Microbial Transformations of Chalcones: Hydroxylation, *O*-Demethylation, and Cyclization to Flavanones

M. Sanchez-Gonzalez and J. P. N. Rosazza*

Division of Medicinal and Natural Products Chemistry, Center for Biocatalysis and Bioprocessing, College of Pharmacy, University of Iowa, Iowa City, Iowa 52242

Received October 16, 2003

Microorganisms were examined for their potential to catalyze biotransformation reactions that mimic plant biosynthetic processes. Specifically, microorganisms were screened for their abilities to transform selected chalcones to flavonoid and other products. *Aspergillus alliaceus* UI 315 efficiently transformed 3-(2″,3″-dimethoxyphenyl)-1-(2′-hydroxyphenyl)propenone (2′-hydroxy-2,3-dimethoxychalcone) (**1**) to several products, all of which were characterized by UV, NMR, and mass spectral analyses. *A. alliaceus* cyclized **1** to three flavanones and to *O*-demethylated and hydroxylated chalcones, some of which functioned as intermediates in the cyclization process. Inhibition studies using SKF525A, metyrapone, and phenylthiocarbamide with whole cell reactions showed that as many as three cytochrome P450 enzymes may be involved in these reactions. One enzyme catalyzed chalcone cyclization; another, *O*-demethylation; and a third, hydroxylation of chalcones. Flavonoid products are racemic, unlike the same products that are stereoselectively cyclized in plants. This work shows that microorganisms are capable of cyclizing chalcones to form flavonoid products, thus affording a mimic of plant biosynthetic processes.

Flavonoids are a diverse group of natural products that play important roles in plant growth and development and in defenses against microorganisms and pests.[1] Apart from their physiological roles in plants, flavonoids are important components in the human diet as antioxidants that scavenge free radicals.[2] Biosynthetically, flavonoids are derived from chalcone precursors that in turn are derived from the condensation of *p*-coumaroyl CoA and three malonyl CoA's by the enzyme chalcone synthase (CHS).[1] CHSs are members of the family of plant polyketide synthases (PKS), which together form a large variety of natural products.[1]

Although CHSs were once believed to be specific to plants, an *rppA* gene from the bacterium *Streptomyces griseus* encoded a 372 amino acid protein that showed significant similarity to plant CHS.[3] The discovery of bacterial PKSs belonging to the plant CHS superfamily of condensing enzymes linked the biosynthetic capabilities of plants and bacteria.[4] Bioinformatic approaches showed that genes homologous to *rppA* could be found in fungi.[5] Similar genes coding for the enzymes known to cyclize chalcones to flavanones, chalcone isomerase (CHI), have not yet been found in microorganisms.

Microorganisms are well known for their abilities to catalyze many types of useful biotransformation reactions with flavonoids including hydroxylations, reductions (double bonds and ketones), *O*-alkylations and dealkylations, glycosylations, and sulfations.[6−13] Surprisingly, there has been only one report concerned with microbial transformation of chalcones.[14] We postulated that microorganisms could catalyze valuable biosynthetic type reactions similar to those found in plants, such as chalcone cyclization. In this paper, we describe fungal *O*-demethylation and hydroxylation of chalcones and confirm that microorganisms can cyclyze chalcones to flavanones.

Results and Discussion

Metabolite Isolation and Identification. Twenty five cultures were initially screened for their abilities to catalyze interesting biotransformation reactions with **1** as substrate. Cultures screened included different species of *Streptomyces, Amycolata, Amycolaptosis, Bacillus, Nocardia, Pseudomonas,* and *Actinoplanacete,* and fungi including species of *Aspergillus, Absidia, Beauveria, Cunninghamella, Curvularia, Gliocadium, Mortierella, Mucor,* and *Thamnidium*. Of these, *A. alliaceus* UI315 reproducibly transformed **1** to metabolites in good yield. Therefore, this organism was selected for a comprehensive study on the biotransformation of **1**.

Preparative scale *A. alliaceus* biotransformation reactions gave different mixtures of products versus time. Incubations using 500 mg of **1** as substrate gave several new metabolites that were isolated by ethyl acetate extraction and subsequent column and preparative layer chromatography. *A. alliaceus* reactions at 144 h gave **2**, **5**, and **6**, while those from shorter reaction times afforded the same flavanones plus modified chalcones **3** and **4** (Figure 1). Metabolite identifications were based on ^{1}H and ^{13}C NMR, HMBC, HMQC, UV, and mass spectral analysis and by comparison to values for known compounds where possible.

Modified novel chalcones **3** and **4** were isolated in 6.5 and 7.2% yield, respectively. ^{1}H NMR spectra of these products contained two sets of doublets at δ 8.20−7.80 ppm (J = 15.5−15.7), signals characteristic of chalcone olefinic protons. Both chalcones had single methoxy group signals at δ 3.83, indicating that they were *O*-demethylated products of **1**. The ^{1}H NMR spectrum of **3** showed seven proton signals in the aromatic region and gave $C_{16}H_{15}O_4$ [*m*/*z* 271.0961, $(M + H)^+$] by HRMS, which confirmed that **3** was an *O*-demethylated derivative of **1**. The *O*-methyl resonance was correlated with the carbon resonance at δ 149 (C-2″) by HMBC. All other resonances were similar to those for **1**,[15] permitting the assignment of structure **3** as 3-(2″-methoxy-3″-hydroxyphenyl)-1-(2′-hydroxyphenyl)propenone. The ^{1}H NMR spectrum of **4** had six aromatic proton signals. HRMS gave $C_{16}H_{13}O_5$ [*m*/*z* 285.0755 $(M - H)^-$], indicating that **4** was a hydroxylated product of compound **3**. ^{1}H NMR signals for ring B were the same as those of **3**, but protons in ring A were represented as doublets of

* To whom correspondence should be addressed. Tel: (319) 335-4902. Fax: (319) 335-4901. E-mail: john-rosazza@uiowa.edu.

