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Biotransformation of (−)-bornyl acetate using submerged cultures of *Collybia velutipes*, *Trametes hirsuta* and *Ganoderma applanatum*

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Abstract: The biotransformation of (−)-bornyl acetate by three Basidiomycetes, *Collybia velutipes, Trametes hirsuta* and *Ganoderma applanatum* was monitored for 7 days. The observed reactions were regio- and stereo-selective hydroxylation, acetate hydrolysis and oxidation of alcohols to carbonyl compounds. Nine cyclic compounds, three of which (6-*exo*-hydroxybornyl acetate, 8-hydroxy-5-*exo*-hydroxybornyl acetate, 9-hydroxy-5-*exo*-hydroxybornyl acetate), not previously described, were isolated from the fermentation broth and therefore were identified. Product types and concentrations varied with strain and incubation time. The toxicity of the substrate was assessed from the variation of fungal biomass in the course of incubation.

Keywords: basidiomycetes; biotransformation; (−)-bornyl acetate; metabolic pathways; 6-*exo*-hydroxybornyl acetate; 8-hydroxy-5-*exo*-hydroxybornyl acetate; 9-hydroxy-5-*exo*-hydroxybornyl acetate

INTRODUCTION

The ability of fungi to metabolize foreign substrates is of considerable interest in organic chemistry because bioreactions proceed under mild conditions and show a high degree of regio- and/or stereo-specificity.[1,2] The bioconversion of easily available monoterpene hydrocarbons[3,4] is of increasing interest in the production of new fragrances for the cosmetic and food industries.[5–9] We chose to investigate three Basidiomycetes for the ability to functionalize bicyclic terpenoids in regio- and/or stereo-specific ways. These fungi, which grow well in various media, were selected because of their conceived ability to make enzymes that can oxidize terpene hydrocarbons.[10,11]

The present study aimed to evaluate *Collybia velutipes, Trametes hirsuta* and *Ganoderma applanatum* for their ability to transform (−)-bornyl acetate (**1**), particularly with regard to biotransformation kinetics. *C velutipes* and *T hirsuta* were chosen because they had given the highest metabolite yields in a previous screening over a range of monoterpene substrates conducted with about 100 Basidiomycete species, using multivariate analysis (Nano and Binello, 2001, unpublished results). *G applanatum* was a promising candidate because of its stereoselective activity, as reported in the literature.[10,12]

Working with submerged cultures, we analysed products every 24 h for 7 days and evaluated the relevant metabolic conversions. To clarify these further, we also studied the metabolization of (−)-borneol (**2**) under the same experimental conditions.

MATERIALS AND METHODS

Mycelia

The Basidiomycetes selected for the present study belonged to the following families: Tricholomataceae (*C velutipes*), Polyporaceae (*T hirsuta*) and Ganodermaceae (*G applanatum*).

Mycelia, obtained from the Department of Plant Biology, University of Turin, Italy, are registered by MUT (Mycotheca Universitatis Taurinensis) with the following MUT accession numbers: *Ganoderma applanatum* 3637, *Collybia velutipes* 3638, *Trametes hirsuta* 3639.

Chemicals

(−)-Bornyl acetate was purchased from Sigma-Aldrich (St Louis, MO, USA) and (−)-borneol from Fluka (St Louis, MO, USA).

Screening method

Mycelia were grown on Petri dishes for 14 days at 24 °C to provide precultures. The culture medium contained 20 g glucose (Merck Whitehouse Station,

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NJ, USA), 20 g malt extract (Merck) and 20 g agar (Merck) per dm^3 of distilled water. pH was not adjusted.

Erlenmeyer flasks (300 cm^3) containing 100 cm^3 of liquid culture medium (20 g dm^{-3} glucose and 20 g dm^{-3} malt extract) were inoculated with fragmented disks (1.6 cm in diameter) of mycelia taken from 7-day-old precultures.

In order to isolate fungal metabolites in sufficient amounts for identification (at least 20 mg), four 1 dm^3 Erlenmeyer flasks containing fragmented mycelia suspended in 500 cm^3 of liquid medium were inoculated with 250 mg of substrate and kept at 24 °C for 7 days on a rotary shaker (Dubnoff BSD, Milano, Italy) at 90 rpm. Yields are reported in Fig 1. For the kinetic study seven 300 cm^3 Erlenmeyer flasks were run in parallel with 100 cm^3 medium and 50 mg substrate. Each experiment was repeated three times. Blanks without mycelium were also run in parallel to rule out the occurrence of spontaneous chemical transformations. Substrate toxicity was evaluated for each mycelium by following the variation of fungal biomass during the biotransformations. Results of dry weight determinations are reported in Table 1. They show that the growth of *T hirsuta* was only moderately slowed. On the other hand the other two mycelia, after 3 days' exposure to (**1**), had suffered a temporary weight loss, but later on growth slowly resumed, possibly following partial detoxification of the substrate.

