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TRANSFORMATION OF AROMATIC COMPOUNDS UNDER THE ACTION OF THE BASIDIOMYCETES *Phanerochaete sanguinea* AND *Coriolus villosus*

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The pathways of the transformation of some aromatic compounds by rhe basidiomycetes Phanerochaete sanguinea *and* Coriolus villosus *have been studied. It has been shown that the degradation of these compounds has an oxidative nature and depends on the type of substituents in the benzene ring and the propane chain. A difference has been found in the mechanisms of the reactions of the two fungi that is a consequence of the different compositions of their enzyme complexes.*

Lignin is a complex biopolymer of irregular structure consisting of phenylpropane units and having various functional groups. In lignin the monomeric units are linked with one another by ether bonds and C—C bonds [1, 2]. Only the fungi of white rot are capable of effecting the biodegradation of lignin to an appreciable degree [3]. At the present time, using the fungus *Phanerochaete chrysosporium* on compounds modeling the fragments and bonds of lignin, it has been shown that biotransformation takes place as the result of a broad spectrum of oxidative reactions leading to the breakdown of the aromatic ring and of C_α—C_β, alkyl—aryl and aryl—aryl bonds [4-7]. In recent years, new active lignolytic cultures have been isolated, and these are being studied intensively, since the biochemistry of their action may differ from that of *P. chrysosporium* [8, 9].

Occupying ourselves with biotechnical processes of obtaining cellulose, we have used little-studied strains of the fungi *Phanerochaete sanguinea* BKMF-2487D and of *Coriolus villosus* 0276 and have shown that they are effective in processes for the biopretreatment of wood chips and the bleaching of cellulose [10-12]. In connection with this, the question arises of the chemical reactions accompanying the degradation of lignin and compounds modeling it.

It has been shown previously [13, 14] that the biodegradation by the fungus *C. villosus* of veratrylpropane substrates modeling lignin fragments substituted at phenolic OH groups takes place through cleavage of the C_α—C_β bond and is accompanied by redox reactions $C_{\alpha,\beta}{=}O \rightleftarrows C_{\alpha,\beta}{-}OH$, between which a dynamic equilibrium is established. With time, it shifts in the direction of the formation and subsequent destruction of compounds with a C_α-OH group. Cleavage of a $C_\alpha - C_\beta$ bond may be preceded by oxidation of the C_β atom. It has been found that a relationship exists between the rate of metabolism of the substrates and the degree of oxidation of the C_α and C_β atoms of the propane chain. Thus, substrates containing a C_α—OH group are readily included in the metabolism of the fungus. In the case of compounds containing only a $C_\alpha{=}O$ group in the propane chain resistance to biotransformation appears, but with the additional presence of a C=O or C—OH group in the C_β position the compounds are degraded just as readily.

The present work is a continuation of these investigations. Its aim consists in the study of possible pathways for the biodegradation of aromatic compounds differing by the type of substitution of the ring (*o*-dihydroxy-, guaiacyl, veratryl, syringyl) and also by the substitution and degree of oxidation of the carbon atoms of the propane chain under the action of the fungi *C. villosus* and *P. sanguinea*.

The compounds modeling lignin are shown below:

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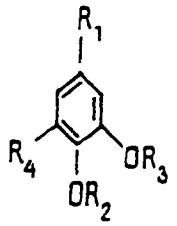
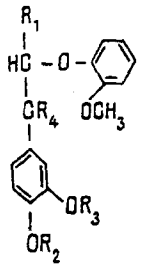


1 — pyrocatechol
$R_1=R_2=R_3=R_4=H$
2 — guaiacol
$R_1=R_2=R_4=H$; $R_3=CH_3$
3 — veratrole
$R_1=R_4=H$; $R_2=R_3=CH_3$
4 — veratryl alcohol
$R_1=CH_2OH$; $R_2=R_3=CH_3$; $R_4=H$
5 — vanillyl alcohol
$R_1=CH_2OH$; $R_2=R_4=H$; $R_3=CH_3$
6 — veratraldehyde
$R_1=CHO$; $R_2=R_3=CH_3$; $R_4=H$
7 — syringaldehyde
$R_1=CHO$; $R_2=H$; $R_3=CH_3$; $R_4=OCH_3$
8 — veratric acid
$R_1=COOH$; $R_2=R_3=CH_3$; $R_4=H$

