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Initial Steps in the Pathway for Bacterial Degradation of Two Tetrameric Lignin Model Compounds

JOUNI JOKELA,* JUKKA PELLINEN,† AND MIRJA SALKINOJA-SALONEN
Department of General Microbiology, University of Helsinki, SF 00280 Helsinki, Finland

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We investigated the metabolic route by which a lignin tetramer-degrading mixed bacterial culture degraded two tetrameric lignin model compounds containing β—O—4 and 5—5 biphenyl structures. The α-hydroxyl groups in the propane chain of both phenolic and nonphenolic tetramers were first oxidized symmetrically in two successive steps to give monoketones and diketones. These ketone metabolites were decomposed through $C_\alpha(=O)—C_\beta$ cleavage, forming trimeric carboxyl acids which were further metabolized through another $C_\alpha(=O)—C_\beta$ cleavage. Dehydrodiveratric acid, which resulted from the cleavage of the carbon bonds of the nonphenol tetramer, was demethylated twice. Four metabolites of the phenolic tetramer were purified and identified. All of these were stable compounds in sterile mineral medium, but were readily degraded by lignin tetramer-degrading bacteria along the same pathway as the phenol tetramer. No monoaromatic metabolites accumulated. All metabolites were identified by mass and proton magnetic resonance spectrometry. The metabolic route by which the mixed bacterial culture degraded tetrameric lignin model compounds was different from the route of the main ligninase-catalyzed $C_\alpha—C_\beta$ cleavage by *Phanerochaete chrysosporium*.

Because of the complexity of the lignin macromolecule (1), different types of synthetic lignin substructure model compounds have been used for the study of the mechanism of lignin biodegradation. Model compound research has mostly focused on white rot fungi, especially *Phanerochaete chrysosporium* (8, 10, 13, 19, 34), which are probably the most efficient lignin degraders (4). The mechanism of lignin degradation is best characterized with *P. chrysosporium*, in which it is a radical reaction catalyzed by a number of extracellular enzymes, the major enzyme being lignin peroxidase (9, 21, 28, 33).

Bacteria can decompose lignin and probably can also further degrade the low-molecular-weight degradation components produced from lignin by fungi (3, 11, 12, 20, 22). Thus, understanding of the metabolic pathways and reaction mechanisms of lignin substructure decomposition by bacteria is needed. Dimers with the β—O—4 ether structure have been used to elucidate the decomposition pathway of lignin by bacteria (2, 23, 29) because of the great abundance of this linkage in native lignin (1). Dimeric model compounds containing β—β, β—5, β—1, and 5—5 linkages were also shown to be decomposed by bacteria (6, 17, 25). Despite the better structural analogy of the larger model compounds with the lignin macromolecule, only a few studies have been made with trimeric or tetrameric model compounds (14–16).

In a previous report (14) we demonstrated the ability of a mixed bacterial culture to decompose tetrameric lignin model compounds. Here we report the metabolic pathway by which the β—O—4 and 5—5 linkages containing tetramers were metabolized by the mixed bacterial culture.

MATERIALS AND METHODS

Model compounds. 1,1′-(4,4′-Dihydroxy-5,5′-dimethoxy-3,3′-biphenylene)bis[2-(2-methoxyphenoxy)-1,3-propanediol] (compound A; molecular weight, 638) was synthesized as described by Pew and Connors (26). (4,4′,5,5′-Tetramethoxy-3,3′-biphenylene)bis 2-(2-methoxyphenoxy)-1,3-propanediol (compound B; molecular weight, 666) was prepared from compound A by methylation with CH_3I in dimethyl formamide (32).

The structures of compounds A and B (see Fig. 6) were confirmed by ^1H nuclear magnetic resonance (NMR) and mass spectrometry (for data, see Fig. 3 and 4 and Tables 1 through 3). The purity of compound A was 90%, and that of compound B was 70% (14).

Reference compounds dehydrodiveratric acid dimethyl ester (compound C) and dehydrodivanillic acid (compound D; structures are shown in Fig. 1) were gifts of G. Brunow, Department of Chemistry, University of Helsinki.

Degradation of compounds A and B. Lignin tetramer-degrading (LTD) culture, media, and culture conditions were as described by Jokela et al. (14), with the following modifications. To isolate milligram amounts of the metabolites from compound A, 20 mg of compound A was dissolved in 40 ml of the mineral medium (MM), and the solution was transferred to eight 10-ml culture bottles and inoculated with 0.4 ml of the LTD culture suspension. After 66 h the amount of the metabolites was maximal, as monitored by high-performance liquid chromatography (HPLC) at 280 nm, and the cultivation was then terminated by adding HCl. To dissolve compound B in MM, 150 mg of compound B was first dissolved in 5 ml of acetonitrile and transferred to a 2.5-liter bottle, and the solvent was evaporated in a flow of N_2 overnight so that compound B formed a film on the wall of the lower part of the bottle. MM (1 liter) was then added, and the bottle was agitated for 2 h at 28°C. The medium was then inoculated with 90 ml of the LTD culture suspension that had been precultured on compound A. After 53 days the culture was acidified with HCl.

