Biocatalytic oxidation of 4-vinylphenol by *Nocardia*

Kyung-Seon Lee and John P.N. Rosazza

Abstract: *Nocardia* species NRRL 5646 stereospecifically hydrates 4-vinylphenol (**15**) to *S*-1-(4′-hydroxyphenyl)ethanol (**17**), and further oxidizes **17** to 4′-hydroxyacetophenone (**18**). Labeled metabolites **17** and **18** obtained from incubations in D_2O and $H_2{}^{18}O$ support initial enzymatic tautomerization of **15** to a reactive quinone methide (**16**), which adds water in the first reaction. Commitment to catalysis is high in the hydration reaction, while the alcohol dehydrogenation reaction appears to be reversible. The stereochemical features of water addition, alcohol oxidations, and ketone reductions with growing culture biocatalysis were established by chiral HPLC. Alcohol oxidations or ketone reductions in 12 000 × *g* supernatants preferentially require $NADP^+$–$NADPH,H^+$ as co-factors. The alcohol dehydrogenase has broad substrate specificity, favoring the oxidation of primary alkanols and 4-hydroxybenzyl alcohols.

Key words : 4-vinylphenol, *Nocardia* sp., enantiospecific hydration, 1-(4′-hydroxyphenyl)ethanol, 4′-hydroxyacetophenone

Résumé : L'espèce *Nocardia* NRRL 5446 hydrate stéréospécifiquement le 4-vinylphénol (**15**) en *S*-1-(4′-hydroxyphénnyl)éthanol (**17**) qu'il oxyde aussi en 4′-hydroxyacétophénone (**18**). Les métabolites **17** et **18** marqués obtenus par incubation dans D_2O et $H_2{}^{18}O$ sont en accord avec un mécanisme impliquant une tautomérisation enzymatique initiale de **15** en une méthoquinone réactive (**16**) qui fixe de l'eau dans la première étape de la réaction. Dans la réaction d'hydratation, le degré de catalyse est élevé alors que la réaction de déshydrogénation de l'alcool semble réversible. Faisant appel à la CLHP chirale, on a déterminé les caractéristiques stéréochimiques de l'addition d'eau, des oxydations de l'alcool et des réductions de cétone par la catalyse de cultures en croissance. Les oxydations d'alcool ou réductions de cétones dans des liquides surnageant 12,000 x *g* requièrent le coupe $NADP^+$–$NADPH,H^+$ comme cofacteurs. La déshydrogénase de l'alcool possède une grande spécificité par rapport au substrat qui favorise l'oxydation d'alcools primaires et d'alcools 4-hydroxybenzyliques.

Mots clés : 4-vinylphénol, *Nocardia* sp., hydratation spécifique, 1-(4′-hydroxyphényl)éthanol, 4′-hydroxyacétophénone.

[Traduit par la Rédaction]

Introduction

Microbial and enzymatic transformations have been developed for nearly every class of natural and synthetic chemical products (1). Microorganisms and their enzymes are biocatalysts, which are now widely used as "reagents" in synthetic organic chemistry. Biocatalysts are valued for their intrinsic abilities to bind organic substrates and to catalyze highly specific and selective reactions under the mildest of reaction conditions. Biocatalytic transformations of abundant renewable natural chemicals afford attractive "green chemistry" alternatives to synthetic chemical approaches, which rely on petrochemically derived starting materials.

Green chemistry (2) presents a continuing need for the discovery of new biocatalysts capable of catalyzing useful chemical transformations by novel mechanisms. An ideal biocatalyst has few technical hurdles, such as the requirement for co-factors. They should be robust, stable, and recyclable, and ultimately be amenable to large-scale adaptation to bioreactor use, even under unusual reaction conditions (3).

p-Coumaric acid (4), ferulic acid (5), and caffeic acid are abundantly occurring phenolic acids commonly found in plants in their esterified or free-acid forms. The acids *p*-coumaric and ferulic compose nearly 4% of the dry weight of maize plant parts, rendering them available in the billions of pounds annually from this source alone (5). The large quantities of these natural compounds render them useful for the biocatalytic production of value-added aromatic natural chemicals. Systematic screening of microorganisms showed that the major and most common biocatalytic reaction with hydroxycinnamates is decarboxylation to the corresponding vinylphenols (hydroxystyrenes) (5, 6). Deuterium-labeling experiments showed that non-oxidative decarboxylations occurred by enzymatic tautomerization of *p*-hydroxycinnamates to doubly vinylogous β-keto acid intermediates that spontaneously decarboxylated (5, 6*a*). Phenolic cinnamate decarboxylation is capable of providing potentially huge quantities of oxygenated styrenes, which are difficult to produce by ordinary chemical means (5, 6*a*, *b*). Phenolic styrenes are valuable starting materials for oxygenated biodegradable polymers, fragrances, and flavors, and chiral intermediates for organic synthesis.

