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Biotransformation of tyrosol by whole-cell and cell-free preparation of *Lentinus edodes*

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Abstract

The phenolic components of waste olive waters are degraded by the white-rot basidiomycete *Lentinus edodes*. Tyrosol is converted by cell-free preparation of the fungus into a dimeric tetracyclic ketone, derived from a Pummerer's ketone-like intermediate. Conversely, the alcohol corresponding to the above ketone was isolated after 7 days from whole cells of *Lentinus edodes* incubated with tyrosol.

Keywords: Waste olive waters; *Lentinus edodes*; Laccase; Tyrosol; Biotransformation; Pummerer's ketone; Oxidoreductase

1. Introduction

Lentinus edodes is a white-rot basidiomycete able to carry out the degradation of lignin [1,2] and lignin-related compounds [3]. Its extracellular ligninolytic complex mainly includes two phenol-oxidizing enzymes, namely laccase (E.C: 1.10.3.2 benzenediol: oxygen oxidoreductase) and manganese-dependent peroxidase (E.C. 1.11.1.13 Mn(II): hydrogen peroxide oxidoreductase) [4].

The former enzyme is a multi-copper oxidase able to perform the one-electron oxidation of several substrates including polyphenols, substituted monophenols, aromatic amines and other easily oxidizable aromatic compounds [5,6].

Mn-dependent peroxidase (MnP) is a heme protein able to bring about the oxidation of phenolic and of some non-phenolic compounds [4,7], oxidizing Mn(II) to Mn(III), which in turn, if suitably chelated, can act as a freely-diffusible oxidizing species [8].

Laccase is expressed during the primary growth by *Lentinus edodes* [1], whereas MnP expression occurs during the secondary metabolism (idiophase) in the presence of nitrogen starvation conditions [9].

In a previous paper we reported on the degradation of waste olive waters by liquid cultures of *Lentinus edodes*. Our studies showed the total phenol content to be lowered by 66% within four days from the incubation with a concomitant extensive decoloration [10]. Whereas polyphenols such as catechol, 4-methyl

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catechol and hydroxytyrosol [11] were easily degraded both by suspended cultures and by extracellular enzyme preparations, the monophenol tyrosol required more drastic conditions of reaction [12].

In this paper we report on the structure elucidation of the two biotransformation products, a dimeric ketone and its corresponding alcohol, obtained by treatment of tyrosol with extracellular preparations (containing laccase but not MnP) and whole-cell enzymes, respectively.

2. Experimental

2.1. Materials

Mps were determined with a Kofler apparatus. Optical rotations were measured on a Perkin Elmer 243 polarimeter. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) were run on a Varian Gemini 300; TMS as int. stand; solvents: pyr-d_5 for **2** and **5**, $CDCl_3$ for all other compounds.

2.2. Microorganism and culture conditions

Lentinus edodes Berk Peg. (strain SC 495) was obtained from Professor T.H. Quimio (Los Banos, Philippines). The inoculum was produced as previously reported [13].

The composition of the liquid growth medium (per 1 liter) was as follows: 25 g sucrose, 2.5 g KNO_3, 0.1 g $MgSO_4$, 0.1 g $CaCl_2$, 0.005 g $FeCl_3$, 0.002 g $MnSO_4$, 0.002 g $CuSO_4$, 0.0001 g $CoCl_2$, 0.0002 $NaMoO_4$ and 20 mM sodium-2,2′-dimethylsuccinate as buffer (pH 4.5) [14].

The cultivation was carried out on a rotary shaker (120 rpm/min) at 30°C for 10 days.

2.3. Cell-free enzyme preparation

Liquid cultures were harvested after 8 days of incubation and centrifuged at 1200 *g* for 30 min. The supernatant was concentrated by ultrafiltration in a stirred cell equipped with a Diaflo PM-10 membrane (Amicon, Danvers). The retentate, containing 85 IU ml^{-1} of laccase activity, was used in the following biotransformation experiments. Such enzymatic preparation did not contain manganese dependent peroxidase activity, since it was obtained from liquid cultures carried out under nitrogen-rich conditions. Thermal stability experiments of the crude laccase, performed at pH 5.0, revealed that it was fully stable at 40°C retaining 90% of its activity after 12 h of incubation.