Degradation of metabolites produced by LTD culture from compounds A and B. Isolated metabolic products A1b, A2, A3, A4, and B4 (see Fig. 2 and 6) were dissolved in $CHCl_3$ and transferred into five separate 2-ml culture bottles. $CHCl_3$ was evaporated for 45 min in a vacuum centrifuge (Savant Instruments, Inc., Hicksville, N.Y.). MM (0.45 ml) was transferred to the bottles, and after shaking for 2 h, 0.05 ml

* Corresponding author.
† Present address: School of Forest Resources, North Carolina State University, Raleigh, NC 27695-8005.

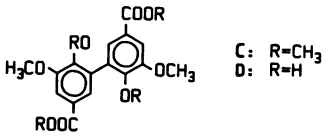

FIG. 1. Structures of the two dimeric reference compounds C and D.

of the LTD culture suspension was added. The degradation of metabolites at 28°C was monitored by HPLC.

Isolation of the metabolites. Metabolites from compounds A and B were isolated by HPLC, as described by Jokela et al. (14). Because of the limited capacity of the HPLC column that was used, the purification involved several successive elutions. The appropriate fractions were pooled, acidified with HCl, and extracted 2 times with 1 ml of ethyl acetate (for compound A) or 2 times with 2 to 5 ml of chloroform (for compound B). The pooled solvents were evaporated under an N_2 flow, and residues (metabolites of B) were dried in vacuo over P_2O_5, KOH, and paraffin chips.

Chromatography. A high-performance liquid chromatograph (Micromeritics Instrument Corp., Norcross, Ga.) with a UV detector was used at 280 nm. Rad-Pak C18 (5 and 10 μm) columns in a compression module (RCM-100; Waters Associates, Inc., Milford, Mass.) were eluted with a concave or a stepwise gradient of 1% (vol/vol) acetic acid in acetonitrile (HPLC grade; Rathburn, Walkerburn, Peeblesshire, Scotland) with 1% (vol/vol) aqueous acetic acid.

Metabolite B1a was analyzed by gas chromatography by using a gas chromatograph (model HP5780; Hewlett-Packard Co., Palo Alto, Calif.) equipped with a capillary column (25 m by 0.32 mm; CP Sil 8 CB; Chrompack, 4330 AA Middelburg, The Netherlands) and a flame ionization detector. Operation conditions was as follows: injector temperature, 270°C; detector temperature, 300 °C; program, 1 min at 60°C, increasing by 20°C min^{-1} to 300°C; carrier gas, H_2.

$NaBH_4$ reduction of metabolite B4. About 0.6 mg of metabolite B4 was dissolved in 0.7 ml of dioxane, and 0.3 ml (2.5 equimolar amount) of freshly prepared $NaBH_4$ solution (0.3 mg of $NaBH_4$ per ml of H_2O) was added. After 23 h at ambient temperature, the reaction mixture was analyzed by HPLC. Compound B, metabolites B3 and B4, and the reaction mixture immediately after $NaBH_4$ addition were used as references.

Derivatization. Isolated compounds were acetylated as described by Pellinen and Salkinoja-Salonen (24), except at the end the sample was washed once with acetonitrile. The reaction mixture was then evaporated in a vacuum centrifuge, and the residue was washed 4 times with toluene and 1 time with acetonitrile.

For mass spectrometry the samples were methylated with an excess of diazomethane reagent (30) in dioxane at the ambient temperature. After a reaction time of 2 to 4 h the solvent was evaporated in a vacuum centrifuge. Reversed-phase HPLC was used to check the formation of the acetyl and methyl derivatives.

Spectrometry. Mass spectra of the metabolites were measured on two different instruments, a Finnigan MAT system 8200 and a Finnigan MAT TSQ-45A GC/MS/MS/DS SYSTEMS with a scanning range of *m/z* 100 to 1,000 and *m/z* 200 to 900, respectively. Underivatized and derivatized samples were ionized by a desorption chemical ionization (DCI) technique (electron energy, 150 eV; emission current, 0.3 mA) by using isobutane as a reactant gas (0.55 torr). Samples were placed on the emitter as a chloroform solution.

^1H NMR spectra were recorded with a spectrometer (200 MHz; Fx-200; JEOL) by using the pulse Fourier mode. Acetylated samples were dissolved in $CDCl_3$ (internal reference, tetramethylsilane) to a concentration of 1 to 5 mg (metabolites A2, A3, A4, and B2) and 15 to 25 mg (compounds A and B and metabolites B3 and B4) in 0.5 ml of solvent.

RESULTS

The HPLC chromatograms from the degradation of compounds A (Fig. 2c and d) and B (Fig. 2a and b) containing five to six major metabolites, respectively, by the LTD culture are shown. Incubation of compound A (40 mg) for 66 h and compound B (150 mg) for 30 days with the LTD culture yielded the following metabolites: A1b (0.1 mg), A1 (0.3 mg), A2 (1.5 mg), A (substrate; 2 mg), A3 (3.5 mg), and A4 (1 mg) from compound A; B1a (0.4 mg), B1 (1 mg), B (substrate; 22 mg), B2 (5 mg), B3 (13 mg), B4 (24 mg), and B5 (2 mg) from compound B.

The tetrameric lignin models used in this study were mixtures of diastereomers. Thus, the metabolites isolated from the culture medium were also diastereoisomeric mixtures. In ^1H NMR spectra this was visible as reduced resolution (see Fig. 4).