9 — veratrylpropan-1-ol
$R_1=CHOH-CH_2-CH_3$; $R_2=R_3=CH_3$; $R_4=H$
10 — veratrylpropan-1-one
$R_1=CO-CH_2-CH_3$; $R_2=R_3=CH_3$; $R_4=H$
11 — veratrylpropan-1-on-2-ol
$R_1=CO-CHOH-CH_3$; $R_2=R_3=CH_3$; $R_4=H$
12 — veratrylpropane-1,2-diol
$R_1=CHOH-CHOH-CH_3$; $R_2=R_3=CH_3$; $R_4=H$
13 — guaiacylpropan-1-ol
$R_1=CHOH-CH_2-CH_3$; $R_2=R_4=H$; $R_3=CH_3$
14 — ferulic acid
$R_1=CH=CH-COOH$; $R_2=R_4=H$; $R_3=CH_3$
15 — acetoguaiacone
$R_1=CO-CH_3$; $R_2=R_4=H$; $R_3=CH_3$



16 — β-guaiacyl ether of veratrylpropan-1-ol $R_1=R_2=R_3=CH_3$; $R_4=H$, OH
17 — β-guaiacyl ether of veratrylpropan-1-one $R_1=R_2=R_3=CH_3$; $R_4=O$
18 — β-guaiacyl ether of veratrylpropane-1,3-diol
$R_1=CH_2OH$; $R_2=R_3=CH_3$; $R_4=H$, OH
19 — β-guaiacyl ether of veratrylpropane-1-on-3-ol
$R_1=CH_2OH$; $R_2=R_3=CH_3$; $R_4=O$

20 — β-guaiacyl ether of guaiacylpropan-1-ol $R_1=R_3=CH_3$; $R_2=H$; $R_4=H$, OH
21 — β-guaiacyl ether of guaiacylpropan-1-one $R_1=R_3=CH_3$; $R_2=H$; $R_4=O$
22 — muconic acid COOH—CH=CH—CH=CH—COOH

Not only veratrylpropane compounds [3] but also more complex structures, such as β-O-4 ethers, underwent biodegradation under the action of *C. villosus*. Also in the group of veratryl-substituted β-O-4-ethers (**16-18**) as well as in the group of veratryl propanone compounds (**9-12**) [13] the dependence of the rate of biodegradation of the substrate on the nature of the substituent in the C_α position of the propane chain was retained. Thus, while substrates (**16**) and (**18**), each containing an OH at C_α, were present in the sample only in trace amounts after 8 days, there was still 47% of (**17**), with a $C_\alpha=O$ group, after 10 days. That is, a C_α-carbonyl-containing β-O-4 substrate is also more resistant to biotransformation.

The biodegradation of substrates (**16—18**) took place with the cleavage of the $C_\alpha—C_\beta$ bond, leading to the formation of veratraldehyde (**6**) and, then, veratryl alcohol (**4**), which were detected in the supernatant after 3-4 days (Fig. 1, *a*, *b*). In addition to the cleavage of the $C_\alpha—C_\beta$ bonds in samples with the substrates (**16**) and (**18**), oxidation of the C_α atoms was observed after 5-7 days, leading to compounds (**17**) and (**19**), respectively. In addition, it was noted that the degree of oxidation of the C_γ atom had no effect on the rate and direction of the process of biodegradation of substrates of the β-O-4 type (compare (**18**) with (**16**)).

As is known [15], a similar type of oxidative reactions can be catalyzed by ligninase. This enzyme was discovered in culture filtrates of *C. villosus* [16]. These reactions begin with the transfer of an electron to the hydrogen-peroxide-oxidized heme of the enzyme, which leads to the formation of cation radicals:

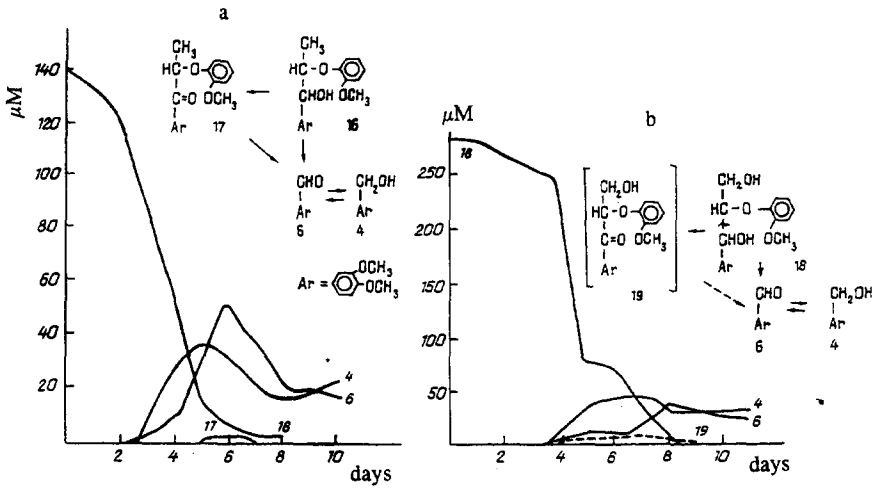
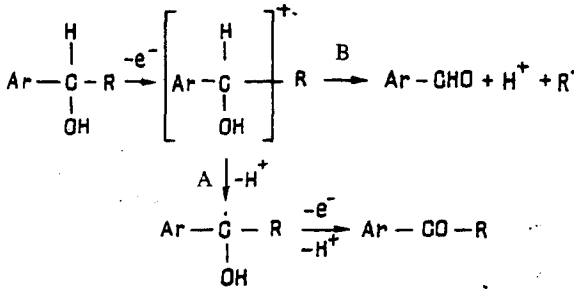


Fig. 1. Amounts of substrates [*a*) the guaiacyl ether of veratrylpropan-1-ol; *b*) the β-guaiacyl ether of veratrylpropane-1,3-diol] and metabolites in culture filtrates of *Coriolus villosus*.



The cation radicals either split out a proton and undergo repeated one-electron oxidation with the formation of carbonyl-containing intermediates (route A) or split into fragments with cleavage of the C_α—C_β bond (route B). Both routes, i.e., not only cleavage of the C_α—C_β bond but also oxidation of the C_α atom, were realized in the biooxidation of the β-O-4 substrates of *C. villosus* that we selected.

Substrates containing free phenolic hydroxyls (**1, 2, 5, 13, 14, 20,** and **21**) underwent transformation by the fungus faster than their substituted-OH analogues. Thus, for example, (**2, 5, 13,** and **14**) were absent from the samples after only one day, and (**1**) after incubation for 36 h (Fig. 2). The predomant direction of biotransformation of these compounds in the initial stages of biooxidation was polymerization.

The molecular-mass characteristics of several substrates were obtained by gel permeation chromatography (Table 1). It was found that all the polymers were polydisperse. Their molecular masses ranged from 400 to 10,000; i.e., the polymers contained an average of from 2 to 35 monomeric units. The guaiacol polymer was an exception. It had a higher molecular mass and was less polydisperse, probably because of a more regular structure.

During further incubation, breakdown of the polymers formed took place. This process occurred particularly clearly in the case of vanillyl alcohol (**5**). Only 2 h after seeding, an aromatic intermediate was detected in the culture filtrate (λ_{max} 285 nm), and this underwent further transformations: chromatograms of one-day samples already showed 13 metabolite peaks. We assumed that the intermediate formed was a dimer of vanillyl alcohol and some of the metabolites that appeared were products of its oxidative degradation.

This pathway of the process was suggested in a study of the electrochemical oxidation of guaiacol [17] and is based on the facts that, in the first place, the one-electron oxidation of phenols forms phenoxy radicals capable of undergoing dimerization (polymerization), and, in the second place, some of the dimers formed possess a lower oxidation potential than the initial monomers and undergo oxidative degradation more readily.

In our experiment, the cause of the appearance of phenoxy radicals may have been the interaction of the phenols both with ligninase [18] and with laccase. The latter is synthesized by the fungus with a fairly high activity [16] and, as is known, catalyzes the oxidation of phenols by a free-radical mechanism [19].

TABLE 1. Molecular-Mass Characteristics of the Polymers Formed as a Result of the Biotransformation of Phenolic Compounds by the Fungi *Coriolus villosus* and *Phanerochaete sanguinea*

Substrate		$\bar{M}_n$	$\bar{M}_w$	U	Interval of M	Interval of n
2	a	850	1670	1.0	400—9300	3—75
	b	800	1650	1.1	250—4000	2—32
13	a	350	1450	3.1	400—6500	2—36
	b	750	1550	1.1	600—10000	3—55
14	a	380	870	1.2	400—5000	2—26
	b	400	940	1.3	400—6300	2—33
21	a	550	1300	1.4	600—10000	2—33
	b	600	1250	1.1	600—4000	2—13

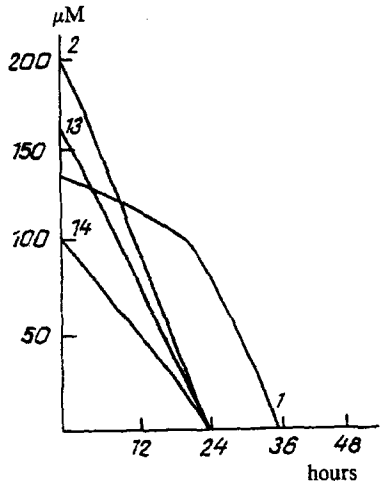


Fig. 2. Changes in the amounts of pyrocatechol (*1*), guaiacol (*2*), guaiacylpropan-1-ol (*13*), and ferulic acid (*14*) in culture filtrates of *Coriolus villosus*.