Extraction

At the end of incubations, cultures were filtered, filtrates were extracted with $CHCl_3$ and the solvent was evaporated to yield crude extracts.

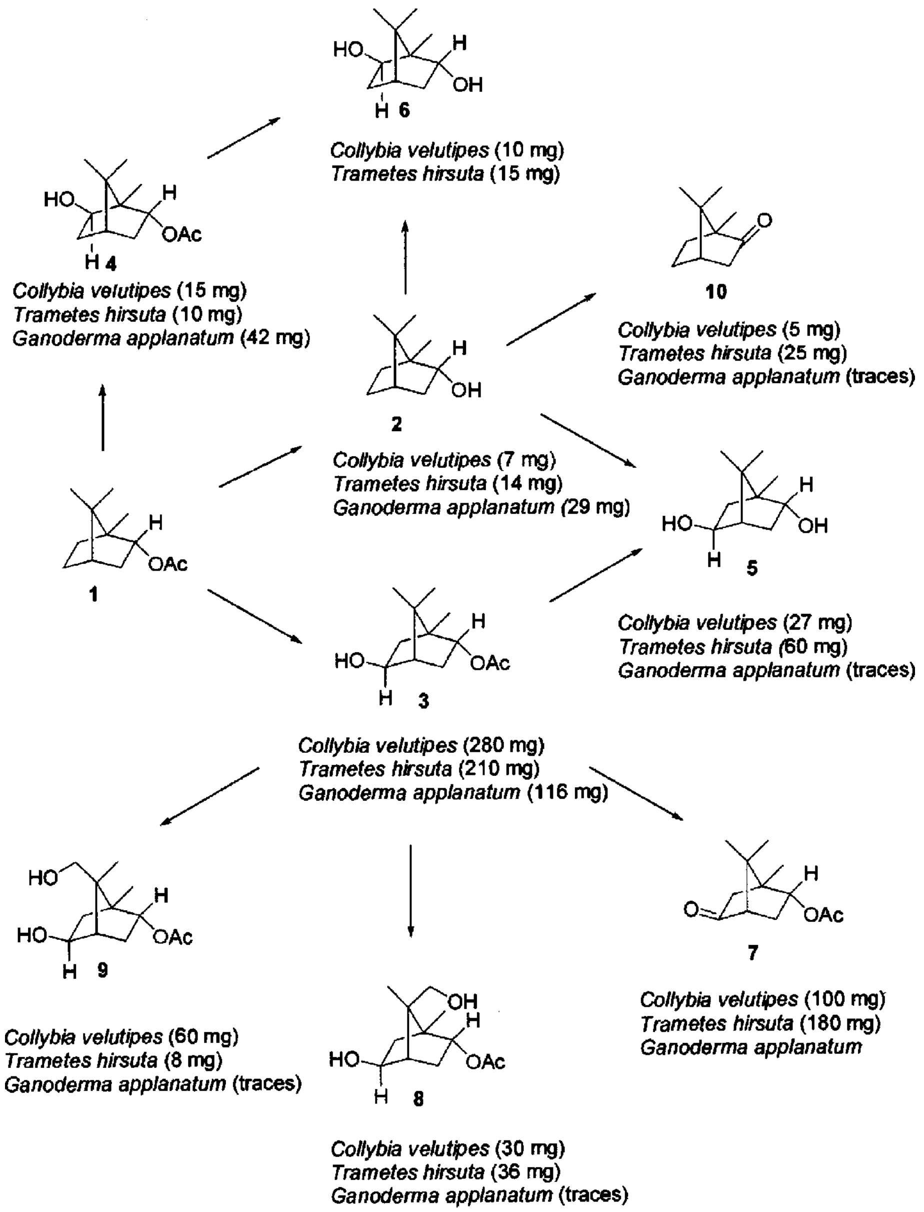

Figure 1. Possible metabolic pathways for compound **1** with *Collybia velutipes, Trametes hirsuta* and *Ganoderma applanatum*. Yields obtained at day seven of incubation with 1g of substrate are reported.

