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Structural Diversity of Peroxidase-Catalyzed Oxidation Products of *o*-Methoxyphenols

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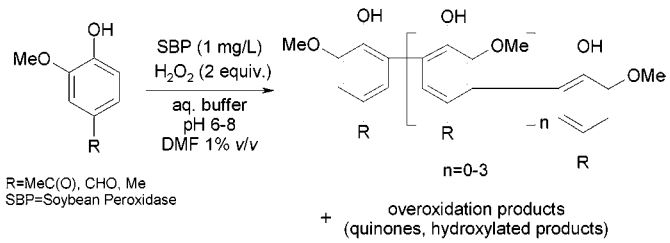
ABSTRACT



The biocatalytic oxidation of *o*-methoxyphenolic compounds led to a variety of oligophenols (dimers to pentamers) and some of their oxidation products. The reaction was carried out in an aqueous medium at room temperature with hydrogen peroxide as the terminal oxidant in a facile and green route to potentially bioactive compounds. Detailed structural information on the products of peroxidase-catalyzed oxidation of *o*-methoxyphenols is presented for the first time.

Peroxidases catalyze the one-electron oxidation of phenols in the presence of H_2O_2.[1] Subsequent, mainly nonenzymatic, radical-driven transformations result in a wide diversity of reaction products. *o*-Methoxyphenols (guaiacols) are a major class of peroxidase substrates and represent a common structural motif found in nature, ranging from lignin precursors (e.g., coniferyl alcohol and ferulic acid[2]) to vanillin and related compounds. In addition to their natural properties, *o*-methoxyphenols have been shown to possess medicinal properties. For example, a combinatorial synthetic approach recently led to identification of a series of *o*-methoxyphenols that induced apoptosis in leukemia U-937 cells.[3] Likewise, apocynin (4-hydroxy-3-methoxyacetophenone) has been shown to be an inhibitor *in vivo* of neutrophil and endothelial cell NADPH oxidases.[4−6] This enzyme is the primary source of reactive oxygen species (ROS), which are implicated in inflammatory diseases, including atherosclerosis, cancer, diabetes, and ischemia-reperfusion lung injury.[7−11] Thus, NADPH oxidase and its inhibition by apocynin and related *o*-methoxyphenols have biomedical significance. These stud-

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Table 1. Products from the SBP-Catalyzed Oligomerization of *ortho*-Methoxyphenols

			products[a]									
	monomer[b]	pH	II	IIHy	IIIHy	IIIHy 2	IV-2 Me	IVHy	IIIQ	III	IIIHyQ	IV
1	A	8			5%	18%		2%			40%	
2	A	7	2%		13%			7%	7%		9%	
3	A	6	18%		7%	4%			2%	8%	5%	
4	V	8	15%		1%		3%					
5	V	7	4%		3%		11%					
6	V	6	1%		4%		6%					
7	G	8	16%	1%	4%					11%		5%
8	G	7	12%	1%	4%					14%		
9	G	6	32%	2%	3%					6%		6%

	monomer[b] (continued)	pH	IVQ	IVQ-2Me	IVQ-Me	V	IVHy 2	IVQ-4M e	VQ2-4Me	VHy	VQ	VQ-Me
1	A	8					3%					
2	A	7	10%			4%	9%				5%	
3	A	6	4%			4%	1%				1%	
4	V	8		27%	10%			4%	9%			
5	V	7		35%	6%			5%	2%			
6	V	6		50%	5%			6%	5%			
7	G	8	5%			7%				4%	5%	8%
8	G	7	7%			9%				6%	9%	7%
9	G	6	5%			4%				5%	4%	5%

[a] Proportion of main products in the first precipitate obtained after centrifugation for 30 min at 3000 rpm, determined by HPLC/UV (λ = 254 nm). Symbols: II, III, IV, V = dimer, trimer, tetramer, pentamer, respectively. Q = quinone (Q2 = 2 quinones), Hy = hydroxylated (Hy2 = twice hydroxylated), −Me = demethylated (−2Me = twice demethylated). [b] A = apocynin, V = vanillin, G = 4-methylguaiacol. Additional precipitates give similar product distributions.

