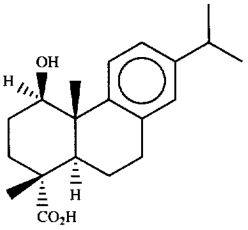

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The substrate **1** obtained by disproportionation from commercial rosin [11] was dissolved in DMF before addition to the culture medium. After a time-course study indicated the optimal length of incubation, a preparative scale fermentation was performed in order to isolate sufficient quantities of the metabolite for structural elucidation and antimicrobial activity determination. After incubation for 7 days at 26–28° no more starting material was transformed when checked by TLC. The metabolite so obtained was recovered from the culture medium by solvent extraction and then isolated and purified by CC using silica gel. The compound was identified as 1β-hydroxydehydroabietic acid (**2**) from its spectral data. The high resolution mass spectrum of **2** was in accordance with the formula $C_{20}H_{28}O_3$. ^1H NMR and

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Table 1. NMR spectral data of compound **2** in $CDCl_3$

Position	δ (^1H)*	δ (^{13}C)†
1	3.98 *dd* (8.8, 7.0)	76.8 CH
2		29.2 CH_1
3		34.7 CH_2
4		47.0 C
5	2.2 *dd* (12.2, 2.8)	44.4 CH
6		21.2 CH_2
7	2.9 *dd* (8.5, 5.0)	29.5 CH_2
8		135.0 C
9		145.9 C
10		43.0 C
11	8.08 *d* (8.3)	126.9 CH
12	6.99 *dd* (8.3, 1.9)	124.0 CH
13		146.2 C
14	6.87 *d* (1.9)	126.8 CH
15	2.87 *septet* (6.9)	33.4 CH
16	1.22 *d* (6.9)	23.9 Me
17	1.22 *d* (6.9)	23.9 Me
18		184.0 C
19	1.29 *s*	15.9 Me
20	1.28 *s*	18.1 Me

* Only assignable signals are listed. *J* (in parentheses) in Hz.

† Signal assignments based on HETCORR (J_{CH} = 145 Hz), COLOC (J_{CH} = 8 and 4 Hz) and DEPT experiments.

COSY spectra (Table 1) indicated that we were dealing with a compound which must be closely related to the 1α-hydroxy stereoisomer, a natural product isolated from *Nepeta teydea* [12]. Absorption at 3340 cm^{-1} in the IR spectrum confirmed the presence of an hydroxyl group, while the ^1H and ^{13}C (DEPT) NMR signals indicated the presence of a secondary hydroxyl group (δ_H 3.98, δ_C 76.8). However, the hydroxyl group at C-1 in **2** was equatorial as could be established by the observed down-field shift of H-11 (δ 8.08), compared with the same proton of **1**. Furthermore, a NOESY phase-sensitive experiment [13] showed dipolar couplings between H-1 (δ 3.98) and H-5 (δ 2.2), that confirmed the α-orientation of H-1. The mass spectrum (see Experimental), ^{13}C (DEPT) (Table 1), COSY, HETCORR and COLOC experiments were in agreement with the proposed structure **2**.

The antibacterial activity of **1** and **2** against Gram positive and Gram negative bacteria are summarized in Table 2. Compound **2** was inactive against almost all bacteria but showed activity only against *Serratia* sp. and *Bacilus subtilis*; compound **1** showed activity against all bacteria, except *Serratia* sp. and *Pseudomonas aeruginosa*.

EXPERIMENTAL

General. Mps are uncorr. ^1H and ^{13}C NMR spectra were recorded at 200.1 and 50.3 MHz, respectively, in $CDCl_3$ with TMS as int. standard. COSY, phase-sensitive NOESY, HETCORR and COLOC expts were obtained using standard Bruker software. EIMS were obtained at 70 eV. Optical rotations were recorded in $CHCl_3$ soln. TLC was performed on Kieselgel 60 GF_{254} coated plates using benzene–EtOAc (7:3). Detection was achieved by spraying with *p*-anisaldehyde–EtOH–HOAc–H_2SO_4 (0.1:17:2:1) followed by heating. CC was performed on Kieselgel 60 H.

Table 2. Antibacterial activity of compounds **1** and **2**

Microorganisms	1	2	V	G
S. aureus (m.r.)*	12‡	R	16	18
S. aureus (m.s.)†	12	R	16	18
S. cohnii	17	R	16	18
S. epidermidis	13	R	16	18
K. pneumoniae	12	R	16	18
Salmonella sp.	18	R	16	18
Serratia sp.	R	12	16	18
E. coli	15	R	16	18
S. aureus	14	R	16	18
P. aeruginosa	R	R	16	18
B. subtilis	12	16	16	18

* Methicillin-resistant.

† Methicillin-sensitive.

‡ Diameter inhibition zone (mm).

R—resistant (no activity); V—vancomycin; G—gentamicin.