Biocatalytically unsubstituted styrene (**1**) is usually converted to styrene epoxides (**2**) of varying enantiomeric

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Dedicated to Professor J. Bryan Jones on the occasion of his 65th birthday.

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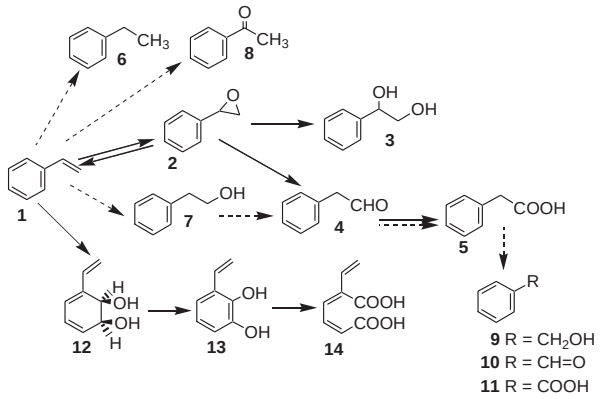
Scheme 1. A summary diagram of known styrene bioconversions. Solid arrows indicate aerobic conditions and dashed arrows indicate anaerobic conditions.



purities by enzymatic activation and insertion of molecular oxygen into the unsaturated styrene side-chain (7–9). Styrene epoxides (**2**) are also formed by hydrogen peroxide dependent-peroxidase mediated epoxidations (10). Styrene epoxides (**2**) are hydrolytically converted to diols (**3**) by epoxide hydrolases (8, 11), to phenylacetaldehyde (**4**) and phenylacetic acid (**5**) (9, 12), and to alkenes in a "reversal" of the epoxidation reaction (13). With anaerobic consortia, after water addition to the double bond, styrene (**1**) is oxidized through the phenylacetate route to yield various products, including ethylbenzene (**6**), phenylethanol (**7**), 1-phenylethanone (**8**), phenylacetaldehyde (**4**), phenylacetic acid (**5**), benzyl alcohol (**9**), benzaldehyde (**10**), and benzoic acid (**11**) (14). Styrene (**1**) is also metabolized via styrene *cis*-glycol (**12**) to 3-vinylcatechol (**13**) and 2-vinylmuconate (**14**), demonstrating double-bond and aromatic-ring oxygenation prior to ring fission (15) (Scheme 1).

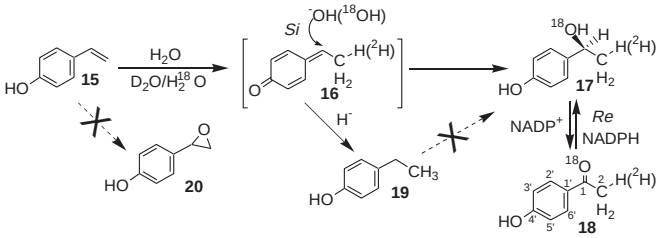
Much less is known about biocatalytic transformations of phenolic styrenes such as **15**. The introduction of oxygen into the styrene side-chain olefin by a hydration mechanism is a novel and relatively unexplored biocatalytic reaction. In this study, we examined the mechanism and disposition of **15** by growing cultures, induced resting cells and cell-free enzyme preparations of the *Nocardia* species, that afforded *S*-1-(4′-hydroxyphenyl)ethanol (**17**) by vinylphenol hydratase, and subsequently, 4′-hydroxyacetophenone (**18**) by an $NADP^+$-dependent alcohol dehydrogenase (Scheme 2).

Experimental

Chemicals

4-Vinylphenol (**15**) (10% solution in propylene glycol), 4′-hydroxyacetophenone (**18**), cinnamyl alcohol (3-phenyl-2-propen-1-ol), 2-hydroxybenzyl alcohol, 3-hydroxybenzyl alcohol, 4-hydroxybenzyl alcohol, oleyl alcohol, and vinyl acetate were obtained from Lancaster Synthesis Inc. (Windham, NH, U.S.A.). D_2O (99.9 atom% D), vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol), cyclohexanol, tetralol (1,2,3,4-tetrahydro-1-naphthol), 4-methoxybenzyl alcohol, benzyl alcohol, 1-phenyl-1-propyl alcohol, 1-phenyl-2-propyl alcohol, and 3-phenyl-1-propyl alcohol were purchased from Aldrich (Milwaukee, WI, U.S.A.). Methanol, ethanol, 1-propanol, 1-butanol, 2-butanol, 2-phenylethyl alcohol, β-NAD^+ (99%), β-$NADP^+$ (sodium salt, 98%), and β-NADPH (tetrasodium salt, 95%) were obtained from Sigma (St. Louis, MO, U.S.A.). Coomassie Plus protein assay reagent and Albumin Standard (bovine serum album fraction V, 2 mg mL^{-1} in a 0.9% aqueous NaCl solution containing sodium azide) were obtained from Pierce (Rockford, IL, U.S.A.). $H_2{}^{18}O$ (96 atom% ^{18}O) and NMR solvents were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, U.S.A.). HPLC solvents were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.), and other reagents were analytical grade from Fisher Scientific unless otherwise indicated.

