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# Regio- and Stereospecific Conversion of 4-Alkylphenols by the Covalent Flavoprotein Vanillyl-Alcohol Oxidase

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**The regio- and stereospecific conversion of prochiral 4-alkylphenols by the covalent flavoprotein vanillyl-alcohol oxidase was investigated. The enzyme was active, with 4-alkylphenols bearing aliphatic side chains of up to seven carbon atoms. Optimal catalytic efficiency occurred with 4-ethylphenol and 4-*n*-propylphenols. These short-chain 4-alkylphenols are stereoselectively hydroxylated to the corresponding (*R*)-1-(4′-hydroxyphenyl)alcohols (F. P. Drijfhout, M. W. Fraaije, H. Jongejan, W. J. H. van Berkel, and M. C. R. Franssen, Biotechnol. Bioeng. 59:171–177, 1998). (*S*)-1-(4′-Hydroxyphenyl)ethanol was found to be a far better substrate than (*R*)-1-(4′-hydroxyphenyl)ethanol, explaining why during the enzymatic conversion of 4-ethylphenol nearly no 4-hydroxyacetophenone is formed. Medium-chain 4-alkylphenols were exclusively converted by vanillyl-alcohol oxidase to the corresponding 1-(4′-hydroxyphenyl)alkenes. The relative *cis-trans* stereochemistry of these reactions was strongly dependent on the nature of the alkyl side chain. The enzymatic conversion of 4-*sec*-butylphenol resulted in two (4′-hydroxyphenyl)-*sec*-butene isomers with identical masses but different fragmentation patterns. We conclude that the water accessibility of the enzyme active site and the orientation of the hydrophobic alkyl side chain of the substrate are of major importance in determining the regiospecific and stereochemical outcome of vanillyl-alcohol oxidase-mediated conversions of 4-alkylphenols.**

Vanillyl-alcohol oxidase (EC 1.1.3.38) is a flavoprotein from *Penicillium simplicissimum* that originally was shown to catalyze the oxidation of vanillyl alcohol to vanillin with the simultaneous reduction of molecular oxygen to hydrogen peroxide (2). The biological function of vanillyl-alcohol oxidase is unknown, but recent studies have indicated that the enzyme is involved in the biodegradation of 4-(methoxymethyl)phenol (6). Vanillyl-alcohol oxidase is a homooctamer, with each 64-kDa subunit containing an 8α-($N^3$-histidyl)-flavin adenine dinucleotide (FAD) as a covalently bound prosthetic group (2). The *vaoA* gene has been cloned (1), and the vanillyl-alcohol oxidase structure has been determined at 2.5-Å resolution (11). These studies, together with sequence alignments, have revealed that the enzyme belongs to a novel oxidoreductase family sharing a conserved FAD binding domain (8). Vanillyl-alcohol oxidase is a versatile biocatalyst, mechanistically. It can convert a wide range of 4-hydroxybenzylic compounds by catalyzing oxidation, hydroxylation, demethylation, deamination, and desaturation reactions (3, 5). Some of these reactions are of particular interest for biotechnological applications (16).

Based on studies with eugenol and 4-(methoxymethyl)phenol, we have proposed that vanillyl-alcohol oxidase catalysis involves the initial transfer of a hydride from the C-α atom of the substrate to N-5 of the flavin cofactor (5, 7). Formation of the resulting *p*-quinone methide intermediate is facilitated by substrate deprotonation upon binding. The *p*-quinone methide product intermediate subsequently reacts with water in the enzyme active site to form the final aromatic product (Fig. 1).

A similar reaction mechanism has been proposed for the conversion of 4-alkylphenols by the related bacterial flavocytochrome *p*-cresol methylhydroxylase (10, 13). Further support for the hydride transfer mechanism comes from crystallographic data (11). The vanillyl-alcohol oxidase structure has revealed that the active site is located in the interior of the protein and contains an anionic binding pocket facilitating substrate deprotonation. His422 was identified as the residue which covalently links the flavin cofactor. The crystal structure also revealed that the side chain of Asp170 is located close to the C-α atom of the substrate. However, the exact function of this residue in catalysis remains to be elucidated.

Vanillyl-alcohol oxidase displays a remarkable reactivity towards short-chain 4-alkylphenols. Recent studies showed that 4-ethylphenol and 4-*n*-propylphenols are stereospecifically converted into the corresponding 1-(4′-hydroxyphenyl)alcohols with an *e.e* of 94% for the *R* enantiomers. During these reactions, considerable amounts of 1-(4′-hydroxyphenyl)alkenes are formed as side products, indicating that rearrangement of the *p*-quinone methide intermediate competes with water addition (3). 4-Methylphenol, the parent substrate of *p*-cresol methylhydroxylase, is a very poor substrate for vanillyl-alcohol oxidase. Crystallographic and kinetic data suggest that this is due to the stabilization of a flavin N-5 adduct (9, 11). To obtain more insight into the catalytic performance of this unusual flavoenzyme, we have addressed the reactivity and stereochemistry of vanillyl-alcohol oxidase with medium-chain 4-alkylphenols, bicyclic phenols, and 4-hydroxyphenyl alcohols. The results of this study are discussed in relation to the recently determined crystal structure.