10.1021/np030448o CCC: $27.50
Published on Web 03/04/2004

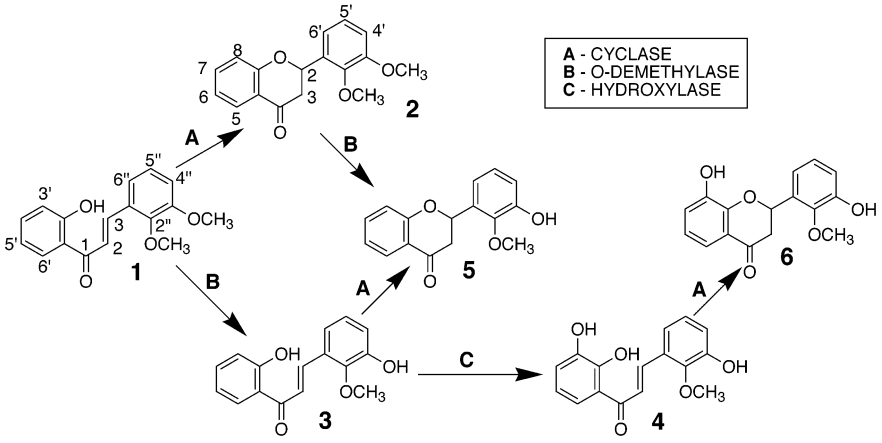

Figure 1. Biocatalytic pathway for the conversion of **1** to flavanone and altered chalcones by *A. alliaceus* UI 315.

doublets at δ 7.60 and 7.08 *m*-coupled (J = 1.4 Hz) with each other and *o*-coupled (J = 8 Hz) to a triplet at δ 6.78, indicating the presence of a hydroxy group at C-3′ or C-6′. HMBC correlations between H-6′ and carbon resonances at δ 195 (C1), 152 (C-2′), and 121.5 (C-4′) confirmed that the hydroxyl group was at C-3′.

Three racemic or slightly levorotatory ($[\alpha]_D$ values of −0.01 to −0.05) flavanones, products **2**, **5**, and **6**, were apparently obtained by cyclizations of **1**, **3**, and **4**, respectively. Flavanones **5** and **6** are new compounds. Specific rotations of pure flavanone isomers from plants similar to **2**, **5**, and **6** are relatively large, showing specific rotations between −9° and −51°.[16] All three compounds showed UV signals characteristic for flavanones. The UV spectra of **2**[15] and **5** contained absorption maxima at 317 and 279 nm. For **2**,[15] the band at 317 nm was evident as a small shoulder that showed no bathochromic or hypsochromic shifts in the presence of NaOMe. Metabolite **6** gave absorption maxima at 335 and 265 nm. UV absorptions at 265 and 279 nm are typical of flavanones and differ from the characteristic single UV absorption maximum at 320 nm for the chalcone substrate **1**.[15,17] For the ^{1}H NMR spectra of **2**,[15] **5**, and **6**, the olefinic protons H-2 and H-3 of the substrate were absent. These signals were replaced by a doublet of doublets at δ 5.80 (J = 13.8/2.8 Hz, H-2) and doublets of doublets at 3.14 ppm (J = 17/13.8 Hz, H-3$_{ax}$) and at 2.80 ppm (J = 17/2.8 Hz, H-3$_{eq}$), showing that a flavanone ring had been formed. Signals for H-3 protons showed geminal and vicinal couplings between H-3 and H-2, respectively. Both **5** and **6** contained single methoxy group signals.

LRMS gave *m*/*z* 285 $(M + H)^+$ for metabolite **2**, indicating that this was a cyclization product of **1**. ^{1}H NMR, ^{13}C NMR, and UV spectra were identical to those reported for 2′,3′-dimethoxyflavanone,[15] confirming the structure.

HRMS gave $C_{16}H_{15}O_4$ [271.0993 $(M + H)^+$] for **5**, confirming the loss of a methyl group. ^{1}H NMR for **5** was similar to **2** for rings A and B except for the loss of one methyl group. By HMBC, the remaining methoxy proton signals were correlated with C-2′ at δ 146, thus showing the structure as **5**.

HRMS gave $C_{16}H_{15}O_5$ [287.0934 $(M + H)^+$] for metabolite **6**, indicating the presence of a hydroxyl group and lack of a methyl group versus **2**. ^{1}H NMR was similar to **5** except for ring A. A hydroxyl group at either position 5 or 8 was indicated by the presence of doublets of doublets at δ 7.37 and 7.07 *m*-coupled to each other and *o*-coupled to a triplet at δ 6.90. The presence of a deshielded H-5 that was correlated by HMBC with C-7 (δ 122.3), C-9 (δ 151.8), and C4 (δ 194.5) showed that the hydroxyl group was at C-8. The remaining methoxy group signal was correlated with C-2′ as for **5**.

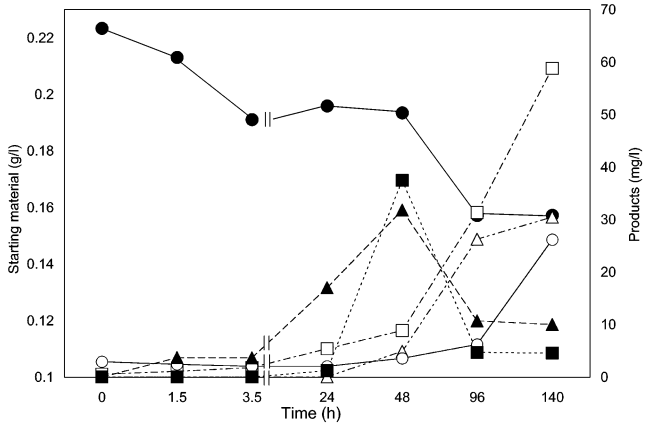

Figure 2. *A. alliaceus* UI 315 biotransformation kinetics. Symbols: −●−, **1**; −○−, **2**; --▲--, **3**; ···■···, **4**; −□−, **5**; --△--, **6**.

