Fungal Biotransformation Products of Dehydroabietic Acid

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Received July 6, 2006

Dehydroabietic acid (DHA) (**1**) is one of the main compounds in Scots pine wood responsible for aquatic and microbial toxicity. The degradation of **1** by *Trametes versicolor* and *Phlebiopsis gigantea* in liquid stationary cultures was followed by HPLC-DAD-ELSD. Both fungi rapidly degraded DHA relative to a control. More breakdown products were observed for *T. versicolor* than for *P. gigantea*. After 13 days, four compounds were identified by means of spectroscopic methods in *P. gigantea* cultures: 1β-hydroxy-DHA (**2**), 1β,7α-dihydroxy-DHA (**3**), 1β,16-dihydroxy-DHA (**5**), and tentatively 1β-hydroxy-7-oxo-DHA (**4**). In *T. versicolor* cultures, 1β,16-dihydroxy-DHA (**5**), 7β,16-dihydroxy-DHA (**6**), 1β,7β,16-trihydroxy-DHA (**7**), 1β,16-dihydroxy-7-oxo-DHA (**8**), 1β,15-dihydroxy-DHA (**9**), and 1β,7α,16-trihydroxy-DHA (**10**) were identified after 9 days of incubation. Thus the biotransformation of **1** by the two fungi was different, with only **5** being produced by both strains. Compounds **3**, **7**, **8**, and **10** are reported for the first time as natural products.

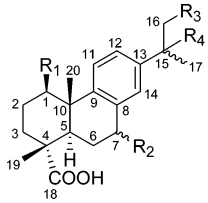
Resin acids are tricyclic diterpenoids found at relatively high concentrations in many coniferous trees. These compounds belong to one of the most abundant diterpenoid families found in higher plants, and they can account for up to 21.4% of the nonstructural lipophilic fraction in coniferous wood or 0.66% of the total wood dry matter.[1] Dehydroabietic acid (DHA) (**1**) is one of the major resin acid constituents. Resin acids are a primary source of toxicity found in pulp mill effluents toward fish and other aquatic organisms in the receiving water ecosystem[2−4] and microorganisms in wastewater treatment systems.[5] The 96 h 50% lethal concentrations of most resin acids toward fish vary between 0.5 and 1.5 mg/L.[2,6,7]

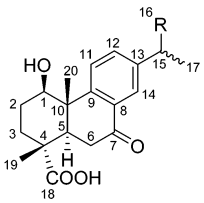
Research on the microbial degradation of resin acids has mainly been driven by interest in the biotransformation of plant diterpenoids and in the role of microorganisms in the detoxification of pulp and paper mill effluents. Resin acids are readily biodegraded by various fungi and aerobic bacteria, but appear to be relatively recalcitrant to degradation in anaerobic environments.[8,9] Extensive studies on the metabolism of **1** by aerobic bacteria have led to the elucidation of some degradation pathways.[8,10−15] In contrast, knowledge on the biotransformation of resin acids by fungi is limited. Several studies have considered degradation of **1** by ascomycetous fungi (molds),[16−20] but very little is known about the metabolism of resin acids by basidiomyceteous wood-degrading fungi. We are aware of only one previous report on the metabolism of **1** by the basidiomycetes *Fomes annosus*, in which several oxidized derivatives were identified.[21]

In the present work we report on the biotransformation of **1** by two common basidiomyceteous wood-degrading fungi, *Trametes versicolor* and *Phlebiopsis gigantea*, and the structural determination of nine degradation products, four of which are reported for the first time as natural products.

Results and Discussion

The white rot fungi *T. versicolor* and *P. gigantea* rapidly metabolized DHA (**1**) in stationary liquid assays. After 9 days of incubation, the elimination of **1** (initial concentration 100 mg/L) by these fungal strains relative to an abiotic control averaged 74% and 54%, respectively. Various biotransformation products, which were not present in abiotic controls, were detected in the cultures of the two white rot fungi. The HPLC-ELSD retention times of the compounds detected in the *T. versicolor* cultures were 4.0 min (**7**), 5.0 min (**10**), 5.4 min (**8**), 6.0 min, 8.4 min (**9**), 9.1 min (**5**), and 13.6 min (**6**) and in the *P. gigantea* cultures 9.1 min (**5**), 12.1 min (**3**), 12.1 min (tentatively **4**), and 18.0 min (**2**). All the breakdown products are more polar than **1**.





1: $R_1 = R_2 = R_3 = R_4 = H$

2: $R_1 = OH$, $R_2 = R_3 = R_4 = H$

3: $R_1 = OH$, $R_2 = \alpha$-OH, $R_3 = R_4 = H$

4: R = Me

5: $R_1 = R_3 = OH$, $R_2 = R_4 = H$

6: $R_1 = R_4 = H$, $R_2 = \beta$-OH, $R_3 = OH$

7: $R_1 = R_3 = OH$, $R_2 = \beta$-OH, $R_4 = H$

8: R = CH_2OH

9: $R_1 = R_4 = OH$, $R_2 = R_3 = H$

10: $R_1 = R_3 = OH$, $R_2 = \alpha$-OH, $R_4 = H$

To identify the degradation products, the incubations were repeated at a larger scale and stopped after 9 and 13 days for *Trametes* and *Phlebiopsis*, respectively. At these times, the yield of products was optimal. After extraction, biotransformation products were separated by SPE and pure fractions were analyzed by NMR and MS. On the basis of the starting compound DHA (**1**), HPLC retention times, LC-DAD spectra, and available literature, the formed metabolites were expected to be DHA with one or more oxidized carbons. To gather as much information as possible from the NMR spectra, for the first time ever the ^{1}H NMR

