Biotransformation of (+)-Catechin to Novel B-Ring Fission Lactones

ARTICLE *in* ORGANIC PROCESS RESEARCH & DEVELOPMENT · AUGUST 2010

Impact Factor: 2.53 · DOI: 10.1021/op100108j

3 AUTHORS, INCLUDING:

Andrew S Lamm
University of Technology, Jamaica
13 PUBLICATIONS **129** CITATIONS
SEE PROFILE

John P N Rosazza
University of Iowa
204 PUBLICATIONS **3,813** CITATIONS
SEE PROFILE

Organic Process Research & Development **XXXX,** *xxx,* 000–000

Biotransformation of (+)-Catechin to Novel B-Ring Fission Lactones

Shuvendu Das,* Andrew S. Lamm, and John P. N. Rosazza

University of Iowa, Center for Biocatalysis and Bioprocessing, 2501 Crosspark Road, MTF-Suite C100, Oakdale Research Park, Coralville, Iowa 52241-3486

Abstract:

Thirty-six microorganisms were screened for their abilities to transform (+)-catechin to metabolites. Of these, *Aspergillus giganteus* UI 10, *A. ochraceous* ATCC 1008, *Cylindrocarpon radicicola* ATCC 11011, *Amycolata autotrophica* ATCC 35203, *Mycobacterium flavescens* ATCC 14474, *M. fortuitum* UI 53378, *Streptomyces rimosus* NRRL 2234, *S. griseolus* ATCC 11796, and *S. griseus* ATCC 13273, converted (+)-catechin to new products. *C. radicicola* and *S. griseolus* were chosen for preparative-scale incubations to produce polar products, which were isolated and characterized by UV, NMR, and mass spectral analyses. The products were new carboxylic acid lactones formed by B-ring fission of catechin. Labeling with $^{18}O_2$ showed incorporation of two oxygen atoms into the new lactone products. Based on $^{18}O_2$ labeling, likely pathways for lactone formation involved initial dioxygenase-mediated meta B-ring cleavage followed either by aldehyde oxidation to a diacid that lactonizes, or by hemiacetal (lactal) formation followed by alcohol oxidation. We believe this to be the first example of microbial B-aryl-ring cleavage in catechins.

Introduction

Flavonoids are among the most ubiquitous groups of polyphenolic compounds in foods of plant origin. As integral constituents of the diet, these flavan-3-ol antioxidants may exert a wide range of beneficial effects on human health versus cancer, inflammatory, and cardiovascular diseases.[1−5]

The topic of microbial and enzymatic transformations of flavonoids was extensively reviewed where biocatalytic dimerizations, carbon−carbon cross couplings, and degradations were described.[6−16] In studies of the bacterial degradation of catechins, a *Pseudomonas* sp. grown on (+)-catechin, catalyzed initial 8-hydroxylation of taxifolin to form the 7,8-catechol derivative, dihydrogossypetin.[9,10] Subsequent *ortho*-dioxygenase A-ring cleavage gave oxaloacetic acid and 5-(3,4-dihydroxyphenyl)-4-hydroxy-3-oxovalero-δ-lactone. Co-oxidations of phloroglucinol and (+)-catechin with mushroom tyrosinase gave an adduct linking phloroglucinol to the 6′-position of (+)-catechin.[11] Hydrogen peroxide-dependent peroxidase oxidations,[12,15] polyphenol oxidase oxidation,[13] and autoxidation[14] of (+)-catechin gave biaryl and biaryl ether dimers.[13] Structurally, dimeric catechins differ in their linkages, positions, and the relative conformations of biphenyl or biphenyl ether moieties.[12−14]

As an extension of our ongoing studies on biotransformations of flavonoids,[6] we carried out a new investigation on microbial oxidations of (+)-catechin. Herein, we report the production, isolation, and characterization of two new B-ring fission oxygenated metabolites of (+)-catechin (**1**) (Figure 1).