Biotransformation Kinetics. With the structures of metabolites identified and standards for analysis available, biotransformation kinetics were evaluated. HPLC analysis (Figure 2) showed that at zero time the concentration of starting material recovered was only 56% of the total amount added, likely due to entrapment of substrate within cells. As **1** was consumed, **3** was formed first, followed by **4**. Maximum levels of **3** and **4** (about 7% yield) were obtained at 48 h, after which these metabolites were consumed, giving rise to **5** and **6**, respectively. At 140 h, metabolites **2**−**6** accounted for approximately 160 mg of product, while 200 mg of **1** was recovered. Mass balance was not 100% between substrate utilized and products formed. Explanations for the lack of mass balance in the analytical biotransformation reaction are speculative. However, substrate and products are likely further metabolized or degraded to undetectable products.

In separate experiments, when **3** and **4** were used as substrates, **5** and **6** were their respective products. Flavanone **2** was the cyclization product of **1**. When **2** was used as substrate, **5** was formed as the major *A. alliaceus* metabolite in 7% yield after 144 h. Compounds **3**, **4**, and **6** were not observed as metabolites of **2**. The conversion of **2** to **5** indicated that this compound could also be formed by *O*-demethylation of **2** after initial cyclization of **1** rather than by initial *O*-demethylation of the chalcone precursor.

***A. alliaceus* Enzyme Activities.** Assays to measure the presence of laccase, tyrosinase, peroxidase, and cyto-

chrome P450 were conducted in cell-free extracts obtained from cultures harvested after 72 h of incubation. Neither laccase nor cytochrome P450 activities could be demonstrated in cell-free extracts. All efforts to detect cytochrome P450 in cell extracts of *A. alliaceus* using metyrapone difference-dithionite reduced cytochrome P450 spectra[18,19] were also unsuccessful. Peroxidase (4 U/mg protein) and tyrosinase (0.026 U/mg protein) activities were detected in cell-free extracts. Peroxidase activity could also be detected in filtered fermentation media. However, chalcones **1**, **3**, and **4** were not converted to products in cell-free extracts containing H_2O_2 or simply sparged with O_2, ruling out the involvement of peroxidase and tyrosinase in the bioconversion processes.

Effects of Cytochrome P450 Inhibitors on Chalcone Conversions. In fungi, direct demonstration of the involvement of cytochrome P450 enzymes in catalysis is rare and rendered difficult because these proteins are usually unstable in cell-free extracts, and their electron transport components are often poorly expressed.[20] Very few examples are known in the literature where product formation can be directly measured by HPLC or GC, for example, with fungal cell-free extracts. Therefore, the participation of P450 enzymes is in most cases concluded from indirect evidence, such as by the use of cytochrome P450 specific inhibitors.

Several types of cytochrome P450 inhibitors were initially screened versus *A. alliaceus* bioconversion reactions. These included octylamine, SKF 525A, metyrapone, and PTC. SKF 525A, metyrapone, and PTC changed the pattern of products obtained in the conversion of **1** to various metabolites. Phenylthiocarbamide belongs to the thionosulfur-containing cytochrome P450 inhibitors and can affect monophenol monooxygenase activities as well.[21,22] Although SKF 525A and metyrapone inhibit several isoforms of cytochrome P450, they too can serve to inhibit the activities of other metalloenzymes.

Reactions containing **1** or **1** plus inhibitor were evaluated by HPLC, and the results are shown in Figure 3. In 72 h controls, **1** was converted into five metabolites, **2**, **3**, **4**, **5**, and **6**. While 1 mM SKF 525A had no effect, 5 mM SKF 525A completely shut down the biotransformation reaction process. In reactions containing 2 mM SKF 525A, conversions of **1** to **2**, **5**, and **6** were either completely eliminated or greatly reduced, indicating selective inhibition of an enzyme involved in chalcone cyclizations. When used in concentrations of 2 and 5 mM, metyrapone had no effect on *A. alliaceus* biotransformations of **1**. With 10 mM metyrapone, conversions of **1** to **4** and **6** were dramatically reduced, while yields of **2**, **3**, and **5** were slightly increased. The same result was obtained in the presence of 15 mM PTC. These results suggested that an enzyme involved in 3′-hydroxylation of chalcone **3** was selectively inhibited. Octylamine inhibition was nonselective.

These results show different inhibition patterns, indicating that more than one enzyme was involved in the biotransformation process. The enzyme catalyzing the cyclization reaction was inhibited by SKF 525A, while that catalyzing C-3′ hydroxylation was inhibited by metyrapone and PTC. The 3′-*O*-dealkylation reaction was blocked, but only with high concentrations of SKF 525A. This result indicated that catalysis involved either a third enzyme with low affinity to SKF 525A or a different type of enzyme that was inhibited by high concentrations of SKF 525A. *A. alliaceus* was unable to convert **1** to any products when cultures were grown under an argon atmosphere, indicating the involvement of oxidative enzymes in chalcone