## MATERIALS AND METHODS

**Chemicals.** 4-Methylphenol, 4-ethylphenol, 4-*n*-propylphenol, 2-methoxy-4-*n*-propylphenol, 4-isopropylphenol, 4-*n*-butylphenol, 4-*sec*-butylphenol, 4-(3′-methylcrotyl)phenol, 5-indanol, 5,6,7,8-tetrahydro-2-naphthol, vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol), vanillin (4-hydroxy-3-methoxybenzaldehyde), tyramine [4-(2-aminoethyl)phenol], *trans*-isoeugenol [1-(4′-hydroxy-3′-methoxyphenyl)propene], 3-(4′-hydroxyphenyl)propanol, 4-phenylphenol, and 4-benzylphenol were from Aldrich. 4-*n*-Pentylphenol, 4-*n*-heptylphenol, 4-*n*-non-

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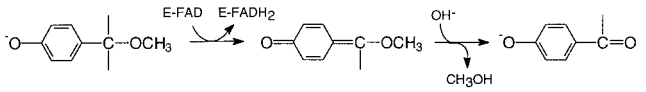


FIG. 1. Proposed reaction mechanism of vanillyl-alcohol oxidase with 4-(methoxymethyl)-phenol.

ylphenol, 4-vinylphenol, and 2-(4′-hydroxyphenyl)ethanol were obtained from Lancaster. Frambinon [1-(4′-hydroxyphenyl)-2-butanone] was from Quest.

4-(3′-Methylcrotyl)phenol (75% pure, based on gas chromatography-mass spectrometry [GC-MS] analysis) was purified to apparent homogeneity by high-performance liquid chromatography (HPLC) with a Lichrospher RP8 reverse-phase column. Racemic 1-(4′-hydroxyphenyl)ethanol and racemic 1-(4′-hydroxyphenyl)propanol were synthesized by Drijfhout et al. (3). (*R*)-1-(4′-Hydroxyphenyl)ethanol was obtained from the enzymatic conversion of 4-ethylphenol (3).

**Enzyme purification.** Vanillyl-alcohol oxidase was purified from *P. simplicissimum* (Oudem.) Thom. CBS 170.90 (ATCC 90172) as described previously (5).

**Analytical methods.** All experiments were performed in air-saturated 50 mM potassium phosphate buffer, pH 7.5, at 25°C, unless stated otherwise. Enzyme concentrations were measured spectrophotometrically by using a molar absorption coefficient, $\varepsilon_{439}$ of 12.5 $mM^{-1}$ $cm^{-1}$ for protein-bound FAD (2). Vanillyl-alcohol oxidase activity was determined by monitoring absorption spectrum changes of aromatic substrates or by oxygen consumption experiments using a Clark electrode. Vanillin production was measured at 340 nm ($\varepsilon_{340}$ = 15.0 $mM^{-1}$ $cm^{-1}$). Formation of 4-vinylphenol and 1-(4′-hydroxyphenyl)ethanol from 4-ethylphenol was measured at 255 nm ($\varepsilon_{255}$ = 14.3 $mM^{-1}$ $cm^{-1}$) and 270 nm ($\varepsilon_{270}$ = 1.2 $mM^{-1}$ $cm^{-1}$), respectively. Formation of 1-(4′-hydroxyphenyl)propanol was monitored at 270 nm ($\varepsilon_{270}$ = 1.3 $mM^{-1}$ $cm^{-1}$). Absorption spectra were recorded on an SLM Aminco DW-2000 spectrophotometer. Dissociation constants of enzyme-inhibitor complexes were determined from flavin absorption perturbation difference spectra by titration of a known concentration of enzyme with the inhibitor.

**Product identification.** HPLC experiments were performed with an Applied Biosystems 400 pump equipped with a Waters 996 photodiode array detector. Enzyme reaction products were separated with a 4.6- by 150-mm Lichrospher RP8 column and isocratic methanol-water mixtures containing 1% acetic acid as the eluent. The methanol/water ratio depended on the type of aromatic compounds to be separated. For resolution of *cis* and *trans* isomers, a 4.6- by 100-mm Microspher $C_{18}$ column was used under conditions similar to those used for the Lichrospher RP8 column. Relative yields of aromatic products were determined by the molar absorption coefficients of 4-vinylphenol ($\varepsilon_{265}$ = 13.0 $mM^{-1}$ $cm^{-1}$), 1-(4′-hydroxyphenyl)ethanol ($\varepsilon_{265}$ = 0.9 $mM^{-1}$ $cm^{-1}$), 1-(4′-hydroxyphenyl)propanol ($\varepsilon_{265}$ = 1.0 $mM^{-1}$ $cm^{-1}$), 4-hydroxyacetophenone ($\varepsilon_{265}$ = 12.5 $mM^{-1}$ $cm^{-1}$), and 4-hydroxypropiophenone ($\varepsilon_{265}$ = 12.9 $mM^{-1}$ $cm^{-1}$) in the appropriate HPLC solvent.

GC-MS analysis was performed on a Hewlett-Packard HP 6090 gas chromatograph and an HP 5973 mass spectrometer equipped with an HP-5 column. Reaction mixtures, containing 0.5 to 1.0 mM aromatic substrate and 0.2 to 0.8 μM enzyme, were incubated at 25°C until the reaction ceased. Samples prepared by extracting the reaction mixtures with 2 volumes of diethylether were injected without derivatization. The temperature program was 5 min isothermal at 50°C, followed by an increase to 240°C at 7°C $min^{-1}$. Relative yields were calculated from the integration of the total ion current peak areas.