Results and Discussion

Microorganism Screening. Thirty-six growing cultures were initially screened for their abilities to catalyze biotransformation reactions with **1** as substrate. Cultures screened included bacterial species of *Streptomyces*, *Amycolata*, *Amycolaptosis*, *Bacillus*, *Candida*, *Mycobacterium*, *Nocardia*, *Pseudomonas*, and *Actinoplanacete*, and fungi including species of *Aspergillus*, *Absidia*, *Beauveria*, *Cunninghamella*, *Cylindrocarpon*, *Curvularia*, *Gliocadium*, *Mortierella*, *Mucor*, *Rhizopus*, *Sepedonium* and *Thamnidium*. Of these, *Aspergillus giganteus* UI 10, *A. ochraceous* ATCC 1008, *Streptomyces rimosus* NRRL 2234, *Cylindrocarpon radicicola* ATCC 11011, *Mycobacterium flavescens* ATCC 14474, *S. griseolus* ATCC 11796, *S. griseus* ATCC 13273, *Amycolata autotrophica* ATCC 35203, and *M. fortuitum* UI 53378 reproducibly transformed **1** to metabolites **2** and **3** in good yield. *C. radicicola* ATCC 11011, and *S. griseolus* ATCC 11796 were selected for a more comprehensive study of the biotransformation of **1** because these two organisms appeared to reproducibly form the metabolites in question and also appeared to form the metabolites in greatest amount.

Biotransformations using resting cells as catalyst have been well documented.[17,18] Resting cells as biocatalysts have several advantages over growing cells or isolated enzymes. These include (a) biocatalyst cell concentrations can be adjusted with

* Author for correspondence: Telephone: (319)-335-4900. Fax: (319)-335-4901. E-mail: shuvendu-das@uiowa.edu.

(1) Gil, B.; Sanz, M. J.; Terencio, M. C.; Ferrándiz, M. L.; Bustos, G.; Payá, M.; Gunasegaran, R.; Alcaraz, M. J. *Life Sci.* **1994**, *54*, 333.
(2) Ferrándiz, M. L.; Alcaraz, M. J. *Agents Actions* **1991**, *32*, 283.
(3) Middleton, Jr. E.; Kandaswami, C. *Biochem. Pharmacol.* **1992**, *43*, 1167.
(4) Cotelle, N.; Bernier, J. L.; Catteau, J. P.; Pommery, J.; Wallet, J. C.; Gaydou, E. M. *Free Radical Biol. Med.* **1996**, *20*, 35.
(5) Rice-Evans, C. A.; Miller, N. J.; Bolwell, P. G.; BramLey, P. M.; Pridham, J. B. *Free Radical Res.* **1995**, *22*, 375.
(6) Das, S.; Rosazza, J. P. N. *J. Nat. Prod.* **2006**, *69*, 499.
(7) Cheynier, V.; Osse, C.; Rigaud, J. *J. Food Sci.* **1988**, *53*, 1729.
(8) Hirose, Y.; Yamaoka, H.; Nakayama, M. *J. Am. Oil Chem. Soc.* **1991**, *68*, 131.
(9) Jeffrey, A. M.; Jerina, D. M.; Self, R.; Evans, W. C. *Biochem. J.* **1972**, *130*, 383.
(10) Jeffrey, A. M.; Knight, M.; Evans, W. C. *Biochem. J.* **1972**, *130*, 373.
(11) Rensburg, W. J.; Ferreira, D.; Malan, E.; Steenkamp, J. A. *Phytochemistry* **2000**, *53*, 285.
(12) Hosny, M.; Rosazza, J. P. N. *J. Agric. Food Chem.* **2002**, *50*, 5539.
(13) Guyot, S.; Vercauteren, J.; Cheynier, V. *Phytochemistry* **1996**, *42*, 1279.
(14) Cheynier, V.; Rigaud, J.; Souquet, J. M.; Barillére, J. M.; Moutounet, M. *Am. J. Enol. Vitic.* **1989**, *40*, 36.
(15) Weings, K.; Huthwelker, D. *Liebigs Ann. Chem.* **1970**, *731*, 161.
(16) Young, D. A.; Young, E.; Roux, D. G.; Brandt, E. V.; Ferreira, D. *J. Chem. Soc., Perkin Trans. 1* **1987**, 2345.

10.1021/op100108j

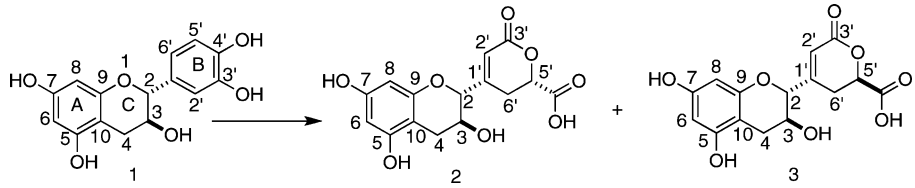

Figure 1. **Structures of (+)-catechin (1) and its B-ring fission lactone products, 2 and 3.**