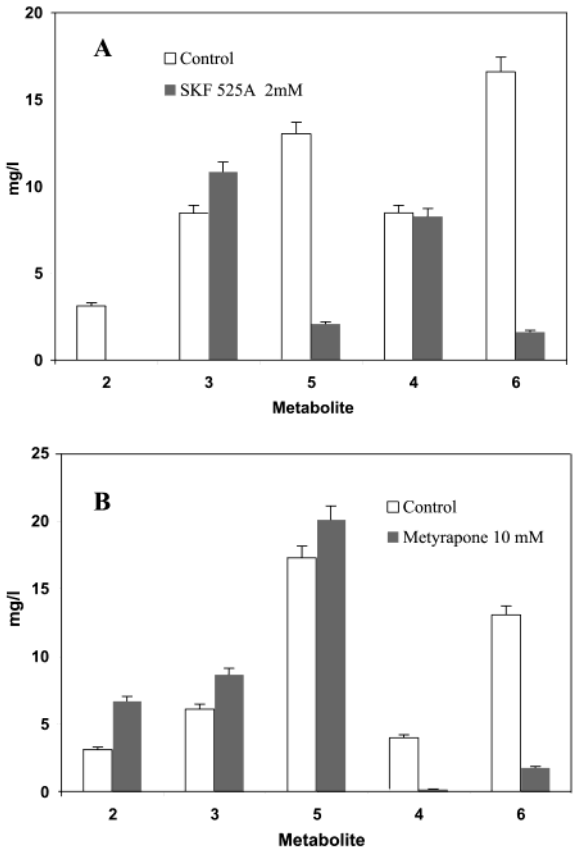

Figure 3. Effects of cytochrome P450 inhibitors on metabolite synthesis by *A. alliaceus* UI 315. (A) Effect of SKF 525A. Hollow bars indicate results with controls; gray bars show results with 2 mM SKF 525A. (B) Effect of metyrapone. Hollow bars indicate results with controls; gray bars, the results obtained with cultures treated with 10 mM metyrapone.

biotransformation reactions. The finding that three separate cytochrome P450 inhibitors perturbed biotransformation reactions suggests that the reactions observed with *A. alliaceus* were catalyzed by this family of heme biocatalysts.

A. alliaceus is a versatile microorganism that hydroxylates aromatic and aliphatic substrates and *O*-dealkylates other aromatic compounds.[23−27] In plants, flavanone synthesis is catalyzed by the enzyme chalcone isomerase (CHI).[28] The proposed plant mechanism involves an ionic intramolecular reaction in which a phenolate ion cyclizes by 1,4-Michael addition with the α,β-unsaturated carbonyl functionality to form flavanones. The architecture of the CHI active site and the nature of the reaction give flavanones in stereospecific fashion.

The cyclization of **1** to flavanones by *A. alliaceus* was not stereoselective. Cytochromes P450 are well known for their abilities to catalyze free radical reactions. We speculate that enzyme systems of *A. alliaceus* catalyze a radical-based intramolecular cyclization of chalcones to flavanones. The initial step likely involves formation of an oxygen radical at position-2′ that undergoes intramolecular cyclization with the α,β-unsaturated carbonyl functionality. The resulting flav-3-en-4-oxy radical intermediate would be reduced to the flavanone. While this type of ring-forming coupling reaction is unusual for cytochrome P450s,[29] phenolic radical couplings mediated by this class of enzyme have been cited before. For example, cytochrome P450s are implicated in the intramolecular cyclization of reticuline to salutaridine in morphine biosynthesis from benzyltetrahydroisoquinolines[29,30] and in the intramolecular cou-

pling of indoxyl.[29,31] While inhibitors indirectly support the proposed pathway, the results cannot rule out a nonstereoselective ionic process in *A. alliaceus* cyclizations.

The formation of catechols such as **4** has been reported before with *A. alliaceus*.[26,27] This fungus efficiently converted 17-β-estradiol to 4-hydroxyestradiol. For the C-3′ hydroxylation reported here, three mechanisms are generally regarded as possible. The hydroxyl group could be introduced by direct insertion of molecular oxygen,[29,32] by initial introduction of molecular oxygen to form an arene oxide intermediate,[33,34] or by way of an *ipso* mechanism where water is inserted into the ring. Mechanisms similar to these have been proposed for the C-10 hydroxylation of 17β-estradiol.[29,33,34] Since unlabeled **4** and **6** were obtained when *A. alliaceus* was incubated with **1** in $H_2{}^{18}O$, the *ipso* mechanism can be ruled out. The precise mechanism for 3′-hydroxylation remains unknown.

This work describes a remarkable series of oxidative chalcone biotransformation reactions. The products of fungal biotransformation are all reminiscent of similar compounds traditionally found in plants. Although the cyclase of *A. alliaceus* differs mechanistically from plant chalcone isomerases, this organism has demonstrated a propensity to mimic plant biosynthetic pathways and to transform chalcones to rare plant products.

Experimental Section

Chemicals. 3-(2″,3″-Dimethoxyphenyl)-1-(2′-hydroxyphenyl)propenone (2′-hydroxy-2,3-dimethoxychalcone) (**1**) (Figure 1) was obtained from Avocado Research Chemicals, LTD, Ward Hill, MA. 2,3-Dimethoxyflavanone (**2**) was obtained from Indofine Chemical Company, Inc., Somerville, NJ. Metyrapone and sodium methoxide were obtained from Aldrich Chemical Co., Milwaukee, WI. $H_2{}^{18}O$ (96 atom % ^{18}O) was purchased from Cambridge Isotope Laboratories, Inc., Andover, MA. SKF 525A, pyrogallol, octylamine, phenylthiocarbamide (PTC), syringaldazine, and tyrosine were obtained from Sigma Chemical Co., St. Louis MO.