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10.1021/np060325e CCC: $37.00
Published on Web 01/13/2007

Table 1. ^{1}H NMR Spectroscopic Data for **1**, **2**, **3**, and **5**

	1		**2**	**3**	**5**
position	δ_H (J in Hz)	δ_H	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)
1α	1.38, ddd (13.2, 12.8, 3.5)	1.51	4.00, dd (10.3, 5.4)	3.93, dd (8, 8)	3.87, dd (9, 7)
1β	2.13, dddd (12.8, 3, 3, 1)	2.31			
2α	1.45, ddddd (14, 3.8, 3.5, 3.5, 3)	1.75	1.57, m	1.70−2.05, m	1.75−1.95, m
2β	1.57, ddddd (14, 13.5, 13.2, 3.4, 3)	1.80	1.90, m	1.70−2.05, m	1.75−1.95, m
3α	1.85, ddd (13.5, 13, 3.8)	1.80	1.95, m	2.25, ddd (13.8, 13.8, 4.5)	1.75−1.95, m
3β	1.64, dddd (13, 3.5, 3.4, 1)	1.71	1.72, ddd (13, 3, 3)	1.70−2.05, m	1.62, ddd (15.7, 2.8, 2.8)
5	2.41, dd (12.7, 2.2)	2.24	2.24, dd (12.5, 2.8)	2.49, bd (13)	2.13, bdd (12.4, 2.6)
6α	1.62, dddd (12.9, 7.5, 2.2, 1)	1.55	1.57, m	1.70−2.05, m	1.75−1.95, m
6β	1.79, dddd (12.9, 12.7, 11.6, 6.5)	1.86	1.85, m	1.70−2.05, m	1.75−1.95, m
7α	2.92, ddd (17.2, 11.6, 7.5)	2.93	2.92, m		2.85, m
7β	2.78, ddd (17.2, 6.5, 1)	2.88	2.92, m	4.84, bd (3.2, 1)	2.85, m
11	7.18, d (8.2)	7.18	8.13, d (8.4)	8.27, d (8.4)	8.12, d (8.3)
12	7.08, dd (8.2, 2.0)	7.00	7.03, dd (8.4, 2.0)	7.14, dd (8.4, 2.0)	6.94, dd (8.3, 1.9)
14	6.92, d (2.0)	6.89	6.91, d (2.0)	7.22, d (2.0)	6.83, d (1.9)
15	2.82, septet (7.0)	2.83	2.84, septet (7.0)	2.88, septet, (7.0)	2.81, tq, (7, 7)
16	1.27, d (7.0)	1.23	1.24, d (7.0)	1.23, d (7)	3.58, dd (11, 7)
16′					3.63, dd (11, 7)
17	1.27, d (7.0)	1.23	1.24, d (7.0)	1.23, d (7)	1.21, d (7.0)
19	1.34, s	1.29	1.30, s	1.28,[a] s	1.22, s
20	1.13, s	1.22	1.30, s	1.24,[a] s	1.22, s
solv	C_6D_6	$CDCl_3$	$CDCl_3$	$CDCl_3$	$CDCl_3$-CD_3OD

[a] Author: Please supply the missing footnote.

spectrum of DHA (**1**) was completely assigned including all coupling constants. This was possible only after recording several 2D NMR spectra in $CDCl_3$ and C_6D_6 at 500 and 600 MHz, respectively (Table 1). Subsequently the NMR spectra of all metabolites were compared with the various spectra of the starting material.

The least polar of the four DHA degradation products (t_R 18.0 min) in the *Phlebiopsis* cultures had a MW of 316.2038, i.e., one oxygen atom more than DHA. The ^{1}H NMR (H-1 and H-11 were especially characteristic[21,22]), and ^{13}C NMR data were in perfect accordance with the literature data for 1β-hydroxy-DHA (**2**).[19] This compound has been reported twice before, namely, as a degradation product of DHA by *Fusarium* species[19] and by *Aspergillus niger*.[18]

The next more polar product (t_R 12.1 min) had its M^+ at *m*/*z* 332.1984, i.e., two oxygens more than **1**. The ^{1}H and ^{13}C NMR spectra showed the presence of hydroxyls at C-1 and C-7 (double doublet at δ 3.93 (J = 8 and 8 Hz), a broad doublet (J = 3 Hz) at δ 4.84, and signals at δ 68.2 and 77.7, respectively). This suggested that the hydroxyl at C-1 had the β-configuration similar to **2**. This was proven by a clear cross-peak between H1 and H5 in a NOESY spectrum. The 7-hydroxyl was confirmed by the 5 ppm upfield shift of C-5 and the 9 ppm downfield shift of C-6 relative to **1** and **2**. The stereochemistry of the 7-hydroxyl was α because of the observed small coupling constant[23−28] and the absence of a cross-peak between H5 and H7 in the NOESY spectrum. The NMR data of **3** differ significantly from those of 1β,7β-dihydroxydehydroabietic acid, a biotransformation product from **1** by *Aspergillus niger*.[18] This is the first report of 1β,7α-dihydroxydehydroabietic acid (**3**). The mass and ^{1}H NMR spectra of **3** also revealed the presence of a minor compound. On the basis of the molecular ion at *m*/*z* 330.1827, a fragment at *m*/*z* 253, and characteristic NMR signals at δ 8.23 (d, J = 8 Hz, H-11), 7.85 (d, J = 2 Hz, H-14),[28−30] 7.43 (dd, J = 8 and 2 Hz, H-12), and 4.14 (dd, J = 11 and 5 Hz, H-1) the coeluting minor compound was tentatively identified as 1β-hydroxy-7-oxo-dehydroabietic acid (**4**). Additional evidence for the presence of a 7-oxo-DHA compound was obtained from the UV spectrum, which showed peaks at 214, 257, and 303 nm corresponding to literature data for this chromophore.[30]

