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Polyguaiacol: a Useful Model Polymer for Lignin Biodegradation Research

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A polymer of ring-labeled [^{14}C]*o*-methoxyphenol ([^{14}C]guaiacol) was prepared by peroxidase-H_2O_2-catalyzed oxidation of the ^{14}C-labeled monomeric compound. The ring-labeled [^{14}C]polyguaiacol contained 67.71% carbon, 5.09% hydrogen, 27.49% oxygen, 25.44% methoxyl, and 8.60% phenolic hydroxyl. The polymer had an average molecular weight of between 5,000 and 15,000, as determined by gel chromatography. A schematic representation of the polymer, similar to previously published structures of polyguaiacols, was devised to meet these and other analytical parameters. The polymer is primarily composed of *o-o* and *p-p*-linked guaiacol moieties, with an occasional *o-p*-biphenyl link and some *p*-diphenoquinone structures. An approximate molecular formula is $[C_{49}O_{14}H_{31}]_n$, where $n \geq 5.8$. Its C_6 formula is $C_6H_{2.3}O_{0.3}^{carbonyl}(OH)_{0.7}(OCH_3)_{1.0}$. Polyguaiacol has many of the characteristics of a synthetic lignin. It is easier and less expensive to prepare than standard synthetic lignins (dehydrogenation polymers of coniferyl alcohol). It is degraded ([^{14}C]polyguaiacol $\rightarrow$ $^{14}CO_2$) by the lignolytic system of the white-rot fungus *Phanaerochaete chrysosporium*. It is suggested that [^{14}C]polyguaiacol may be of value as a substrate for lignin biodegradation research.

In recent years, radioisotopic methods have been applied very successfully to the study of lignin biodegradation (6, 7). Several types of isotopically labeled lignin have been prepared and used for biodegradation studies. Radioactive lignin preparations have included: [^{14}C-lignin]-labeled lignocelluloses (natural lignocellulose complexes labeled specifically in their lignin components), [^{14}C]DHPs (synthetic dehydrogenative polymers of [^{14}C]coniferyl alcohol), and ^{14}C-labeled milled wood lignins purified from ^{14}C-labeled plant tissues. The conversion of these ^{14}C-labeled lignins to $^{14}CO_2$ provides a convenient and unequivocal assay for microbial mineralization of lignin (7).

Of the three types of [^{14}C]lignin available, [^{14}C]DHPs are probably the mose useful for general research. Not only are DHPs very good lignin model polymers, but they have been well characterized as to their chemical and physical properties (6, 9). Unfortunately [^{14}C]DHPs (particularly the very valuable ring-labeled preparations) are somewhat difficult and expensive to prepare (7). These disadvantages are serious enough to preclude the use of [^{14}C]DHPs in many laboratories.

We investigated the potential for using a ^{14}C-labeled polymer of guaiacol (*o*-methoxyphenol) as a lignin model. We report here that ring-labeled [^{14}C]polyguaiacol is (i) an excellent lignin model polymer, particularly as regards lignin's biphenyl structures, (ii) easier and less expensive to prepare than the ring-labeled coniferyl alcohol DHPs, and (iii) degraded by the ligninolytic system of the wood-rotting fungus *Phanaerochaete chrysosporium*.

MATERIALS AND METHODS

Synthesis and characterization of ring-labeled [^{14}C]polyguaiacol. [^{14}C]guaiacol was prepared from [*U*-^{14}C]phenol by the methods of Kratzl and Vierhapper (11). The [^{14}C]guaiacol was then oxidatively polymerized by modification of previously reported methods (3, 12). Three solutions were prepared: (i) 1 liter of 50 mM MOPS (morpholinopropanesulfonic acid) buffer, pH 7.0, containing 1.0 g of guaiacol (~5 μCi of ring-labeled [^{14}C]guaiacol), (ii) 1 liter of distilled water containing 0.3% H_2O_2, and (iii) 50 ml of 50 mM MOPS buffer, pH 7.0, containing 1 to 5 mg of horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo.). Solutions (ii) and (iii) were dripped simultaneously into stirred solution (i) over a period of 8 h. The mixture was then stirred for an additional 40 h. Polyguaiacol precipitated from solution as a brick-red, amorphous solid. The precipitate was collected by centrifugation and washed four times by suspension in water. The final product (0.6 g) was dried by lyophilization from a minimal volume of water. The ring-labeled [^{14}C]polyguaiacol had a specific radioactivity of 8,463 dpm/mg. It was soluble in ethanol (where it lost its color over a period of several hours), acetone, chloroform, dioxane-water (1:1 by volume), 0.1 N