Scheme 2. Oxidation of 4-vinylphenol (**15**) to **17** and **18** by *Nocardia* sp.



Racemic 1-(4′-hydroxyphenyl)ethanol (**17**) was prepared as described by the $NaBH_4$ reduction of 4′-hydroxyacetophenone (**18**) in CH_3OH (16).

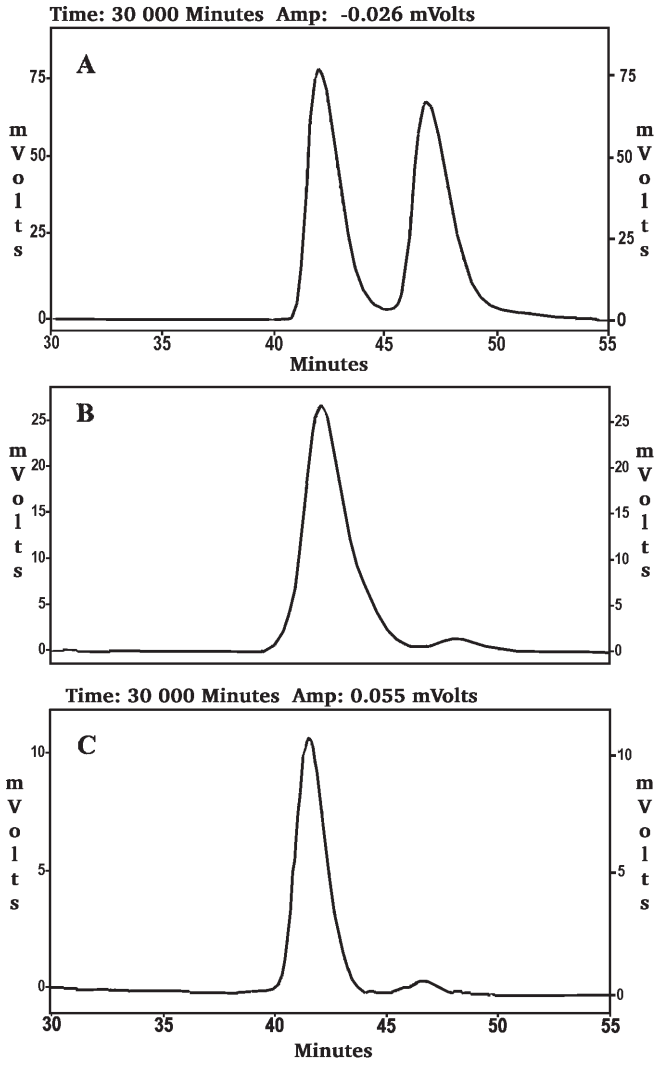
S-(–)-**17** was prepared by a modification of Reeve et al. (17), where the lipase mediated enantioselective esterification of *R*-(+)-**17** occurs, leaving unreacted *S*-(–)-**17** behind. Racemic **17** (4 mg) was dissolved in vinyl acetate (4 mL) as the acyl donor and as the solvent and lipase (4 mg, *Pseudomonas fluorescens* lipase, EC 3.1.1.3, 3097 units mg^{-1}, Fluka Chemical Co., Milwaukee, WI, U.S.A.) were added. After incubation for 12 h at 28°C, lipase was filtered off and the filtrate was evaporated. The residue was dissolved in CH_3OH (2 mL), filtered, and subjected to chiral HPLC analysis, where the enantiomeric excess of unreacted **17** was 98.8% ee of the *S*-(–)-isomer (Fig. 1B).

Analytical methods and instrumentation

TLCs were carried out on 0.25 mm layers of silica gel GF_{254} (Sigma) precoated on polyester plates (20 × 20 cm). The developing solvent was a mixture of CH_2Cl_2–CH_3OH–HOAc (95:5:0.5, v/v/v). Developed chromatograms were directly visualized under 254 and 366 nm UV light for fluorescence quenching or fluorescence. Plates were then spayed with diazotized sulfanilic acid reagent, which consisted of solution A (0.5% sulfanilic acid in 1 N HCl), solution B (0.5% $NaNO_2$ in water), and solution C (0.5% NaOH in 50% (v/v) C_2H_5OH–H_2O). Developed plates were first sprayed with a fresh mixture of equal volumes of solutions A and B, warmed with a heat gun, followed by spraying with solution C to develop the colors. Under these conditions, **15**, **17**, and **18** gave R_f values of 0.7 (scarlet), 0.3 (yellow), and 0.5 (orange), respectively.