2.4. Enzyme assay

Laccase activity was determined spectrophotometrically by oxidation of 2,2′-azinobis-(3-ethylbenzthiazoline)-6-sulfonate (ABTS) [15]. The assay mixture contained 1.0 mM ABTS, 0.1 M sodium acetate and a suitable amount of enzyme. Oxidation of ABTS was monitored determining the increase in absorbance at 420 nm ($\varepsilon_{270} = 3.6 \times 10^4$ M^{-1} cm^{-1}). Enzyme activity was expressed in international units (IU) defined as follows: 1 IU = 1 μmol of ABTS oxidized per min. Manganese-dependent peroxidase was assayed by the method of Waarishi et al. [8]. The assay mixture (1 ml) contained 0.5 M $MnSO_4$, 75 μM H_2O_2 in 50 mM malonate buffer pH 4.5 and the formation of Mn(III)–malonate complex was kinetically followed at 270 nm ($\varepsilon_{270} = 11590$ M^{-1} cm^{-1}).

2.5. Biotransformation with cell-free enzyme preparation

Tyrosol (**1**, 20 mg) was incubated at pH 5.0 (100 mM acetate buffer) with the above mentioned extracellular enzyme preparation (2 ml, 170 IU of laccase) at 40°C for 6 h. The pooled EtOAc extracts of five biotransformation reactions were evaporated and the residue on preparative TLC ($CHCl_3$–EtOAc–AcOH, 49:49:2) gave compound **2** (6 mg, yield 6%); mp 94–95°C; $[\alpha_D] = 0$ (c 0.56, $CHCl_3$); ^{1}H NMR and ^{13}C NMR in Table 1. EIMS *m/z* (rel. int.): 274 $[M]^+$ (63), 256 $[M\text{-}H_2O]^+$ (2), 243 $[M\text{-}CH_2OH]^+$ (100), 225 $[243\text{-}H_2O]^+$ (9),

Table 1
^{1}H- and ^{13}C-NMR spectral data of biotransformation products **2** and **5**

Position	**2**		**5**	
	δ_C	δ_H	δ_C	δ_H
1	205.85	—	63.44	4.28 tt (9, 4.5)
2	39.65	2.75 dd (18, 35)	33.66	2.15 m
		2.32 dd (18, 2.5)		1.90 dt (13.5, 8.5 × 2)
3	83.70	3.92 br t (3.5) [a]	82.86	4.06 dd (9, 5.5)
4	53.05	—	53.36	—
5	88.22	4.76 br t (3) [a]	86.61	4.89 t (4)
6	40.06	3.04 dd (18, 3)	36.51	2.60 m
		2.94 dd (18, 3)		2.15 m
8	67.28	4.04 dt (8.5 × 2, 6.5)	65.90	4.16 dt (9 × 2, 6.5)
		3.94 dt (9 × 2, 6.5)		4.10 dt (9, 5 × 2)
9	38.24	2.60 ddd (13.5, 9.5, 6.5)	35.77	2.15 m
		2.00 ddd (13.5, 8.5, 6.5)		
11	158.33	—	157.59	—
12	130.04	—	129.37 [b]	—
13	124.50	7.34 d (2)	124.16	7.37 d (2)
14	133.68	—	133.12	—
15	130.24	7.26 dd (8,2)	129.37 [b]	7.23 dd (8, 2)
16	109.24	6.92 d (8)	109.57	6.94 d (8)
17	39.84	3.08 t (7)	39.84	3.06 t (7)
18	63.63	4.14 t (7)	63.79	4.12 t (7)

The signals showed the appropriate integral intensities. Coupling constants (in Hz) are given in parentheses. Proton and carbon signals were connected by HETCOR experiments.

[a] Broadness is due to mutual long-range coupling (~ 1 Hz).

[b] The two signals were coincident.

213 (6), 201 (27), 199 (7), 197 (11), 171 (13), 157 (16), 149 (13), 144 (12), 128 (18), 115 (21); HRMS: found; 274.1254, calculated for $C_{16}H_{18}O_4$; 274.1205.

2.6. Acetylation of compound 2

Treatment of **2** with pyr/Ac_2O overnight gave products **3** (55%) and **4** (40%).