The DCI mass spectrometric technique was used to assay the molecular weights of the metabolites, because direct electron ionization was found to completely fragment compound A, resulting in a spectrum with a base peak at *m/z* 109 and a molecular ion at *m/z* 638 (M^+) with a low relative intensity (0.03%). In the DCI mode, the majority of the metabolites and substrates formed the quasimolecular ion MH^+ (Tables 1 and 2), as was expected when isobutane was used as a reactant gas. However, acetylated compound A, metabolite A1b, acetylated metabolite B3, and compound B formed the molecular ion M^+ instead of MH^+.

Mass spectral data of the underivatized metabolites A3 and B3 (Tables 1 and 2 and Fig. 3) show MH^+ (*m/z*) of 637 and 665, respectively, which were only 2 mass units lower than the MH^+s of the parent compounds A and B. Therefore, metabolites A3 and B3 were structurally most closely related to the respective parent compounds. The cleavage of

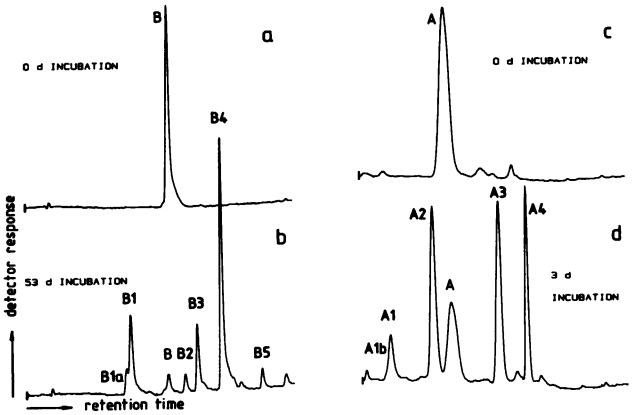

FIG. 2. HPLC analysis of the metabolites formed from compounds B (a and b) and A (c and d) by LTD culture. Elution conditions were as follows: 20-min concave gradient (1.5 ml/min) from 25% (c and d) or 30% (a and b) CH_3CN to 100% CH_3CN with 1% (vol/vol) acetic acid in 1% (vol/vol) aqueous acetic acid.

TABLE 1. DCI mass spectra of underivatized and derivatized compounds of the tetramer compound A and metabolites A1b to A4

Compound or metabolite	Derivative[a]	Molecular ion and the most significant fragment ions (type of molecular ion and relative intensity [%])
A		639 (MH^+; 15), 621 (13), 603 (23), 573 (60), 516 (100), 497 (60)
A	A	890 (M^+, 25), 831 (100), 789 (2), 771 (26), 711 (10), 651 (6)
A1b		486 (M^+, 1), 125 (100)
A1b	A	654 (M^+, 15), 595 (100), 535 (11), 239 (12)
A1b	A+M	668 (M^+, 62), 609 (100), 549 (13)
A2		485 (MH^+, 22), 467 (20), 454 (6), 423 (2), 317 (100)
A2	A	611 (MH^+, 35), 579 (6), 551 (100), 509 (5)
A2	A+M	625 (MH^+, 100), 565 (22), 501 (7), 354 (46), 302 (69), 285 (73)
A3		637 (MH^+, 51), 619 (100), 601 (10), 589 (84), 559 (8), 513 (8)
A3	A	846 (M^+, 17), 787 (100), 745 (9), 727 (87), 667 (7)
A4		635 (MH^+, 45), 617 (95), 599 (42), 587 (38), 467 (44), 449 (100)
A4	A	803 (MH^+, 58), 785 (6), 761 (8), 743 (100), 701 (12), 683 (53)

[a] Abbreviations: A, acetylated compound; A+M, acetylated and methylated compound.

only three aliphatic acetyl groups as acetic acid from acetylated metabolites A3 and B3 (Tables 1 and 2) indicates the loss of one aliphatic hydroxyl group. The ^1H NMR spectra showed the absence of one aliphatic α-proton and a shift of two aromatic protons downfield (7.50 to 7.80 ppm) because of the α-carbonyl group (Fig. 4 and Table 3), which confirms that these compounds are monoketones (see Fig. 6). Metabolites A3 and B3 thus were produced from the parent compounds by dehydrogenation.

The underivatized metabolites A4 and B4 showed MH^+s (*m/z*) of 635 and 663 (Fig. 3), respectively, of which the MH^+s were also near the molecular ions of the parent compounds (MH^+_A = 639, M^+_B = 666) and 2 mass units lower than the MH^+s of metabolites A3 and B3. The formation of the quasimolecular ions $MH^+_{A4\ (acetylated)}$ = 803 and $MH^+_{B4\ (acetylated)}$ = 747 and the cleavage of two aliphatic acetyl groups (Tables 1 and 2) were in accordance with the diketone structure (see Fig. 6). ^1H NMR data confirmed the diketone structure by showing the absence of α-protons and a shift of four aromatic protons downfield (7.60 to 7.85 ppm) because of the two α-carbonyl groups that were formed (Fig. 4 and Table 3). Additional confirmation of the presented diketone structures was obtained by reducing metabolite B4 with $NaBH_4$. HPLC chromatograms showed that immediately after $NaBH_4$ addition, metabolite B4 was partially reduced to metabolite B3 (Fig. 5b and d). After 23 h the amount of metabolite B3 was increased and a part of metabolite B3 was further reduced to compound B (Fig. 5a and e).