HPLC was performed with a Shimadzu liquid chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with dual pumps (LC-6A) and a variable wavelength UV detector (SPD-6AV). Eluted peaks were detected at 280 nm and identified by comparison with standard compounds. Separations were carried out under isocratic

Fig. 1. Chiral HPLC resolution of *R*-(+)- and *S*-(–)-1-(4′-hydroxyphenyl)ethanol (**17**): (A) racemic **17**; (B) *S*-(–)-**17** resolved by *Pseudomonas fluorescens* lipase; (C) **17** isolated from reaction of *Nocardia* cultures with 4-vinylphenol (**15**).



conditions over a Hypersil C18 column (250 × 4.6 mm ID, 5 μm particle size, Alltech Associates, Inc., Deerfield, IL, U.S.A.) protected with a guard column (10 × 4.6 mm ID, 5 μm particle size, Alltech) eluted with CH_3OH–CH_3CN–H_2O (7:3:10, v/v/v) at a flow rate of 0.8 mL min^{-1} to give retention volumes (*Rv*) of 3.6 mL for **17**, 4.5 mL for **18**, and 10.1 mL for **15**. Standard curves were established over the range of 1–10 μg for **15**, 1–8 μg for **17**, and 0.2–0.8 μg for **18**.

Chiral HPLC was carried out with a Chiralcel OJ column (250 × 4.6 mm ID, 10 μm particle size, Chiral Technoloies Inc., Exton, PA, U.S.A.) linked to a Chiralcel OJ guard column (50 × 4.6 mm ID, 10 μm particle size) at a flow rate of 0.8 mL min^{-1} with *n*-hexane–2-propanol (9:1, v/v), which gave retention volumes (*Rv*, mL) of 16.6 for **15**, 16.8 for **18**, 33.6 for *S*-(–)-**17**, and 37.5 for *R*-(+)-**17**.

Flash column chromatography was conducted over silica gel, 40 μm for flash columns (J.T. Baker, Phillipsburg, NJ, U.S.A.).

Mass spectra were obtained by direct inlet probe mass spectrometry (DIPMS) performed on a Voyager mass spectrometer (ThermoQuest, Manchester, England). One dimensional ^1H and ^{13}C NMR spectra were obtained with a Bruker DMX-400 (operating at 400 MHz for ^1H and 100 MHz for ^{13}C) or an AMX-600 spectrometer (operating at 600 MHz for ^1H, Karlsruhe, Germany), equipped with an IBM Aspect 2000 processor and with VNMR version 4.1. Impurities in deuterated NMR solvents were used as internal standards. Chemical shift values (δ) are reported in parts per million (ppm) and coupling constants (*J* values) are given in Hz. Abbreviations for NMR are s, singlet; d, doublet; and q, quartet.

Fermentation and biocatalysis

Nocardia sp. NRRL 5646 was grown and maintained on slants of Sabouraud-dextrose agar or sporulation agar (ATCC No. 5 medium) (18). For analytical scale reactions, cultures were grown by a two-stage protocol (19) in 25 mL of sterile soybean meal-glucose medium held in stainless steel-capped 125-mL DeLong culture flasks. The medium contained 2% glucose, 0.5% yeast extract, 0.5% soybean meal, 0.5% NaCl, and 0.5% K_2HPO_4 in doubly distilled water, and was adjusted to pH 7.0 with 6 N HCl before being autoclaved at 121°C for 15 min. Cultures were incubated with shaking at 250 rpm at 28°C on an Innova 5000 Gyrotory tier Shaker (New Brunswick Scientific Co., Edison, NJ, U.S.A.). A 10% inoculum derived from a 72-hour-old first-stage culture was used to initiate the second-stage cultures, which were incubated as above for 24 h. For bioconversions, **15** and **17** were added directly, usually at concentrations of 1 mg mL^{-1}, and **18** was added at 0.4 mg mL^{-1} of media.

Induced *Nocardia* resting cells were prepared by adding 1 mg mL^{-1} **15** to 24-hour-old stage-two cultures. After an additional 24 h, incubations were filtered through a cheese cloth to remove soybean meal solids, and the cells were concentrated by centrifugation (Sorvall RC 26 Plus centrifuge with a SLA-600TC rotor, Kendro Laboratory Products, Newton, CT, U.S.A.) at 10 000 × *g* at 4°C for 20 min. Cell pellets were suspended and washed in cold phosphate buffer (pH 7.0, 0.1 M) and centrifuged again. Typical wet cell yields ranged from 20 to 24 g L^{-1}.