$^1$H-nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AMX 400-MHz spectrometer at 24°C. Samples were prepared by freeze-drying the isolated products obtained from HPLC separations and dissolving the products in $CD_3OD$ or $D_2O$.

## RESULTS

**Substrate specificity.** Earlier studies revealed that vanillyl-alcohol oxidase has a relaxed substrate specificity towards 4-hydroxybenzylic compounds (3, 5) (Fig. 2). 4-Ethylphenol, 4-*n*-propylphenol, and 2-methoxy-4-*n*-propylphenol are efficiently oxidized by vanillyl-alcohol oxidase (9). In this study, we investigated the substrate specificity in further detail. Medium-chain 4-alkylphenols with aliphatic side chains of up to seven carbon

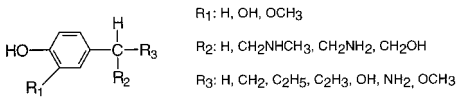


FIG. 2. Substrate specificity of vanillyl-alcohol oxidase from *P. simplicissimum*.

TABLE 1. Steady-state kinetic parameters for vanillyl-alcohol oxidase from *P. simplicissimum*[a]

| Substrate | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($10^3$ $s^{-1}$ $M^{-1}$) |
|---|---|---|---|
| 4-Ethylphenol[b] | 9 | 2.5 | 280 |
| 4-*n*-Propylphenol[b] | 4 | 4.2 | 1,050 |
| 2-Methoxy-4-*n*-propylphenol[b] | 6 | 4.9 | 820 |
| 4-Isopropylphenol | 16 | 1.3 | 81 |
| 4-*sec*-Butylphenol | 72 | 0.5 | 7 |
| 4-*n*-Butylphenol | 2 | 1.2 | 600 |
| 4-*n*-Pentylphenol | 8 | 0.3 | 38 |
| 4-*n*-Heptylphenol | 42 | <0.001 | <0.02 |
| 4-(3′-Methylcrotyl)phenol | 65 | 1.4 | 21 |
| 1-(4′-Hydroxyphenyl)-2-butanone | 128 | 0.3 | 2 |
| 5-Indanol | 77 | 0.5 | 7 |
| 5,6,7,8-Tetrahydro-2-naphthol | 94 | 0.7 | 7 |
| (*R,S*)-1-(4′-Hydroxyphenyl)propanol | 30 | 3.0 | 100 |
| (*R*)-1-(4′-Hydroxyphenyl)ethanol | 222 | 0.7 | 3 |
| (*S*)-1-(4′-Hydroxyphenyl)ethanol | 26 | 4.4 | 170 |
| 2-(4′-Hydroxyphenyl)ethanol | 100 | 0.004 | 0.04 |
| 3-(4′-Hydroxyphenyl)propanol | 8 | 0.1 | 13 |

[a] Standard errors of kinetic parameters were less than 10% except for 4-*n*-heptylphenol and 2-(4′-hydroxyphenyl)ethanol, which had standard errors of about 25%.

[b] Data from Drijfhout et al. (3).

atoms were converted by vanillyl-alcohol oxidase. 4-Alkylphenols with longer aliphatic side chains, like 4-*n*-nonylphenol, did not react. Table 1 shows that straight-chain 4-alkylphenols interact tightly with vanillyl-alcohol oxidase and that the catalytic efficiency (expressed as $k_{cat}/K_m$) is optimal at an alkyl chain length of three carbon atoms. Substituents in the aliphatic side chain significantly affected the catalytic efficiency of vanillyl-alcohol oxidase. Like 4-isopropylphenol and 4-*sec*-butylphenol, frambinon [1-(4′-hydroxyphenyl)-2-butanone] was converted at a significant rate (Table 1). As 4-allylphenols are among the best substrates of vanillyl-alcohol oxidase (5), it was of interest to study the reactivity of 4-(3′-methylcrotyl)phenol. Table 1 shows that this branched-chain 4-allylphenol is indeed a good substrate. Vanillyl-alcohol oxidase was also active with bicyclic phenols. Both 5,6,7,8-tetrahydro-2-naphthol and 5-indanol were readily converted (Table 1). In contrast, no activity was found with 4-benzylphenol.

In an earlier study, we reported that 1-(4′-hydroxyphenyl)alcohols, produced from the enzymatic conversion of short-chain 4-alkylphenols, are not readily oxidized to the corresponding alkanones. This might be due to the high enantioselectivity of the initial hydroxylation reaction, which predominantly yields the *R* isomer (3). Therefore, it was of interest to study the enzymatic conversion of pure enantiomers. Table 1 shows that (*S*)-1-(4′-hydroxyphenyl)ethanol is a far better substrate than (*R*)-1-(4′-hydroxyphenyl)ethanol. This explains why in the reaction of vanillyl-alcohol oxidase with 4-ethylphenol, hardly any 1-(4′-hydroxyphenyl)acetophenone is formed. For the enzymatic conversion of 4-*n*-propylphenol and 2-methoxy-4-*n*-propylphenol, the *R* isomer of the corresponding aromatic alcohol was identified as the main product as well (3). This suggests that for these substrates, the same kinetic resolution mechanism is operative. Vanillyl-alcohol oxidase reacted poorly with C-β and C-γ hydroxylated 4-hydroxyphenyl alcohols (Table 1). In line with this, the enzyme was not active with 4-(aminoalkyl)phenols including tyramine.

