Microbial Transformations of *p*-Coumaric Acid by *Bacillus megaterium* and *Curvularia lunata*

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p-Coumaric acid (**1**) is an abundant phenolic natural product that exhibits both chemoprotectant and antioxidant properties. Microbial transformation screening studies showed that **1** was converted to 4-vinylphenol (**4**), 4-hydroxybenzoic acid (**2**), caffeic acid (**5**), protocatechuic acid (**6**), and other unidentified metabolites over 144 h. 4-Vinylphenol (**4**) and its dimer, (2*R*,2*S*)-4-(2,3-dihydro-5-hyroxy-2-benzofuranyl) phenol (**11**), were produced by *Bacillus megaterium*, and 5-[(*E*)-2-carboxyethenyl]-2,3-dihydro-2-(4-hydroxyphenyl)-3-benzofurancarboxylic acid (**15**) and 4-hydroxybenzoic acid (**2**) were produced by *Curvularia lunata*. On the basis of deuterium-labeling experiments, *B. megaterium* catalyzes the nonoxidative enzymatic tautomerization of **1** to a vinylogous *β*-keto acid intermediate that decarboxylates to **4**. The presence of peroxidase and laccase activities in *C. lunata* extracts suggests that these enzymes may be involved in one-electron, *p*-coumaric acid dimerization in this organism.

p-Coumaric acid (**1**) is the central intermediate in the biosynthesis of many plant phenols, and it is present in esterified or free acid forms in many fruits, vegetables, and Graminaceous plants.[1,2] Results from our laboratory indicate that it composes approximately 4% of the dry weight of maize plant parts.[3] The large quantities of **1** that are available naturally render it useful as a precursor for the biocatalytic production of value-added aromatic natural products.

Although no systematic screening of microorganisms for the purpose of identifying metabolic products of **1** has been reported, 4-hydroxybenzoic acid (**2**) and protocatechuic acid (**6**),[4−6] 4-vinylphenol (**4**),[7−11] caffeic acid (**5**),[12,13] and 4-ethylphenol[9] have all been identified as metabolites of **1**. *p*-Coumaric acid hydroxylases[12,13] and decarboxylases[7,8,14,15] are involved in the conversions of **1** to **5** and **4**, respectively. *p*-Coumaric acid was examined as a sole carbon source for the growth of fungi and yeasts,[16] but no degradation pathway intermediates were identified.

This paper presents the first comprehensive results of screening bacteria, yeasts, and filamentous fungi for their abilities to transform **1** to a range of products. The mechanism of 4-vinylphenol formation by *Bacillus megaterium* was examined, and preparative scale biotransformation reactions were used to isolate and characterize two new phenolic dimers produced by *B. megaterium* and *Curvularia lunata*.

Results and Discussion

Screening Studies. Microbial transformation screening studies identified cultures that reproducibly metabolized **1** to new products. On the basis of TLC analysis (system A), 21 microorganisms (Table 1) converted **1** to **2**, **4**, **5**, **6**, and other unidentified metabolites (Figure 1). These include oxidative decarboxylation of **1** to **2** by the fungi *Curvularia lunata*, *Memnoniella echinulata*, and *Aspergillus alliaceus* (each within 6 h). 4-Vinylphenol (**4**) was the most commonly observed metabolite of **1**. Five *Bacilli*, *Caldariomyces fumago*, *Candida lipolytica*, *Cylindrocarpon radicicola*, *Penicillium purpurogenum*, and *Rhodotorula rubra* completely metabolized **1** to **4** in addition to other products. Catechol metabolites (**5** or **6**) had the same TLC mobilities and were indistinguishable from each other. Catechols were formed by *A. giganteus*, *C. radicicola*, *Gliocladium deliquescens*, and *Streptomyces griseus*. Caffeic acid (**5**) was isolated and spectrally identified as a metabolite from *S. griseus* (24 h, 5% yield, 16% by HPLC) and *G. deliquescens* (48 h, 6% yield), which also gave **6** (48 h isolated yield, 2%). Most organisms screened gave at least one unknown metabolite from **1** with a range of TLC mobilities and colors when developed plates were sprayed with diazotized sulfanilic acid reagent.

Table 1. Results of Screening Microorganisms for Conversions of *p*-Coumaric Acid (**1**)

species	strain[a]	**2**	**4**	**5/6**	**U**[b]
Aspergillus alliaceus	UI-315	+			
A. giganteus	UI-10			+	
A. niger	UI-X-172	+	+		+
Bacillus lichenformis	UI-A-5A		+		+
B. megaterium	ATCC 14581		+		+
B. pumilus	DRVUI-DRV		+		+
B. subtilis	ATCC 6633		+		
B. subtilis var. niger	IFO-3108		+		+
Caldariomyces fumago	ATCC 16373	+	+		
Candida lipolytica	UI-MR−Can		+		+
Curvularia lunata	NRRL 2178	+			+
Cylindrocarpon radicicola	ATCC 11011		+	+	
Gliocladium deliquescens	NRRL 1086			+	+
Memnoniella echinulata	UI-MR-188	+			
Nocardia sp.	NRRL 5646				+
Penicillium notatum	ATCC 36740	+			+
P. purpurogenum	UI-MR-193				+
P. purpurogenum	ATCC 9777		+		+
Rhodotorula rubra	ATCC 20129		+		
Streptomyces griseolus	ATCC 11796				+
S. griseus	UI-L-103			+	

[a] ATCC, American Type Culture Collection, Rockville, MD; NRRL, Northern Regional Research Laboratories, Peoria, IL; IFO, Institute for Fermentation, Osaka, Japan; UI, University of Iowa, College of Pharmacy, Iowa City, IA. [b] Unknown products by TLC.