Cell-free extracts were prepared from induced cells. Typically, 15 g wet weight of cells were suspended in cold phosphate buffer (45 mL, pH 7.0, 0.1 M) containing dithiothreitol (1 mM) (Aldrich) and disrupted by a Sonifier Cell Disrupter 350 (Branson Sonic Power Co., Danbury, CT, U.S.A.) operating at 10 W with a 60% intermittent duty cycle for 5 min. Cell debris was removed by centrifugation at 12 000 × *g* for 30 min at 4°C to provide a supernatant as the cell-free extract. The cell-free extract was assayed for total-protein content by the Bradford protein microassay (20) with bovine serum albumin as the standard.

For preparative-scale conversions, stage-two incubations were carried out in 200 mL of medium held in 1-L DeLong flasks. After 24 h of incubation in the second stage, **15** was added as the substrate and incubated with shaking at 250 rpm at 28°C for 24 h. The reaction mixture was harvested, acidified to pH 2 with 6 N HCl, followed by centrifugation at 10 000 × *g* at 4°C for 20 min to remove

Table 1*a*. ^{1}H NMR and mass spectral analyses for 1-(*4′*-hydroxyphenyl)ethanol (**17**) isolated from reactions of 4-vinylphenol (**15**) with *Nocardia* conducted in H_2O- or D_2O-containing buffers.

	Relative intensity at *m/z* (%)					
D_2O amount (%)	138 $[M]^+$	139 $[M + 1]^+$	140 $[M + 2]^+$	123 $[M - CH_3]^+$	124 $[M + 1 - CH_3]^+$	^{1}H NMR relative intensity – C-2H at δ 1.35
0	100	9	1	100	14	3.0
50	100	18	2	100	9	2.7
100	100	69	11	100	8	1.6

Table 1*b*. ^{1}H NMR and mass spectral analyses for 4′-Hydroxyacetophenone (**18**) isolated from reactions of 4-vinylphenol (**15**) with *Nocardia* conducted in H_2O- or D_2O-containing buffers.

	Relative intensity at *m/z* (%)					
D_2O amount (%)	136 $[M]^+$	137 $[M + 1]^+$	138 $[M + 2]^+$	121 $[M - CH_3]^+$	122 $[M + 1 - CH_3]^+$	^{1}H NMR relative intensity – C-2H at δ 2.51
0	100	24	7	100	9	3.0
50	100	23	6	100	8	2.8
100	100	83	18	100	10	1.6

cells. The supernatant was extracted wiht EtOAc (3 × 200 mL), the combined EtOAc extracts were dried over anhydrous Na_2SO_4 and concentrated by rotary evaporation to give 431 mg of crude extract. The extract was dissolved in CH_3OH (5 mL), adsorbed onto 2 g of silica gel, and loaded onto a flash column (2 × 25 cm). The column was eluted stepwise with $CHCl_3$ and $CHCl_3$–CH_3OH (99:1 to 97:3) to give fraction A (39 mg) and fraction B (173 mg). These fractions were separately resolved by flash column chromatography over silica gel using mixtures of *n*-hexane–EtOAc (95:5) for fraction A, and *n*-hexane–EtOAc (8:2 to 7:3) for fraction B. The resulting pure products were subjected to chromatographic and spectral analyses.

Induced resting cell conversions of 4-vinylphenol

Induced resting-cell reactions were performed by suspending 0.6 g wet weight of *Nocardia* cells in phosphate buffer (25 mL, pH 7.0, 0.1 M) containing glucose (0.5%) and **15** (25 mg). Samples of 2.0 mL were diluted with CH_3OH (2.0 mL), filtered through 0.2-μm nylon membrane filters, and 10 μL of the filtrates were injected for HPLC analysis. The same procedure was used in induced cell conversions of **17** at 1 mg mL^{-1} and **18** at 0.4 mg mL^{-1}.

Reactions of 4-vinylphenol with *Nocardia* sp. in D_2O and 20% $H_2{}^{18}O$

Deuterium-incorporation experiments used induced resting cells of *Nocardia* sp. as described, except that flasks held phosphate buffer (25 mL, pH 7.0, 0.1 M) containing glucose (0.5%, prepared with 50 and 100% D_2O) and **15** (1 mg mL^{-1}). For each reaction, incubations were stopped after 24 h, acidified and pelleted as before, and supernatants were extracted with EtOAc (3 × 25 mL). The combined extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness. The extracts were purified on glass preparative-TLC plates coated with 1-mm layers of silica gel GF_{254} (20 × 20 cm, Sigma). UV-quenching bands were scraped from plates, extracted with CH_3OH (3 × 20 mL), and the extracts were evaporated to dryness for ^{1}H NMR and MS analysis.