The most polar *Phlebiopsis* metabolite (t_R 9.1 min) showed its molecular ion at *m*/*z* 332.19875, suggesting again a dihydroxylated DHA structure. The ^{1}H NMR spectrum with signals at δ 3.9 and a 2H AB pattern at δ 3.6 in combination with only one methyl doublet at δ 1.2 suggested a 1β-hydroxy function and a hydroxyl at C-16. This was confirmed by the ^{13}C NMR spectrum, with a signal at δ 69.0 with two attached protons and only three instead of four methyl groups. This compound was thus identified as 1β,16-dihydroxydehydroabietic acid (**5**). It was first described by Ekman and Sjöholm as a degradation product from DHA by *Fomes annosus*.[21] Our data do not allow a distinction between the almost identical 15*S* and 15*R* stereoisomers. The selective synthesis of methyl (15*S*)- and (15*R*)-16-hydroxydehydroabietate has been reported, but the ^{1}H NMR data of the two isomers were fully identical.[31] When administered to rabbits, **1** was converted stereoselectively to the 15*S* isomer.[31]

The most nonpolar of the three *Trametes* DHA degradation products (t_R 13.6 min) that were isolated in pure form showed its molecular ion peak at *m*/*z* 332.1979, i.e., 32 amu higher than DHA, indicating the presence of two additional oxygens. In the ^{1}H NMR spectrum a 2H multiplet at δ 3.7 and a double doublet (J = 9.4 and 7.2 Hz) at δ 4.90 suggested the hydroxyls to be located at C-16 and C-7. This was confirmed by signals at δ 69.0 (CH_2) and 71.0 (CH) in the ^{13}C NMR spectrum. In contrast to **3**, the 7-hydroxyl group by comparison with literature data was now deduced to possess the 7β configuration.[23−28] Thus, this compound was identified as 7β,16-dihydroxy-DHA (**6**). This compound has been reported before as a DHA biotransformation product of the fungus *Chaetomium cochlioides*.[16]

The next most polar product in the *Trametes* incubation was identical to the most polar product isolated from the *Phlebiopsis* cultures: 1β,16-dihydroxy-DHA (**5**).

The most polar DHA degradation product in the *Trametes* cultures eluted after 4 min in the HPLC system. No clear molecular ion peak could be observed in the EI mass spectrum. Fragments at *m*/*z* 332, 331, and 330 suggested that the molecular weight was 348, i.e., a trihydroxylated DHA. Confirmation was obtained by negative mode LC-ESIMS which showed a pseudomolecular ion peak at *m*/*z* 347. In the NMR spectra two 1H double doublets at δ 3.72 and 4.72 and a 2H multiplet at δ 3.54 together with two CH signals at δ 76.9 and 70.2 and one CH_2 signal at δ 68.7 indicated the presence of three hydroxyl groups at C-1, C-7, and C-16. In the TOCSY spectrum that was recorded by LC-NMR cross-peaks between H7−H5−H6, H1−H2−H3, and H15−H16−H17 confirmed the proposed hydroxylation sites. The coupling constants of H-1 (J = 7.5 and 5.0 Hz) in combination with the diagnostic chemical shift of δ 8.16 (*vide supra*) of H-11 indicated that the 1-hydroxyl possessed the β-configuration. Because of the coupling constants of H-7 (J = 7 and 7 Hz) and the characteristic shift of C-5 at δ 43.4, the 7-hydroxyl was deduced to have the β-config-