Chromatography. Flash column chromatography was performed using 40 μm J. T. Baker silica gel (Mallinckrodt Baker, Inc., Phillipsburg, NJ) as the stationary adsorbent phase. Thin-layer chromatography (TLC) was performed on silica gel GF_{254} plates (Merck). Layers of 0.5 mm of thickness were used for analytical and 1 mm thickness for preparative TLC. Plates were developed using $CHCl_3$/EtOAc/HOAc (30:10:0.2, v/v/v) as a solvent system. The developed plates were visualized under 254 and 360 nm UV light before being sprayed with Pauly's reagent.[35] Pauly's reagent consisted of three separate solutions: 0.5% sulfanilic acid in 2% HCl, 0.5% $NaNO_2$, and 5% KOH in 50% ethanol. Equal volumes of $NaNO_2$ and sulfanilic acid solutions were mixed immediately prior to use, and plates were sprayed with this mixture and then with NaOH before being warmed with a heat gun to give burnt-orange and red colored phenolic compounds.

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 (Kyoto, Japan). Separations were carried out over an Econosil C18 column (250 mm × 4.6 mm i.d., 10 μm particle size, Alltech, Deerdfield, IL). The mobile phases consisted of CH_3CN/H_2O (10:90) containing 5% of HCOOH (A) and CH_3CN/H_2O (90:10) containing 5% of HCOOH (B). The gradient was 0−100% B over 65 min at a flow rate of 1 mL/min. UV absorbances of eluting peaks were recorded at 328 nm.

Quantitation of metabolites in biotransformation samples was based upon comparison of metabolite peak areas to standard curves obtained by duplicate injections of the following amounts of pure metabolites: 0.125−6 μg for **1**, 1.6−20 μg for **2**, 0.175−15 μg for **3**, 0.25−15 μg for **4**, 0.1−20 μg for **5**, and 0.58−23.3 μg for **6**.

Spectral Analysis. Ultraviolet (UV) spectra were determined with a Shimadzu UV-2101PC UV−vis scanning spectrophotometer (Shimadzu Corporation, Kyoto, Japan). ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with a Bruker NMR 360 spectrometer (Bruker Instruments, Billerica, MA), operating at 360 and 90.56 MHz, respectively. All NMR spectra were obtained in methanol-d_4 using tetramethylsilane (TMS) as internal standard, with chemical shifts expressed in parts per million (δ) and coupling constants (J) in hertz. HMBC and HMQC NMR experiments were carried out using a Bruker AMX-600 high-field spectrometer equipped with an IBM Aspect-2000 processor. Low-resolution mass spectra (LRMS) and high-resolution mass spectra (HRMS) were determined with a Micromass Spec M317 spectrometer.

Organism and Culture Conditions. *Aspergillus alliaceus* UI 315 is maintained in the University of Iowa, College of Pharmacy culture collection and was grown and stored on Sabouraud maltose agar in sealed screw-cap tubes at 4 °C. Organisms were cultivated in two stages in a medium composed of 20 g of dextrose, 5 g of soybean meal, 5 g of yeast extract, 5 g of NaCl, and 5 g of K_2HPO_4 in 1 L of distilled water, which was adjusted to pH 7 with 6 N HCl before autoclaving. Media were sterilized in an autoclave at 121 °C for 20 min. Cultures were grown in steel-capped DeLong culture flasks containing one-fifth of their volumes of culture medium. Incubations were conducted at 28 °C on a New Brunswick Scientific (Edison, NJ) Innova 5000 Gyrotory tier shaker, operating at 250 rpm. Stage I cultures were inoculated from fresh *A. alliaceus* slants and were grown as described. A 10% vol/vol inoculum derived from a 72-h-old, first-stage culture was used to initiate the second-stage culture, which was incubated as described. After 24 h, **1** dissolved in *N*,*N*-dimethylformamide (DMF) (50 mg/mL) was added to second-stage cultures to a final concentration of 0.5 mg/mL. The progress of microbial transformation reactions was monitored by TLC or HPLC by the following sampling procedure.

Samples (2 mL) were withdrawn from substrate-containing cultures. These were extracted with EtOAc (1 mL), and extracts were spotted (10 μL) onto TLC plates for analysis. For HPLC analysis, EtOAc extracts were dried over Na_2SO_4, evaporated, dissolved in an equal volume of HPLC MeOH (Fischer Scientific), and finally filtered through a 13 mm, 0.2 μm Nylon syringe filter (Gelman Laboratory).

To observe the influence of dissolved oxygen on biotransformation reactions, flasks were sparged with argon for 2 min prior to chalcone addition and sealed with rubber caps.

Preparation of Cell-Free Extracts. Cells from 72-h-old, stage II cultures induced by adding 0.5 mg/mL of **1** were washed successively with 0.5% aqueous NaCl and then with pH 7.6, 0.1 M phosphate buffer. Cells (2.5 g) suspended in 12.5 mL of the same buffer containing 0.01 M EDTA, 0.01 M glutathione, and 0.25 M sucrose at 4 °C were homogenized by making three passes through a French pressure cell at 1100 psig (18 000 psi) (SLM Aminco, SLM Instruments, Inc. Urbana, IL). Homogenized cells were centrifuged at 30000*g* for 1 h at 4 °C, and the supernatant, or cell-free extract, was stored over an ice bath and assayed for protein, tyrosinase, laccase, peroxidase, and cytochrome P450 activities.