**Product identification.** Vanillyl-alcohol oxidase produces different amounts of 1-(4′-hydroxyphenyl)alcohols and 1-(4′-hydroxyphenyl)alkenes from short-chain 4-alkylphenols (3).

TABLE 2. Relative yields of products formed from the conversion of 4-alkylphenols by vanillyl-alcohol oxidase from *P. simplicissimum*

| Substrate | Relative yield (%)[a] | | |
|---|---|---|---|
| | 4-Hydroxyphenyl alcohol | 4-Hydroxyphenyl alkanone | 4-Hydroxyphenyl alkenes |
| 4-Ethylphenol[b] | 76 | <1 | 24 |
| 4-*n*-Propylphenol[b] | 68 | <1 | 32 |
| 2-Methoxy-4-*n*-propylphenol[b] | 90 | <1 | 10 |
| 4-Isopropylphenol | 20 | <1 | 80 |
| 4-*sec*-Butylphenol | 26 | 0 | 74 |
| 4-*n*-Butylphenol | 0 | 0 | 100 |
| 4-*n*-Pentylphenol | 0 | 0 | 100 |
| 4-*n*-Heptylphenol | 0 | 0 | 100 |
| 4-(3′-Methylcrotyl)phenol | 40 | 0 | 60 |
| 5-Indanol | 16 | 60 | 24 |
| 5,6,7,8-Tetrahydro-2-naphthol | 4 | 2 | 94 |

[a] Products were identified by HPLC, GC-MS, and $^1$H-NMR analyses, and relative yields, calculated from HPLC and GC-MS analyses, were determined at saturating substrate concentrations.
[b] Data from Drijfhout et al. (3).

TABLE 3. Relative yields of *cis* and *trans* isomers of 4-hydroxyphenyl alkenes formed from the conversion of 4-alkylphenols by vanillyl-alcohol oxidase from *P. simplicissimum*

| Substrate | Relative yield (%)[a] | |
|---|---|---|
| | *cis*-4-Hydroxyphenyl alkene | *trans*-4-Hydroxyphenyl alkene |
| 4-*n*-Propylphenol | 45 | 55 |
| 2-Methoxy-4-*n*-propylphenol | <1 | >99 |
| 4-(3′-Methylcrotyl)phenol | 0 | 100 |
| 4-*sec*-Butylphenol | 100 | 0 |
| 4-*n*-Butylphenol | 93 | 7 |
| 4-*n*-Pentylphenol | 60 | 40 |
| 4-*n*-Heptylphenol | 50 | 50 |

[a] Products were identified by HPLC, GC-MS, and $^1$H-NMR analyses, and relative yields were calculated from HPLC analysis.

HPLC analysis of the enzymatic conversion of 4-*n*-propylphenol showed that the relative yield of 1-(4′-hydroxyphenyl)propene was 32% (Table 2). Further examination of this fraction on a Microspher $C_{18}$ column revealed two peaks with comparable peak areas near the expected elution time of the aromatic alkene (Fig. 3A). The two products showed slightly different absorption characteristics, with maxima at 251 and 256 nm, respectively, and minor differences in the characteristic aromatic alkene shoulder near 290 nm (Fig. 3B). The mass spectra of the products were identical, with a molecular ion at *m/z* (relative intensity) $(M)^+$ 134 (100%) and the following diagnostic fragments with more than 25% abundance: 132 (77%) and 107 (30%). These results indicate that a mixture of *cis-trans* isomers of 1-(4′-hydroxyphenyl)propene is formed during the vanillyl-alcohol oxidase-mediated conversion of 4-*n*-propylphenol (Table 3). The vanillyl-alcohol oxidase catalyzed conversion of 2-methoxy-4-*n*-propylphenol predominantly yielded the alcoholic product (Table 2). Moreover, from the identical HPLC elution time and absorption spectrum of the main alkenylic product and the reference compound *trans*-isoeugenol, it is evident that *trans*-isoeugenol is formed in large excess over the *cis* isomer (Table 3).

HPLC analysis of the enzymatic conversion of 4-*sec*-butylphenol revealed a low yield of the alcoholic product (Table 2). In addition to 2-(4′-hydroxyphenyl)-*sec*-butanol, two aromatic butenes were produced. However, these two products were not *cis-trans* isomers, because their mass spectra showed different fragmentation patterns. The first eluted aromatic butene contained a molecular ion at *m/z* (relative intensity) $(M)^+$ 148 (96%) and the following diagnostic fragments with more than 25% abundance: 147 (35%), 133 (100%), and 105 (37%). This fragmentation pattern is indicative of 2-(4′-hydroxyphenyl)-*sec*-butene (Fig. 4A). The second alkenylic product contained a molecular ion at *m/z* (relative intensity) $(M)^+$ 148 (100%) and the following diagnostic fragments with more than 25% abundance: 147 (26%), 133 (76%), 119 (88%), and 91 (38%). This points to the production of 1-(4′-hydroxyphenyl)-*sec*-butene (Fig. 4B). Furthermore, comparisons of the absorption spectrum of 2-(4′-hydroxyphenyl)-*sec*-butene with identified *cis-trans* isomers of medium-chain 1-(4′-hydroxyphenyl)alkenes (see below) indicate that only *cis*-2-(4′-hydroxyphenyl)-*sec*-butene is formed.