Labeled-oxygen incorporation experiments also used induced *Nocardia* resting cells that were suspended in a phosphate buffer (10 mL, pH 7.0, 0.1 M) containing glucose (0.5%, prepared with 20% $H_2{}^{18}O$) and contained in 50-mL DeLong flasks. After addition of **15** (30 mg), the incubation was carried out at 28°C with spinning at 250 rpm. The control consisted of an identical reaction mixture, except the buffer was prepared with unlabeled H_2O. The 24-hour-old culture solutions were extracted with EtOAc (10 mL), the extracts were concentrated and purified by preparative TLC, as previously mentioned, to give products that were subjected to spectral analysis.

Enantiospecificity of 4-vinylphenol hydration

The enantiospecificity of the hydration reaction was evaluated by adding **15** (25 mg) to 25 mL volumes of 24-hour-old stage-two cultures. Incubations were conducted in 125-mL DeLong flasks. Samples of 3 mL were taken after 24, 48, 72, and 144 h and each was extracted with EtOAc (3 mL). After evaporation, crude extracts were redissolved in CH_3OH to concentrations of 2 mg mL^{-1}, passed through syringe filters (Acrodisc 13 mm with 0.45 μm nylon membrane filter, Gelman Science, Ann Arbor, MI, U.S.A.), and the filtrate samples (20 μL) were injected for chiral HPLC analysis.

Substrate specificity of alcohol dehydrogenase in *Nocardia* cell-free extracts

Incubations in quartz cuvettes were conducted in phosphate buffer (1.0 mL, pH 7.0, 0.1 M) containing the substrates (3 mM), $NADP^+$ or NAD^+ (0.25 mM), and cell-free extract (500 μL, 1.70 mg protein). Substrates were dissolved in dimethylformamide (DMF), and 10 μL of the DMF solutions were added to incubation mixtures. Reactions were

Table 2*a*. Mass spectral data for 1-(4′-hydroxyphenyl)ethanol (**17**) isolated from reactions of 4-vinylphenol (**15**) with *Nocardia* conducted in H_2O- and 20% $H_2{}^{18}O$-containing buffers.

	138	139	140	123	125
	$[M]^+$	$[M + 1]^+$	$[M + 2]^+$	$[M - CH_3]^+$	$[M + 2 - CH_3]^+$
Control H_2O	100	10	0	100	2
20% $H_2{}^{18}O$	100	23	18	100	23

Table 2*b*. Mass spectral data for 4′-hydroxyacetophenone (**18**) isolated from reactions of 4-vinylphenol (**15**) with *Nocardia* conducted in H_2O- and 20% $H_2{}^{18}O$-containing buffers.

	136	137	138	121	123
	$[M]^+$	$[M + 1]^+$	$[M + 2]^+$	$[M - CH_3]^+$	$[M + 2 - CH_3]^+$
Control H_2O	100	11	3	100	3
20% $H_2{}^{18}O$	100	16	17	100	20

monitored under initial-rate conditions by measuring changes in absorbance at 340 nm for 5 min at 20°C (Shimadzu 2101-PC equipped with UVPC Optional Kinetics Software v 2.5). The molar extinction coefficient used for NADPH at pH 7.0 was $\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$.

Results and discussion

Conversion of 4-vinylphenol by *Nocardia* sp.

The reaction of *Nocardia* sp. with 4-vinylphenol (**15**) gave two major products identified as **17** and **18**. Characterization data for **17**: R_f = 0.3. ^1H NMR (CD_3OD) δ: 7.19 (d, ^2J = 8.4 Hz, 2H, Ar-2′, 6′H), 6.76 (d, ^2J = 8.4 Hz, 2H, Ar-3′, 5′H), 4.73 (q, ^2J = 6.5 Hz, 1H, C-1H), 1.35 (d, ^2J = 6.5 Hz, 3H, C-2H); identical to the results of Everhart and Craig (21). MS *m*/*z* (%): 138 ($[M]^+$, 15.4), 123 ($[M - CH_3]^+$, 63.5). Characterization data for **18**: R_f = 0.5. ^1H NMR (CD_3OD) δ: 7.87 (d, ^2J = 8.8 Hz, 2H, Ar-2′, 6′H), 6.82 (d, ^2J = 8.8 Hz, 2H, Ar-3′, 5′H), 2.51 (s, 3H, C-1H); spectrum was identical to that for authentic **18**. MS *m*/*z* (%): 136 ($[M]^+$, 35.2), 121 ($[M - CH_3]^+$, 100).