Isolation and Characterization of 4-Vinylphenol. Initial screening results were confirmed and showed that **1** was transformed by *B. megaterium* to **4** as the sole metabolite within 6 h, and then at 24 h, three new products were observed at R_f 0.55 (brown), R_f 0.45 (orange), and at

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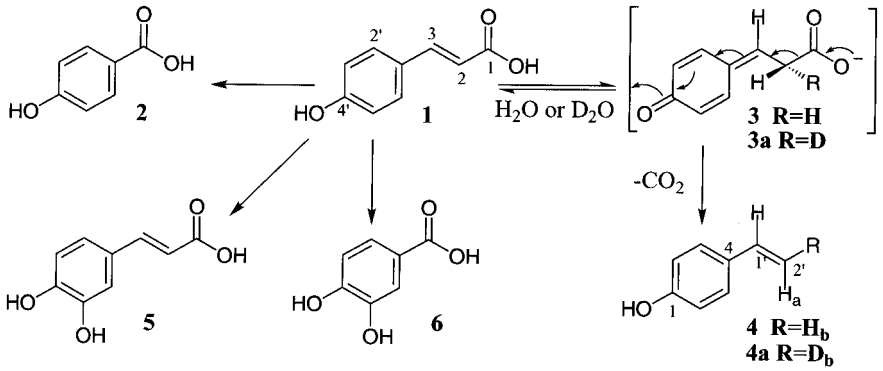

Figure 1. Compounds formed by whole cell biotransformations of *p*-coumaric acid (**1**) in the screening study and by *B. megaterium* cultures incubated with *p*-coumaric acid in solutions containing D_2O.

Table 2. GC/MS and ^1H NMR Results of **4** Isolated from Cultures of *B. megaterium* Incubated with **1** in H_2O, H_2O-D_2O, and D_2O

D_2O (v/v %)	MS % relative intensity *m/z* 120	121	122	^1H NMR relative intensity for H_b-2′ (δ 5.03)
0	100	5.4	0.2	1.00
50	100	39	3.1	0.71
100	47	100	8.7	0.24

R_f 0.38 (orange). When 4-vinylphenol (**4**) was incubated in uninoculated medium as a control, products at R_f 0.45 and R_f 0.38 were observed as nonenzymatically formed artifacts. 4-Vinylphenol (**4**) was isolated from preparative-scale cultures 24 h after substrate addition. Cells were discarded when TLC analysis of acetone extracts showed only traces of substrate or product. The supernatant afforded a brown oil that gave **4** as an off-white powder (13% yield) after silica gel column chromatography. The product was chromatographically (TLC system A) and spectrally (^1H NMR) identical to standard **4**.

Conversion of 1 to 4 and 4a in D_2O. The conversion mechanism of **1** to **4** by *B. megaterium* was examined in incubations containing H_2O, H_2O-D_2O (1:1, v/v), or D_2O. MS and NMR analyses (Table 2) of unlabeled **4** show *m/z* 120 for the molecular ion C_8H_8O ($[M]^+$), and **4a** shows *m/z* 121 for C_8H_7DO ($[M]^+$). GC/MS results (Table 2) show 34% and 67% deuterium incorporations into **4a** for the H_2O-D_2O (1:1, v/v) and D_2O incubations, respectively. Deuterium incorporations were less than 100% in pure D_2O likely due to dilution by H_2O in cells. ^1H NMR (Figure 2, Table 2) analysis indicated that the ^1H signal for H_b-2′ at δ 5.0 decreased as the concentration of D_2O in the incubation increased. In deuterated **4a**, a second doublet at δ 5.6 was observed δ 0.01 upfield from the signal for H_a-2′ of **4**. This signal was in proportion to the amount of deuterium incorporated into **4**. Furthermore, the signal for H-1′ became a broad doublet (J = 17.7 Hz), indicating coupling with H_a-2′ but loss of coupling with H_b-2′. These results indicated the specific incorporation of a single deuterium atom into **4a** at position H_b-2′. ^1H NMR analysis indicated deuterium incorporations of 29% and 76% for H_2O-D_2O (1:1, v/v) and 100% D_2O incubations, respectively.

The results of the D_2O-incorporation experiments suggest a nonoxidative decarboxylation mechanism for the formation of **4** by *B. megaterium* (Figure 1). Enzymatic tautomerization of **1** would give a doubly vinylogous β-keto acid, which nonenzymatically decarboxylates to **4**. An alternative mechanism for forming **4a**, involving enzymatic nucleophilic attack at C-3 of **1**[11] and subsequent decarboxylation, cannot be differentiated by these experiments.

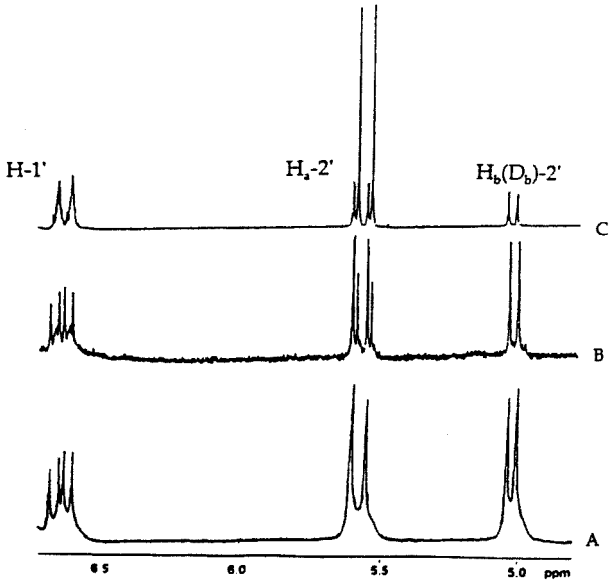

Figure 2. ^1H NMR spectra of selected protons of 4-vinylphenol (**4**) isolated from *B. megaterium* decarboxylation reactions conducted in 0.1 M KH_2PO_4 prepared with (A) H_2O, (B) H_2O-D_2O (1:1, v/v), and (C) D_2O.