NaOH (weakly soluble), and dimethyl formamide. It was insoluble in distilled water and 0.1 N HCl. Elemental analyses (Galbraith Laboratories, Inc., Knoxville, Tenn.) yielded: 67.71% carbon, 5.09% hydrogen, 27.49% oxygen, and 25.44% methoxyl. After methylation with diazomethane, methoxyl content increased to 38.84%. The brick-red polymer was decolorized immediately in the presence of sodium borohydride. Phenolic hydroxyl content of the polymer (8.60 ± 1.05%) was estimated by the ΔE_i method of Aulin-Erdtman (2). The molecular weight distribution of the polyguaiacol was examined by gel exclusion chromatography (Sephadex G-25, packed in 0.1 N NaOH), as described in Results and Discussion. Proton nuclear magnetic resonance spectra of polyguaiacol were used to estimate aromatic/aliphatic proton ratios. Spectra were obtained in deuteroacetone, using a Brüker 270-MHz spectrometer run in the Fourier transform mode.

Microbiological experiments. Strain ME-446 (U.S. Department of Agriculture, Forest Products Laboratory, Madison, Wis.) of *P. chrysosporium* was used for biodegradation experiments. The growth media used contained the following components (grams per liter of water): NH_4Cl (0.033 [low-nitrogen medium] or 0.66 [high-nitrogen medium]), KH_2PO_4 (0.2), $MgSO_4 \cdot 7H_2O$ (0.05), $CaCl_2$ (0.01), L-asparagine–water (0.1), thiamine (0.0001), polyacrylic acid buffer (0.72), and glucose (10.0). In addition, 1 ml of a mineral elixer (10) per liter was added as a source of trace elements. The final pH of all media was adjusted to 4.3. These are essentially the media described by Kirk et al. (10), which they optimized for lignin degradation by *P. chrysosporium*. Ring-labeled [^{14}C]polyguaiacol (1.85 mg = 15,630 dpm) was added to 100 ml of growth medium in a flask equipped with ports for periodic flushing of its gas phase. The [^{14}C]polyguaiacol was added in a small volume (about 30 μl) of acetone. All cultures were inoculated with an equal number of fungal spores. Evolution of $^{14}CO_2$ was monitored by flushing flasks periodically and trapping evolved $^{14}CO_2$ in an ethanolamine-supplemented liquid scintillation cocktail (5). Cultures were incubated without agitation at 37°C.

RESULTS AND DISCUSSION

Our data suggest that our preparation of polyguaiacol may be represented by the schematic shown in Fig. 1, which is a modification of the schematic of "tetraguaiacoquinone" published previously (3, 12). Analytical data calculated for this structural scheme match very closely data obtained by actual analysis of the polymer (Table 1). The proposed structure also agrees with previous observations of peroxidase-hydrogen peroxide-catalyzed oxidations of methoxyl-substituted phenols, including guaiacol. When guaiacol is oxidized by phenol oxidases, a C-C-coupled polymer is formed (12). The polymer is known to be linked predominantly by *o-o* and *p-p* bonds, with occasional *o-p* links and some formation of *p*-diphenoquinone structures (imparting the brick-red color; 3, 14). Our observed decolorization of the polymer by