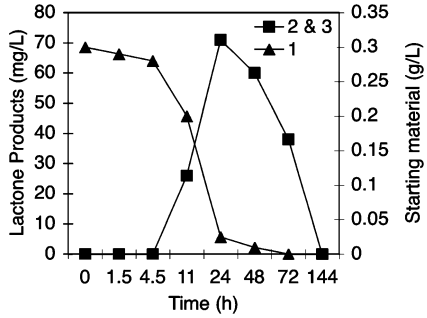

Figure 2. **Rates of formation of 2 and 3 and consumption of 1 by *C. radicicola* ATCC 11011.**

resting cells, whereas cell concentrations in shake flask growing cultures are difficult to control; (b) resting cells can be suspended in appropriate buffers to control pH, modified culture media, distilled water, or even nonaqueous solvent mixtures for biocatalysis; (c) simpler isolation and purification of biotransformation products from resting cell reactions versus growing cultures, which often contain organic acids, phenols, amino acids, lipids, and other secondary metabolites; (d) resting cells can accomplish multistep enzymatic reactions without the addition of exogenous and expensive coenzymes, cofactors, and stabilizing agents; (e) and, enzymes in resting cells are in their native state, and generally more stable than isolated enzymes that are recombined in incubation medium.

Biotransformation Kinetics. A kinetic study was carried out with *C. radicicola* resting cell suspensions. Products (**2** and **3**) formed at different time points were always equal in quantity. HPLC analysis gave zero time concentrations of **1** at about 60% of the actual amount of substrate added, likely due to entrapment of substrate within cells (Figure 2). As **1** was consumed, formation of **2** and **3** was first observed at about 11 h, giving a maximum yield of 7% each at 24 h, after which **2** and **3** gradually disappeared (Figure 2). Substrates and metabolites were completely undetectable at 72 h, and 144 h, respectively, and no other intermediates or products were observable by HPLC or TLC at any time. Mass balance between the amount of substrate utilized and products formed was never 100%. To recover the products from inside the cells, cells were extracted with acetone, and TLC of the extracts showed very little remaining extractable product. The lack of any additional metabolites would strongly suggest that **2** and **3** may have been produced at low steady-state levels as intermediates in a degradation pathway that mineralizes the compounds, giving undetectable products. To address such a point, mutants blocked in further catechol degradation as in Gibson's work[19] or cell-free systems capable of catalyzing the biotransformation reaction would have to be developed. These experiments are beyond the scope of this work.

Preparative-Scale Biotransformation and Metabolite Isolation. Preparative-scale biotransformation reactions were done with *C. radicicola* and *S. griseolus* using 200 mg of **1** to give quantities of metabolites **2** and **3**. Modified lactones **2** and **3** were isolated by solvent extraction, subsequent flash column chromatography over silica gel and sephadex LH 20, and finally by preparative C-18 reverse phase HPLC in 7.0% yield from *C. radicicola* and 6.5% yield from *S. griseolus*. Although not optimized, these steps provided a good basis for developing scalable and practical large-scale processes. The time course was monitored by TLC for this preparative biotransformation. Similar results were obtained in screening and preparative-scale reactions, including recovery of low mass balance. Yields of **2** and **3** from these organisms were always equal in quantity. The reaction stopping point was selected on the basis of TLC, when the apparent maximum amount of product was formed.

Metabolite Identification. Structures of **2** and **3** were established by spectroscopic analysis. Lactones **2** and **3** gave *m/z* $[M - H]^-$ 321.0627 and 321.0617, respectively indicating empirical formulae of $C_{15}H_{13}O_8$. Thus, the metabolite structures each contained two oxygen atoms more than **1** from which they were obtained. ^1H and ^{13}C NMR spectra of **2** and **3** contained all signals for the A and C rings of **1** indicating that structural changes had occurred only in the B-rings of the metabolites. UV spectra showed λ_{max} 240 nm, characteristic for α,β-unsaturated carbonyl compounds. The structural changes obtained were identical for both **2** and **3**, and analyses used to assign structures for these compounds are described below in detail for **2**.

Four new proton signals replaced the three aromatic proton signals of ring-B. These included signals for a new olefinic proton at 6.14 ppm, two 6′-position methylene protons as a dd at 3.04 ppm ($J = 16.16$ Hz, 4.61 Hz) and 2.54 ppm ($J = 16.1$ Hz, 8.7 Hz) and a ddd at 5.62 ppm ($J = 8.3$, 3.5, and 1.8 Hz) for H-5′ coupled to the methylene group signals. The ^{13}C NMR spectrum of **2** contained all signals for rings A and C. In addition, the spectrum of **2** contained signals for two new carbonyl groups at 170.0 and 171.0 ppm, two olefinic carbons at 170 ppm (C-2′) and 117 ppm (C-1′), and signals at 37 and 80 ppm for C-6′ and C-5′, respectively. Signal assignments and connectivities were confirmed by using COSY, DEPT, HMBC, and HMQC spectral editing. COSY analysis correlated H-2′ with H-6′ and H-2, and correlated H-5′ with H-6′. HMBC showed three-bond correlations between H-2 and C-2′ and C-6′; H-5′ between C-3′ and C-1′; and H-6′ between C-2, C-2′, and C−COOH, thus confirming the structure of the lactone products.