Table 3. NMR Data (methanol-d_4) for (2*R*,2*S*)-4-(2,3-Dihydro-5-hydroxy-2-benzofuranyl)phenol (**11**)

position	^1H (δ, J in Hz)	^{13}C (δ)	HMBC correlations (C#)
1′		158.3	
2′	6.76 (d, 8.6)	116.1	1′, 2′, 4′, 6′
3′	7.21 (d, 8.4)	128.4	2, 1′, 2′, 3′, 4′, 5′, 6′
4′		134.3	
5′	7.21 (d, 8.4)	128.4	2, 1′, 2′, 3′, 4′, 5′, 6′
6′	6.76 (d, 8.6)	116.1	1′, 2′, 4′, 6′
2	5.58 (dd, 8.8, 8.8)	85.6	3, 7a, 3′, 4′, 5′
3 (H_a-3)	3.47 (dd, 9.1,15.7)	39.8	2, 3a, 4, 7a, 4′
3 (H_b-3)	3.08 (dd, 8.5,15.7)	39.8	2, 3a, 4, 7a, 4′
3a		129.0	
4	6.66 (br d, 2.4)	113.1	3a, 5, 6, 7a
5		152.4	
6	6.54 (dd, 2.5,8.0)	115.1	4, 5, 7a
7	6.57 (d, 8.5)	109.9	3a, 5, 7a
7a		154.2	

Isolation and Characterization of a 4-Vinylphenol Dimer (11). The R_f 0.55 product (**11**, 5% yield by TLC, system A) was isolated from culture supernatants of *B. megaterium* 24 h after substrate addition. HREIMS gave an exact mass of 228.0802 for $C_{14}H_{12}O_3$, indicating a product formed by coupling of **4** with a second six-carbon moiety. By ^1H NMR (Table 3) an AB system of doublets at δ 7.21 and 6.76 indicated a *p*-substituted aromatic ring, while a *m*-substituted aromatic ring gave *o*-coupling of the

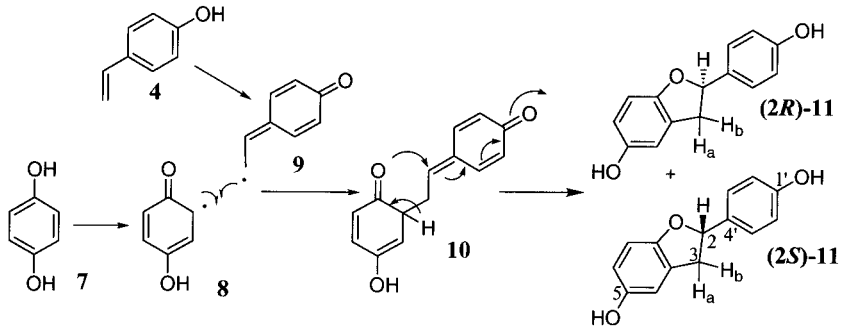

Figure 3. Conversion of 4-vinylphenol (**4**) and hydroquinone (**7**) by whole cell cultures of *B. megaterium* to the modified dimer of 4-vinylphenol (**11**).

^{1}H signal at δ 6.54 (dd) with that at δ 6.57 and *m*-coupling with that at δ 6.66. A pair of double doublets at δ 3.47 and 3.08 indicated coupling between geminal methylene protons (J = 15.7 Hz) that were vicinally coupled with a ^{1}H signal at δ 5.58 (dd, J = 8.8, 8.8 Hz). ^{13}C NMR indicated that among 12 aromatic carbons, three at δ 158.3 (C-1′), 154.2 (C-7a), and 152.4 (C-5) were oxygen-substituted, two at δ 134.3 (C-4′) and 129.0 (C-3a) were carbon-substituted, and the remaining aromatic carbons were methines. ^{13}C signals at δ 128.4 (C-3′, C-5′) and 116.1 (C-2′, C-6′) corresponded to carbons of the *p*-substituted aromatic ring, while those at δ 115.1 (C-6), 113.1 (C-4), and 109.9 (C-7) composed the *m*-substituted ring. A nonaromatic oxygenated carbon at δ 85.6 (C-2) and a methylene carbon at δ 39.8 (C-3) were also detected. ^{1}H and ^{13}C NMR showed that the vinyl group of **4** was absent in the metabolite and that it contained a 2,3-dihydrobenzofuran ring.[17,18] Key HMBC data (Table 3) supported the presence of the 2,3-dihydrobenzofuran structure with substitution at positions 2 and 5. NMR analysis confirmed the structure of **11** as 4-(2,3-dihydro-5-hydroxy-2-benzofuranyl)phenol, a previously unknown compound. The specific optical rotation of 0° for **11** showed that the dimer was a (±)-(2*R*,2*S*)-racemate.

On the basis of its structure, **11** can form by a 1:1 dimerization of **4** and hydroquinone (**7**) (Figure 3). The origin of hydroquinone (**7**) in the biotransformation product **11** is unknown. It is possible that **7** is derived by vinyl-group elimination from **4**,[19] or it may be a biosynthetic product of *B. megaterium*. Similar oxidative couplings have been proposed for the dimerization of ferulic acid.[20] The final aromatization step is accompanied by 2,3-dihydrobenzofuran ring formation giving a 1:1 racemate of (2*R*)- and (2*S*)-(**11**). The dimer **11** was prepared biomimetically by $K_3Fe(CN)_6$ oxidation of a mixture of 4-vinylphenol (**4**) and hydroquinone (**7**) in chloroform and water.[21] Pure **11** gave HREIMS and ^{1}H NMR data identical to those for **11** isolated from *B. megaterium*. The specific optical rotation of 0° indicated that synthetic **11** was also a (±)-(2*R*,2*S*)-racemate.

Proposed initial oxidative steps of such a pathway are supported by the $K_3Fe(CN)_6$-mediated synthesis of **11**.[20,22] The exact enzymatic process by which *B. megaterium* catalyzes the oxidation reaction remains unknown. *B. megaterium* contains a well-known cytochrome P450 enzyme system that could be capable of catalyzing such a process.[23]

Metabolism of 1 by *C. lunata*. *C. lunata* transformed **1** to a new metabolite (**15**) (17% yield) and **2** (10% yield) within 6 h. A preparative-scale *C. lunata* culture gave 31 mg of **2** and 110 mg of **15** from culture filtrates. The structure of **2** as 4-hydroxybenzoic acid was confirmed by TLC (system A, R_f 0.42), and ^{1}H NMR and EIMS comparison with data for authentic **2**.