Metabolites A1b, A2, and B2 gave molecular ions at *m/z* 486, 485, and 513, respectively. These results suggest that the metabolites are produced from compounds A and B by elimination of a moiety containing one aromatic ring (Tables 1 and 2). Methylation of the acetylated metabolites A1b, A2, and B2 resulted in increasing *m/z* of the molecular ions by 14 mass units, indicating the presence of one carboxyl group in each of metabolites A1b, A2, and B2 (Tables 1 and 2). ^1H NMR data of metabolites A2 and B2 showed the absence of the α-protons (Fig. 4 and Table 3). It is evident from comparison of the structures of the metabolites described above that metabolites A1b, A2, and B2 were formed from metabolites A3, A4, and B4, respectively, through cleavage in the propane chain. No monoaromatic products accumulated to a concentration level that made detection of the products possible.

The mass spectrum of the methylated metabolite mixture B1a-B1 (Fig. 3) showed a base peak at *m/z* 391, which was considered to be also the MH^+ peak based on the behavior of the other nonphenolic metabolites B2 to B4, showing MH^+ as a base peak (Fig. 3 and Table 2). Also, synthetic compound C (Fig. 1) gave a mass spectrum with a MH^+ (*m/z* 391) as a base peak (Table 2). The retention time of 8.24 ± 0.01 min of the methylated metabolite B1 and compound C by RP-18 HPLC (isocratic elution with 60% [vol/vol] acetonitrile with 1% [vol/vol] acetic acid in 1% [vol/vol] aqueous acetic acid at 1.5 ml/min) supported the identity of metabolite B1 as dehydrodiveratric acid. This indicates that metabolite B2 is metabolized by propane chain cleavage, similar to that in the previous step.

When the metabolite B1a-B1 mixture was first acetylated and then methylated, a mass spectrum with two major peaks at *m/z* 447 and 391 was obtained. These peaks were probably molecular ion peaks of metabolites B1a and B1 since there was no fragmentation in the mass spectrum of compound C under the same ionization conditions (Table 2). Because acetylation resulted in an increase of the *m/z* for the molecular ion of metabolite B1a by 56 mass units more than that by methylation, which corresponded to two acetyl groups, it is likely that metabolite B1a contains two hydroxyl groups that are formed by the demethylation of metabolite B1. The retention time of B1a (acetylated and methylated) on gas chromatography was 11.56 min, whereas that of authentic

TABLE 2. DCI mass spectra of underivatized and derivatized compounds of the tetramer compound B, metabolites B1a to B4, and compound C

Compound or metabolite	Derivative[a]	Molecular ion and the most significant fragment ions (type of molecular ion and relative intensity [%])
B		666 (M^+, 37), 649 (55), 631 (28), 601 (100), 571 (26), 498 (14)
B	A	834 (M^+, 61), 775 (100), 715 (80), 697 (3), 655 (32), 595 (4)
B1a-B1	M	391 (MH^+, 100), 359 (6)
B1a-B1	A+M	447 (MH^+) and 391 (MH^+)
B2		513 (MH^+, 100), 495 (75), 345 (11), 299 (6)
B2	A	555 (MH^+, 100), 513 (1), 495 (33), 373 (12), 279 (16)
B2	A+M	569 (MH^+, 100), 509 (51), 387 (78)
B3		665 (MH^+, 100), 647 (83), 617 (65), 587 (8), 496 (22), 329 (14)
B3	A	791 (MH^+, 9), 749 (1), 731 (100), 671 (19), 611 (4), 609 (20)
B4		663 (MH^+, 100), 633 (17), 495 (6), 465 (1), 355 (2), 300 (1)
B4	A	747 (MH^+, 26), 687 (74), 627 (100), 523 (20), 505 (32), 463 (21)
C		391 (MH^+, 100), 359 (1), 331 (1)

[a] Abbreviations: A, acetylated compound; M, methylated compound; A+M, acetylated and methylated compound.