(17) Goodhue, C. T.; Rosazza, J. P. N.; Peruzzotti, G. P. *Manual of Industrial Microbiology and Biotechnology*; ASM: Washington, D.C., 1986.

(18) Michels, P. C.; Rosazza, J. P. N. *Manual of Industrial Microbiology and Biotechnology*; ASM: Washington, D.C., 1999.

(19) Gibson, D. T.; Mahadevan, V.; Jerina, D. M.; Yagi, H.; Yeh, H. J. C. *Science* **1975**, *189*, 295.

Table 1. **Relative intensities of mass spectral ions of 3 obtained from incubations of 1 in H_2O, 19% $H_2{}^{18}O$ and 33% $^{18}O_2$**

oxygen source	% relative intensity at *m/z*						
	321 $[M - H]^-$	323 $[M - H + 2]^-$	325 $[M - H + 4]^-$	327 $[M - H + 6]^-$	303 $[M - H]^-$-18	305 $[M - H + 2]^-$-18	307 $[M - H + 4]^-$-18
controls in air, $H_2{}^{16}O$	100	5	1	1	42	3	1
$H_2{}^{18}O$	100	3	0	0	35	2	0
$^{18}O_2$	1	5	100	2	1	14	22

The polar, catechin carboxylic acid lactones are new products, and the first obtained by microbial B-ring fission.

The specific rotations ($[\alpha]^{25}_D$) of lactone products **2** and **3** are −12° and +28° (*c* 0.143, MeOH), respectively. Identical masses and skeletal structures of lactones **2** and **3** suggest that they are diastereomers. As **2** and **3** are not enantiomers, their specific rotation values are not exactly opposite. The lactone diastereomers **2** and **3** are epimeric at the C-5′ position where the arrangements of the COOH group and the H atom in 3D space are opposite. Except for the orientation of C-5′ substituents, lactones **2** and **3** are exactly identical.

^{18}O Incorporation. The structures of **2** and **3** indicated that microbial ring-B fission products were likely formed by the actions of dioxygenases. Dioxygenases are not well-known in the organisms used in this work. $^{18}O_2$−Labeling studies were performed in order to assess the possible involvement of dioxygenases in the B-ring fission process. We also conducted biotransformation reactions in buffers containing $H_2{}^{18}O$ to determine whether hydrolytic processes might be involved in the formations of **2** and **3**. For these experiments, resting cells of *C. radicicola* were prepared in buffers containing either H_2O or 19% $H_2{}^{18}O$ plus (+)-catechin (**1**); or in similar resting cell suspensions incubated in an artificial N_2 and $^{18}O_2$ atmosphere also containing **1**. After 24 h of incubation, reactions were stopped, mixtures were extracted, organic extracts were evaporated to dryness, and the residues were dissolved in methanol and analyzed by LC−MS. Mass data including fragmentation patterns for **2** and **3** were identical, and values are reported for **3**. As shown in Table 1, **3** from $H_2{}^{18}O$ was unlabeled showing a molecular ion at *m/z* 321 for $(M - H)^-$ with a similar mass spectral fragmentation pattern to that obtained with unlabeled **3**. On the other hand, **3** obtained from cultures in an $^{18}O_2$ atmosphere gave a molecular ion at *m/z* 325 for $(M - H + 4)^-$, or four mass units higher than unlabeled **3**, indicating the incorporation of two atoms of $^{18}O_2$ into the lactone. Major fragment ions at *m/z* 303 for $(M - 18 - H)^-$ for unlabeled **3** and *m/z* 307 for $(M - 18 - H)^-$ for labeled **3** confirmed the presence of two oxygen atom labels in **3**. A fragment at *m/z* 272 is also the major fragment ion of **1** likely formed by loss of water from the C-ring, showing that no oxygen label occurred in this ring.