Table 4. NMR Data (methanol-d_4) for 5-[(*E*)-2-Carboxyethenyl]-2,3-dihydro-2-(4-hydroxyphenyl)-3-benzofurancarboxylic Acid (**15**) Isolated from *Curvularia lunata*

			correlations	
position	^{1}H (δ, J in Hz)	^{13}C (δ)	HMBC (C#)	NOESY (H#)
2	5.99 (d, 7.3)	89.0	3, 7a, 1′, 2′, 6′, 1″	3, 2′, 6′
3	4.26 (d, 7.3)	57.9	2, 3a, 4, 7a, 1′	2, 4, 2′, 6′
3a		128.6		
4	7.66 (br d)	125.4	3, 6, 7a, 1″	3, 2″
5		128.6		
6	7.51 (dd, 1.5,8.4)	131.2	4, 7a, 1″	7, 1″, 2″
7	6.88 (d, 8.4)	109.3	3a, 5, 7a	6
7a		162.4		
1′		132.8		
2′	7.21 (d, 8.4)	128.0	2, 2′, 3′, 4′, 5′, 6′	2, 3, 3′
3′	6.79 (d, 8.6)	116.0	1′, 3′, 4′, 5′	2, 2′
4′		158.4		
5′	6.79 (d, 8.6)	116.0	1′, 3′, 4′, 5′	2, 6′
6′	7.21 (d, 8.4)	128.0	2, 2′, 3′, 4′, 5′, 6′	2, 5′
1″	7.65 (d, 15.7)	146.0	4, 6, 2″, 3″	6, 2″
2″	6.34 (d, 15.7)	116.1	5, 3″	4, 6, 1″
3″		171.0		
1‴		175.5		

Although **15** was mentioned once as a synthetic analogue of a product from barley, no structural data have ever been given.[24] Negative-ion HRFABMS gave $C_{18}H_{13}O_6$ (*m/z* 325.0723), indicating a metabolite mass approximately twice that of **1**. ^{1}H NMR (Table 4) supported a three-ring structure similar to that of **11**. One *p*-disubstituted aromatic ring was indicated by an AB system (*o*-coupled doublets at δ 7.21 and 6.79), and a *m*-substituted aromatic ring was indicated by an ABX system, for which the ^{1}H signal at δ 7.51 (dd) was *o*-coupled to that at δ 6.88 (d) and *m*-coupled to that at δ 7.66 (d). *trans*-Coupling of signals at δ 7.65 (d, J = 15.7 Hz) and 6.34 (d, J = 15.7 Hz) identified an *E*-substituted vinyl group. Vicinal protons at δ 5.99 (d, J = 7.3 Hz) and 4.26 (d, J = 7.3 Hz) provided strong evidence for a 2,3-dihydrobenzofuran ring.[20,25−27] ^{13}C NMR signals (Table 4) at δ 175.5 (C-1‴) and 171.0 (C-3″) confirmed the presence of two carboxylic acid groups. Signals at δ 162.4 (C-7a) and 158.4 (C-4′) showed two oxygen-substituted aromatics and three carbon-substituted aromatics δ 132.8 (C-1′), 128.6 (for C-3a and C-5), and the remaining aromatic carbons were methines. Signals at δ 128.0 (C-2′, C-6′) and 116.0 (C-3′, C-5′) indicated a *p*-substituted aromatic ring, while those at δ 109.3 (C-7), 125.4 (C-4), and 131.2 (C-6) composed the aromatic ring with three substitutions. Carbons of an acrylic acid side chain were identified by signals at δ 171.0 (C-3″), 146.0 (C-1″), and 116.1 (C-2″), and two methine carbons at δ 57.9 (C-3) and 89.0 (C-2) were also observed. Key HMBC and

NOESY data (Table 4) supported the presence of the 2,3-dihydrobenzofuran structure with substitution at positions 2, 3, and 5. The ^{1}H signals for H-2 and H-3 and ^{13}C signals for C-2 and C-3 were very similar to those for the dehydrodimers of ferulic acid[25,26] and the methyl ester of **1**,[27] confirming the presence of the 2,3-dihydrobenzofuran ring and the phenol substitution at C-2. Therefore, the structure of the R_f 0.30 product was 5-[(*E*)-2-carboxyethenyl]-2,3-dihydro-2-(4-hydroxyphenyl)-3-benzofurancarboxylic acid (**15**).

The relative configurations of 2,3-dihydrobenzofurans about H-2 and H-3 can be determined by ^{1}H NMR. In the *cis*-configuration, H-2 is shifted downfield by approximately 0.5 ppm vs *trans* H-2.[28] $K_3Fe(CN)_6$-mediated phenol coupling of the methyl ester of **1** gave the (±)-*trans* dimethyl ester of **15**.[29] ^{1}H NMR and HRFABMS confirmed that our synthetic dimer and **15** isolated from *C. lunata* were identical. While the specific optical rotation of 0° indicated that synthetic **15** was a racemate of (2*R*,3*R*)/(2*S*,3*S*)-*trans* diastereomers, **15** isolated from *C. lunata* gave a specific rotation of +3.8°, indicating a degree of stereospecificity in the biotransformation reaction. Compound **15** is very similar in structure to dimers of *p*-coumaryl alcohols and ferulic acid formed in lignin and lignans.[25,30] All of the 2,3-dihydrobenzofuran phenol dimers formed by in vitro oxidative phenol couplings by peroxidases or chemical oxidations have the (±)-*trans* relative configuration between H-2 and H-3.[17,20,25,27,30,31] However, lignans are formed in vivo by stereospecific phenol coupling reactions,[30] as in (+)-geodin from *Aspergillus terreus*.[32] Conclusive determination of the absolute configuration of **15** will require X-ray crystallography, which has successfully proven the absolute configuration of other phenolic 2,3-dihydrobenzofuran dimers, including that of dehydrodiferulate.[20,29,33]

***C. lunata* Enzymes.** Assays were conducted with culture filtrates and cell-free extracts of *C. lunata* for laccase and peroxidase activities. Laccases are copper oxidases that oxidize phenols through the reduction of molecular O_2 to H_2O by single electron transfer reactions. Peroxidases also oxidize phenols by H_2O_2-dependent one-electron oxidation. Oxidation of syringaldazine indicates laccase activity.[34] *C. lunata* culture filtrates rapidly oxidized syringaldazine to the deeply purple syringaldazine quinone,[34] while cell-free extracts were only weakly positive for laccase. Peroxidase activity was present in both the cell-free extract (35 nmol/min) and culture filtrates (23 nmol/min), demonstrating that either laccase or peroxidase could catalyze the dimerization of **1** to **15**.