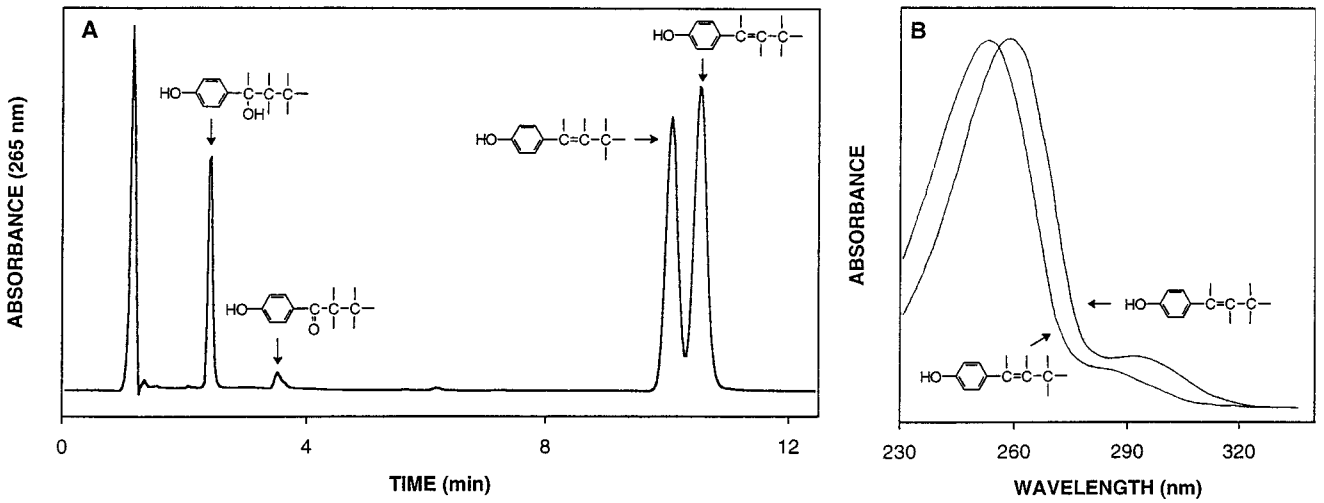


FIG. 3. (A) HPLC analysis of aromatic products formed from the conversion of 4-*n*-propylphenol by vanillyl-alcohol oxidase from *P. simplicissimum*. The elution solvent was methanol-water-acetic acid (50:50:1). (B) Absorption spectrum of *cis*-1-(4′-hydroxyphenyl)propene and *trans*-1-(4′-hydroxyphenyl)propene.

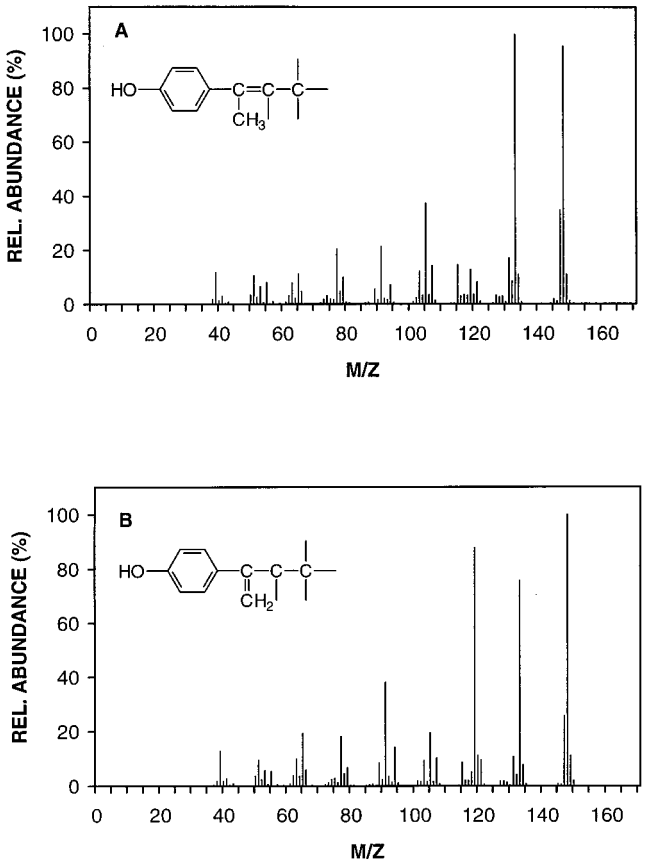


FIG. 4. Mass-spectral analysis of aromatic butenes formed from the conversion of 4-*sec*-butylphenol by vanillyl-alcohol oxidase from *P. simplicissimum*. (A) *cis*-2-(4′-Hydroxyphenyl)-*sec*-butene. (B) 1-(4′-Hydroxyphenyl)-*sec*-butene.