In the case of the oxidation of substrates possessing a double bond in the aliphatic chain and electron-accepting groups — for example (**14**) — the dimers (polymers) formed clearly have a lower oxidation potential, which permits oxidation products, especially vanillaldehyde, to be detected even during the first few days of incubation.

The fungus *P. sanguinea*, just like *C. villosus*, includes veratrylmethane substrates (**4, 6, 8**) in its metabolism, performing redox ractions at the C_α group (Fig. 3, *a*, *b*, *c*). Likewise, in the group of veratrylpropane compounds (**9—11**) only the C_α atom is affected; for example veratrylpropan-1-one (**10**) is formed from veratrylpropan-1-ol (**9**) (Fig. 4, *a*), and veratrylpropane-1,2-diol (**12**) from veratrylpropan-1-on-2-ol (**11**) (Fig. 4, *b*). It is interesting that the fungus *P. sanguinea*, while oxidizing veratryl alcohol (**4**) to veratraldehyde (**6**), which serves as a test for ligninase activity, is incapable of cleaving the C_α—C_β bond in veratryl-substituted compounds, i.e., of effecting the typical reaction catalyzed by ligninase. In view of the fact that by the term "ligninase" is meant the sum of a number of isoenzymes [20], it may be assumed that we had a variety of their combination.

Further investigations showed that the degree of inclusion of substrates in the metabolism of the fungus *P. sanguinea* depends on the presence of free phenolic OH groups and on the type of substitution of the aromatic ring. Thus, while veratruylpropan-1-ol (**9**) underwent only 40% conversion in 28 days, guaiacylpropan-1-ol (**13**) was completely absent after 21 days (Fig. 5), and, while loss of veratryl alcohol (**4**) in 30 days amounted to about 30%, no vanillyl alcohol (**5**) remained in the samples after 12-14 days. Veratrole (**3**) did not suffer degradation, but the replacement of the methoxy groups by hydroxy

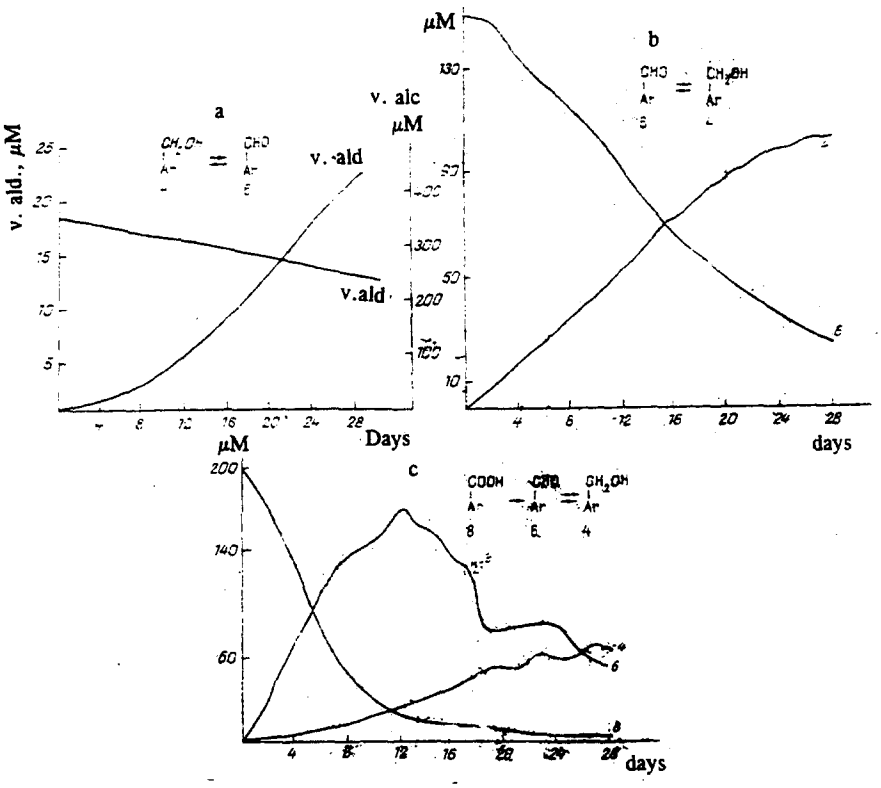
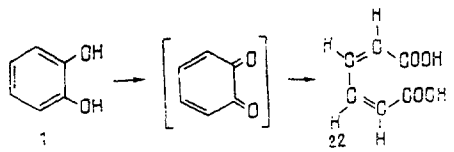