The presence of laccase[35] and peroxidase[36] activities in *C. lunata* and the $K_3Fe(CN)_6$ -mediated synthesis of **15** from **1** support an initial radical-based coupling process (Figure 4) involving phenoxy radicals (**12**), quinone methide, and semiquinone radicals (**13**) that couple to give a mixture of (*R*)- and (*S*)-**14**. Subsequent cyclization would give racemic *trans*-substituted dihydrobenzofurans **15**.[20]

Microbial transformation of *p*-coumaric acid (**1**) afforded 4-hydroxybenzoic acid (**2**), 4-vinylphenol (**4**), caffeic acid (**5**), protocatechuic acid (**6**), and other products by several microorganisms for which these conversions had not been previously reported. Additionally, dimers **11** and **15** from *B. megaterium* and *C. lunata* are new and previously unidentified biotransformation products. The isolation of **11** and **15** from microorganisms demonstrates the microbial formation of phenolic acid dimers similar to those found in plant cell walls, lignin, and lignans. Only two dimers of **1**, both of different structures from **15**, have been previously characterized. These include the photodimer 4,4′-dihydroxytruxillic acid and its cyclobutane stereoisomers (from various grass cell walls),[2] and 4-hydroxy-3-(4′-cinnamyloxy)cinnamic acid, the product of *p*-coumaric acid coupling by an extracellular laccase from the basidiomycete *Trametes versicolor*.[37] Further investigation into the enzymes involved in the biotransformation of *p*-coumaric acid by *C. lunata* would be interesting, especially since the product is not a racemate.

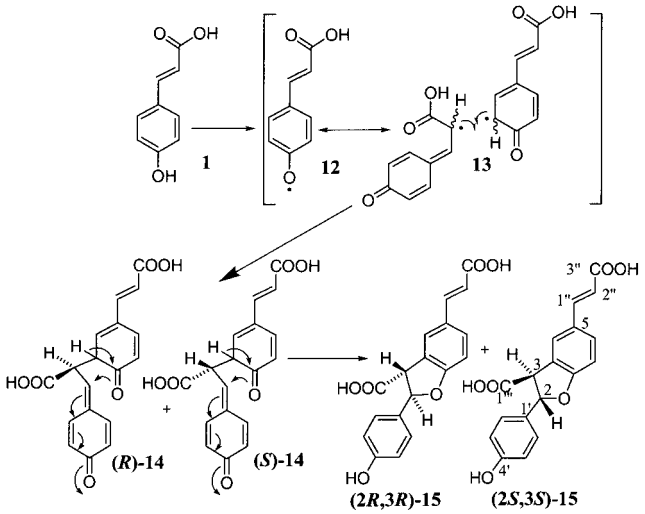

Figure 4. Proposed mechanism for the formation of the *p*-coumaric acid dimer (**15**) from *p*-coumaric acid (**1**) by whole cell cultures of *C. lunata*.

Experimental Section

General Experimental Procedures. One-dimensional ^{1}H and ^{13}C NMR spectra were recorded with a Bruker WM-360 MHz, a Bruker DRX-400 MHz, or a Bruker AMX-600 MHz instrument (Karlsruhe, Germany). Samples were analyzed by heteronuclear multiple bond correlation (HMBC) or by nuclear Overhauser effect spectroscopy (NOESY) experiments by the Bruker AMX-600 spectrometer. Chemical shifts are recorded in δ value (ppm) downfield from TMS. Coupling constants (*J* values) are recorded in hertz. Electron-impact mass spectrometry (EIMS) was performed with either a Trio-1 mass spectrometer (VG Analytical, Manchester, England) or a Voyager mass spectrometer (ThermoQuest, Manchester, England). For direct inlet probe mass spectrometry (DIPMS), the ionization temperature of the probe was held at 30 °C for 1 min and increased linearly over 2 min to 320 °C. Gas chromatography−mass spectrometry (GC/MS) was carried out by the Trio-1 mass spectrometer at 70 eV connected to a Hewlett-Packard 5890 A gas chromatograph (Palo Alto, CA) using a DB-1 methylsilicone column (15 × 0.32 mm i.d., J & W Scientific, Folsom, CA). The initial analysis temperature of 50 °C was increased at a rate of 20 °C/min to 250 °C and held at this temperature. Negative ion fast atom bombardment mass spectrometry (FABMS) was carried out with a ZAB-HF mass spectrometer (VG Analytical, Manchester, England). The ionizing matrix was triethanolamine.

Caffeic acid (**5**), *p*-coumaric acid (**1**), deuterium oxide, 4-hydroxybenzoic acid (**2**), potassium(III) ferricyanide ($K_3Fe(CN)_6$), protocatechuic acid (**6**), pyrogallol, syringaldazine, and tetramethylsilane (TMS) were purchased from Aldrich (Milwaukee, WI). Phenylmethylsulfonylfluoride (PMSF) was obtained from Amresco (Solon, OH), dithiothreitol (DTT) was purchased from GIBCO Laboratories (Grand Island, NJ), and NMR solvents were purchased from Isotec, Inc. (Miamisburg, OH). 4-Vinylphenol [**4**, a 10% solution (w/w in propylene glycol)] was purchased from Lancaster, Inc. (Windham, NH), and 100% EtOH was obtained from Pharmco Products (Brookfield, CT). All other chemicals were purchased from Fisher Chemicals (Fair Lawn, NJ).