With the oxygen-labeling results in hand and the structures of **2** and **3** fully identified, it was possible to propose a dioxygenase pathway by which **1** was converted to its lactones (Figure 3). Ortho metabolic cleavage where diatomic oxygen is inserted "between" the carbons bearing phenolic hydroxyl groups (in ring B) can be ruled out as such an initial oxidation would lead to diacid intermediates with insufficient oxygen in the ring-cleaved product. Meta dioxygenase cleavage of B-ring catechols could occur by insertion of diatomic oxygen into either 2′,3′- or 4′,5′-positions. Meta cleavage at the 2′,3′-positions can be ruled out by the structural analysis for **2** and **3** that clearly shows two carbon atoms flanking C-1′ where formerly the ring-B was attached to ring-C at position-2. Initial B-ring cleavage would give putative intermediate **4** bearing aldehyde and carboxylic acid functionalities at original B-ring positions 5′ and 4′, respectively. Oxidation of the putative aldehyde and reduction of one double bond to diacid **5**, and subsequent lactonization would afford **2** and **3**. A pathway involving initial monooxygenase hydroxylation of **1** to a putative 3′,4′,5′-trihydroxy ring-B derivative followed by ortho ring dioxygenase cleavage can be ruled out because such a path would lead to randomization of the oxygen abel through C-3′ and C-5′ symmetry. Incubations of a 3′,4′,5′-trihydroxy ring-B derivative,

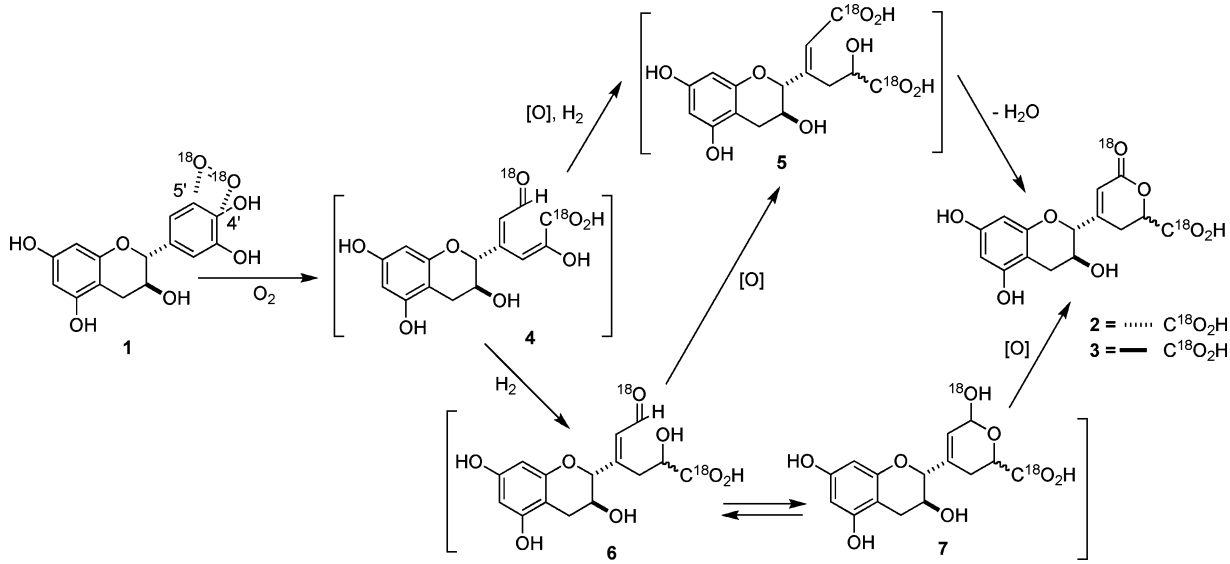

Figure 3. **Proposed pathways for the formation of B-ring fission lactones from 1.**

such as (+)-gallocatechin with growing cultures and resting cells of *C. radicicola* and *S. griseolus* gave no products, further suggesting that trihydroxylated intermediates like these were not involved in the ring-B biodegradation process. An alternative pathway, from intermediate **6** (one double bond reduced), would involve formation of a diastereomeric intermediate hemiacetal **7** (lactal), which upon oxidation would provide lactones **2** and **3**. Intermediate **6** could be oxidized to diacid **5**, which upon lactonization would afford **2** and **3**. Oxidations of hemiacetals to afford lactones find precedence in the work of Jones et al.[20] where horse liver alcohol dehydrogenase (HLADH) oxidized hemiacetals to give lactone products.

The observed reaction represents a new pathway for the multistep conversion of catechins. Initial meta cleavage, double bond reduction, aldehyde or lactal oxidation, and lactonization to products **2** and **3** are novel and apparently find no chemical counterpart for such a regioselective, oxidative cleavage, and accompanying multistep reactions. It is likely that the novel multistep process reported here may have application with other substituted catechols.