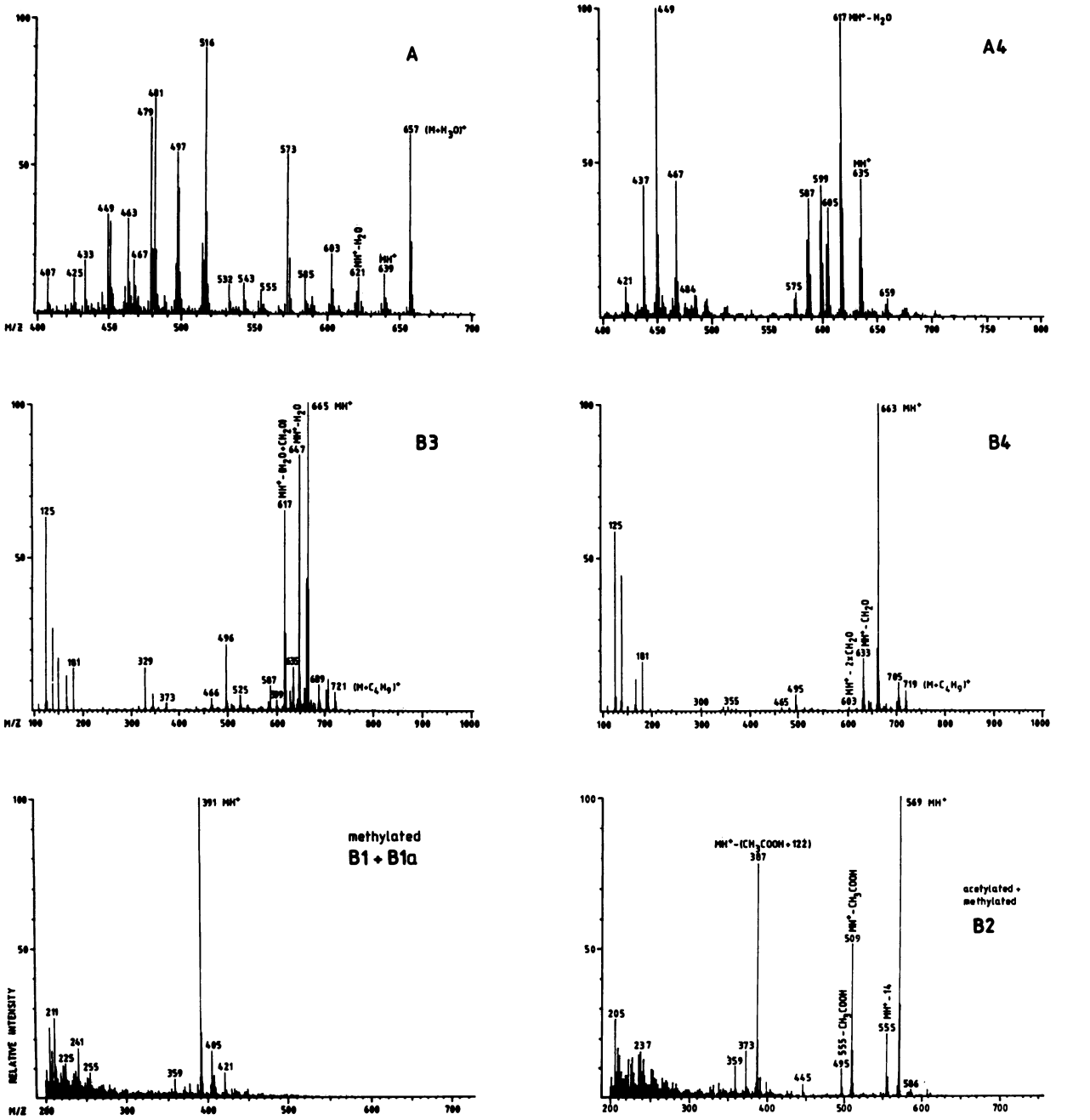

FIG. 3. DCI mass spectra of the underivatized, acetylated, or methylated substrates and metabolites. A small amount of H_2O was present in the ionization of compound A, as seen from the prominent $(M + H_3O)^+$ peak in the spectrum.

compound D (acetylated and methylated) was 11.72 min. Therefore, metabolite B1a (Fig. 6) is not identical to compound D (Fig. 1) but, rather, represents an isomer of it.

Metabolite B1b was isolated from metabolite B1 in trace amounts. ^1H NMR spectrometric characterization of metabolite B1b (Table 3) suggested a phenolic trimer structure (Fig. 6), indicating cleavage at C_α—C_1 of the tetramer compound B.

The role of the LTD bacteria in the metabolic pathway summarized in Fig. 6 was further clarified by refeeding purified metabolites A1b, A2, A3, A4, and B4 as a sole source of carbon to the LTD culture. All these metabolites proved to be stable during 150 h of incubation in MM in the absence of the LTD culture. The inoculated cultures were analyzed by HPLC, and the retention times of the peaks were compared with those of the previously isolated and identified metabolites. It was found that metabolite A4 is transformed into A2 and metabolite A3 is transformed into both metabolites A2 and A4. Metabolites A1b and A2 were degraded without accumulation of any further intermediates. Metabolite B4 was not transformed during the 150 h of incubation. Results from the experiments in which isolated metabolites were used as substrates indicate that every step in the metabolic pathway in Fig. 6 was catalyzed by the LTD