Table 2. ^{1}H NMR Spectroscopic Data for **6**, **7**, **8**, **9**, and **10**

	6	**7**	**8**	**9**	**10**
position	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)
1α	1.45, m	3.72, dd (7.5, 5.0)	4.02, dd (9, 6)	3.82, d (10, 5)	3.83, dd (9.7, 5.3)
1*β*	2.30, m				
2α	1.70−1.95, m	1.58, m	1.70−1.90, m	1.75−1.85, m	1.64, ddd (12.8, 3, 3)
2*β*	1.70−1.95, m	1.80, m	1.70−1.90, m	1.75−1.85, m	1.77−1.87, m
3α	1.70−1.95, m	1.80, m	1.70−1.90, m	1.92, m	1.77−1.87, m
3*β*	1.70−1.95, m	1.80, m	1.70−1.90, m	1.63, bd (11)	1.77−1.87, m
5	2.30, m	2.13, dd (12.0, 1.9)	2.55, dd (13.8, 3.9)	2.02, m	2.30, bd (13)
6α	1.70−1.95, m	1.80, m	2.88, dd (18.8, 13.8)	1.46, m	1.46, bd (15)
6*β*	1.70−1.95, m	1.80, m	2.33, dd (18.8, 3.9)	1.46, m	2.21, ddd (15, 13, 5.0)
7α	4.90, dd (9.4, 7.2)	4.72, dd (7, 7)		2.85, m	
7*β*				2.85, m	4.73, bs
11	7.21, d (8.2)	8.16, d (8)[a]	8.15, d (8.2)	8.02, d (8)	8.13, d (8.3)[a]
12	7.12, bd (8.2)	6.97, dd (8, 2)[a]	7.49, d (8.2)	7.15, dd (8, 2)	7.14, dd (8.3, 1.8)[a]
14	7.41, bs	7.24, d (2)[a]	7.70, s	7.08, d (2)	7.17, d (1.8)[a]
15	2.93, m	2.79, tq (7, 7)[a]	2.90, tq (6.7, 6.7)		2.84, tq, 7.0, 7.0)
16	3.70, m	3.54, m[a]	3.57, dd (10.8, 7.2)	1.45, s	3.57, dd (10.9, 7.0)[a]
16′	3.70, m	3.54, m[a]	3.61, dd (10.8, 7.2)		3.63, dd (10.9, 7.0)[a]
17	1.23, d (7.4)	1.16, d (7.0)[a]	1.18, d (6.7)	1.45, s	1.16, d (7.3)
19	1.30, s[a]	1.25, s	1.27, s	1.20, s	1.19, s
20	1.31, s[a]	1.18, s	1.27, s	1.16, s	1.12, s
solv	$CDCl_3$	$CDCl_3$−CD_3OD	MeCN−D_2O	MeCN−D_2O	MeCN−D_2O

[a] Extra signal for second C-15 isomer can be observed.

Table 3. ^{13}C NMR Spectroscopic Data of Compounds **1**−**3** and **5**−**10** (δ_C)

position	**1**	**2**	**3**	**5**	**6**	**7**	**8**	**9**	**10**
1	37.9 CH_2	77.2 CH	77.2 CH	77.1 CH	36.6 CH_2	76.9	75.4	77.5	76.8
2	18.5 CH_2	29.6 CH_2	30.0 CH_2	29.6 CH_2	18.8 CH_2	29.2	28.1	29.0	28.7
3	36.7 CH_2	35.1 CH_2	34.7 CH_2	35.2 CH_2	38.3 CH_2	35.0[a]	34.8	35.5	34.8
4	47.5 qC	47.5 qC	47.3 qC	47.3 qC	47.4 qC	nd[b]	nd	nd	nd
5	44.6 CH	44.7 CH	39.8 CH	44.8 CH	43.8 CH	43.4	43.8	45.0	40.1
6	21.8 CH_2	21.7 CH_2	30.9 CH_2	21.6 CH_2	33.0 CH_2	32.2[a]	37.3	22.0	30.6
7	30.0 CH_2	30.0 CH_2	68.7 CH	29.9 CH_2	71.0 CH	70.2	nd	30.0	67.3
8	134.7 qC	135.5 qC	136.1 qC	135.9 qC	141.7 qC	nd	nd	nd	nd
9	146.8 qC	146.7 qC	146.2 qC	147.5 qC	147.9 qC	nd	nd	nd	nd
10	36.9 qC	43.4 qC	43.8 qC	43.5 qC	38.3 qC	nd	nd	nd	nd
11	124.2 CH	127.5 CH	127.3 CH	127.8 CH	125.0 CH	126.2[c]	126.8	127.7	127.6
12	123.9 CH	124.5 CH	128.1 CH	125.3 CH[c]	127.1 CH	127.2[c]	135.0	122.0	128.6
13	145.8 qC	146.4 qC	147.5 qC	141.3 qC	137.8 qC	nd	nd	nd	nd
14	126.9 CH	127.2 CH	128.3 CH	128.3 CH[c]	127.3 CH	127.9	126.1	125.5	127.6
15	33.5 CH	33.8 CH	33.9 CH	42.2 CH	42.4 CH	42.2	41.5	nd	41.4
16	24.0 CH_3	24.4 CH_3	24.3 CH_3[a]	69.0 CH_2	69.0 CH_2	68.7[c]	67.4	32.0	67.7
17	24.0 CH_3	24.4 CH_3	24.5 CH_3[a]	17.9 CH_3	18.0 CH_3	17.7[c]	17.4	32.0	17.4
18	185.4 qC	185.0 qC	182.3 qC	182.3 qC	183.4 qC	nd	nd	nd	nd
19	16.2 CH_3	16.3 CH_3	16.4 CH_3	16.5 CH_3	16.7 CH_3	16.3	16.0	16.0	16.1
20	25.2 CH_3	18.7 CH_3	18.5 CH_3	18.4 CH_3	25.8 CH_3	19.4	nd	18.0	18.0
solv	$CDCl_3$	$CDCl_3$	$CDCl_3$	$CDCl_3$	$CDCl_3$−MeOH	MeCN−D_2O	MeCN−D_2O	MeCN−D_2O	MeCN−D_2O

[a] Assignment can be interchanged. [b] nd = not detectable in HMQC spectrum. [c] Extra signal for second C-15 isomer can be observed.

uration (*vide supra*). Some signals in the ^{1}H NMR and ^{13}C NMR spectra were duplicated with a minimal chemical shift difference between the two signals, indicating the presence of both the 15*R* and 15*S* stereoisomers. This suggested the oxidation at C-16 to be non-stereospecific. Thus, the structure of this unknown degradation product was formulated as 1*β*,7*β*,16-trihydroxy-DHA (**7**).