Enzyme Assays. Enzyme assays were conducted by adding cell-free extracts to 0.1 M phosphate buffers at the indicated pH. Controls consisted of reaction blanks prepared in buffer but without added enzyme. Laccase activity was assayed at 37 °C at pH 7.0 by monitoring the oxidation of syringaldazine (4.44 mM in MeOH), evidenced by an increase in absorbance at 526 nm.[36] Tyrosinase activity was assayed at pH 7 and 37 °C for 15−20 min by monitoring the oxidation of L-DOPA (2 mM), evident by an increase in absorbance at 475 nm.[37] One unit (U) of tyrosinase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of DOPAchrome per minute under these conditions. Peroxidase activity was determined at pH 7 and 25 °C in reactions containing 4 mM H_2O_2 and 42 mM pyrogallol by monitoring purpurogallin synthesis evidenced by an increase in absorbance at 420 nm for 10 min.[38] Controls consisted of reactions without H_2O_2, or containing heat-inactivated and cell-free extract, or using

buffer instead of samples. One unit (U) of peroxidase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of purpurogallin per minute under these conditions.

Cytochrome P450 spectroscopic analyses were determined in subcellular fractions prepared by differential centrifugation of cell-free extracts by the method of Ghosh and Samanta.[18] Cytochrome P450 was estimated by the method of Luu-The, Cumps, and Dumont.[19] Metyrapone difference-dithionite reduced cytochrome P450 spectra were recorded following the same procedure. Metyrapone dissolved in H_2O was added to a concentration about 1 mM for these assays. Difference spectra were obtained with a Shimadzu UV-2101PC UV−vis scanning spectrophotometer.

Protein was determined by Coomassie plus protein assay reagent (Pierce, Rockford, IL)[39] using bovine serum albumin as standard.

In Vitro Biotransformation of 1 with *A. alliaceus* Cell-Free Extracts. Cell-free extracts in pH 7.6, 0.1 M phosphate buffer containing substrate (1.8 mM), 1 mM NADPH, or 0.4 mM H_2O_2, or O_2 were incubated at 30 °C for 144 h. Reactions without substrate, or cell-free extract, or heat denaturated cell-free extracts were used as controls. Samples were analyzed by HPLC.

Effects of Cytochrome P450 Inhibitors on Chalcone Biotransformation. The effects of inhibiting cytochrome P450 activity on chalcone metabolism by *A. alliaceus* UI 315 were studied by incubating 25 mL, 24-h-old second-stage cultures containing 0.5 mg/mL substrate and inhibitors for 72 h at 28 °C and 250 rpm. The inhibitors used were SKF 525A (1, 2, and 5 mM), metyrapone (2, 5, and 10 mM), PTC (1.5 and 15 mM), and octylamine (2 and 5 mM). Inhibitors were dissolved in 250 μL of DMF and filtered through 0.45 μm membranes before being added to cultures. Control flasks consisted of cultures containing substrate without inhibitors. Reaction mixtures were analyzed by HPLC. All reactions were conducted in triplicate, and the results are the averages of three determinations for each case.

Attempted $H_2{}^{18}O$ Incorporation. Second-stage culture medium (5 mL) was prepared using one part of $H_2{}^{18}O$ (96% atom) and four parts of H_2O (^{18}O 19.2% atom final concentration). Cultures harvested 48 h after receiving substrate were extracted three times with EtOAc (5 mL), and the extracts were concentrated and purified by preparative TLC to give products that were subjected to MS analysis.

Preparative Scale Biotransformation. Second-stage *A. alliaceus* cultures were grown in 1 L of medium held in 40, 125 mL DeLong flasks. A total of 500 mg of **1** in 10 mL of DMF was evenly distributed among the 24-h-old second-stage cultures. Substrate containing cultures were incubated for 48 or 144 h; the fermentation beers were combined, and the cells and other solids were removed by filtration through cheesecloth. Solids containing only trace amounts of metabolites were discarded, and the filtrates were exhaustively extracted with EtOAc. The extract was dried over anhydrous Na_2SO_4 and evaporated to a brown oil. The concentrated crude extract was chromatographed over a silica gel flash column (2 × 35 cm), eluting with a hexanes/EtOAc gradient (8:2 → 5:5 v/v). Fractions (6 mL) were collected and analyzed by TLC. Similar fractions were combined, dried, and further purified by preparative TLC to give metabolites.

2,3-Dimethoxyflavanone (2): 30 mg, 6%; $[\alpha]^{25}_D$ 0.0, UV and NMR spectra as in the literature.[15]

3-(2″-Methoxy-3″-hydroxyphenyl)-1-(2′-hydroxyphenyl)-propenone (3): 31 mg, 6.5%; ^{1}H NMR (methanol-d_4, 600 MHz) δ 8.20 (1H, d, J = 15.5 Hz, H-3), 8.10 (1H, dd, J = 8/1.4 Hz, H-6′), 7.90 (1H, d, J = 15.5 Hz, H-2), 7.52 (1H, dt, J = 8/1.4 Hz, H-4′), 7.35 (1H, dd, J = 8/1.4 Hz, H-6″), 7.02 (1H, t, J = 8 Hz, H-5″), 6.98 (1H, dt, J = 8/1.4 Hz, H-5′), 6.97 (1H, dd, J = 8/1.4 Hz, H-3″), 6.96 (1H, dd, J = 8/1.4 Hz, H-4″), 3.83 (3H, s, 2″-OCH_3); ^{13}C (methanol-d_4, 90.56 MHz) δ 195 (C, C-1), 164 (C, C-2′), 153 (C, C-3″), 149 (C, C-2″), 141.5 (CH, C-3), 137.5 (CH, C-4′), 131 (CH, C-6′), 129 (C, C-1″), 125.3 (CH, C-5″), 122 (CH, C-2), 121 (C, C-1′), 120 (CH, C-5′), 119.8 (CH, C-4″), 119.7 (CH, C-6″), 118.9 (CH, C-3′), 61.7 (CH_3, OCH_3); HRMS (M + H)$^+$ 271.0961 for $C_{16}H_{15}O_4$, calcd 271.0970.