2.6.1. Compound 3

^{1}H NMR: δ 7.07 (1H, d, $J = 2$ Hz, H-13), 7.05 (1H, dd, $J = 7$ and 2 Hz, H-15), 6.76 (1H, d, $J = 7$ Hz, H-I 6), 4.77 (1H, t, $J = 3$ Hz, H-5), 4.25 (2H, t, $J = 7$ Hz, H_2-18), 4.15 (1H, ddd, $J = 15$, 9 and 6.5 Hz, H-8a), 4.5 (1H, ddd, $J = 15$, 9 and 7 Hz, H-8b), 3.92 (1H, t, $J = 3$ Hz, H-3), 2.94 (1H, dd, $J = 18$ and 3 Hz, H-6a), 2.91 (2H, t, $J = 7$ Hz, H_2-17), 2.81 (1H, dd, $J = 17.5$ and 3 Hz, H-2a), 2.70 (1H, dd, $J = 18$ and 2-5 H-8b), 2.29 (1H, dd, 17.5 and 3 Hz, H-2b), 2.21, 2.16 (1H each, ddd, $J = 13$, 7.5 and 6 Hz, H_2-9), 2.05 (3H, s, COMe); ^{13}C NMR: δ 205.82 (s, C-1), 171.01 (s, OCO), 158.22 (s, C-11), 130.99, 129.85 (s each, C-12, C-14), 129.93 (d, C-15), 123.43 (d, C-13), 110.09 (d, C-16), 87.56 (d, C-5), 83.58 (d, C-3), 67.22 (t, C-8), 65.10 (t, C-18), 52.76 (s, C-4), 39.61 (t, C-6), 39.22 (t, C-2), 38.57 (t, C-9), 34.59 (t, C-17), 21.00 (q, Me); EIMS m/z (rel. int.): 316 $[M]^+$ (6), 256 $[M\text{-}AcOH]^+$ (100), 243 $[M\text{-}CH_2OAc]^+$ (6), 225 (3), 214 (8), 201 (7), 199 (7), 186 (15), 171 (26), 157 (21), 128 (12), 115 (13).

2.6.2. Compound 4

^{1}H NMR: δ 7.21 (1H, dd, $J = 8$ and 2 Hz, H-15), 7.18 (1H, d, $J = 2$ Hz, H-13), 7.05 (1H, d, $J = 8$ Hz, H-16), 6.82 (1H, dd, $J = 10.5$ and 2 Hz, H-5), 6.12 (1H, d, $J = 10.5$ Hz, H-6), 4.59 (1H, m, $\sum J = 9$ Hz, H-3), 4.26 (2H, t, $J = 7$ Hz, H_2-18), 4.02, 3.97 (1H each, dt,

$J = 15$ and 7×2 Hz, H_2-8), 2.94 (2H, t, $J = 7$ Hz, H_2-17), 2.85, 2.71 (1H each, dd, $J = 17$ and 3.5 Hz, H_2-2), 2.68, 2.34 (1H each, dt, $J = 13.5$ and 7×2 Hz, H_2-9), 2.34 (3H, s, 11-OCOMe), 2.04 (3H, s, 18-OCOMe); ^{13}C NMR: δ 193.56 (s, C-1), 173.66 (s, 18-OCO), 167.45 (s, 11-OCO), 150.24 (s, C-11), 129.09 (d, C-15), 128.61, 127.67 (s each, C-12, C-14), 127.71, 126.68 (d each, C-5, C-6), 124.65 (d, C-13), 101.19 (d, C-16), 80.73 (d, C-3), 66.47 (t C-8), 64.52 (t, C-18), 42.75 (s, C-4), 39.27, 39.18 (t each, C-2, C-9), 34.60 (t, C-17), 22.68, 21.49 (q each, $2 \times$ Me); EIMS m/z (rel. int.): 358 $[M]^+$ (12), 316 $[M\text{-}CH_2CO]^+$ (8), 298 $[M\text{-}AcOH]^+$ (65), 256 $[M\text{-}AcOHCH_2CO]^+$ (100), 243 $[316\text{-}CH_2OAc]^+$ (3), 225 (47), 212 (21), 211 (50), 210 (24), 199 (16), 197 (17), 185 (8), 183 (17), 171 (16), 157 (19), 128 (21), 115 (21).

2.7. Biotransformation with whole cells

Tyrosol (**1**, 65 mg) was aseptically added to the growth liquid medium (130 ml) [14] and incubated with *Lentinus edodes*.

After 7 days the reaction mixture was extracted with EtOAc ($\times 3$). Separation of the residue by preparative TLC ($CHCl_3$–EtOAc–AcOH, 49:49.2) gave compound **5** (R_F lower than **2**; 4 mg; yield 6%); mp 127–128°C; $\alpha_D = +3.1$ (c 0.35, $CHCl_3$); ^1H and ^{13}C NMR in Table 2; EIMS m/z (rel. int.): 276 $[M]^+$ (85), 258 [M-H2O]$^+$ (11), 245 [M-CH2OH]$^+$ (100), 227 (4), 213 (11), 201 (6), 199 (7), 197 (5), 185 (12), 183 (8), 171 (12), 157 (15), 129 (28), 115 (20); HRMS: found; 276.1374, calculated for $Cl_6H_{20}O_4$; 276.1362.