Three SPE fractions consisted of mixtures of compounds, and these were investigated by LC-NMR and LC-MS. The most nonpolar mixture was found to consist of **5** and another compound with molecular weight 332. On the basis of 1D and 2D NMR data (Tables 2 and 3) and comparison with literature data,[21,27] it was deduced to be 1*β*,15-dihydroxy-DHA (**9**). This compound was previously isolated and identified as a metabolite of the conversion of **1** by liquid cultures of the wood-degrading fungus *Fomes annosus*.[21]

The most polar mixture consisted of three major compounds. The most polar one was identified as **7**. The ESIMS of the peak at 5.0 min showed a pseudomolecular ion peak at *m*/*z* 347, suggesting that it was an isomer of **7**. By LC-NMR a ^{1}H NMR, a COSY, and an HMQC spectrum could be recorded. The double doublet at *δ* 3.72 and H-11 at *δ* 8.13 confirmed the presence of a 1*β*-hydroxy group, while a broad singlet at *δ* 4.73 and ^{13}C NMR shifts at *δ* 40.1, 30.6, and 67.3 for C-5, C-6, and C-7, respectively, provided proof for the presence of a 7α-hydroxy group. Two 1H double doublets around *δ* 3.6 and a 3H doublet (J = 7.3 Hz) at *δ* 1.16 indicated the presence of the third hydroxyl at C-16. The COSY and HMQC spectra were in accordance with the structure of 1*β*,7α,16-trihydroxy-DHA (**10**), the only difference with **7** being a different stereochemistry at C-7. The peak at t_R = 5.4 min showed, in negative mode in nondeuterated solvents, a pseudomolecular ion peak at *m*/*z* 345 and in deuterated solvents in positive mode a pseudomolecular ion at *m*/*z* 351 (MW = 346), suggesting the presence of either a double bond or a keto function relative to **7** and **10**. The UV was characteristic of a 7-oxo-DHA chromophore. By means of LC-NMR it was possible to record 1D and 2D (TOCSY, HMQC) NMR spectra. H-1 was observed at *δ* 4.02 with coupling constants of 9 and 6 Hz, indicating in combination with H-11 at *δ* 8.15 the presence of a 1*β*-hydroxyl group. Two strongly coupled protons at *δ* 3.6 that showed cross-peaks with signals at *δ* 2.9 and 1.2 in the TOCSY spectrum provided proof for the presence of a hydroxyl at C-16. The HMQC spectrum showing only two OH signals and three aromatic carbons confirmed the earlier

conclusions based on MS and UV data. The characteristic downfield shift of H-14 to δ 7.70[28,30] in combination with a distinctive ABC pattern for H-5, H-6α, and H-6β at δ 2.55, 2.88, and 2.33[28] proved the presence of a keto function at C7. 1β,16-Dihydroxy-7-oxo-DHA (**8**) has not been described as a natural product before.

Figures 1 and 2 (Supporting Information) illustrate the formation of metabolites from the degradation of **1** by *P. gigantea* and *T. versicolor*, respectively, as a function of time. Figure 3 (Supporting Information) shows the possible degradation pathways of **1** by the two fungi evaluated in this study based on studies of their metabolic intermediates. The latter figure lists the names of microorganisms previously reported to produce some of the hydroxylated metabolites detected in our study.

Hydroxylation of **1** at the C-1 position appears to be the first degradation step in *P. gigantea* cultures. Further hydroxylation at the position C-7 or C-16 leads to the formation of the dihydroxylated compound **3** or **5**.

DHA was rapidly hydroxylated by *T. versicolor* at C-1 and C-16 or C-7 and C-16, leading to the dihydroxylated compounds **5** and **6**. These underwent further hydroxylation at C-1 or C-7, yielding the trihydroxylated **7** and **10**. Further oxidation of the 7-hydroxyl group to a carbonyl function leading to **4** and **8** was also observed. An increase in the concentration of **7**, **8**, **10**, and an unidentified degradation product eluting at 6.0 min coincided with a decrease in the concentration of **5** and **6**.