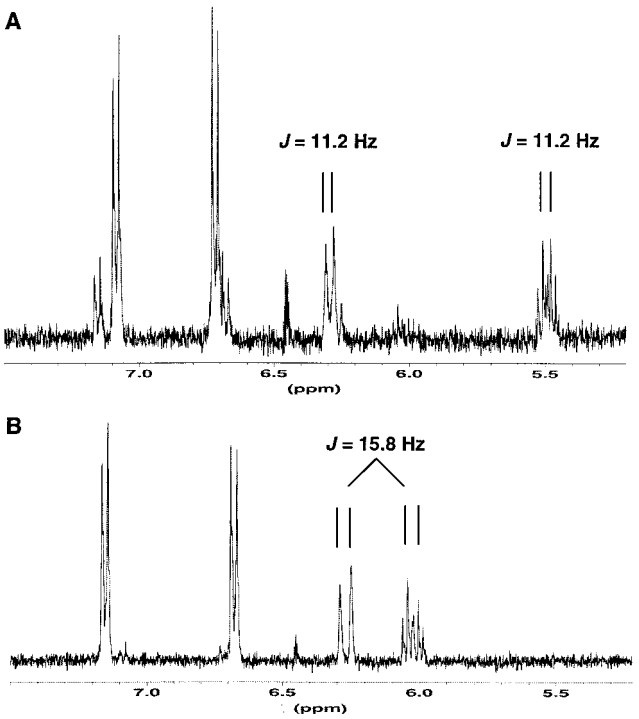


FIG. 5. Expanded $^1$H-NMR spectra of aromatic products formed from the conversion of 4-*n*-pentylphenol by vanillyl-alcohol oxidase from *P. simplicissimum*. (A) *cis*-1-(4′-Hydroxyphenyl)pentene. (B) *trans*-1-(4′-Hydroxyphenyl)pentene.

No alcoholic products are formed in the reaction of vanillyl-alcohol oxidase with aromatic substrates having an alkyl side chain of at least four carbon atoms (Table 2). With 4-*n*-butylphenol, mostly one 1-(4′-hydroxyphenyl)butene isomer was formed. The $^1$H-NMR spectrum of this compound showed vinylic bands at 5.52 ppm (dt, $J$ = 11.5 Hz, 7.0 Hz, 1H) and 6.30 ppm (d, $J$ = 11.5 Hz, 1H) for the C-β proton and C-α proton, respectively. This established that the aromatic butene has a *cis*-relative stereochemistry (Table 3). HPLC analysis of the enzymatic conversion of 4-*n*-pentylphenol showed a mixture of two alkenylic products. The mass spectra of the compounds were identical, with a molecular ion at *m*/*z* (relative intensity) $(M)^+$ 162 (30%) and the following diagnostic fragments with more than 25% abundance: 133 (100%) and 105 (27%). The two products had slightly different absorption characteristics with respect to the absorption maximum and the aromatic alkene shoulder (not shown). These results point to the formation of a mixture of *cis*- and *trans*-1-(4′-hydroxyphenyl)pentene. $^1$H-NMR analysis revealed differences between the two compounds concerning the coupling constants of the vinylic protons: *trans* isomer, 6.03 ppm (dt, $J$ = 15.8 Hz, 7.1 Hz, 1H) and 6.27 ppm (d, $J$ = 15.8 Hz, 1H); *cis* isomer, 5.49 ppm (dt, $J$ = 11.2 Hz, 7.1 Hz, 1H) and 6.29 ppm (d, $J$ = 11.2 Hz, 1H) (Fig. 5). In order to rule out possible *cis-trans* isomerization in the enzyme active site, the isolated isomers of 1-(4′-hydroxyphenyl)pentene were incubated with vanillyl-alcohol oxidase in 50 mM potassium phosphate buffer, pH 7.5. HPLC analysis clearly demonstrated that the configurations of the *cis* and *trans* isomers of 1-(4′-hydroxyphenyl)pentene did not change with time. In analogy to the reaction of 4-*n*-pentylphenol, enzymatic conversion of 4-*n*-heptylphenol resulted in the formation of equal amounts of the *cis* and *trans* isomers of 1-(4′-hydroxyphenyl)heptene (Table 3).

Conversion of eugenol by vanillyl-alcohol oxidase results in the stoichiometric formation of coniferyl alcohol (5). Interestingly, enzymatic oxidation of 4-(3′-methylcrotyl)phenol resulted in the formation of two aromatic products (Table 2). The mass spectrum of the most polar product contained a molecular ion at *m*/*z* (relative intensity) $(M)^+$ 178 (24%) and the following diagnostic fragments with more than 25% abundance: 163 (71%), 145 (63%), 127 (37%), 115 (26%), 107 (100%), and 43 (47%). This points towards the formation of the C-γ hydroxylated product 4-(3′-methyl-1′-butene-3′-ol) phenol (Fig. 6A). Further evidence for the structure of the product was obtained by $^1$H-NMR analysis. The $^1$H-NMR spectrum in $D_2O$ gave bands at δ 1.80 (s, 6H, methyl), 6.20 (d, $J$ = 16.4 Hz, 1H, vinylic), 6.43 (d, $J$ = 16.4 Hz, 1H, vinylic), 6.77 (d, $J$ = 8.4 Hz, 2H, aromatic), and 7.28 (d, $J$ = 8.4 Hz, 2H, aromatic). The coupling constants of the vinylic protons also established that the product has a *trans*-relative stereochemistry. Besides this alcoholic compound a second, more hydrophobic product was formed with a molecular ion at *m*/*z* (relative intensity) $(M)^+$ 160 (46%) and the following diagnostic fragments with more than 25% abundance: 145 (100%), 127 (44%), and 115 (35%) (Fig. 6B). The $^1$H-NMR spectrum in $D_2O$ gave bands at δ 1.85 (3H, s, methyl), 4.99 (d, $J$ = 2.0 Hz, 1H, vinylic), 5.03 (d, $J$ = 2.1 Hz, 1H, vinylic), 6.52 (d, $J$ = 16.6 Hz, 1H, vinylic), 6.78 (d, $J$ = 8.5 Hz, 2H, aromatic), 6.81 (d, $J$ = 16.6 Hz, 1H, vinylic), and 7.34 (d, $J$ = 8.5 Hz, 2H, aromatic). The above data are consistent with the second product being 4-(3′-methylbutadiene)phenol. Furthermore, the coupling