Compounds were separated by thin-layer chromatography (TLC) on either Merck silica gel GF_{254} spread over glass plates with a Quickfit Industries (London, England) spreader (0.25 mm thickness, activated at 120 °C for 20 min) or aluminum foil-backed (Alltech) Kieselgel 60 F_{254} plates. Plates were developed by one of the following systems (v/v): (A) CH_2Cl_2−MeOH−HCOOH (95:5:0.5), (B) C_6H_{14}−EtOAc (1:1), (C) CH_2Cl_2−MeOH−HCOOH (93:7:0.5). Compounds were visualized by viewing the plates at 254 and 366 nm and by spraying with diazotized sulfanilic acid reagent, which was prepared by mixing (1:1, v/v) solution A [0.5% (w/v) sulfanilic acid in 2% HCl] with solution B [0.5% (w/v) $NaNO_2$ in H_2O] and heating the plate, followed by spraying with solution C [5% NaOH in 50% EtOH] and heating. Optical rotations were measured with a JASCO P-1020 polarimeter (Kyoto, Japan), and UV spectra were obtained with a UV-2101PC scanning spectrophotometer (Shimadzu). Melting points were obtained with a Mel-Temp apparatus.

Microbial cells were broken by an Aminco French Pressure Cell (American Instrument Co., Silver Spring, MD). Whole and homogenized cells were centrifuged with either a Sorvall RC-5 superspeed refrigerated centrifuge (Dupont Instruments, Newton, CT), an Eppendorf 5415C microcentrifuge (Germany), or an L8-55 preparative ultracentrifuge (Beckman Instruments, Palo Alto, CA).

For HPLC, a Shimadzu LC-6A dual pumping system was connected to a Shimadzu SPD-6AV UV/vis detector. Samples were introduced onto the column (Versapack C18 10μ, 250 mm × 4.6 mm, Alltech, Deerfield, IL) through a Shimadzu 7161 injection port. Chromatograms were recorded and processed by the Shimadzu ClassVP program. Chemicals were diluted in Optima grade MeOH and injected in triplicate. *C. lunata* culture samples were centrifuged at 14 000 rpm for 1 min in an Eppendorf centrifuge. The supernatant was passed through a 0.45 mm filter, diluted 1:1 (v/v) with Optima grade MeOH, and injected in triplicate (20 μL each). Controls included cultures without substrate and substrate added to uninoculated medium. Standard curves were constructed by injecting in triplicate at least three concentrations of each compound. Culture samples were eluted isocratically with MeOH−2% HCOOH, 1:1 (v/v), at 1 mL/min (265 nm). Retention volumes (R_v, mL) of eluted compounds were 5.0 for 4-hydroxybenzoic acid (**2**), 6.4 for **1**, and 16.4 for **15**.

Microorganisms. All microorganisms were maintained on Sabouraud maltose agar except *G. deliquescens* (Czapek's agar, American Type Culture Collection medium no. 312). All cultures were stored at 4 °C prior to use. Fifty-two microorganisms of the following genera were used, including three *Aspergillus* spp., nine *Bacillus* spp., one *Caldariomyces* sp., one *Candida* sp., seven *Cunninghamella* spp., one *Curvularia* sp., one *Cylindrocarpon* sp., one *Gliocladium* sp., one *Memnoniella* sp., three *Mucor* spp., one *Mycobacterium* sp., four *Nocardia* spp., three *Penicillium* spp., five *Pseudomonas* spp., one *Rhizopus* sp., one *Rhodotorula* sp., one *Saccharomyces* sp., one *Sarcina* sp., one *Schizosaccharomyces* sp., five *Streptomyces* spp., and an unidentified soil microorganism.

Screening and Analytical Scale Culture Conditions. A two-stage fermentation protocol[38] was used for screening and preparative isolation of *p*-coumaric acid (**1**) metabolites. The incubation medium consisted of 0.5% (w/v) yeast extract, 0.5% NaCl, 0.5% K_2HPO_4, 0.5% soybean meal, and 2% dextrose per 1 L of distilled water, adjusted to pH 7.0 with 6 N HCl. Stage I cultures were grown in 25 mL of sterile soybean meal−glucose medium held in 125 mL stainless steel-capped DeLong flasks and autoclaved at 121 °C for 15 min. Flasks were incubated at 28 °C and 250 rpm on New Brunswick Scientific (New Brunswick, NJ) G10, G25, or Innova 5000 Gyrotory shakers. After 72 h, 25 mL stage II fermentations were started in the same medium with a 10% inoculum of stage I culture. Stage II cultures were incubated for 24 h, when *p*-coumaric acid (**1**) was added to flasks to 1 mg/mL culture medium (250 μL of a 0.1 g/mL solution of **1** in EtOH). Controls included uninoculated sterile medium containing 1 mg/mL **1** and inoculated medium without substrate. Samples (4 mL) were withdrawn for analysis 6, 24, 72, and 144 h after substrate addition, acidified to pH 2.0, and extracted with 1 mL of EtOAc−propanol (9:1, v/v). The organic layer was separated by centrifugation to 1200*g* in a desktop centrifuge, and 40 μL samples were spotted onto TLC plates and developed in system A.

Preparative Transformation of *p*-Coumaric Acid (1) to 4-Vinylphenol (4) by *B. megaterium*. After incubation of stage II cultures (10 1 L DeLong flasks, 200 mL medium each) for 24 h at 28 °C, **1** was added (0.8 mg/mL). Controls consisted of 25 mL of medium in 125 mL flasks containing either **1** (0.8 mg/mL), 4-vinylphenol (**4**, 1 mg/mL), or no substrate incubated under the same conditions. Samples (4 mL) from each flask were taken and assayed by TLC (system A) to show **4** (R_f 0.70, red) in estimated 40% and 50% yields after 6 and 24 h, respectively.