ies have proposed that apocynin is not a direct inhibitor but is metabolized by peroxidases *in vivo* to yield oligomeric oxidation products that possess biological activity.[12,13]

Despite the emergence of apocynin and related compounds as potential prodrugs against the NADPH oxidase target, the products of peroxidase-catalyzed apocynin oxidation both *in vitro* and as metabolites of *in vivo* peroxidase action remain poorly characterized. For this reason, we embarked on a study of the peroxidase-generated oxidation products of apocynin and related *o*-methoxyphenols, paying particular attention to oligomeric species that may hold promise as therapeutic candidates.

Peroxidase-catalyzed oxidation of apocynin was performed as follows, using soybean peroxidase (SBP) as a commercially available and highly active enzyme:[14] 1.0 μg/mL SBP was dissolved in 0.5 L of aqueous buffer (20 mM, pH 6, 7, or 8) containing 12 mM apocynin and 1% (v/v) DMF (to aid in substrate solubility). The reactions were initiated by the slow feed (0.1 mL/h from a 30%, w/v, H_2O_2 solution) of H_2O_2 to the reaction flask, which also prevented oxidant-induced inactivation of SBP.[15] The molecular weight of peroxidase-generated oligomers and polymers is strongly dependent on the presence and concentration of organic solvents[16,17] or the presence of surfactants.[18] Because our interest was focused on small oligophenols (2−5 units), the presence of 1% (v/v) DMF was deemed sufficient. The reactions proceeded for 12 h, following which the solution was centrifuged to recover the precipitated products. The overall yield of apocynin oxidation products was >80% with a very high conversion (see Supporting Information).

The precipitate was then fractionated via flash chromatography over silica to yield a range of products that were analyzed by LC-MS (APCI), UV spectroscopy, and $^1H/^{13}C$ NMR. Positive- and negative-mode LC-MS yielded proton and sodium adducts (M + 1 and M + 23, respectively) and the conjugated base (M − 1) of the multiple products. As a result, oligomers were identified to be formed via both ortho−ortho and ortho−meta (to the phenolic moiety) coupling and in some cases contained hydroxylated and quinone products. A small fraction of demethylated products were obtained through further peroxidase catalysis, which likely led to the corresponding catechols or to quinone compounds. These kinds of products are consistent with known peroxidase chemistries.[19−23]

The oxidation of apocynin was strongly influenced by reaction pH. Slightly acidic conditions favored the formation of the dimer, diapocynin, as the primary product (Table 1),

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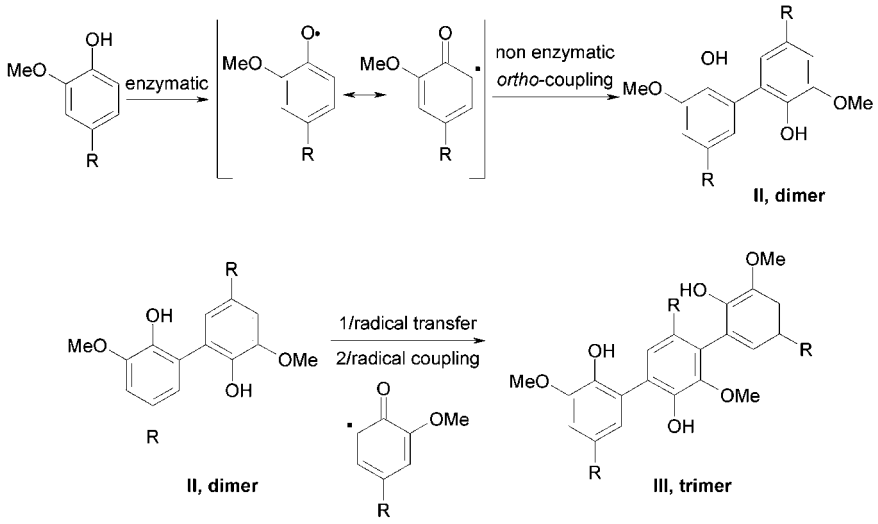
Scheme 1. Enzyme-Catalyzed Radical Formation Followed by Nonenzymatic Ortho Coupling in Solution