Table 1. Growth of mycelia in 100 cm^3 of culture medium inoculated with (−)-bornyl acetate. Biomass is expressed as dry weight and parallel data are given for blanks

	Day				
Mycelia	1	3	3 (blank)	7	7 (blank)
Collybia velutipes	0.39 g	0.27 g	0.64 g	0.41 g	0.98 g
Ganoderma applanatum	0.42 g	0.28 g	0.57 g	0.35 g	0.78 g
Trametes hirsuta	0.39 g	0.43 g	0.58 g	0.61 g	0.83 g

For the kinetic study, one 300 cm^3 Erlenmeyer flask containing 100 cm^3 of culture was removed each day from the rotary shaker, and extraction was performed as described.

Isolation and identification of metabolites

Crude extracts from the larger Erlenmeyer flasks were combined and chromatographed first on a Silica gel 60 (Merck) column with a petroleum ether/EtOAc gradient, then by semipreparative HPLC to complete the separation of metabolites. HPLC analyses were run on a system consisting of a Waters (Milford, MA, USA) M-6000 pump, a Waters R-401 differential refractometer and a MicroPorasil 7.8 × 300 mm column (Waters), with 60/40 *n*-hexane/EtOAc isocratic elution at room temperature. Extracts, as well as isolated metabolites, were examined by gas chromatography using a Shimadzu (Duisburg, Germany) 14BPSF instrument. A fused-silica chiral capillary column (25 m × 0.25 mm id, MEGA—Milan, Italy) of DMP-β-CD (2-dimethyl-3-pentyl-β-cyclodextrin, 30% in PS 086) was used under the following conditions: injection, split; split ratio, 1:20; temperature, 230 °C; FID temperature, 250 °C; temperature programme, from 50 °C (2 min) to 140 °C (10 min) at 2 °C min^{-1} then up to 200 °C (10 min) at 5 °C min^{-1}; the carrier gas was hydrogen at a flow-rate of 1.5 cm^3 min^{-1}.

Isolated products were identified on the basis of their spectroscopic data: IR (Shimadzu FT-IR 8101M), MS (Finnigan Mat TSQ 70, Thermo Electron Corp, Rodano-MI, Italy) and ^{1}H, ^{13}C NMR (Bruker AM 300, Milan, Italy). Spectra were compared with published data.[13–15]

Time courses of biotransformations were determined from gas chromatograms on the basis of relative abundance for each metabolite. All products were found to be optically pure.

Table 2. Time course for biotransformation of **1** by *Collybia velutipes* (metabolite amounts, expressed as percentages of substrate inoculum, were estimated by GC)

	Day						
Compound	1	2	3	4	5	6	7
1	81.4	49.4	18.7	10.5	0.4	0.1	0.3
2	0.4	4.6	0.9	2.9	2.7	1.1	0.1
3	16.4	41.0	66.1	76.4	85.2	81.1	62.4
4	1.1	2.1	2.5	3.5	4.0	3.5	1.5
5	0.0	0.2	3.5	1.3	1.8	2.1	4.2
6	0.0	0.2	0.1	0.1	0.1	0.1	0.2
7	0.5	2.1	6.7	3.5	3.1	5.9	17.0
8	0.0	0.1	0.5	0.6	1.0	2.8	5.2
9	0.2	0.3	1.0	1.2	1.7	3.2	9.1
10	0.0	0.1	0.1	0.1	0.1	0.1	0.1

Table 3. Time course for biotransformation of **1** by *Trametes hirsuta* (metabolite amounts, expressed as percentages of substrate inoculum, were estimated by GC)

	Day						
Compound	1	2	3	4	5	6	7
1	70.8	36.5	23.1	10.2	7.1	0.1	0.1
2	10.9	13.1	10.5	18.5	21.3	9.4	2.4
3	14.3	39.9	51.5	52.0	50.8	49.1	38.1
4	2.1	2.8	2.9	3.1	4.0	3.0	1.2
5	0.1	0.3	0.5	1.4	1.6	3.5	10.4
6	0.0	0.1	0.2	0.3	0.3	0.7	2.7
7	1.5	6.4	13.6	11.6	11.4	28.4	33.2
8	0.0	0.1	0.2	0.5	0.6	2.2	6.3
9	0.0	0.4	0.4	0.4	0.4	0.8	1.4
10	0.2	0.4	0.8	1.9	2.5	2.9	4.4