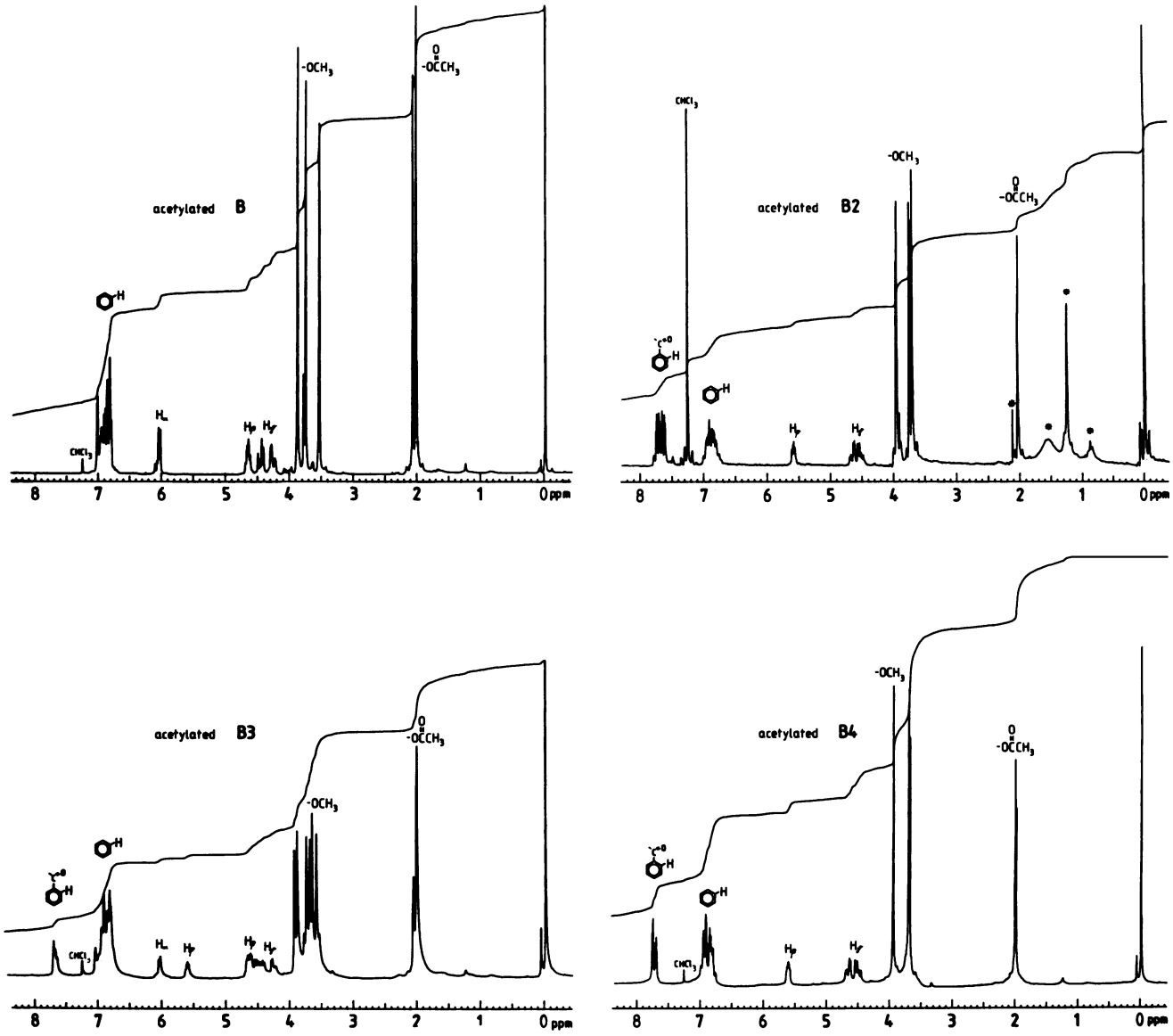

FIG. 4. ^{1}H NMR spectra of the acetylated nonphenolic tetramer compound B and the corresponding acetylated metabolites B2 to B4. The positions of the α, α′, β, β′, γ, and γ′ protons in the molecules are shown in Fig. 6 (compound B). The asterisks indicate impurities.

culture, and that the pathway proceeded via the ketone metabolites which were not metabolized by the reversed reduction reaction back to the starting substrates.

DISCUSSION

The structures of the lignin model tetramer metabolites identified by mass spectrometry, ^{1}H NMR, gas chromatography, and HPLC indicate that both the phenolic (compound A) and the nonphenolic (compound B) tetramers were metabolized by the LTD bacteria through the pathway illustrated in Fig. 6. The molecular weights obtained from the mass spectra of the metabolites are in good accordance with the results from size-exclusion chromatography described by Jokela et al. (14).

At the first stage, both α-hydroxyls of the tetramers were oxidized to carbonyl groups. Analogous oxidation at the α-carbon of dimeric and trimeric β—O—4 model compounds has been described for different species of *Pseudomonas*, *Streptomyces viridosporus*, and *Fusarium solani* M-13-1 (5, 7, 16, 18, 29). *Phanerochaete chrysosporium* has been shown to degrade β—O—4 model compounds primarily without C_α oxidation, although a part of the substrate was C_α oxidized (8). The resulting α-carbonyl compound was not rereduced to the α-hydroxyl compound, suggesting that *P. chrysosporium* degraded these hydroxy and ketone compounds through different pathways.