In agreement with the results obtained in this study, existing evidence indicates that hydroxylation is the dominant mechanism of resin acid degradation by fungi.[8,9] Several studies suggest that microbial hydroxylation of diterpenoids might involve P450 monooxygenases.[15,20] Stereoselective hydroxylation of **1** at the C-1 position appeared to be the first degradation step by *P. gigantea* and *T. versicolor*. Initial attack of **1** at C-7 by *Trametes* is also suggested by the detection of the 7β-hydroxylated metabolite **6**. Hydroxylation of **1** at the C-1 position has only been reported in cultures of two different *Fusarium* species[19] and *Aspergillus niger*.[18] The C-7 and/or C-16 hydroxylations produced in the incubation of **1** with *T. versicolor* and *P. gigantea* have also been observed in the biotransformation of **1** by the fungi *Chaetomium cochliodes*,[16] *Fomes annosus*,[21] *Aspergillus flavipes*,[32] *A. niger*,[18] *Whetzelinia sclerotiorum*,[32] and *Mortierella isabellina*.[17] Hydroxylation of **1** at C-7 has also been reported in studies with the aerobic bacteria *Alcaligenes* sp.,[12] *Pseudomonas* sp.[12] and *Pseudomonas abietaniphila*.[15,30] In the latter studies, oxidation at the C-7 position was shown to be first step in the DHA biodegradation pathway. Further oxidation of the hydroxyl group at C-7 to a carbonyl function, as in the degradation products **4** and **8**, has also been observed in studies of the conversion of **1** by the aerobic bacteria *Flavobacterium resinovorum*[11] and *Moraxella* sp. HR6,[20] and in assays investigating the degradation of **1**, abietic acid and pimaric acid by *Pseudomonas abietaniphila*.[10,30] 7-Oxo-DHA has been detected in pulp mill effluents[33] and receiving waters, suggesting that this is a common pathway of degradation.[34]

Experimental Section

General Experimental Procedures. All solvents used for extraction, partitioning, and fractionation purposes were redistilled, with the exception of MeOH and MeCN, which were HPLC grade. Dehydroabietic acid (DHA) was purchased from ICN Biochemicals. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter with a 10 cm 1 mL cell with a sodium lamp in EtOH. UV spectra were recorded by HPLC-DAD. One-dimensional off-line NMR spectra were recorded on a Bruker AC200, Bruker AM-400, or Bruker DRX-600 spectrometer using the residual solvent peaks as internal standard. Two-dimensional off-line spectra (COSY, HMQC, and HMBC) were all recorded at 400 MHz ($CDCl_3$) or 600 MHz (C_6D_6). LC-NMR was carried out with a Bruker LC22 pump, HP 1100 autosampler, DAD detector, BPSU-36 loop storage system, and 500 MHz NMR spectrometer equipped with an LC-NMR probe. The system was controlled by HystarNT software. Off-line EI and CI (positive and negative mode, reaction gas CH_4) mass spectra were recorded on a MAT 95 mass spectrometer. On-line LC-ESI-mass spectra were recorded on a Bruker Esquire-LC ion trap MS. The data were acquired in positive and negative ionization mode under the following conditions: the capillary exit was set to $\pm$60 V while the skimmer 1 was set to $\pm$20 V. The scan range was 100 to 1000 *m/z*. The nebulizer was set to 50 psi, and the dry gas was set to 8 L/min at 300 °C. Solvents were removed at 12−35 mmHg pressure with a Büchi RE 11 rotary evaporator combined with a Vacuubrand CVC2 vacuum pump and Büchi 461 water bath.

Microorganisms and Culture Conditions. *Trametes versicolor* strain LaVec94-6 [CBS 114372] was kindly supplied by Dr. J. A. Field from the Division of Industrial Microbiology, Wageningen University, The Netherlands. *Phlebiopsis gigantea* CBS 429.72 was obtained from the Centraal Bureau voor Schimmelcultures (CBS), Baarn, The Netherlands. Cultures were maintained at 4 °C on glucose and peptone yeast (GPY) extract slants (per liter: 20 g of glucose, 5 g of peptone, 2 g of yeast extract, and 15 g of agar). For inoculation, strains were cultured at 27 °C for 5 days in glucose malt extract (GME) plates (per liter: 10.0 g of glucose, 3.5 g of malt extract, and 15 g of agar). Agar plugs (diameter = 6 mm) from the leading edge were used to inoculate culture flasks that were utilized to grow fungal mycelium for the inoculation of the biodegradation experiment.

The basal culture medium used to grow fungal mycelium contained 2.2 mM N as diammonium tartrate, 56 mM glucose and BIII mineral medium,[35] and 20 mM 2,2-dimethylsuccinate (pH 4.5) buffer. Basal medium (175 mL) was placed in 1000 mL culture flasks. Following sterilization, 2 mg L^{-1} thiamine was added and each flask was inoculated using colonized agar plugs obtained from the GME plates. Flasks were incubated at 27 °C for 10−12 days. Subsequently, the culture medium was decanted under aseptic conditions, and the mycelium mat was washed and resuspended in sterilized water and then dispersed into a slurry by gentle blending (Waring commercial blender).

Microbial Biotransformation. Fungal biotransformation of DHA (**1**) was investigated in stationary batch assays. Experiments were conducted in 100 mL Erlenmeyers supplied with 20 mL of Kirk medium[35] and 100 mg L^{-1} **1**. Kirk medium contains 10 g L^{-1} glucose. A concentrated stock solution of DHA in acetone was prepared, and 80 μL of the stock solution containing 25 mg of DHA mL^{-1} was added to the culture flasks to attain the desired final concentration. Following sterilization, culture flasks were equilibrated at ambient temperature, 2 mg L^{-1} thiamine was added, and each flask was inoculated using 1 mL of mycelium slurry (containing 3.1 mg oven dry weight). Non-inoculated abiotic controls were run in parallel. All assays were performed in triplicate and incubated at 27 °C for up to 12 days. Fungal treatment flasks as well as abiotic control flasks were retrieved after 0, 3, 5, 7, 9, and 12 days for extraction and analysis of DHA and its fungal metabolites. Samples were filtered to remove hyphal growth and analyzed by HPLC/ELSD for DHA and fungal biotransformation products.