With induced resting cells, **15** was transformed to **17** (25%) and **18** (1%) within 48 h. Trace amounts of 4-ethylphenol (**19**) were detected as a peak at *Rv* 12.1 mL, but only after 144 h, indicating this to be a very minor biotransformation pathway. With racemic **17**, induced resting cells gave **18** in 10% yield, while **15** was undetectable. In the reverse direction, 4′-hydroxyacetophenone (**18**) gave **17** (33%) in 48 h, again producing trace amounts of 4-ethylphenol (**19**). Neither **17** nor **18** gave **15** as a product, indicating the irreversibility of the initial hydration reaction.

Resting cell incubations in 50% D_2O, 100% D_2O, and 20% $H_2{}^{18}O$ containing buffers were conducted to examine the source of oxygen and the mechanism by which *Nocardia* oxidized **15** to **17** and **18**. Reaction products isolated from these incubations were analyzed by mass spectrometry and ^1H NMR spectroscopy to determine the extent and positions of isotope incorporations. Tables 1*a* and 1*b* show the results of deuterium incorporation. For **17**, when compared with control reactions conducted in H_2O-containing buffer, ions at *m*/*z* 139 and 140 for $[M + 1]^+$ and $[M + 2]^+$ were enhanced by 9% and 1%, respectively, from 50% D_2O-containing buffer reaction, and 60% and 10%, respectively, from the 100% D_2O-containing buffer reaction. Fragment ions at *m*/*z* 123 and 124, where methyl groups were lost from the molecular ions, were essentially identical to those from unlabeled **17**, showing that all the deuterium in labeled **17** resided in the methyl groups. ^1H NMR of relative peak intensities for C-2H showed approximately 10 and 47% deuterium incorporations vs. signals for Ar-2′ and 6′-H, respectively, similar to the mass spectral findings. Similar analyses of **18** showed that all deuterium labeling resided in the C-2 methyl group. Deuterium incorporations of 59 and 11%, respectively, for $[M + 1]^+$ and $[M + 2]^+$ from 100% D_2O-containing reactions matched very well with the results for **17** from 100% D_2O. ^1H NMR results for **18** again gave 6.6% incorporation from the 50% D_2O reaction and 47% from the 100% D_2O buffer reaction. Results of deuterium incorporation from 50% D_2O buffers were inexplicably lower than expected.

Comparisons of $[M + 2]^+$ ions for **17** and **18** from reactions conducted in buffers prepared with 20% $H_2{}^{18}O$ (Tables 2*a* and 2*b*) revealed oxygen-incorporation levels of 18 and 14%, respectively. Relative intensities of $[M + 2 - CH_3]^+$ ions revealed 21 and 17% increases in similar ions vs. unlabeled **17**. Both results are consistent with complete incorporation of oxygen from water and not from molecular oxygen.

Labeling results permit a description of the reaction process as shown in Scheme 2. *Nocardia* contains a hydratase that binds and tautomerizes 4-vinylphenol (**15**) to a quinoid intermediate (**16**), which adds water to afford **17**. The tautomerization reaction is apparently partially reversible, because both **17** and its oxidation product **18** contain 1.4 deuterium atoms on the C-2 methyl group. Addition of oxygen from water and not air was clearly confirmed by the $H_2{}^{18}O$ labeling experiment, which gave nearly theoretical incorporations of ^{18}O into **17** and **18**. Slightly lower values than expected in deuterium and $H_2{}^{18}O$ incorporations can be explained by dilutions of isotopically labeled water by intracellular stores of water during the hydration reaction. Reactions conducted under an argon atmosphere with induced resting cells and in cell free extracts without cofactors gave similar products (data not shown), supporting

Table 3. Influence of time on ee% of **17** from hydration of 4-vinylphenol (**15**), reduction of 4′-hydroxyacetophenone (**18**), and oxidation of racemic **17** by *Nocardia* cultures.

	From hydration of **15**		From reduction of **18**		From oxidation of *R,S*-**17**	
Time (h)	*S*-(–)-**17** ee%	Yield (%)	*S*-(–)-**17** ee%	Yield (%)	*R*-(+)-**17** ee%	Yield of **18** (%)
24	90	4	98	0.5	1.4	16
48	90	10	90	1.6	2.2	29
72	88	13	90	2.4	4.9	36
144	84	24.6	86	5.7	7.7	52

Table 4. Substrate specificity of *Nocardia* sp. alcohol dehydrogenase.

Substrate (3 mM)	Relative activity (%)[a]
Primary alcohols	
Vanillyl alcohol	100
Benzyl alcohol	10
2-Hydroxybenzyl alcohol	15
3-Hydroxybenzyl alcohol	20
4-Hydroxybenzyl alcohol	65
4-Methoxybenzyl alcohol	11
2-Phenylethyl alcohol	4
Cinnamyl alcohol	23
3-Phenyl-1-propyl alcohol	2
Methanol	59
Ethanol	25
1-Propanol	39
1-Butanol	39
Oleyl alcohol	0
Secondary alcohols	
1-(4′-Hydroxyphenyl)ethanol	12
2-Butanol	9
Cyclohexanol	9
1-Phenyl-1-propyl alcohol	2
1-Phenyl-2-propyl alcohol	3
Tetralol	3

[a]Vanillyl alcohol was oxidized by cell free extracts containing $NADP^+$ at a rate of 101.2 μM sec^{-1} with a ΔmAbs sec^{-1} of 0.630.

the proposed hydration mechanism. The addition of water to a styrene double bond through a hydration mechanism and not by activation and insertion of molecular oxygen through an epoxide intermediate has not been observed before, except with a mixed anaerobic microbial consortium (14).