Microorganisms. Fusarium moniliforme (NRRL 13616), *F. graminearum* (NRRL 5883), *F. proliferatum* (NRRL 6332), *F. oxysporum* (from Fusarium Research Center, University of Pennsylvania), *Trichoderma reesei* (CM 9441), *Rhyzopus arrhizus* (ATCC 11145), *R. stolonifer* (ATCC 6227B) and *Cunninghamella echinulata* (from the culture collection of Instituto de Biotecnología, Universidad Nacional de San Juan), were used in screening expts. *Fusarium oxysporum* and *F. moniliforme* were used on a prep. scale. *Fusarium* strains were stored on glucose–potato agar, other organisms on Czapeck agar slants at 4°. For antibacterial activity, the organisms used were *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis*, *Staphylococcus aureus* (methicillin resistant and methicillin sensitive), *S. cohnii*, *S. epidermidis*, *Serratia* sp., *Klebsiella pneumoniae* and *Salmonella* sp. strains (from Laboratorio de Microbiología, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo).

Fermentation. Screening experiments were carried out in flasks (250 ml) containing 40 ml of culture medium placed on a rotary shaker at 200 rpm and incubated at 26–28° during 24 hr. Media were adjusted to pH 5 and sterilized at 121° for 15 min. A standard two-stage fermentation protocol was employed in all expts. Yeast extract (5 g l^{-1}) was added to Czapek medium then inoculated with a suspension in H_2O (10 ml) of the mycelium from an agar slant prepared from a 5-day-old slant and incubated for 48 hr (stage I cultures). The first stage culture broth (5 ml) was then used as inoculum for the same medium without yeast extract (stage II cultures). After 24 hr incubation of stage II, dehydroabietic acid (**1**) (10 mg) dissolved in DMF was added at 0.2 mg ml^{-1}. Medium controls were composed of sterile media, sterile media with substrate and sterile media with DMF and then incubated. Culture controls consisted of fermentation blanks, in which the organisms were grown under identical conditions but without adding the substrate and adding DMF. Fermentations were sampled from the stage II cultures at 24 hr intervals for analysis. Samples (2 ml) were extracted with EtOAc (1 ml), the organic layer washed with 10% H_3PO_4 (0.5 ml), then with H_2O (0.5 ml) and examined by TLC. The prep. scale fermentation was done in two 1 l Erlenmeyer flasks each containing 120 ml of culture medium and incubated at 26–28°. Dehydroabietic acid (**1**) was added to obtain a final concn of 2 mg ml^{-1}. Samples were taken every 24 hr from the culture and fermentation continued until no more starting material was transformed.

Extraction and purification. After 7 days, liquors were combined, mycelium filtered off and washed with H_2O (100 ml), which was combined with the filtrate then extracted with EtOAc (3 × 50 ml). Organic layers were combined, washed with 10% H_3PO_4 (2 × 20 ml), dried (Na_2SO_4) and evapd to dryness. The resulting solid was chromatographed on a silica gel column and eluted with mixts of benzene–EtOAc (10:0–1:1). The metabolite was finally purified by CC on silica gel using petrol–EtOAc (9:1). In this way, 1β-hydroxydehydroabietic acid (**2**) (45 mg), mp = 138.3° was obtained. $[\alpha]_D + 1.34$ ($CHCl_3$, *c* 0.38). EIMS *m/z* (rel. int.): 316 $[M]^+$ (92), 301 (5), 298 (2), 272 (2), 257 (15), 239 (7), 186 (95), 43 (100); HRMS *m/z* 316.2038 (calcd: 316.2051). IR ν_{max}^{KBr} cm^{-1}: 3340 (—OH), 2990, 2935, 2860, 2820, 1690 (>C=O), 1610, 1490, 1455, 1440, 1420, 1385, 1345, 1310, 1225, 1180, 1140, 1025, 1010, 925, 900, 710, 605, 530, 490. ^1H NMR (200.1 MHz, $CDCl_3$): Table 1. ^{13}C NMR (50.3 MHz, $CDCl_3$): Table 1.

Antibacterial activity. Antibacterial activity of compound **2** was determined by the agar well diffusion method [14] and compared directly with compound **1**. Inoculum containing 10^5–10^6 bacteria ml^{-1} was incorporated into molten Müeller–Hinton agar plates. Plates were allowed to solidify and six wells of 8 mm diameter were cut in each plate using a sterile cork-borer. Stock solns of compounds **1** and **2** containing 0.5 mg ml^{-1} and standard reference antibiotics (gentamicin 10 μg ml^{-1} and vancomycin 30 μg ml^{-1}) were poured into the wells. An aq. 10% soln of DMF was the vehicle. Plates were then incubated at 37° for 24 hr. Four assays under identical conditions were carried out and inhibition zones measured. Results obtained are shown in Table 2.

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