Chromatography. Fungal biotransformation of **1** was monitored by high-pressure liquid chromatography with evaporative light scattering detection (HPLC-ELSD). Aqueous samples of filtered medium were acidified with HCl to pH < 2 and then extracted with an equal volume of *tert*-butyl methyl ether in three successive steps. The organic phase was dried with $MgSO_4$, concentrated to dryness in a rotary evaporator, and then dissolved in a solvent containing MeOH−H_2O (60:40) prior the analysis with HPLC-DAD-ELSD using an Alltima C-18 5 μm end-capped reversed-phase column (Alltech Netherlands). The composition of the eluent was increased linearly from MeOH−H_2O−HOAc (60:40:0.1) to MeOH−HOAc (100:0.1) in 30 min. The eluent flow rate was 1 mL min^{-1}, and the temperature of the column was 30 °C.

For LC-NMR investigations, the sample was dissolved in D_2O−MeCN (1:1). The separation was carried out on the same column as described above with a flow rate of 1 mL/min at 35 °C with a mobile phase that consisted of MeCN (A) and D_2O with 0.1% formic acid (B) using the following gradient: 80% B starting condition, then linear gradient to 50% B in 20 min. The diode array detector was operated at a wavelength of 254 nm.

After loop storage, the peaks were transferred into an DRX 500 NMR spectrometer equipped with a 4 mm LC SEI 13C 1H probe head with an active volume of 120 μL from Bruker BioSpin (Rheinstetten, Germany).

^{1}H NMR spectra were acquired using a pulse sequence based on the 1D version of the NOESY sequence, with multiple presaturation suppressing residual water and MeCN signals using a shaped pulse. The second channel was used to selectively decouple the ^{13}C satellites of MeCN. A total of 32K data points were recorded with a sweep width of 10 000 Hz and an acquisition time of 1.64 s. To process the data acquired, a line broadening of 1 Hz was used. Two-dimensional techniques (^{1}H−^{1}H TOCSY and ^{1}H−^{13}C HSQC) were also used for the structure elucidation of the compounds. The parameters for the phase-sensitive (TPPI mode) ^{1}H−^{1}H TOCSY spectra using a multiple WET solvent suppression were as follows: spectral width, 7003 Hz; acquisition time, 0.15 s; relaxation delay, 2 s; spin-locking pulse, 20 ms; and 24 scans for each of the 160 increments. ^{1}H−^{13}C HSQC experiments were acquired using a multiple WET solvent suppression with a spectral width of 6010 Hz in the F_2 (^{1}H) dimension and 25 000 Hz in the F_1 (^{13}C) dimension; acquisition time was 0.09 s, relaxation delay was 2 s, and there were 64 scans per increment (256 increments). Samples were also investigated by negative mode LC-ESIMS under chromatographic conditions identical with those in the LC-NMR measurements.

Large-Scale Incubation. After 9 and 13 days of incubation for *Trametes* and *Phlebiopsis*, respectively, for each fungus 40 Erlenmeyers containing 20 mL of medium were pooled and extracted as described above. The collected degradation products were dissolved in MeOH−H_2O (1:1) and transferred to an SPE column containing 10 g of Bakerbond 40 μm RP-C18 stationary phase. For *Phlebiopsis* the column was eluted with 25 mL portions of MeOH−H_2O in varying ratios starting with (55:45) and ending with 100% MeOH. Each fraction contained 5% more MeOH than the previous one. For *Trametes* the column was eluted with 20 mL portions of MeOH−H_2O in varying ratios starting with 100% H_2O and ending with 100% MeOH. Each fraction contained 5% more MeOH than the previous one. From the 60% MeOH eluent composition onward, two fractions of 20 mL each were collected.

For *Phlebiopsis* three fractions containing DHA degradation products were collected as amorphous solids: the 65% MeOH fraction contained 2.6 mg of pure **5**, the 70% MeOH fraction consisted 2.0 mg of **3** (containing 25% of **4**), and the 75% MeOH fraction contained 7.1 mg of pure **2**. For *Trametes* six fractions with degradation products were collected. The 45% MeOH fraction contained 5.1 mg of pure **7**, the 60% MeOH fraction contained 4.0 mg of **5**, and the 65% MeOH fraction contained 5.2 mg of pure **6**. The fractions containing more than one degradation product were further investigated by LC-NMR and LC-MS.

Dehydroabietic acid (1): t_R (HPLC) 28.5 min; $[\alpha]^{22}_D$ +62 (*c* 0.51, EtOH); UV (MeOH−H_2O−HOAc) λ_{max} 214, 266, 275 nm; ^{1}H NMR and ^{13}C NMR, see Tables 1 and 3, respectively; NMR data are in agreement with those published;[26] EIMS identical to that published by NIST;[36] HREIMS *m*/*z* 300.2087 (calcd for $C_{20}H_{28}O_2$, 300.2089).