Fig. 3. Amounts of substrates [*a*) veratryl alcohol; *b*) veratraldehyde; *c*) veratric acid] and metabolites in culture filtrates of *Phanerochaete sanguinea*

groups led to a decrease in the resistance of the substrates to biotransformation — guaiacol (**2**) was present in only trace amounts after 20-22 days, while pyrocatechol (**1**) had disappeared after incubation for only 14 days. A dependence of the rate of biodegradation of the substrates on the degree of oxidation of the C_α atom was also traced — while, as shown above, C_α-hydroxyl-containing substrates, were absent from the samples after 21 days, substrate (**15**), having a C_α=O group, had undergone complete transformation only after incubation for 31 days. β-Ethers having an OH group in ring *A* (**20**, **21**) are also included in the metabolism of *Phanerochaete sanguinea*, but more slowly than in the case of *C. villosus* — their loss in 27 days amounted to about 55%, substrate (**20**) being partially oxidized to compound (**21**).

The biodegradation of pyrocatechol (**1**) took place with the formation of single metabolite, which was isolated preparatively and was identified as *cis,cis*-muconic acid (**22**) from its UV absorption (λ_{max} 262 nm), retention time in reversed-phase chromatography in comparison with a standard speciment, melting point (187—188°C), and the mass spectrometry of its methyl ester (*m/z* 170). The presence of this metabolite showed that the enzyme complex of *P. sanguinea* is capable of cleaving the aromatic ring of *ortho*-dihydroxy-substituted structures. The oxidation of pyrocatechol by the fungus may be represent schematically in the following way:



The presence of *o*-quinone as an intermediate of this reaction was shown indirectly by the brown coloration of the culture liquid. No further degradation of the metabolite (**22**) was observed, as for the *trans,trans*-muconic acid used as a comparison substrate.

The biotransformation of vanillyl alcohol (**5**) by the fungus *P. sanguinea* took place in the same way as in the case of *C. villosus* but at a lower rate. Syringaldehyde (**7**) was also included in the metabolism of the fungus — it was present in only trace amounts after incubation for 12-14 days (Fig. 5)..

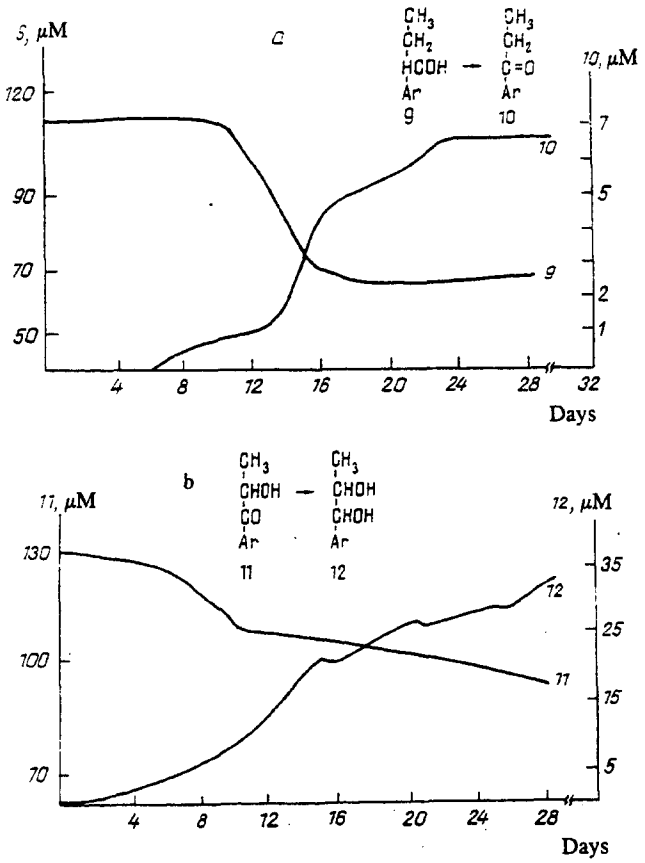


Fig. 4. Amounts of substrates [*a*) veratrylpropan-1-ol; *b*) veratrylpropan-1-on-2-ol] and metabolites in culture filtrates of *Phanerochaete sanguinea*.