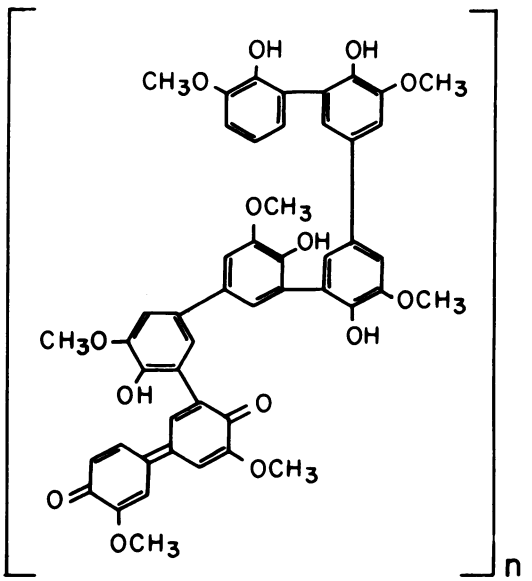

FIG. 1. *Schematic representation of polyguaiacol. The polymer has a molecular formula of* $[C_{49}O_{14} \cdot H_{41}]_n$, *where* $n \geq 5.8$ *for the excluded fractions. Its* C_6 *formula is* $C_6H_{2.3}O_{0.3}^{carbonyl}(OH)_{0.7}(OCH_3)_{1.0}$, *which agrees well with the* C_6 *formula of "tetragaiacoquinone" proposed by Lindgren (12). For schematic representations of lignin, see references 1, 6, and 14.*

borohydride probably reflects reduction of these diphenoquinone structures (12).

Elemental and functional group analyses of polyguaiacol approximate those for a typical spruce milled wood lignin (Table 1); however, there are some significant differences. Polyguaiacol contains 3.8% less oxygen and 0.8% less hydrogen than spruce lignin. It contains 5.4% more carbon, about 10% more methoxyl, and about 8.4% more phenolic hydroxyl than a typical spruce lignin. These differences apparently are not sufficient to prevent *P. chrysosporium* from recognizing polyguaiacol as lignin (see below).

Figure 2 shows the polymer's molecular weight distribution. Much of the polyguaiacol was excluded in the void volume of a Sephadex G-25 column, suggesting an average molecular weight for the excluded fraction of >5,000 (*n* in Fig. 1 being ≥5.86). The polyguaiacol also contained some lower-molecular-weight components in the range of 500 to 1,000. These are probably accurate molecular weight estimates, since gel chromatography was performed in 0.1 NaOH, which prevents aggregation of lignin and lignin-like polymers (4). Also, polymeric lignins run as standards behaved similarly to polyguaiacol under our chromatographic conditions (Fig. 2). Chromatography on Sephadex G-50 indi-

TABLE 1. *Analytical characteristics of polyguaiacol*

Basis of value	Determination						
	% C	% O	% H	% OCH_3	ArH/OCH_3[a]	% OCH_3 after methylation[b]	% Phenolic OH[c]
Fig. 1	68.9	26.2	4.9	25.4	0.80	38.52	8.7
Analysis of polyguaiacol	67.71	27.49	5.09	25.44	0.85	38.83	8.6 ± 1.05
For typical spruce milled wood lignin[d]	62.3	31.3	5.9	15.0			0.24

[a] Ratio of aromatic to methoxyl protons.
[b] Methylation with diazomethane.
[c] Average of three replicates.
[d] From reference 6.

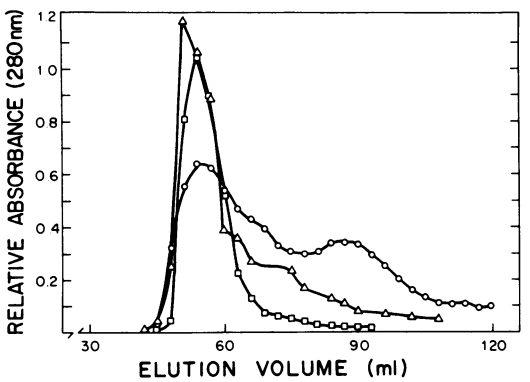

FIG. 2. *Gel exclusion chromatography of polyguaiacol and lignin. Elution patterns were* (△) *spruce milled wood lignin;* (□) *Westvaco Indulin AT kraft lignin, purified by precipitation into ether and water;* (○) *polyguaiacol. The column (63 by 2.8 cm) was packed with Sephadex G-25 in 0.1 N NaOH. Polymers (1.5 mg) were eluted with 0.1 N NaOH at a flow rate of 0.66 ml/min. The column void volume was approximately 48 ml. Elution of these aromatic polymers was monitored by observing their ultraviolet absorbance at 280 nm.*