3-(2″-Methoxy-3″-hydroxyphenyl)-1-(2′,3′-dihydroxyphenyl)propenone (4): 37 mg, 7.2%; ^{1}H NMR (methanol-d_4, 600 MHz) δ 8.15 (1H, d, J = 15.7 Hz, H-3), 7.80 (1H, d, J = 15.7 Hz, H 2), 7.60 (1H, dd, J = 8/1.4 Hz, H-6″), 7.32 (1H, dd, J = 8/1.4 Hz, H-6″), 7.08 (1H, dd, J = 8/1.4 Hz, H-4′), 7.02 (1H, t, J = 8 Hz, H-5″), 6.96 (1H, dd, J = 8/1.4 Hz, H-4″), 6.78 (1H, t, J = 8 Hz, H-5′), 3.83 (3H, s, 2″-OCH_3); ^{13}C (methanol-d_4, 90.56 MHz) δ 195 (C, C-1), 152 (C, C-2′), 151 (C, C-3″), 148 (C, C-2″), 148 (C, C-3′), 142 (CH, C-3), 130 (C, C-1″), 125 (CH, C-5″), 122 (CH, C-2), 121.5 (CH, C-4′), 121 (C, C-1′), 121 (CH, C-4″), 120 (CH, C-6″), 121 (CH, C-6′), 119.5 (CH, C-5′), 60.8 (CH_3, OCH_3); HRMS (M − H)$^-$ 285.0755 for $C_{16}H_{13}O_5$, calcd 285.0763.

3′-Hydroxy-2′-methoxyflavanone (5): 75 mg; 15.8%; $[\alpha]^{25}_D$ −0.01 (*c* 0.14, MeOH); UV (MeOH) λ_{max} 317, 279 + NaOMe 320, 296 (increased intensity); ^{1}H NMR (methanol-d_4, 600 MHz) δ 7.88 (1H, dd, J = 8/1.7 Hz, H-5), 7.54 (1H, dt, J = 8/1.7 Hz, H-7), 7.07 (1H, dt, J = 8/1.7 Hz, H-6), 7.05 (1H, dd, J = 8/1.7 Hz, H-8), 7.05 (1H, dd, J = 8/1.6 Hz, H-6′), 6.99 (1H, t, J = 8 Hz, H-5′), 6.87 (1H, dd, J = 8/1.6 Hz, H-4′), 5.77 (1H, dd, J = 13.8/2.8 Hz, H-2), 3.84 (3H, s, 2′-OCH_3), 3.13 (1H, dd, J = 17/13.8 Hz, H-3$_{ax}$) 2.79 (1H, dd; J = 17/2.8 Hz, H-3$_{eq}$); ^{13}C (methanol-d_4, 90.56 MHz) δ 194 (C, C-4), 164 (C, C-9), 152 (C, C-3′), 146 (C, C-2′), 137 (CH, C-7), 133 (C, C-1′), 127 (CH, C-5), 125.3 (CH, C-5′), 122.3 (CH, C-6), 121.2 (C, C-10), 119.6 (CH, C-8), 117.8 (CH, C-6′), 117.5 (CH, C-4′), 76 (CH, C-2), 61.4 (CH_3, OCH_3), 44.5 (CH_2, C-3); HRMS (M + H)$^+$ 271.0993 for $C_{16}H_{15}O_4$, calcd 271.0970.

3′,8-Dihydroxy-2′-methoxyflavanone (6): 40 mg, 7.6%; $[\alpha]^{25}_D$ −0.05 (*c* 0.13, MeOH); UV (MeOH) λ_{max} 336, 265, + NaOMe 375, 286 (increased intensity); ^{1}H NMR (methanol-d_4, 600 MHz) δ 7.37 (1H, dd, J = 8/1.6 Hz, H-5), 7.12 (1H, dd, J = 8/1.6 Hz, H-6′), 7.07 (1H, dd, J = 8/1.6 Hz, H-7), 7.02 (1H, t, J = 8 Hz, H-5′), 6.90 (1H, t, J = 8 Hz, H-6), 6.89 (1H, d, J = 8 Hz, H-4′), 5.80 (1H, dd, J = 13.5/2.8 Hz, H-2), 3.87 (3H, s, 2′-OCH_3), 3.19 (1H, dd, J = 17/13.5 Hz, H-3$_{ax}$) 2.83 (1H, dd, J = 17/2.8 Hz, H-3$_{eq}$); ^{13}C (methanol-d_4, 90.56 MHz) δ 194.5 (C, C-4), 151.8 (C, C-9), 151.2 (C, C-3′), 147.4 (C, C-8), 146.3 (C, C-2′), 133 (C, C-1′), 125.3 (CH, C-5′), 122.5 (C, C-10), 122.3 (CH, C-7), 122 (CH, C-6), 118.7 (CH, C-6′), 117.9 (CH, C-4′), 117.7 (CH, C-5), 75 (CH, C-2), 61.4 (CH_3, OCH_3), 44.3 (CH_2, C-3); HRMS (M + H)$^+$ 287.0934 for $C_{16}H_{15}O_5$, calcd 287.0919.

Acknowledgment. M.S. acknowledges support from the Iowa Biotechnology Byproducts Consortium through USDA and CONACyT postdoctoral fellowships.