Experimental Section

Instrumentation. Thin-layer chromatography (TLC) was carried out on precoated Si gel 60 F_{254} plates (Merck). Developed chromatograms were visualized by fluorescence quenching under 254-nm UV light and by spraying with Pauly's reagent[21] ($NaNO_2$ 0.5%, sulfanilic acid 0.5% in HCl 2%, and NaOH 5% in 50% ethanol). Equal volumes of $NaNO_2$ and sulfanilic acid solutions were mixed immediately prior to use and sprayed onto plates, followed by NaOH and warming with a heat gun for 3 min to turn isoflavone compounds burnt-orange. Flash column chromatography was performed using J.T. Baker glassware with 40 μm Si gel (Baker) as the stationary phase. Ultraviolet (UV) spectra were determined with a Shimadzu UV-2101PC UV−vis scanning spectrophotometer (Shimadzu Corporation, Kyoto, Japan). ^{1}H and ^{13}C NMR spectra were obtained with Bruker AMX-600 and AMX-400 spectrometers (Bruker Instruments, Billerica, MA). All NMR spectra were obtained in acetone-d_6 using TMS as the internal standard, with chemical shifts expressed in parts per million (δ) and coupling constants (J) in Hz. COSY, HMBC, and HMQC experiments were carried out using a Bruker AMX-600 high field spectrometer equipped with an IBM Aspect-2000 processor. High-resolution electrospray mass spectrometry (HRESIMS) was done using a Fisons VG-ZAB-HF reversed geometry (BE configuration, where B is a magnetic sector and E is an electrostatic analyzer) mass spectrometer (VG Analytical, Inc.). High-performance liquid chromatography (HPLC) was performed with a Shimadzu LC-6A dual pumping system connected to a Shimadzu SPD-6AV UV/vis detector and a Shimadzu SCL-6B system controller (Shimadzu Corporation, Kyoto, Japan). For quantitative analyses, separations were carried out over a Platinum EPS C18 column (150 mm × 4.6 mm i.d., 5 μm particle size, Alltech, Deerfield, IL). Preparative HPLC was carried out over a Econsil C18 column (250 mm × 22.5 mm i.d., 10 μm particle size, Alltech, Deerfield, IL). Samples were eluted with a mobile phase consisting of CH_3CN/H_2O (10:90, v/v) containing 0.05% HCOOH at a flow rate of 0.3 mL/min (3.0 mL/min, for preparative scale) while eluting peaks were detected at 209 nm. Liquid chromatography−mass spectrometry (LC−MS) was performed using an Agilent 1100 MSD (Agilent, U.S.A.) system. The column, solvent system, and flow rate were the same as those used in the HPLC analysis.

Quantitation of metabolites in biotransformation samples was based upon comparison of metabolite peak areas to standard curves obtained by triplicate injections of known amounts of pure compounds.

Substrates. (+)-Catechin (**1**) and (+)-gallocatechin were obtained from Sigma Chemical Company, St. Louis, MO. The purities of substrates were determined by TLC, UV, and ^{1}H NMR spectral analyses.

Microorganisms. Cultures of *A. giganteus* UI 10, *A. ochraceous* ATCC 1008, *S. rimosus* NRRL 2234, *C. radicicola* ATCC 11011, *M. flavescens* ATCC 14474, *S. griseous* ATCC 11796 and ATCC 13273, *A. autotrophica* ATCC 35203, and *M. fortuitum* UI 53378 from the University of Iowa, Center for Biocatalysis and Bioprocessing Culture Collection were maintained on Sabouraud maltose agar slants and stored in a refrigerator at 4 °C prior to use.

Screening Procedure. Cultures were grown according to our standard two-stage procedure in 25 mL of Iowa Medium held in stainless steel-capped, 125-mL DeLong culture flasks. This medium contained (in g/L) 20 dextrose, 5 yeast extract, 5 soybean flour, 5 NaCl, and 5 K_2HPO_4 in distilled H_2O and adjusted to pH 7.0 with 6 N HCl before being autoclaved at 121 °C for 15 min. Cultures were incubated with shaking at 240 rpm at 28 °C on a New Brunswick Scientific, Innova 5000 Gyrotory tier shaker. A 10% inoculum from 72 h stage I cultures was used to initiate stage II cultures, which were incubated for 24 h before receiving 10 mg of substrates (**1**) in 0.1 mL of *N*,*N*-dimethylformamide (DMF) and incubated as before. Substrate controls consisted of sterile medium incubated under the same conditions but without microorganism. Substrate-containing cultures were generally sampled by removing 4 mL of the entire culture at 24, 72, and 144 h after addition of substrate. After determination of pH, they were acidified to pH 2.0 with 6 N HCl and extracted with equal volumes of (EtOAc/*n*-BuOH, 9:1 (v:v)). Samples were subjected to centrifugation at 1000 × g for 1 min in a desktop centrifuge, and 30−40 μL samples of the organic extracts were spotted on TLC plates, which were developed in $CHCl_3/MeOH/H_2O$ (50:50:2, v:v:v) and visualized by spraying with Pauly's reagent. When plates were warmed with a heat gun, (+)-catechin (**1**) gave an intensely yellow spot at R_f 0.64, while metabolites **2** and **3** together gave overlapping, bright yellow spots at R_f 0.37.