cated that polyguaiacol synthesized by our procedure has a maximum molecular weight of <15,000.

Polyguaiacol appears to be an excellent lignin model polymer. It is a water-insoluble, amorphous, high-molecular-weight, methoxy-aromatic polymer containing primarily biphenyl linkages between monomeric units. Biphenyl bonds are among the most important intermonomer linkages in lignin. For example, 9.5 to 11% of the phenylpropane subunits of spruce lignin are connected to an adjacent subunit by a biphenyl bond (1). Since polyguaiacol lacks many other important types of intermonomer linkages known to occur in lignin, it is probably best considered as a polymeric lignin "substructure" model. Studies using polyguaiacol as a lignin model polymer thus will address metabolism of biphenyl-related aromatic structures to the exclusion of side chains and monoaromatic moieties.

If polyguaiacol is to be used in microbiological experiments as a lignin substructure model, it must be recognized as lignin by lignin-degrading microorganisms. Thanks to the pioneering work of Kirk and his colleagues (8–10), who have studied lignin degradation by the white-rot fungus *P. chrysosporium*, it is possible to experimentally test whether polyguaiacol is recognized as lignin by a ligninolytic microorganism.

P. chrysosporium degrades to CO_2 all of the structural components of lignin, including its biphenyl moieties. Operation of the *Phanaerochaete*'s ligninolytic system is readily recognized because the system behaves in a unique physiological manner. The lignin-degrading system of *P. chrysosporium* is one manifestation of secondary metabolism in this organism. The system appears only after primary growth has ended, usually as a result of nitrogen starvation (8, 10). In addition, the system is optimally active when the fungus is cultivated under an 80% O_2 atmosphere and at a pH of about 4.3 (8).

If ring-labeled [^{14}C]polyguaiacol is degraded by the ligninolytic system of *P. chrysosporium*, then under our growth conditions (see reference 10), $^{14}CO_2$ evolution should become significant after nitrogen depletion at about 5 to 6 days of growth in low-nitrogen (1.2 mM) cultures. In high-nitrogen (24 mM) cultures, $^{14}CO_2$ evolution should be repressed for at least several weeks because these cultures are not likely to become nitrogen or carbon limited in this time period. Addition of NH_4Cl to nitrogen-starved, $^{14}CO_2$-evolving cultures should in turn significantly decrease the rate of $^{14}CO_2$ evolution by reimposing nitrogen repression of the ligninolytic system (8, 10). These are the results we have observed.

[^{14}C]polyguaiacol degradation ([^{14}C]polyguaiacol → $^{14}CO_2$) by *P. chrysosporium* com-

menced after about 5 days of growth in the low-nitrogen medium (Fig. 3). The rapid $^{14}CO_2$ evolution after day 5 could be repressed by addition of NH_4Cl (24 mM) to relieve nitrogen limitation of growth. Evolution of $^{14}CO_2$ from nitrogen-rich cultures never became significant despite good growth of the fungus. The pH of all cultures slowly decreased from the initial pH of 4.3 to ~3.4 over the course of the experiments. Thus, ring-labeled [^{14}C]polyguaiacol degradation by *P. chrysosporium* behaves similarly to what has been observed for [^{14}C]DHP (10) and [^{14}C-lignin]labeled lignocellulose (13) degradation under similar cultural conditions.