After 24 h, the contents of five flasks were centrifuged at 10 000*g* for 10 min. Pellets containing only traces of **1** or **4** were discarded. The supernatant (1 L) was adjusted to pH 2.0 with 6 N HCl and extracted with three 500 mL volumes of EtOAc. The organic layers were pooled, dried over Na_2SO_4, filtered through sintered glass, and evaporated to a brown oil (1.5 g). A 0.5 g sample of oil was dissolved in 25 mL of EtOAc and extracted with three 25 mL volumes of 5% (w/v) $NaHCO_3$. The organic layer was dried over Na_2SO_4 and concentrated to afford 85 mg of red oil that was resolved over 11 g of 60−200 mesh silica gel (1.7 × 10.0 cm) with $CHCl_3$−MeOH (95:5) to provide 25 mg of 4-vinylphenol (**4**, 13%) after evaporation of pooled fractions.

Metabolite 4: off-white powder; ^{1}H NMR (Me_2CO-d_6, 360 MHz) δ 7.30 (2H, d, J = 8.6 Hz, H-2, H-6), 6.81 (2H, d, J = 8.6 Hz, H-3, H-5), 6.65 (1H, dd, J = 10.8, 17.6 Hz, H-1′), 5.59 (1H, dd, J = 1.1, 17.6 Hz, H_a-2′), 5.03 (1H, dd, J = 1.1, 10.8 Hz, H_b-2′); EIMS (70 eV) m/z 121 $[M + 1]^+$ (5%), 120 $[M]^+$ (100%), 91 (36%), 65 $[C_5H_5]^+$ (8%); spectral data were indistinguishable from that from a commercial standard of **4** and from the literature.[39]

Conversion of 1 to 4 (Vinylphenol) and 4a in D_2O. Stage I and II cultures of *B. megaterium* (25 mL of medium in 125 mL flasks) were incubated at 28 °C as described above. After 24 h, eight cultures were combined and centrifuged at 10 000*g* for 15 min. The supernatant was decanted, and the pellet was washed with 0.1 M KH_2PO_4, pH 7.0 (120 mL), and recentrifuged at 10 000*g* for 15 min. The resulting pellet was divided into four equal portions, and each was resuspended in 25 mL of one of the following solutions: (1) 0.1 M KH_2PO_4, pH 7.0; (2) 0.1 M KH_2PO_4, pH 7.0, 1 mg/mL *p*-coumaric acid (**1**); (3) 0.1 M KH_2PO_4 prepared in D_2O/H_2O (1:1, v/v), pH 7.0, 1 mg/mL **1**; (4) 0.1 M KH_2PO_4 prepared in D_2O, pH 7.0, 1 mg/mL **1**. Suspended cells were incubated in 125 mL DeLong flasks at 28 °C, 250 rpm. The reaction progress (solution 2) was monitored by TLC system A, which indicated that **4** was produced in nearly 100% yield after 6 h. After 6 h, cultures were centrifuged at 10 000*g* for 15 min, and the supernatants were extracted with 3 × 12 mL of EtOAc. After concentration, 3 mg (D_2O−H_2O, 1:1, v/v) and 9 mg (D_2O) of residues were isolated. These two residues were chromatographed separately over 60−200 mesh silica gel (8.0 × 0.6 cm, 2 mL/min) with C_6H_{14}−EtOAc−HCOOH (75:25:0.5). Seven 0.5 mL fractions from each column were pooled and evaporated to yield 3 mg mixtures of **4** and **4a** from D_2O−H_2O (1:1) and D_2O flasks. A similar workup gave 6 mg of **4** from H_2O incubations. All samples were subjected to low-resolution GC/MS and ^{1}H NMR spectral analyses (Table 2 and Figure 2).

Isolation of (2*R*,2*S*)-4-(2,3-Dihydro-5-hydroxy-2-benzofuranyl)phenol (11). After 24 h, stage II *B. megaterium* cultures (400 mL, 25 mL in sixteen 125 mL flasks) received 4-vinylphenol (**4**, 1 mg/mL, 400 mg total). Controls included cultures without **4** and uninoculated medium with **4** added. Dimer **11** (brown, R_f 0.55, TLC system A) was formed in an estimated 5% yield in 24 h. Cultures were combined and centrifuged (10 000*g*, 10 min), and 125 mL of 1 M $NaHCO_3$ was added to adjust 375 mL of the supernatant to pH 8.0, before being extracted with 2 × 500 mL of CH_2Cl_2. Extracts were dried over Na_2SO_4 and concentrated to give 353 mg of a yellow-red oil.

The oil was chromatographed over 60−200 mesh silica gel (26.5 × 2.3 cm) with 96:4 CH_2Cl_2−MeOH. Fractions were pooled and concentrated to give 31 mg of a yellow residue, which was separated over a second silica gel column (11.5 × 1.5 cm) with C_6H_{14}−EtOAc (60:40) to yield 1.5 mg (0.4%) of **11**.

Metabolite 11: off-white powder; $[\alpha]^{25}_D$ 0.0° (*c* 0.05, MeOH); UV (MeOH) λ_{max} 226, 283, 300 nm; ^{1}H and ^{13}C NMR, HMBC (Table 3); EIMS (70 eV) *m/z* 228 $[M]^+$ (100), 211 $[M - OH]^+$ (13), 181 (12), 134 $[M - C_6H_6O]^+$ (5), 77 $[C_6H_5]^+$ (14); HREIMS *m/z* 228.0802 (calcd for $C_{14}H_{12}O_3$, 228.0786).

Preparation of 11 by $K_3Fe(CN)_6$ Oxidation of 4 and Hydroquinone (7).[21,29] 4-Vinylphenol (**4**, 121 mg, 1.0 mmol) in 200 mL of $CHCl_3$ and **7** (111 mg, 1.0 mmol) in 20 mL of acetone were combined, and 97 mL of an aqueous solution of $K_3Fe(CN)_6$ (578 mg, 1.8 mmol) and Na_2CO_3 (3 g, 27.4 mmol) were added. The resulting mixture was stirred under N_2 in the dark and monitored by TLC (system B) to show an estimated 50% yield of **11** in 22 h. Chloroform and aqueous layers were separated, and the aqueous layer was extracted with 3 × 48 mL volumes of EtOAc. The organic layers were combined, dried over Na_2SO_4, and evaporated to give 32 mg of dark brown oil.

The oil was resolved over a 5 g, 40 μm SiO_2 column (13.5 cm × 1.1 cm) with 4:1 C_6H_{14}−EtOAc (1 mL fractions). After evaporation, one fraction contained 9 mg of **11** (4% yield).