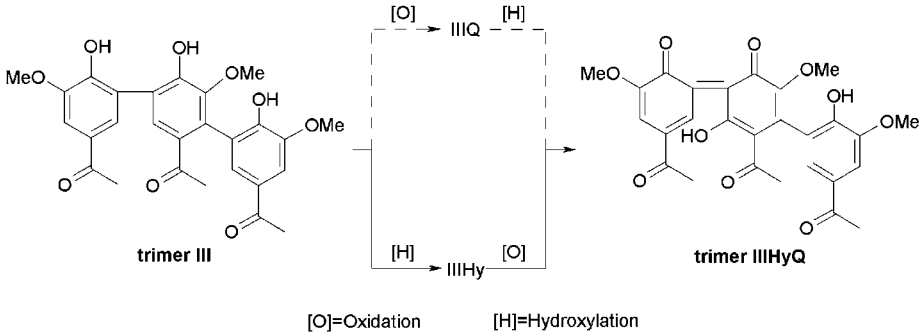
although several trimeric species were obtained. A small fraction of tetramers and pentamers were also formed. The reaction at pH 7 shifted the product spectrum to a nearly equal fraction of trimers and tetramers, including a large total fraction of hydroxylated products. While not unknown in peroxidase chemistry,[20,21] hydroxylation reactions in the H_2O_2 system are not common and may be a result of the unique characteristics of apocynin. Products of the pH 8 reaction were different still, and a major trimeric hydroxylated quinone was obtained in 40% yield (compound IIIHyQ in Table 1). This quinone had an *m*/*z* value of 508 and the potential structure shown in Scheme 2. To explain the formation of this compound, we propose first the usual formation of the trimer III by radical coupling. This trimer may then be hydroxylated to give IIIHy followed by oxidation to IIIHyQ. The lack of IIIQ at pH 8 suggests that this compound is not likely to be along the major route to IIIHyQ.

The tautomeric equilibrium of polyphenolic quinones has been well described.[24−26] IIIHyQ contains three carbonyl groups from the apocynin side chain, two carbonyl groups resulting from the oxidation to quinone, and two phenolic moieties. All these functional groups are conjugated to each other on the aromatic trimeric backbone with the five carbonyl groups virtually locating on any of the seven C−O bonds available.

The ^{13}C NMR spectra of compound IIIHyQ did not reveal corresponding quaternary carbons. The ^{1}H NMR spectra did not resolve the location of the specific hydroxyls oxidized to quinones. Four signals were observed as doublets between 7.70 and 7.20 ppm accounting for four hydrogen atoms. We took these signals as evidence of a central position for the added hydroxyl group in IIIHyQ to give a unique ^{4}J coupling for each proton. Methyl group (three methoxy and three methyl ketone) chemical shifts were observed as expected, in both ^{1}H and ^{13}C NMR analysis. The quinone products are significant, because of their potential reactivity toward critical thiol residues in biological targets[27] such as critical sequences of the protein subunits of NADPH oxidase.

In addition to reaction pH, we evaluated the influence of the nature of the R substituent in the para position of the

Scheme 2. Secondary Reactions Likely to Occur during the Peroxidase-Catalyzed Oxidation of *ortho*-Methoxy Phenols (Case of a Trimer of Apocynin, Leading to Compound IIIHyQ of Mass 508)[a]



[a] Dashed lines on the top route indicate that it is proposed to be a minor route to IIIHyQ.

phenolic monomer on peroxidase-catalyzed *o*-methoxyphenol oxidation. To that end, we studied the oxidation of vanillin (4-hydroxy-3-methoxybenzaldehyde) and 4-methylguaicol. Reactions were performed under conditions identical to those used for apocynin at pH 6−8. As summarized in Table 1, vanillin yielded a product spectrum that was significantly distinct from that obtained from apocynin. At all three pHs, vanillin underwent oxidation to predominantly quinone products with minimal hydroxylation. Unlike apocynin, significant ortho demethylation was observed (anywhere from 53−72% of the converted vanillin was oxidized to a demethylated product), such that at pH 6, ca. 50% of the vanillin oxidation products was a didemethylated tetrameric quinone (IVQ-2Me).