Hopper and co-workers (22) reported **17** and **18** as the products of *Pseudomonas* sp. derived, flavoprotein-catalyzed oxidations of 4-ethylphenol (**19**). 4-Ethylphenol methylenehydroxylase, *p*-cresol methylhydroxylase, and vanillyl alcohol oxidase (17, 22, 23) are all flavoproteins that oxidize 4-ethylphenol (**19**) to a proposed quinoid intermediate (**16**) similar to that proposed here for *Nocardia* sp. with **15**. The *R* vs. *S* stereochemistry of **17** produced in flavoprotein-mediated reactions varies with the enzyme used, and may be affected by the addition of electron acceptors (17). Heresztyn (24) and Eldin et al. (25) showed that 4-vinylphenol (**15**) could be microbiologically reduced to 4-ethylphenol (**19**). To rule out a combination of the 4-vinylphenol reduction and coupled flavoprotein oxidation of 4-ethylphenol (**19**) to **17**, *Nocardia* sp. was incubated with **19**. No metabolites were obtained, thus ruling out this oxidative pathway and the benzylic hydroxylation of **19** to **17**.

Enantiospecificity of 4-vinylphenol hydration

The stereospecificity of *Nocardia* 4-vinylphenol hydration was established by chiral HPLC comparison of **17** formed by *Nocardia* cultures to *S*-(–)-**17** prepared by *Pseudomonas fluorescens* lipase resolution of racemic **17** (17, 26). Chromatograms (Fig. 1) for racemic **17** (Fig. 1A), lipase resolved *S*-(–)-**17** (Fig. 1B), and **17** from incubation reactions (Fig. 1C.) clearly show that **17** produced by *Nocardia* is predominantly *S*-(–)-**17**. We then compared the enantiomeric purity of **17** formed by hydration of **15** vs. time (Table 3). From 24 to 144 h, yields of **17** ranged from 4% to about 25%, while the ee of *S*-(–)-**17** decreased from 90% at 24 h to 84% at 144 h. Over time, the reduction of **18** to **17**, a less favorable reaction over all, progressed from 0.5% at 24 h to about 6% at 144 h, while the ee of *S*-(–)-**17** ranged from 98 to 86% (Table 3). These results indicate that hydration occurs preferentially by *si*-face delivery of water to **16**, and that the reduction of **18** occurs preferentially by *re*-face hydride delivery. As both reactions progressed, the ee% decreased, suggesting the enhancement of *R*-(+)-**17** by reaction reversibility, or the involvement of more than one alcohol dehydrogenase with varying enantiospecificity or selectivity. In this regard, when racemic **17** was oxidized to **18**, small excesses of *R*-(+)-**17** (7.7% ee) accumulated as the reaction time and yield progressed (52% yield at 144 h) (Table 3).

Substrate specificity of alcohol dehydrogenase in cell-free *Nocardia* extracts

Both $NADP^+$ and NAD^+ were co-factors in the oxidation of **17** to **18** in *Nocardia* cell free extracts. The alcohol oxidation reaction, however, was nearly 17 times more favorable with $NADP^+$. The range of substrates used by the crude enzyme preparation was broad, as shown in Table 4. Among the primary alcohols, phenolic benzyl alcohols were favorable substrates with vanillyl alcohol giving the highest relative oxidation rates. Among the mono-phenolic benzyl alcohols, 4-hydroxybenzyl alcohol was most readily oxidized, indicating a preference for and a possible participation of the 4-phenolic moiety in the oxidation reaction. As a group of compounds, aliphatic alcohols including methanol, ethanol, 1-propanol, and 1-butanol were oxidized more readily than secondary alcohols. We compared the relative rates of oxidation vs. reduction of 4-hydroxybenzaldehyde–4-hydroxybenzyl alcohol and benzaldehyde–benzyl alcohol using crude cell free *Nocardia* extracts prepared from cells induced with 4-vinylphenol (**15**). In these preparations, the rate of alcohol oxidation was 13 times greater than aldehyde reduction, while the benzaldehyde reduction was greater by 14 times than the alcohol oxidation of benzyl

alcohol. We have initiated the purification and characterization of vinylphenol hydratase.

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