Oxidation product 11: white powder; mp 161−163.5 °C (decomposition, corrected); $[\alpha]^{25}_D$ 0.0° (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ϵ) 228 (4.07), 286 (3.37), 302 (3.52) nm; NMR and MS spectral analyses were essentially identical to those for **11** isolated from *B. megaterium* (Table 3).

Preparative Transformation of 1 by *C. lunata*. Six 24-h-old stage II cultures (200 mL soybean meal−glucose medium in 1 L flasks) received 1.2 g of *p*-coumaric acid (**1**, 1.0 mg/mL). After 24 h, TLC system C indicated the presence of two new metabolites, **15** (R_f 0.35, brown) and **2** (R_f 0.47, yellow), which were present in approximately 20% and 10% yields, respectively. Cultures were harvested by filtration through cheesecloth, and the filtrate was passed through Whatman No. 1 filter paper. The culture filtrate was adjusted to pH 2.0 with 6 N HCl and extracted with 3 × 600 mL of 9:1 EtOAc−propanol to give 1 g of red oil after evaporation.

The red oil was resolved over 100 g of 40 μm silica gel (32.0 × 3.5 cm), eluted with CH_2Cl_2−MeOH (9:1). Similar fractions were pooled to give 203 mg of a dark orange oil containing **2** and 232 mg of impure **15**. Chromatographic resolution of the oil over 20 g of 40 μm silica gel (17.4 × 1.9 cm) with $CHCl_3$−MeOH (95:5) gave 31 mg (0.22 mmol, 3% yield) of 4-hydroxybenzoic acid (**2**) as an off-white powder: UV (MeOH) λ_{max} (log ϵ) 253 (4.10) nm; ^{1}H NMR and EIMS analyses were in agreement with data for authentic **2**;[40] HREIMS *m/z* 138.0321 (calcd for $C_7H_6O_3$, 138.0317).

Pure **15** was obtained by further resolution of the impure fraction over Sephadex LH-20 (22.5 × 2.0 cm) with MeOH to give 110 mg (0.34 mmol, 9%) of **15**.

Metabolite 15: tan powder; mp 212−215.5 °C (decomposition, corrected); $[\alpha]^{25}_D$ +3.8° (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ϵ) 227 (4.27), 300 (4.21), 317 (4.24); ^{1}H, ^{13}C NMR, HMBC, NOESY (Table 4); EIMS (70 eV) *m/z* 326 $[M]^+$ (0.3%), 282 $[M - CO_2]^+$ (13), 264 $[M - CO_2, H_2O]^+$ (4), 238 $[M - 2CO_2]^+$ (100), 115 (13); FABMS *m/z* 325 $[M - H]^-$ (48), 281 $[M - H - CO_2]^-$ (100), 255 $[M - H - C_2H_2CO_2]^-$ (13), 237 $[M - H - 2CO_2]^-$ (29); HRFABMS *m/z* 325.0723 (calcd for $C_{18}H_{13}O_6$, 325.0712).

Preparation of 15 by $K_3Fe(CN)_6$ Oxidation of 1.[21,29] *p*-Coumaric acid (**1**, 179 mg, 1.1 mmol) in 30 mL of acetone was added to 270 mL of $CHCl_3$. $K_3Fe(CN)_6$ (450 mg, 1.37 mmol) and Na_2CO_3 (2.25 g, 21.0 mmol) were dissolved in 75 mL of distilled water. The organic and aqueous solutions were combined and stirred under N_2 in the dark for 30 h, after which TLC (system A) showed **15** (R_f 0.30) in about 20% yield. The aqueous layer was removed, adjusted to pH 2.0 with 6 N HCl, and extracted with three 35 mL volumes of EtOAc. Pooled extracts were dried over Na_2SO_4 and concentrated to 214 mg of an orange-yellow residue, which was separated over 25 g of 40 μm silica gel (20.5 × 2.0 cm) and eluted with 9.5:0.5 to 9:1 CH_2Cl_2−MeOH. Fractions containing a tan solid **15** (34 mg, 0.10 mmol, 19% yield) gave $[\alpha]^{25}_D$ 0.0° (*c* 0.5, MeOH); UV, NMR (Table 4) and MS spectral properties essentially identical to those of **15** isolated from *C. lunata*; HRFABMS gave *m/z* 325.0710.

Analysis of *C. lunata* Enzymes. Five *C. lunata* stage II cultures (each with 200 mL Iowa medium/1 L DeLong flask) were incubated for 24 h (28 °C). One flask received **1** (1 mg/mL). Twenty-four hours later, the substrate-containing flask was assayed for conversion products of **1** (TLC system A) to confirm the presence of metabolites. After 48 h, the other four stage II culture flasks were harvested by filtration through cheesecloth. Filtered mycelia were washed with 350 mL of pH 5.8, 10 mM phosphate buffer containing 0.5% NaCl, and combined mycelia were frozen at −70 °C. Culture filtrates were stored at 4 °C. Mycelium (15 g) was thawed and suspended in 45 mL of sodium phosphate buffer (30 mM, pH 8.1) containing 2 mM DTT, 2 mM PMSF, and 20% glycerol (v/v),[41] and passed six times through a French press (1100 psig, 18 000 psi). Homogenized cells were centrifuged at 25 000*g* for 30 min at 4 °C to produce a cell-free extract. Laccase activity was qualitatively determined in 0.5 mL of culture filtrate or cell-free extract by adding 10 μL of syringaldazine (4.44 mM in MeOH), mixing vials by inversion, and viewing the development of a deep pink-purple color over 2−10 min.[34] Controls contained only MeOH.

Peroxidase activity was assayed by monitoring the increase in absorbance at 420 nm at 20 °C for 5 min.[42] Peroxidase assay mixtures contained 42 mM pyrogallol, and cell-free extract, or culture filtrate in 3 mL of sodium phosphate buffer (0.1 M, pH 5.8) and 160 μL of H_2O_2 (8 mM). H_2O_2 was replaced by 160 μL of buffer in the blank.

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