1β-Hydroxydehydroabietic acid (2): t_R (HPLC) 18.0 min; $[\alpha]^{22}_D$ +7 (*c* 0.70, EtOH); UV (MeOH−H_2O−HOAc) λ_{max} 215 (sh), 265, 274 nm; ^{1}H NMR and ^{13}C NMR, see Tables 1 and 3, respectively; EIMS *m*/*z* 316 $[M]^+$ (100), 187 (68), 186 (99), 185 (39), 171 (34), 169 (24), 143 (57), 130 (37), 69 (25); HREIMS *m*/*z* 316.2038 (calcd for $C_{20}H_{28}O_3$, 316.2038).

1β,7α-Dihydroxydehydroabietic acid (3): t_R (HPLC) 12.1 min; ^{1}H NMR and ^{13}C NMR, see Tables 1 and 3, respectively; EIMS *m*/*z* 332 $[M]^+$ (100), 314 (42), 289 (20), 209 (31), 201 (68), 200 (52), 185 (39), 159 (57), 143 (56), 130 (29), 169 (100), 155 (53); HREIMS *m*/*z* 332.1984 (calcd for $C_{20}H_{28}O_4$, 332.1988).

1β-Hydroxy-7-oxodehydroabietic acid (4): t_R (HPLC) 12.1 min; UV (MeOH−H_2O−HOAc) λ_{max} 214 (sh), 257, 303 nm; ^{1}H NMR, see text; EIMS *m*/*z* 330 $[M]^+$; HREIMS *m*/*z* 330.1827 (calcd for $C_{20}H_{28}O_4$, 330.1831).

1β,16-Dihydroxydehydroabietic acid (5): t_R (HPLC) 9.1 min; $[\alpha]^{22}_D$ +17 (*c* 0.39, EtOH); UV (MeOH−H_2O−HOAc) λ_{max} 215 (sh), 265, 274 nm; ^{1}H NMR and ^{13}C NMR, see Tables 1 and 3, respectively; EIMS *m*/*z* 332 $[M]^+$ (97), 317 (15), 314 (30), 301 (53), 200 (25), 185 (29), 171 (31), 162 (43), 143 (100), 83 (35); HREIMS *m*/*z* 332.19875 (calcd for $C_{20}H_{28}O_4$, 332.1988).

7β,16-Dihydroxydehydroabietic acid (6): t_R (HPLC) 13.6 min; $[\alpha]^{22}_D$ +22 (*c* 0.51, EtOH); UV (MeOH−H_2O−HOAc) λ_{max} 214 (sh), 265, 274 nm; ^{1}H NMR and ^{13}C NMR, see Tables 2 and 3, respectively; EIMS *m*/*z* 332 $[M]^+$ (34), 314 (61), 301 (100), 284 (87), 283 (43), 281 (47), 253 (46), 235 (35), 195 (25), 169 (35); HREIMS *m*/*z* 332.1979 (calcd for $C_{20}H_{28}O_4$, 332.1988), *m*/*z* 314.1876 (calcd for $C_{20}H_{26}O_3$, 314.1882), *m*/*z* 301.1799 (calcd for $C_{19}H_{25}O_3$, 301.1804).

1β,7β,16-Trihydroxydehydroabietic acid (7): t_R (HPLC) 4.0 min; ^{1}H NMR and ^{13}C NMR, see Tables 2 and 3, respectively; LC-ESIMS (neg. mode) *m*/*z* 695.3 [2M − H], 347.1 [M − H]; EIMS *m*/*z* 332 (73), 331 (82), 313 (35), 301 (42), 300 (45), 227 (59), 226 (64), 209 (42), 208 (39), 195 (88), 183 (78), 169 (100), 155 (53), 131 (38); HREIMS *m*/*z* 331.1886 (calcd for $C_{20}H_{27}O_4$, 331.1909).

1β,16-Dihydroxy-7-oxodehydroabietic acid (8): t_R (HPLC) 5.4 min; UV (MeOH−H_2O−HOAc) λ_{max} 210, 257, 305 nm; ^{1}H NMR and ^{13}C NMR, see Tables 2 and 3, respectively; LC-ESIMS (neg. mode) *m*/*z* 691.3 $[2M - H]^-$, 345.3 $[M - H]^-$.

1β,15-Dihydroxydehydroabietic acid (9): t_R (HPLC) 8.4 min; ^{1}H NMR and ^{13}C NMR, see Tables 2 and 3, respectively; LC-ESIMS (neg. mode) *m*/*z* 663.3 $[2M - H]^-$, 331.3 $[M - H]^-$.

1β,7α,16-Trihydroxydehydroabietic acid (10): t_R (HPLC) 5.0 min; ^{1}H NMR and ^{13}C NMR, see Tables 2 and 3, respectively; LC-ESIMS (neg. mode) *m*/*z* 695.5 $[2M - H]^-$, 347.1 $[M - H]^-$.

Acknowledgment. We wish to thank Dr. J. J. C. Vervoort (Lab. of Biochemistry) for his help in obtaining the LC-NMR data, Dr. P. P. Lankhorst (DSM) for recording the 600 MHz spectra of DHA, Dr. P. de Waard (WnmrC) for recording 500 MHz spectra of DHA and a NOESY measurement, Mr. C. Teunis for MS measurements, and Mr. E. van de Klift for technical support and measuring all optical rotations.

Supporting Information Available: Figures illustrating the formation of metabolites from the degradation of DHA by *P. gigantea* and *T. versicolor* as a function of time. A figure showing the possible degradation pathways of DHA by the two fungi. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP060325E