Biotransformation Kinetics. Biotransformation kinetics with **1** was determined with resting cell suspensions of *C. radicicola* ATCC 11011 in 25 mL volumes. Resting cells were prepared by harvesting 900 mL of 24-h old stage-II cultures by filtration through cheesecloth. Cells were washed twice with pH 7.0, 50 mM phosphate buffer, and were filtered through cheese cloth. Washed cell pellets were resuspended in 450 mL of pH 7.0, 50 mM phosphate buffer containing 0.5% (w/v)

(20) Jakovac, I. J.; Goodbrand, H. B.; Lok, K. P.; Jones, J. B. *J. Am. Chem. Soc.* **1982**, *104*, 4659.
(21) Bobbitt, J. M. *Thin Layer Chromatography*; Reinhold Publishers: New York, 1963.

dextrose. Eighteen 125-mL DeLong culture flasks, each containing 25 mL of cell suspension, received a total of 225 mg of **1** (12.5 mg/flask added in 50 μL DMF). Controls consisted of flasks without substrates. Earlier experiments had shown that substrates alone were not converted to products by shaking under incubation conditions. These substrate-containing, resting cell suspensions were incubated with shaking at 240 rpm at 28 °C. For analysis, duplicate flasks were withdrawn at time intervals ranging from 0 to 144 h. Samples were adjusted to pH 2.0 with 6 N HCl, and extracted with three, 13-mL volumes of EtOAc/*n*-BuOH (9:1 (v/v)). The organic extracts were washed with distilled H_2O, dried over anhydrous Na_2SO_4, and concentrated in vacuo to yield viscous brown residues that were dissolved in 5 mL of MeOH and analyzed by HPLC.

Preparative-Scale Resting Cell Biotransformations of 1. *C. radicicola* ATCC 11011 and *S. griseolus* ATCC 11796 were used for preparative biotransformation reactions. After 24 h, cells from 800 mL stage-II cultures of *C. radicicola* were harvested by filtration through cheese cloth, and cells from a similar culture of *S. griseolus* were obtained by centrifugation at 10,000 × *g* for 15 min. Cells of each organism were washed twice with pH 7.0, 50 mM phosphate buffer, repelleted, and resuspended in 400 mL of pH 7.0, 50 mM phosphate buffer containing 0.5% (w/v) dextrose. Four 1-L DeLong flasks containing 100 mL of cell suspension each received 50 mg of substrate dissolved in 200 μL of DMF. These biotransformation mixtures were incubated with shaking for 24 h after which the flasks for each organism and substrate were combined, adjusted to pH 2.0 with 6 N HCl, and extracted with three, 200 mL volumes of EtOAc/*n*-BuOH (9:1 (v/v)). The organic extracts were washed with distilled H_2O, dried over anhydrous Na_2SO_4, and concentrated in vacuo to yield viscous brown residues from *C. radicicola* (210 mg) and *S. griseolus* (206 mg).

Isolation of Metabolites. The extracts were subjected to Si gel flash chromatography over 2 cm × 45 cm columns eluted with $CHCl_3$/MeOH/H_2O (80:37:2 → 50:50:2) while 5-mL fractions were collected. Similar fractions (by TLC) were combined, dried, and further purified over 1.5 cm × 20 cm Sephadex LH-20 columns eluted with MeOH. Finally, the products were purified over preparative Econosil C18 column to afford metabolites **2** (14 mg) and **3** (14 mg) from *C. radicicola* and **2** (13 mg) and **3** (13 mg) from *S. griseolus*. Spectral properties of **2** and **3** from the two organisms were identical, and the data are reported below.