The α-ketone metabolites A3, A4, and B4 were cleaved at the propane chain by the LTD bacteria. This was deduced from the structures of the downstream metabolites A1b, A2, and B2 (Fig. 6). Similar C_α oxidation of β—O—4 model compounds, followed by C_α—C_β cleavage, has been observed in *Pseudomonas* sp. strain TMY-1009 and *S. viridosporus* (5, 29). Samejima et al. (29) showed that in

TABLE 3. Chemical shifts and amount of protons deduced from the ^1H NMR spectra of the acetylated compounds A and B and the corresponding metabolites A2 to A4 and B1b to B4 formed by the LTD culture

Compound or metabolite	Chemical shifts (ppm [no. of protons]) of:						$J_{\alpha\beta}$ (Hz)
	$\delta_{aromatic\ protons}$	δ_{H_α}	$\delta_{H_{\beta,\beta'}}$	δ_{H_γ}	δ_{OCH_3}	δ_{OCOCH_3}	
A	7.05–6.70 (12H)	6.07 (2H)	4.63 (2H)	4.35 (4H)	3.90–3.65 (12H)	2.15–1.90 (18H)	5.37
A2	7.85–7.55 (2H[a]) 7.05–6.70 (2H)[a]		5.58 (1H)	4.59 (2H)	3.90–3.60 (9H)	2.12, 2.07, 2.03 (9H)	
A3	7.80–7.50 (2H), 7.10–6.55 (10H)	6.05 (1H)	5.58 (1H)	4.70–3.95 (5H[b])	3.90–3.60 (12H)	2.10–1.90 (15H)	
A4	7.85–7.60 (4H), 7.05–6.70 (6–7H)		5.60[c] 2H,	4.57 (3–4H)	3.90–3.60 (12H)	2.10–1.90 (12H)	
B	7.00–6.70 (12H)	6.04 (2H)	4.67 (2H)	4.36 (4H)	3.89, 3.76, 3.55 (18H)	2.08, 2.03 (12H)	5.37
B1b	7.05–6.70 (8H)	6.05 (1H)	4.66 (1H)	4.37 (2H)	3.91, 3.90, 3.77, 3.64, 3.57 (15H)	2.11, 2.08, 2.03 (9H)	5.37
B2	7.80–7.55 (4H), 7.00–6.70 (4H)		5.60 (1H)	4.60 (2H)	3.97, 3.96, 3.75, 3.72, 3.71 (15H)	2.04 (3H)	
B3	7.75–7.55 (2H), 7.05–6.65 (10H)	6.04 (1H)	5.61 (1H), 4.65 (1H)	4.35 (4H)	3.94, 3.89, 3.75, 3.68, 3.66, 3.59 (18H)	2.07, 2.01 (9H)	5.37
B4	7.80–7.65 (4H), 7.05—6.70 (8H)		5.61[c] (2H)	4.58 (4H)	3.95, 3.71, 3.70, 3.69 (18H)	2.02, 2.01 (6H)	

[a] Low intensity (probably an experimental error).
[b] Chemical shifts of $H_{\beta'}$ and $H\gamma$ overlapped.
[c] Poorly resolved peak.

Pseudomonas sp. the cleavage of the β—O—4 linkage preceded the cleavage at C_α—C_β linkage of guaiacylglycerol-β-guaiacyl ether (compound E). The LTD culture may have depolymerized compound A, which is the dehydrodimer of compound E, through a similar pathway, but no metabolite accumulated to show the cleavage of the β-ether linkage or any other reactions preceding the C_α—C_β bond cleavage.

Metabolite B2 was further cleaved at the remaining propane chain, as seen from the structure of metabolite B1. It is evident that metabolite A2 from the phenolic tetramer compound A also was cleaved at C_α—C_β, as was metabolite B2 from the nonphenolic tetramer compound B; but compound D, which was formed from this reaction, did not accumulate in sufficient quantities to be detected.

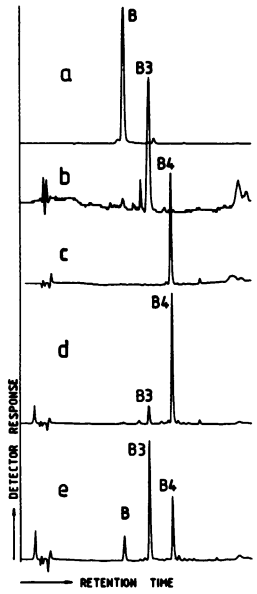

FIG. 5. HPLC analysis of the $NaBH_4$ reduction of metabolite B4. Starting substrate B (a) and isolated metabolites B3 (b) and B4 (c) were used as references. Samples were taken from the reaction mixture immediately after $NaBH_4$ addition (d) and after 23 h of reaction time (e). Elution conditions were as follows: 20-min linear gradient (1.5 ml/min) from 35% CH_3CN to 100% CH_3CN with 1% (vol/vol) acetic acid in 1% (vol/vol) aqueous acetic acid.

The structure obtained for metabolite B1b indicates that compound B was also cleaved at the C_1—C_α bond of the propane chain to some extent. We consider this a side reaction since the amount of metabolite B1b isolated from culture medium was small, and inhibitors which blocked the processing of compounds A and B via ketone metabolites did not cause accumulation of B1b or any other new metabolite (14).

Aromatic hydroxyl groups that are essential for aromatic ring opening were formed after cleavage at the propane chain, as seen from the structure of metabolite B1a in Fig. 6. The two hydroxyls with unknown ring locations were formed via two de-O-methylations. Also, Rast et al. (27) described a C_α—C_β cleavage in the absence of de-O-methylation and without preceding α-oxidation of a nonphenolic dimer, veratrylglycerol-β-phenyl ether, by various gram-positive bacteria. However, the degradation of veratrylglycerol-β-guaiacyl ether by *Streptomyces acidovorans* (4) was different, since side chain cleavage at C_α—C_β linkage took place only after de-O-methylation at the *para* position.