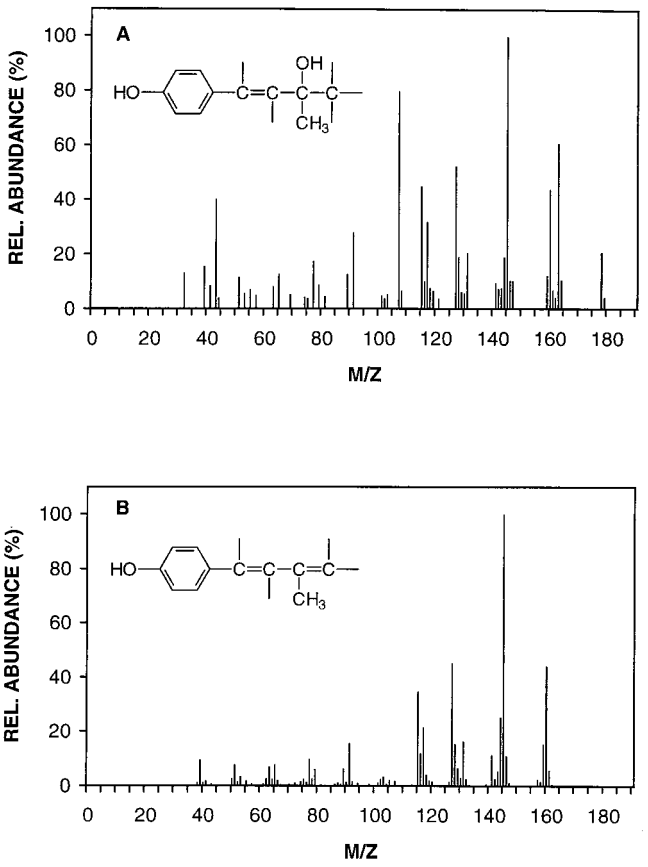


FIG. 6. Mass-spectral analysis of aromatic products formed from the conversion of 4-(3′-methylcrotyl)phenol by vanillyl-alcohol oxidase from *P. simplicissimum*. (A) 4-(3′-Methyl-1′-butene-3′-ol)phenol. (B) 4-(3′-Methylbutadiene)-phenol.

constants of the vinylic protons established that the C-α and C-β protons have a *trans*-relative stereochemistry.

Substantial differences in the ratios of aromatic products were observed when vanillyl-alcohol oxidase was incubated with bicyclic phenols. With 5-indanol, the initial alcoholic product was readily further oxidized to the alkanone, and only small amounts of the alkenylic product were formed (Table 2). However, with 5,6,7,8-tetrahydro-2-naphthol, dehydrogenation of the putative *p*-quinone methide product intermediate was clearly favored over water addition (Table 2). Because the bicyclic products were rather unstable, no attempt was made to study their identity in further detail.

**Product inhibition.** The enzymatic conversion of 4-alkylphenols revealed product inhibition, particularly with medium-chain 4-alkylphenols. This is in agreement with the earlier observation that the alkenylic compounds coniferyl alcohol and isoeugenol are strong competitive inhibitors of vanillyl-alcohol oxidase (5). 4-Vinylphenol, one of the products formed from the enzymatic conversion of 4-ethylphenol (3), appeared to be a very strong competitive inhibitor for vanillyl-alcohol oxidase ($K_i = 3 \pm 1$ μM [Fig. 7]). Similar inhibition during the conversion of 4-ethylphenol was reported for *p*-cresol methylhydroxylase (13). No inhibition constants of other aromatic alkenes were determined. However, the product inhibition observed during the enzymatic conversion of medium-chain 4-alkylphenols suggests that 4-alkenylphenols interact tightly with the enzyme. This is supported by the crystal structures of vanillyl-alcohol oxidase in complex with isoeugenol and 1-(4′-hydroxyphenyl)heptene (11).

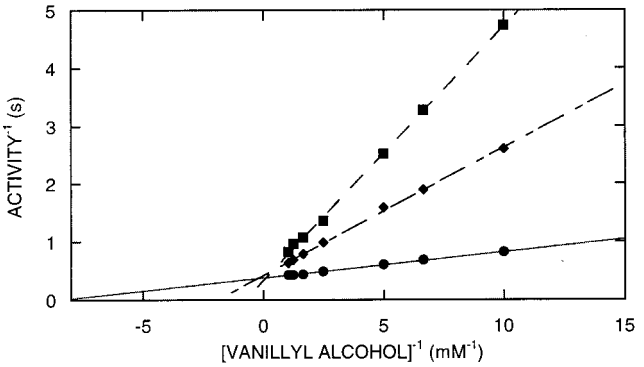


FIG. 7. Competitive inhibition of vanillyl-alcohol oxidase by 4-vinylphenol. The enzymatic conversion of vanillyl-alcohol at pH 7.5, 25°C, was measured by the increase in absorbance at 340 nm. The presence of the following are indicated: no inhibitor (●); 10 μM 4-vinylphenol (◆); 20 μM 4-vinylphenol (■).