Figure 4 summarizes additional evidence to support our suggestion that polyguaiacol is degraded by the ligninolytic system of *P. chrysosporium*. [^{14}C]polyguaiacol is degraded ([^{14}C]-polyguaiacol → $^{14}CO_2$) about 40% faster when cultures are grown under 80% O_2 than when they

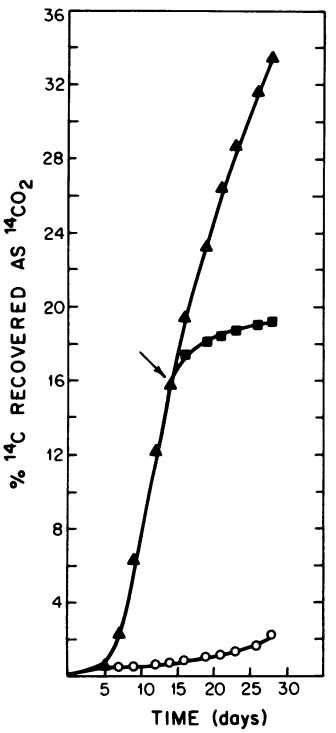

FIG. 3. *Degradation of [^{14}C]polyguaiacol by P. chrysosporium ME-446. Symbols: (▲) Low-nitrogen (1.2 mM) medium (points are averages of three replicates until 14 h, after which single determinations were made); (○) high-nitrogen (24 mM) medium (points are averages of three replicates); (■) after supplementation (at arrow) of the $^{14}CO_2$-evolving low-nitrogen cultures with 24 mM NH_4Cl (points are averages of two replicates). Final pH values of all three culture media were between 3.2 and 3.4.*

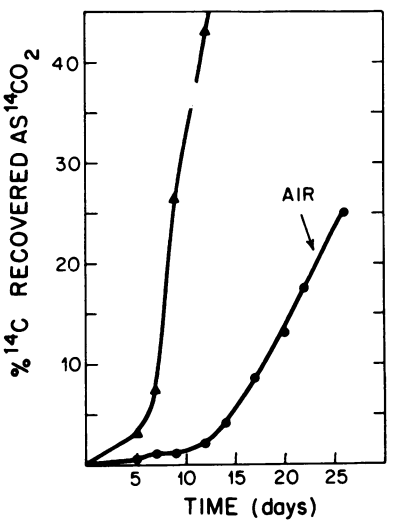

FIG. 4. *Degradation of [^{14}C]polyguaiacol by P. chrysosporium ME-446: effect of O_2 concentration. Symbols: (▲) 80% O_2–20% N_2 atmosphere; (●) ~21% O_2 (air) atmosphere. Each point is the average of two replicates. All replicates were performed with the low-nitrogen (1.2 mM) basal medium.*

are grown under 20% O_2. This type of response is also characteristic of *P. chrysosporium*'s ligninolytic system (8).

It is possible that *P. chrysosporium* preferentially degrades particular molecular fractions of our polyguaiacol. This cannot be determined by the $^{14}CO_2$ evolution experiments reported here. It is possible to chromatographically separate [^{14}C]polyguaiacol into low-, medium-, and high-molecular-weight fractions (Fig. 2) and then examine degradation of each fraction individually. Such experiments are in progress.

Polyguaiacol should become very useful as a substitute for DHPs in many microbiological experiments. It is certainly less difficult and less expensive to prepare (either ^{14}C labeled or non-labeled) than are DHPs. In many systems (e.g., pure culture work with ligninolytic fungi), polyguaiacol should behave similarly to DHPs. However, new applications of polyguaiacol as a means to examine lignin biodegradation mechanisms should always take into account the fact that polyguaiacol contains only biphenyl bonds. Some microorganisms undoubtedly will be found to degrade these biphenyl structures more slowly (or even not at all) than other parts of the lignin macromolecule (e.g., side chains).

[^{14}C]polyguaiacol also can be prepared so that the ^{14}C is located in only methoxyl groups. This would be accomplished by oxidatively polymerizing [*methoxyl*-^{14}C]guaiacol (11). A simple system thus appears available to compare rates and

mechanisms of microbial degradation of lignin-like biphenyl carbons and methoxyl carbons.

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