Thus, as demonstrated by the metabolites that were identified, the degradation pathway (Fig. 6) of the nonphenolic β—O—4 lignin model compound by LTD bacteria is unique, because the nonphenolic substrate was α-oxidized before cleavage of the C_α—C_β bond.

The consumption of tetramer compound A continued in extracellular medium, from which the cells were removed by centrifugation, indicating the involvement of extracellular enzymes in the degradation (J. Pellinen, Academic dissertation, University of Helsinki, Finland, 1986). SKF 525 A, a mixed-function oxygenase inhibitor, totally blocked the oxidation of metabolite A3 to metabolite A4 (14). This was unexpected, because SKF 525 A did not block the analogous oxidation of compound A to metabolite A3. This was found

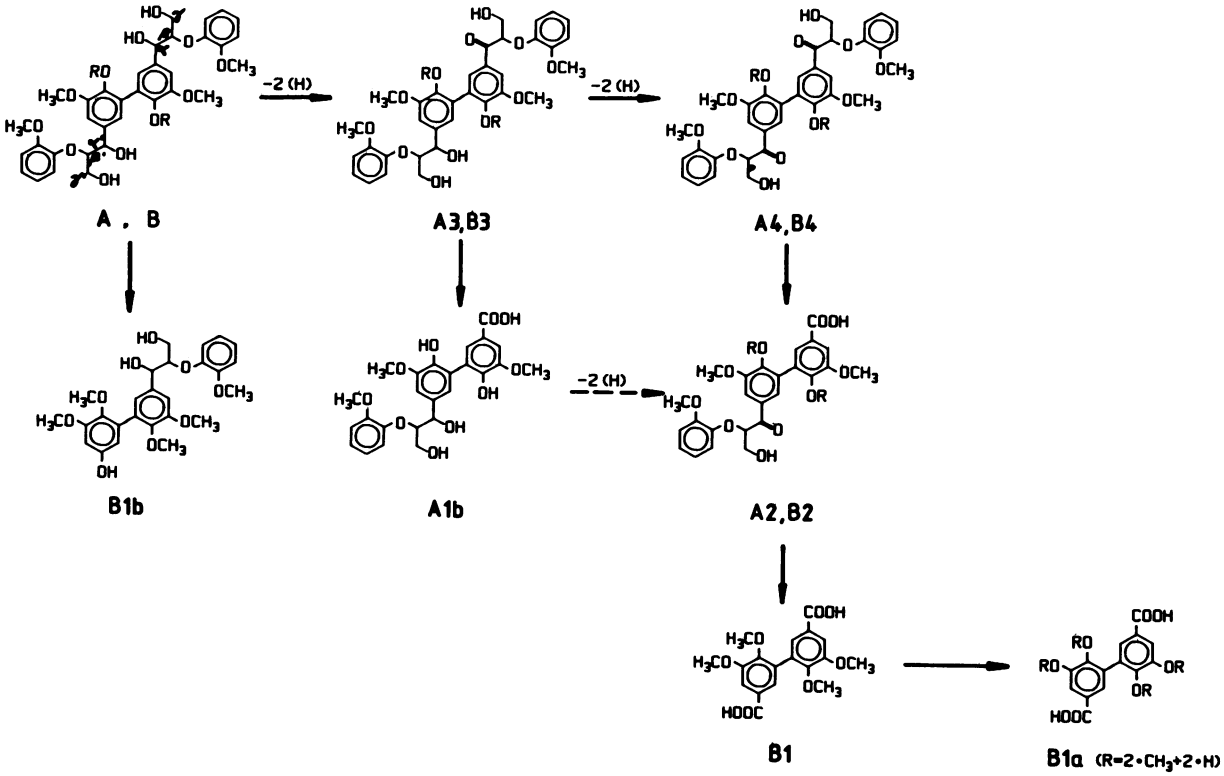

FIG. 6. Proposed metabolic pathway of the tetrameric lignin model compounds A and B preceding the aromatic ring opening. R = H for compound A and the corresponding metabolites A1b to A4, and R = CH_3 for compound B and the corresponding metabolites B1 to B4.

with both the phenolic and nonphenolic tetramers. Mixed-function oxygenases are known as cell-bound enzymes, so if this class of enzymes is responsible for the oxidation of metabolite A3 to metabolite A4, this reaction should take place inside the cell, and thus, the lignin substructure molecule with a molecular weight of 666 g/mol should be capable of penetrating the cell wall. Alternatively, the enzyme catalyzing oxidation of metabolite A3 to metabolite A4 may be a new type of extracellular mixed-function oxygenase. Although it is known that phenol-oxidizing enzymes catalyze α-oxidation of phenolic compounds (31), the participation of the phenol-oxidizing enzymes in the first oxidative reactions of the propane chain is unlikely because these reactions were observed with the nonphenolic tetramer compound B also.

In summary, the enzyme system of the LTD bacteria resembles the ligninase enzyme complex of *P. chrysosporium* with respect to α-oxidation, degradation of nonphenolic lignin model compounds, and cleavage of propane chains at the C_α—C_β linkage. However, the metabolic route depicted in Fig. 6 is different from the route of the main ligninase-catalyzed C_α—C_β cleavage by *P. chrysosporium*, because α-oxidation preceded C_α—C_β cleavage.

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