Table 4. Time course for biotransformation of **1** by *Ganoderma applanatum* (metabolite amounts, expressed as percentages of substrate inoculum, were estimated by GC)

	Day						
Compound	1	2	3	4	5	6	7
1	92.8	85.8	78.8	61.7	56.9	52.8	42.7
2	6.7	12.1	14.1	15.1	12.2	10.6	7.3
3	0.5	1.8	3.5	15.6	18.8	20.0	29.3
4	0.0	0.0	2.4	5.4	6.3	9.3	10.6
5	0.0	0.0	0.0	0.0	0.0	0.0	0.2
6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
7	0.1	0.2	1.0	1.9	4.9	6.0	7.9
8	0.0	0.0	0.0	0.1	0.2	0.3	0.4
9	0.0	0.1	0.1	0.2	0.6	0.7	0.9
10	0.0	0.0	0.0	0.1	0.1	0.4	0.9

RESULTS AND DISCUSSION

On day seven of the incubation period, **1** had been almost entirely metabolized by *Collybia velutipes* and by *Trametes hirsuta*, but only partially (about 50%) by *Ganoderma applanatum*, as shown in Tables 2, 3 and 4.

Each mycelium proved capable of performing hydroxylation, oxidation and hydrolysis, yielding the following known products: borneol (**2**), 5-*exo*-hydroxybornyl acetate (**3**), 5-*exo*-hydroxyborneol (**5**), 6-*exo*-hydroxyborneol (**6**) (not produced by *G applanatum*), 5-oxo-bornyl acetate (**7**) and camphor (**10**). We also isolated 6-*exo*-hydroxybornyl acetate (**4**), 8-hydroxy-5-*exo*-hydroxybornyl acetate (**8**), 9-hydroxy-5-*exo*-hydroxybornyl acetate (**9**), all of which, to our knowledge, had not previously been described.

IR and MS spectra of compound **4** gave evidence for a single hydroxylation; the location of the hydroxy group was established as C-6 *exo* on the basis of COSY interaction of H-6 with the H-5 β-hydrogen and by careful comparison of these spectra with those of relevant hydroxybornyl acetates. This compound was obtained as white crystals, mp 65 °C; FT-IR ν_{max} (KBr): 3495, 1713, 1267 cm^{-1}; EI-MS *m/z* (rel int): 212 $[M]^+$ (5), 152 (10), 137 (15), 119 (15), 108 (90), 93 (40), 69 (15), 49 (25), 43 (100); ^1H NMR ($CDCl_3$)δ: 0.84 (1H, overlapped with CH_3, 3-*endo*-H), 0.88, 0.93, 1.04 (3H each, *s*, CH_3), 1.79 (1H, *m*, 3-*exo*-H), 1.82 (1H, *m*, H-4), 2.02 (3H, *s*, CH_3CO_2-), 2.33 (2H, *m*, H-5), 4.28 (1H, *dd*, $J = 7.52$ and 4.04 Hz, 6-*endo*-H), 4.90 (1H, *dd*, $J = 9.88$ and 3.84 Hz, 2-*exo*-H).

IR and MS spectra of compounds **8** and **9** indicated the presence of two hydroxyl groups. ^1H NMR spectra of these metabolites differed only in two regards: (a) the chemical shifts of the two diastereotopic CH_2OH hydrogens, respectively found at 3.41, 3.64 (**8**) and 3.29, 4.01 (**9**) ppm; (b) those of the two methyl groups, that fell respectively at 0.90, 1.23 (**8**) and 0.86, 1.02 (**9**) ppm.

A comparison of ^{13}C NMR spectra showed that the signal for one methyl group was shifted downfield, owing to the proximity of the CH_2OH. All data from ^1H, ^{13}C, COSY and HETCOR NMR spectra indicate that these compounds are epimers at C-7 and we suppose them to be 8-hydroxy-5-*exo*-hydroxybornyl acetate (**8**) and 9-hydroxy-5-*exo*-hydroxybornyl acetate (**9**). The structure of **9** obtained by molecular modelling (Hyperchem 7.0, 2003, Hypercube Inc, Gainesville, FL, USA) showed that the observed downfield shift could be due to an interaction between one of the two CH_2OH hydrogens and the OH group in position 5 (distance 2.5397 Å as calculated by the Fletcher–Reeves Conjugate Gradient method), while no interference would be predicted with the methyl group at position 1. Likewise for compound **8**, molecular modelling suggested that the downfield shift of the same CH_3 group is probably caused by the presence of the hydroxyl group at C-8.