References and Notes

(1) Dixon, R.; Steele, C. *Trends Plant Sci.* **1999**, *4*, 394−400.
(2) Pietta, P.-G. *J. Nat. Prod.* **2000**, *63*, 1035−1042.
(3) Funa, N.; Onishi, Y.; Fuji, I.; Shibuya, M.; Ebizuka, Y.; Horinouchi, S. *Nature* **1999**, *400*, 897−899.
(4) Moore, B. S.; Hertweck, C.; Hopke, J.; Izumikawa, M.; Kalaitzis, J.; Nilsen, G.; O'Hare, T.; Piel, J.; Shipley, P.; Xiang, L.; Austin, M. B.; Noel, J. P. *J. Nat. Prod.* **2002**, *65*, 1956−1962.
(5) van Wageningen, A. M. A., Kirkpatrick, P. N.; Williams, D. H.; Harris, B. R.; Kershaw, J. K.; Lennard, N. J.; Jones, M.; Jones, S. J.; Solenberg, P. J. *Chem. Biol.* **1998**, *5*, 155−162.
(6) Blanco, S. E., Segovia, R. F.; Sanz de Tosetti, M. I.; Ferretti, F. H. *Rev. Latinoam. Microbiol.* **1988**, *30*, 369−374.
(7) Hosny, M.; Rosazza, J. P. N. *J. Nat. Prod.* **1999**, *62*, 1609−1612.
(8) Hosny, M.; Dhar, K.; Rosazza, J. P. N. *J. Nat. Prod.* **2001**, *64*, 462−465.
(9) Ibrahim, A. R. *Phytochemistry* **2000**, *53*, 209−212.
(10) Ibrahim, A. R.; Abul-Hajj, Y. J. *Xenobiotica* **1990**, *20*, 363−373.
(11) Ibrahim, A. R.; Galal, A.; Mossa, A.; El-Feraly, F. *Phytochemistry* **1997**, *46*, 1193−1195.
(12) Ibrahim, A. R.; Abul-Hajj, Y. J. *J. Nat. Prod.* **1990**, *53*, 644−656
(13) Udupa, S. R.; Banerji, A.; Chadha, M. S. *Tetrahedron* **1969**, *25*, 5415−5419.
(14) Herath, W. H. M. W.; Ferreira, D.; Khan, I. A. *Phytochemistry* **2003**, *62*, 673−677.
(15) Imafuku, K.; Honda, M.; McOmie, J. F. W. *Synthesis* **1987**, *2*, 199−201.
(16) Kojima, K.; Gombosurengyin, P.; Odognyi, P.; Begzsurengyin, D.; Zevgeegyin, O.; Hatano, K.; Ogihara, Y. *Phytochemistry* **1997**, *44*, 711−714.
(17) Mabry, T.J.; Markham, K. R.; Thomas, M. B. *Systematic Identification of Flavonoids*; Springer-Verlag: New York, 1970.

(18) Ghosh, D.; Samantha, T. B. *J. Steroid Biochem*. **1981**, *15*, 1063−1067.
(19) Luu-The, V.; Cumps, J.; Dumont. P. *Biochem. Biophys. Res. Commun*. **1980**, *93*, 776−781.
(20) van den Brink, H. J. M.; van Gorcom, R. F. M.; van den Hondel, C. A. M. J.; Punt, P. J. *Fungal Genet. Biol*. **1998**, *23*, 1−17.
(21) Testa, B.; Jenner, P. *Drug Metab. Dispos.* **1981**, *12*, 1−117.
(22) Zollner, H. *Handbook of Enzyme Inhibitors*; Wiley-VCH: Germany, 1991; Part A, pp 940−942.
(23) Chien, M. M.; Rosazza, J. P. N. *Drug Metab. Dispos.* **1979**, *7*, 211−214.
(24) Chien, M. M.; Rosazza, J. P. N. *Appl. Environ. Mirobiol.* **1980**, *40*, 741−745.
(25) Rosazza, J. P.; Kammer, M.; Youel, L. *Xenobiotica* **1977**, *7*, 133−143.
(26) Williamson, J.; Van Orden, D. E.; Rosazza, J. P. *Appl. Environ. Microbiol*. **1985**, *49*, 563−567.
(27) Williamson, J.; Van Orden, D. E.; Rosazza, J. P. *Appl. Environ. Microb*. **1989**, *55*, 3029−3031.
(28) Jez, J. M.; Bowman, M. E.; Dixon, R. A.; Noel, J. P. *Nat. Struct. Biol.* **2000**, *7*, 786−791.
(29) Guengerich, P. F. *Chem. Res. Toxicol*. **2001**, *14*, 611−650.
(30) Amann, T.; Roos, P. H.; Huh, H.; Zenk, M. H. *Heterocycles* **1995**, *40*, 425−440.
(31) Kodaira, H.; Lisek, C. A.; Arimura, A.; Jardine, J.; Spector, S. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 716−719.
(32) Ohe, T.; Hirobe, M.; Mashino. T. *Drug Metab. Dispos*. **2000**, *28*, 110−112.
(33) Numazawa, M.; Nambara, T. *J. Steroid Biochem*. **1977**, *8*, 835−840.
(34) Kirchner, G. J. In *Techniques of Organic Chemistry: Thin-layer Chromatography*; Perry, E. S., Weissberger, A., Eds.; Interscience Publishers: New York, 1967; Vol. XII, Chapter 7, pp 147−184.
(35) Peter, H.; Jung, R.; Bolt, H. M.; Oesch, F. *J. Steroid Biochem*. **1981**, *14*, 83−90.
(36) Kawai, S.; Umezawa, T.; Higuchi, T. *Wood Res.* **1989**, *76*, 10−16.
(37) Solano, F.; Garcia, E.; Perez de Egea, E.; Sanchez-Amat, A. *Appl. Environ. Microbiol*. **1997**, *63*, 3499−3506.
(38) Mehly, A. C. In *Methods in Enzymology*; Colowick, S. P., Kaplan, N. O., Eds.; Academic Press: New York, 1955; Vol. II, pp 807−815.
(39) Bradford, M. M. *Anal. Biochem*. **1976**, *72*, 248−254.

NP030448O