Table 2
Connectivities (INEPTL) and vicinities (DIF NOE) in compound **2**

Irradiated proton	Connected carbons (^nJ_c)	Neighbouring proton
H-2a	$^2J_1$	H-3
H-2b	$^2J_1$	
H-5	$^3J_1$, $^3J_3$, $^3J_9$	
H-9a	$^3J_3$, $^3J_4$, $^3J_5$	H-3, H-13
H-9b	$^3J_3$, $^3J_4$, $^3J_5$	H-5
H-10	$^2J^{11}$, $^2J_{12}$	H-3, H-9a, H-17a

2.8. Acetylation of compound 5

Treatment of compound **5** by pyr/Ac_2O (4 days) gave only compound **6** (94%); mp 97–98°C; $\alpha_D = -2$ (c 0.38, $CHCl_3$); ^1H NMR: δ 7.01 (1H, dd, $J = 8$ and 2 Hz, H-15), 6.98 (1H, d, $J = 2$ Hz, H-13), 6.74 (1H, d, $J = 8$ Hz, H-16), 5.07 (1H, dq, $J = 9$ and 5×3 Hz, H-1), 4.72 (1H, t, $J = 4.5$ Hz, H-5), 4.23 (2H, t, $J = 7$ Hz, H_2-18), 4.09 (2H, t, $J = 7$ Hz, H_2-8), 3.97 (1H, t, $J = 5$ Hz, H-3), 2.87 (2H, t, $J = 7$ Hz, H_2-17), 2.38, 2.19 (1H each, dd, $J = 13.5$ and 7 Hz, H_2-9), 2.07, 2.05 (3H each, s, $2 \times OCOCH_3$), 1.93 (1H, dt, $J = 15$ and 5×2 Hz, H-2a), 1.88 (1H, dt, $J = 15$ and 5.5×2 Hz, H-2b); ^{13}C NMR: δ 171.04, 170.49 (s each, $2 \times$ OCO), 157.54 (s, C-11), 132.31 (s, C-14), 130.55 (s, C-12), 129.30 (d, C-15), 123.26 (d, C-13), 109.87 (d, C-16), 85.77 (d, C-5), 80.99 (d, C-3), 66.15 (d, C-1), 66.10 (t, C-8), 65.20 (t, C-18), 52.81 (s, C-4), 38.29 (t, C-17), 34.55 (t, C-9), 30.76, 30.52 (t each, C-2, C-6), 21.32, 21.00 (q each, $2 \times$ Me); EIMS m/z (rel. int.): 360 $[M]^+$ (8), 300 $[M\text{-}AcOH]^+$ (100), 287 $[M\text{-}CH_2OAc]^+$ (8), 240 $[M\text{-}2AcOH]^+$ (22), 227 (6), 213 (8), 201 (7), 199 (12), 149 (15), 115 (18).

2.9. Biotransformation of compound 2 with whole cells

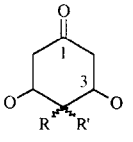
Compound **2** (5 mg) was aseptically added to the growth liquid medium (10 ml) [14] and incubated with *Lentinus edodes*.

After 7 days the reaction mixture was extracted with EtOAc ($\times 3$). The residue by preparative TLC ($CHCl_3$-EtOAc-AcOH, 49:49:2) gave compound **5** (3 mg, 60%), identical in all respects with the sample previously obtained.

3. Results and discussion

When tyrosol (**1**, *p*-hydroxyphenylethanol) was treated at 40°C for 65 h with a cell-free enzyme preparation from *Lentinus edodes* a new metabolite was obtained, albeit in poor yield (6%), from the EtOAc extract of the reaction mixture.