Compound **8** was obtained as a pale yellow powder, mp 136 °C; FT-IR ν_{max} (KBr): 3314, 1732, 1257, 1037 cm^{-1}; EI-MS *m/z* (rel int): 228 $[M]^+$ (2), 168 (10), 150 (47), 137 (42), 124 (58), 109 (100), 95 (75), 67 (30), 49 (25), 43 (98); ^1H NMR ($CDCl_3$)δ: 0.86 (1H, overlapped with CH_3, 3-*endo*-H), 0.90, 1.23 (3H each, *s*, CH_3), 1.48 (1H, *d*, $J = 11.3$ Hz, 6-*exo*-H), 2.03 (3H, *s*, CH_3CO_2-), 2.3–2.4 (2H, *m*, 3-*exo*-H and 6-*endo*-H), 3.41 (1H, *d*, $J = 11$ Hz, H-8β), 3.64 (1H, *d*, $J = 11$ Hz, H-8α), 3.89 (1H, *dd*, $J = 7.88$ and 3.48 Hz, 5-*endo*-H), 4.80 (1H, *m*, 2-*exo*-H).

Compound **9** was obtained as a white powder, mp 85 °C; FT-IR ν_{max} (KBr): 3377, 1740, 1253, 1039 cm^{-1}; EI-MS *m/z* (rel int): 228 $[M]^+$ (2), 168 (5), 150 (20), 137 (15), 124 (20), 109 (20), 94 (100), 67 (15), 43 (65); ^1H NMR ($CDCl_3$)δ: 0.81 (1H, *dd*, $J = 14.3$ and 3.64 Hz, 3-*endo*-H), 0.86, 1.02 (3H each, *s*, CH_3), 1.50 (1H, *d*, $J = 14.4$ Hz, 6-*exo*-H), 2.03 (3H, *s*, CH_3CO_2-), 2.3–2.4 (2H, *m*, 3-*exo*-H and 6-*endo*-H), 3.29 (1H, *d*, $J = 11.36$ Hz, H-9β), 3.90 (1H, *dd*, $J = 7.68$ and 2.4 Hz, 5-*endo*-H), 4.01 (1H, *d*, $J = 1.36$ Hz, H-9α), 4.75 (1H, *m*, 2-*exo*-H).

The time courses of these biotransformations were followed by gas chromatography; results are reported in Tables 1, 2 and 3. GC profiles of samples taken every 24 h during incubation led us to propose for **1** the hypothetical metabolic pathways that are shown in Fig 1.

A common feature of all mycelia tested is the predominant formation of compound **3**. This was partially converted to hydroxylated compounds **8** and **9**, oxidized to **7** and hydrolysed to **5**. 6-*exo*-Hydroxyborneol (**6**), derived from hydrolysis of **4**, was virtually only produced by *T hirsuta*. However, part of metabolites **5** and **6** might have derived from the direct hydroxylation of borneol (**2**).

To investigate this possibility, we studied (−)-borneol under the same experimental conditions. Results showed that *C velutipes* selectively introduced a hydroxyl group at the C-5 position, yielding **5** as the predominant metabolite, while *T hirsuta* yielded more than one hydroxylated derivative (**5, 6**) and other oxidation products as well (camphor, 5-*exo*-hydroxycamphor and 6-*exo*-hydroxycamphor). With *G applanatum*, only **10** was isolated, which suggests a narrower substrate specificity for its hydroxylation and oxidation enzymes.

Finally, towards the end of the incubation with (−)-bornyl acetate each mycelium formed the minor metabolite **10,** probably by oxidation of borneol. We are now carrying out additional experiments to confirm this hypothesis.

CONCLUSION

The identification of metabolites showed that all three mycelia are able to transform (−)-bornyl acetate into a variety of oxygenated compounds, *C velutipes* possessing the highest efficiency, regiospecificity and stereoselectivity.

Gas chromatograms indicated 5-*exo*-hydroxybornyl acetate (**3**) as the major metabolite. This reflects the greater accessibility to enzymic attack of the C-5 position and confirms that the introduction of hydroxyl or carbonyl groups in non-activated positions of the bornyl moiety is a characteristic feature of these, as of many other, biosynthetic sequences. These investigations are now being extended to cyclic saturated monoterpenes and α,β-unsaturated ketones.

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