In contrast, the major products from the oxidation of 4-methylguaiacol, which bears an electron-donating side chain, were simple oligomers (dimer II and trimer III) with relatively little influence of reaction pH (Figure 1).

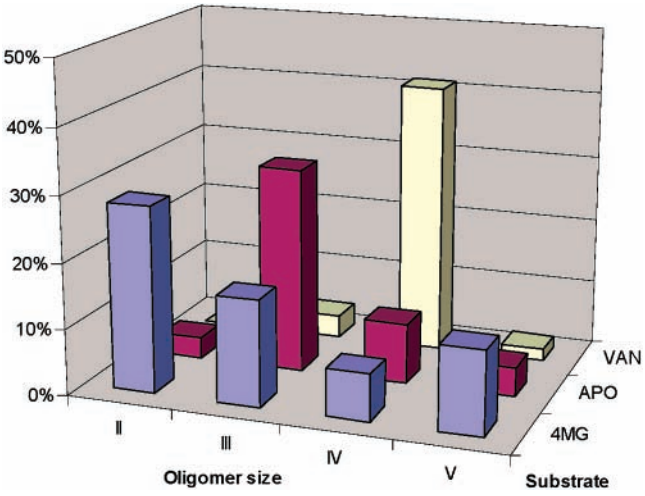


Figure 1. Final percentage of the total product of dimer, trimer, tetramer, and pentamer as a function of the substrate (4-methylguaiacol, apocynin, vanillin).

The different product spectra obtained from these three closely related peroxidase substrates is due to the nature of the side chain. An obvious difference lies in the electron-donating/withdrawing character of the para substituent. 4-Methylguaiacol has an electron-donating substituent and leads to relatively simple dimers, trimers, and higher oligomers. Apocynin and vanillin contain electron-withdrawing para substituents, which appear to favor quinone formation and demethylation. We and others have performed Hammett analysis of peroxidase catalysis.[28,29] In aqueous solutions, the Hammett coefficient is negative, indicating increased enzyme activity on electron-donating para substituents. Most of these substrates undergo ortho−ortho coupling to give simple oligomers and polymers. The less reactive electron-withdrawing substituents also undergo oligomerization; however, their slower oxidation rate may favor noncoupling reactions such as demethylation, hydroxylation, and oxidation to quinones (mainly 1,4-quinones, but 1,2-quinones are possible following demethylation).

Further work is needed to elucidate the precise mechanism of product formation. In any event, the uniqueness of the *o*-methoxy group is highlighted by using the apocynin analogue 4-hydroxy-3-methylacetophenone. This compound, which was poorly reactive (98% of the starting material was recovered unchanged), formed primarily the dimer and did not produce a complex mixture of oligomers.[30]

In summary, peroxidase catalyzes the oxidation of *o*-methoxyphenols to a wide array of oligomeric products, including demethylated, quinones, and demethylated quinones. This structural richness appears to be promoted by electron-withdrawing substituents in the para position. The combination of the two has served nature well as a common structural motif for a range of biologically important compounds, including some with therapeutic potential.

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Note Added after ASAP. One author name, Lakshmi Santhanam, was missing in the version posted ASAP May 7, 2004; the corrected version was posted May 13, 2004.

Supporting Information Available: Detailed general procedure for SBP-catalyzed oxidation of phenols, full MS data for compounds given in Table 1, and spectral data of selected compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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