## DISCUSSION

In this study, we have described the reactivity of the covalent flavoprotein vanillyl-alcohol oxidase with medium-chain 4-alkylphenols, bicyclic phenols, and 4-hydroxyphenyl alcohols. The enzyme was active, with 4-alkylphenols bearing aliphatic side chains of up to seven carbon atoms. This agrees perfectly with structural data which showed that the active-site cavity of vanillyl-alcohol oxidase is completely filled upon binding of the inhibitor 1-(4′-hydroxyphenyl)heptene (11).

During the conversion of short-chain 4-alkylphenols by vanillyl-alcohol oxidase, (*R*)-1-(4′-hydroxyphenyl)alcohols are formed as major products (3). In this paper, clear evidence is provided that the low yield of 1-(4′-hydroxyphenyl)alkanones is due to a preferred stereospecific oxidation of (*S*)-1-(4′-hydroxyphenyl)alcohols (Fig. 8). The low catalytic efficiency of the conversion of the *R* isomers suggests that the binding of 1-(4′-hydroxyphenyl)alcohols is energetically unfavored when the C-α hydroxyl group of the substrate faces the flavin ring. Interestingly, differences in the stereospecificities of oxidation of 1-(4′-hydroxyphenyl)alcohols were reported for the related flavocytochromes 4-ethylphenol methylenehydroxylase (15) and *p*-cresol methylhydroxylase (12).

Besides catalyzing stereoselective hydroxylation reactions, vanillyl-alcohol oxidase is regioselective as well. Whereas 4-alkylphenols are exclusively hydroxylated at the C-α atom, 4-allylphenols, like eugenol, are hydroxylated at the C-γ atom (5). These differences in regioselective hydroxylation suggest that the site of water attack is dependent on the delocalization of

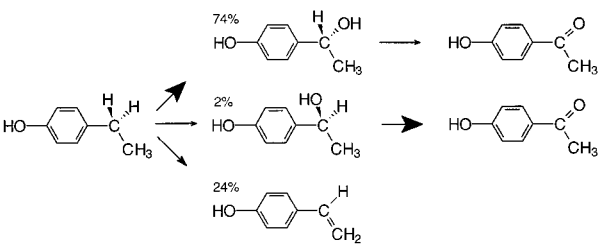


FIG. 8. Reaction pathway for the conversion of 4-ethylphenol by vanillyl-alcohol oxidase.

charge in the enzyme-bound *p*-quinone methide product intermediate. As noted before (11), Asp170 might activate the water, thereby acting as an active-site base. Interestingly, the eugenol derivative 4-(3′-methylcrotyl)phenol is not exclusively hydroxylated at the C-γ position. With this 4-allylphenol, dehydrogenation at the C-δ position occurs to a significant extent.

In contrast to short-chain 4-alkylphenols, medium-chain 4-alkylphenols are exclusively converted by vanillyl-alcohol oxidase to the corresponding 1-(4′-hydroxyphenyl)alkenes. This points to rearrangement of the *p*-quinone methide product intermediate and suggests that the efficiency of water addition to this highly reactive electrophilic species is dependent on the water accessibility of the enzyme active site. The presence of reduced glutathione during turnover of vanillyl-alcohol oxidase with medium-chain 4-alkylphenols did not influence the stoichiometric formation of the alkenylic products, indicating that rearrangement of the *p*-quinone methide intermediate occurs in the enzyme active site. Based on the crystal structures of vanillyl-alcohol oxidase-inhibitor complexes (11), we assume that no significant conformational changes are induced upon binding of the more bulky 4-alkylphenols. Moreover, the crystallographic data show that the carboxylate oxygen atoms of Asp170 are located at about 3.5 Å from the C-β atom of the inhibitors (11). Therefore, rearrangement of the *p*-quinone methide intermediate might be induced by proton abstraction by Asp170, again acting as an active-site base.

In this paper, we have demonstrated that vanillyl-alcohol oxidase dehydrogenates medium-chain 4-alkylphenols stereospecifically. This suggests that the *p*-quinone methide intermediates formed with these substrates are rigidly bound in a specific orientation in the enzyme active site. This *cis-trans* stereospecificity is not unique among flavoenzymes. For example, acyl-coenzyme A dehydrogenases introduce a *trans* double bond between C-2 and C-3 of their coenzyme A substrates (14), whereas glycolate oxidase shows specificity for *re* hydrogen abstraction when prochiral glycolate is used as a substrate (4). In contrast to these enzymes, the relative *cis-trans* stereochemistry of vanillyl-alcohol oxidase is strongly dependent on the nature of the alkyl side chain of the substrate. However, no correlation was found between the stereochemical preference and the bulkiness or length of the 4-alkylphenol side chains.

In summary, the results presented here show that vanillyl-alcohol oxidase is active with a wide range of 4-alkylphenols. Short-chain 4-alkylphenols are mainly hydroxylated to aromatic alcohols, whereas medium-chain 4-alkylphenols are exclusively dehydrogenated to aromatic alkenes. We conclude that the regio- and stereospecificity of the vanillyl-alcohol oxidase-mediated reactions is mainly determined by (i) the intrinsic reactivity of the enzyme-bound *p*-quinone methide intermediate, (ii) the water accessibility of the enzyme active site, and (iii) the orientation of the hydrophobic alkyl side chain of the substrate.

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