Metabolite 2: $[\alpha]^{25}_D$ −12° (*c* 0.143, MeOH); UV (MeOH) λ_{max} 209, 240 nm; ^{1}HNMR (CD_3COCD_3, 600 MHz) δ 6.14 (1H, t, J = 1.5 Hz, H-2′), 5.97 (1H, d, J = 2.2 Hz, H-8), 5.86 (1H, d, J = 2.2 Hz, H-6), 5.62 (1H, ddd, J = 8.3, 3.5, and 1.8 Hz, H-5′), 4.60 (1H, dd, J = 8.5, 1.4 Hz, H-2), 3.87 (1H, td, J = 8.8, 5.8 Hz, H-3), 3.04 (1H, dd, J = 16.16, 4.61 Hz, H-6′), 2.95 (1H, dd, J = 16.16, 4.61 Hz, H-4_{eq}), 2.54 (1H, dd, J = 16.1, 8.7 Hz, H-6′), 2.45 (1H, dd, J = 16.1, 8.7 Hz, H-4_{ax}); ^{13}C NMR (CD_3COCD_3, 400 MHz) δ 171 (C, COOH), 170 (CO, C-3′), 170 (C, C-1′), 157 (C, C-9), 156 (C, C-5), 155 (C, C-7), 117 (CH, C-2′), 99 (C, C-10), 96 (CH, C-8), 95 (CH, C-6), 80 (CH, C-5′), 76 (CH, C-2), 67 (CH, C-3), 37 (CH_2, C-6′), 29 (CH_2, C-4); ESIMS *m*/*z* 321 $[M - H]^-$ (100), 322 (16), 303 (4), 277 (27), 278 (4), 259 (15), 260 (3), 233 (26), 234 (4), 215 (8), 167 (5); HRESIMS *m*/*z* $[M - H]^-$ 321.0627 (calcd for $C_{15}H_{13}O_8$, 321.0610).

Metabolite 3: $[\alpha]^{25}_D$ + 28° (*c* 0.143, MeOH); UV (MeOH) λ_{max} 209, 240 nm; ^{1}HNMR (CD_3COCD_3, 400 MHz) δ 6.31 (1H, t, J = 1.9 Hz, H-2′), 5.96 (1H, d, J = 2.3 Hz, H-8), 5.81 (1H, d, J = 2.3 Hz, H-6), 5.42 (1H, dddd, J = 8.0, 3.8, 1.7, and 0.73 Hz, H-5′), 4.74 (1H, d, J = 7.8, H-2), 4.11 (1H, J = 8.0, 5.7 Hz, H-3), 3.15 (1H, dd, J = 16.6, 3.8 Hz, H-6′), 2.93 (1H, dd, J = 16.3, 5.7 Hz, H-4_{eq}), 2.58 (1H, dd, J = 16.5, 8.1 Hz, H-6′), 2.44 (1H, dd, J = 16.5, 8.1 Hz, H-4_{ax}); ^{13}C NMR (CD_3COCD_3, 600 MHz) δ 171 (C, COOH), 170 (CO, C-3′), 167 (C, C-1′), 157 (C, C-9), 154 (C, C-5), 156 (C, C-7), 119 (CH, C-2′), 99 (C, C-10), 96 (CH, C-8), 95 (CH, C-6), 79 (CH, C-5′), 76 (CH, C-2), 65 (CH, C-3), 37 (CH_2, C-6′), 29 (CH_2, C-4); ESIMS *m*/*z* 321 $[M - H]^-$ (100), 322 (16), 303 (4), 277 (27), 278 (4), 259 (15), 260 (3), 233 (26), 234 (4), 215 (8), 167 (5); HRESIMS *m*/*z* $[M - H]^-$ 321.0617 (calcd for $C_{15}H_{13}O_8$, 321.0610).

$H_2{}^{18}O$- and $^{18}O_2$-Labeling Experiments. Labeled $H_2{}^{18}O$ incorporation experiments were performed using resting cells of *C. radicicola* in 5-mL volumes where buffers were prepared using 19-atom % $H_2{}^{18}O$ and 81% double-distilled water, and 2.5 mg of **1** was used as substrate. For $^{18}O_2$ incorporation experiments, resting cells in 5 mL of buffer were incubated as before, but in an artificial closed atmosphere comprising 33% $^{18}O_2$ (99 atom %) in nitrogen. Incubations were conducted for 24 h with shaking at 240 rpm at 28 °C after receiving substrate. After acidification to pH 2, the reaction mixtures were extracted with EtOAc/*n*-BuOH (9:1, v/v), and the separated organic phases were evaporated to dryness. Concentrates were dissolved in 1.5 mL of MeOH and subjected to LC−MS analysis.

Acknowledgment

This work was supported by the Cooperative State Research, Education and Extension Service, U.S. Department of Agriculture under Agreement Nos. 2002-34188-12035 and 2004-34188-15067, and by Archer Daniel Midland Inc., Decatur, IL.

Received for review April 19, 2010.

OP100108J