The compound showed a molecular peak at m/z 274 in the mass spectrum and a molecular formula $C_6H_{18}O_4$ (by HREIMS), the latter corresponding to two units of the substrate less two hydrogen atoms. The characteristic signals of one hydroxymethyl side chain present in the substrate were evident from the ^{1}H- and ^{13}C-NMR spectra, which showed also the presence of an aromatic nucleus but now with an *ortho*, *orthometa*, *meta* substitution pattern. In the ^{1}H NMR spectrum a series of decoupling experiments revealed the presence of a CH_2CH_2O and two CH_2CHO groupings, as confirmed by an HETCOR measurement (Table 1). The last two signals, at ~53 and 206 ppm in the ^{13}C NMR spectrum were assigned to a quaternary carbon and to a ketonic carbonyl group, respectively. The large difference in chemical shifts suggested the geminal ($J = 16$ Hz) protons of the CH-linked methylenes to be connected to the carbonyl group. Moreover, the absence of further couplings required the methine signal to be linked to the quaternary carbon, thus establishing the presence of a partial structure **a** i.e. a 3,5-dioxy-4,4-disubstituted cyclohexanone.



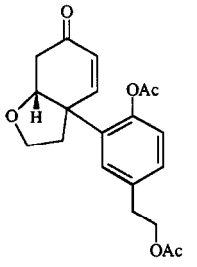
a

One of the two C-4 substituents of the cyclohexanone ring was identified with the non-oxygenated methylene of the CH_2CH_2O grouping, which forms a tetrahydrofuran ring with C-5 and C-4 carbon atoms. Conversely, the second C-4 substituent was identified with the aromatic moiety. In particular, the NMR data for the aromatic ring require the C-4 quaternary carbon of the cyclohexanone unit to be linked to the aromatic ring at a position *ortho* to the oxygen-bearing carbon. In effect this establishes the presence of a dihydrofuran unit linking the aromatic ring and the atoms C-4 and C-3 of the cyclohexanone unit. A series of INEPTL experiments (Table 2) confirmed the connectivties of the C-4 quaternary carbon with the C-3 and C-5 methine groups and with the C-9 methylene (see **2** for numbering system). The selective irradiation of the *meta*-coupled aromatic proton did not show any connectivity with the quaternary spiro carbon. This result may be attributed to the coplanarity of the two units. On consideration of these findings the biotransformation product was tentatively assigned structure **2**. DIF NOE experiments (Table 2) confirmed the assignment and, in particular, evidenced the proximity of the C-13 aromatic proton and one of the C-9 methylene protons. These data further established that the tetrahydrofuran ring (with O-7) is perpendicular to the plane formed by the aromatic and the tetrahydrofuran (with O-10) rings. As a confirmation, protons H-3 and H-5 did not show any mutual influence in DIF NOE experiments, being in a *transoid* position.

The mass fragmentation of compound **2** is characterized by the loss of CH_2OH, as in the case of the starting *p*-hydroyphenylethanol, to give a tropylium ion as the base peak. The successive losses of CH_2CO and C_2H_4O may be ascribed to the cyclohexanone and the tetrahydrofuran rings, respectively.

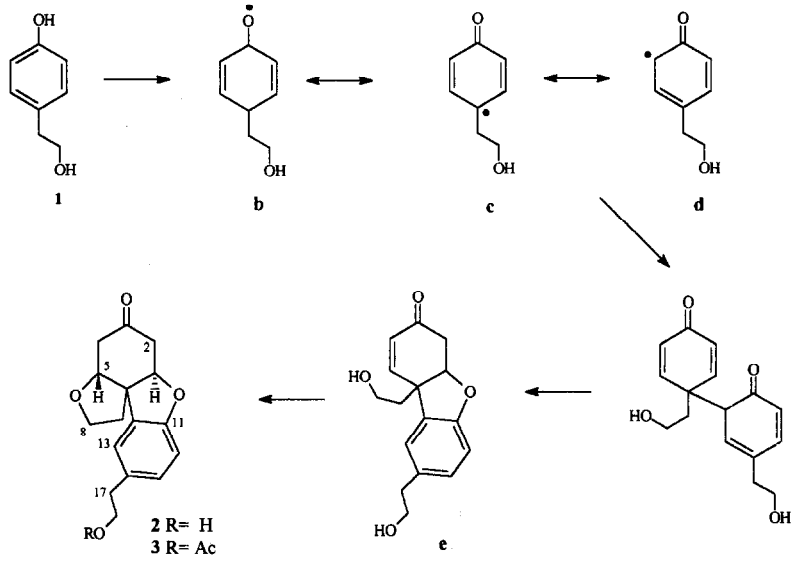
Compound **2** by treatment with acetic anhydride in pyridine gave two products, the former being the expected acetyl derivative **3**, as shown by a three-protons singlet at δ 2.05 in the ^{1}H NMR spectrum and by the molecular peak (at m/z 316) in the mass spectrum. The other product showed a molecular peak at m/z 358 in the mass spectrum and the signals of two acetyl groups in ^{1}H NMR (three-protons singlets at 2.05 and 2.34 ppm) and ^{13}C NMR (two

singlets at ~ 167 ppm and two quartets at ~ 22 ppm) spectra. The upfield shifts shown by the signals of C-11, C-12 and C-16 carbons, and the H-16 proton (see numbering system in **4**) as well as the losses of CH_2CO from both $[M]^+$ and $[M\text{-}AcOH]^+$ ions in the mass spectrum that a second acetyl function was now situated on a phenolic hydroxyl group, which very likely arised via ring opening of the oxide bridge in **2**. In agreement with these findings the signals of C-5 and C-6 protons and carbons in **2** were not present in the ^1H- and ^{13}C-NMR spectra of **4** and were replaced by two new olefinic signals. The formation of a double bond in the cyclohexanone ring was also substantiated by the highfield shift of the signals attributed to the carbonyl (C-1) group and the quaternary carbon (C-4) as well as by the long range coupling of the C-3 and C-5 protons. In conclusion, the other acetylation product was assigned structure **4**, derived as suggested earlier by the ring opening in alkaline medium of the C-5–C-14 oxide bridge in **2** and the loss of one of the protons of the C-6 methylene to generate the olefinic linkage. In the mass spectra of the acetyl derivatives **3** and **4**, the tropylium ion at m/z 243 and its fragments (at m/z 201 and 157) are still present, although of lower abundance. The base peak at m/z 256 in **4** is originated by the losses from the molecular ion of the acetyl groups, the aliphatic one being lost as AcOH and the phenolic one as CH_2CO.



4

The formation of compound **2**, via microbial transformation of *p*-hydroxyphenylethanol, may be ascribed to the presence in the cell-free extract of *Lentinus edodes* of a laccase, capable of generating from the substrate radicals such as **b**, **c** and **d** (Scheme 1). The coupling of **c** and **d** gives via a pathway suggested in Scheme 1 an intermediate **e**, very similar with the so called Pummerer's ketone (**e**; CH_3 instead of CH_2CH_2OH throughout), obtained by the oxi-



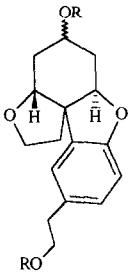
Scheme 1.

dation of *p*-cresol by ferricyanide or peroxidase [16]. The closure of the neighbouring alcohol function on the double bond of the cyclohexenone ring affords finally the product **2**.

Clearly the radicals **b**, **c** and **d** may proceed via a variety of reaction paths thereby affording **2** in only low yield, a result obtained in the present study.

While **2** was formed as the exclusive product from the experiments involving the biotransformation of *p*-hydroxyphenylethanol with the cell-free extract of *Lentinus edodes*, different results were obtained when the same substrate was incubated with whole cells of the fungus: a new biotransformation product was formed exclusively.

The compound showed a molecular peak at m/z 276 (two mass units higher than **2**) and a molecular formula $C_{16}H_{20}O_4$ (by HREIMS). Although the majority of the ^{13}C NMR spectrum of the new compound was very similar to the corresponding spectrum of **2**, the carbonyl group signal was replaced by the resonance of a third oxygenated methine, whose presence was in turn confirmed by a one-proton multiplet at 4.28 ppm in the ^{1}H NMR spectrum. These findings suggested that the new product was the optically active ($\alpha_D = -3$) alcohol corresponding to the ketone **2** and was thus assigned structure **5**. The ^{1}H- and ^{13}C-NMR spectra of compound **5** are reported in Table 1. INEPTL and DIFNOE experiments gave results similar to those reported in Table 2 for compound **2**. Notably, selective irradiation of the C-13 proton gave, as a result of a INEPTL measurement, a ^{3}J connectivity with the quaternary spiro carbon atom. Examination of molecular models showed that now the C-13 and C-4 protons are not coplanar. The formation of compound **5** may be attributed to an initial oxidation by the same laccase system which gave **2**, followed by reduction by means of an oxidoreductase-type enzyme. Since this reduction step is not observed in the studies with cell-free preparation, it appears that the oxidoreductase enzyme is cell associated. In order to confirm this hypothesis, the cell culture of *Lentinus edodes* was incubated with the precursor **2** and the alcohol **5** was isolated in a 60% yield.



5 R= H
6 R= OAc

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