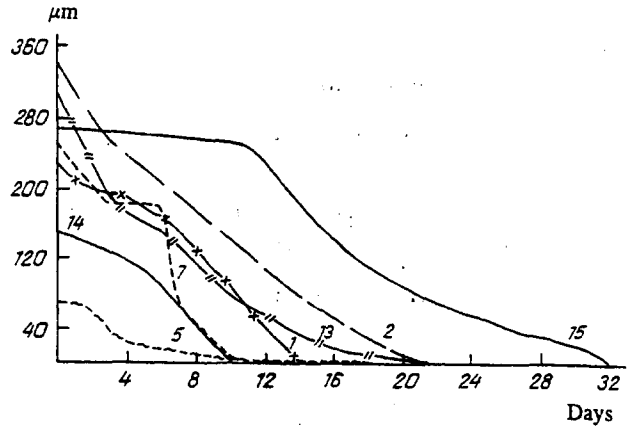


Fig. 5. Changes in the amounts of pyrocatechol (*1*), guaiacol (*2*), vanillyl alcohol (*5*), syringaldehyde (*7*), guaiacylpropan-1-ol (*13*), ferulic acid (*14*), and acetoguaiacone (*15*) in culture filtrates of *Phanerochaete sanguinea*.

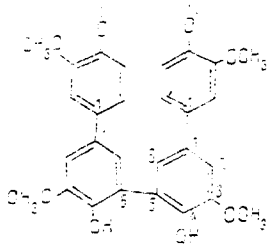
The biotransformation of the majority of substrates with an unsubstituted phenolic hydroxyl by the fungus *P. sanguinea*, just like the fungus *C. villosus*, took place through competing polymerization-degradation reactions. The molecular mass characteristics of some of the polymers are given in Table 1.

Such results of the action of fungi on phenolic compounds are obviously connected with the presence of laccase in the enzyme complex of *C. villosus* and of Mn-peroxidase [16] in that of *P. sanguinea*, these enzymes being similar in their action on these substrates.

In order to investigate the directivity of the polymerization processes brought about by these fungi, we studied the structures of the polymers of guaiacol (**2'**), guaiacylpropanol (**13',13''**), and ferulic acid (**14',14''**) by ^{1}H and ^{13}C NMR spectroscopies. The assignment of the resonance signals in the ^{1}H and ^{13}C NMR spectra of the initial substrates (monomers) was made on the basis of literature information [21-23] and is given in the Experimental part.

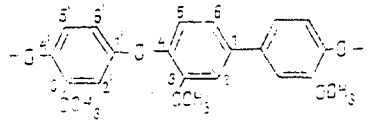
The ^{1}H NMR spectra of the polymeric metabolits of *C. villosus* (**2', 13', 14'**) and of *P. sanguinea* (**13'', 14''**) differed substantially from the spectra of the initial substrates. The resonance signals of the hydrogen atoms of the aromatic rings were highly broadened, which may be connected both with the polymeric nature of the substances under investigation and with the appearance of a large number of new alkyl-aromatic fragments differently substituted in the aromatic ring. The resonance signals of the OCH_3 groups were also broadened.

The ^{13}C NMR spectrum of polymer (**2'**) had none of the resonance signals of the initial substrate. Resonance signals were observed in the following regions: 154-149, 145-142, 140-137, 132, 129-125, 112-108, and ~60 and 56 ppm. The signals with chemical shifts (CSs) in the 140-137 ppm interval showed the formation of C_{ar}—C bonds [21-23]. The probability of the appearance of only C_5—C_5 bonds is low, since there were no signals characteristic for such fragments: 144.6 (C-5), 124.6 (C-1), and 118.8 ppm (C-2) [21-23]. However, the simultaneous formation of C_5—C_5 and C_1—C_1 bonds in structures of the type



can fully explain the presence in the spectrum of (**2'**) of resonance signals with ppm values of 131.9 (C-1), 111-108 (C-2), 143.3 (C-4), and 128.6 (C-5) [23].

Polymerization could take place not only through C—C bonds but also through C_{ar}—O—C_{ar} bonds. The formation of the following fragments in polymer (**2'**) is the most probable:

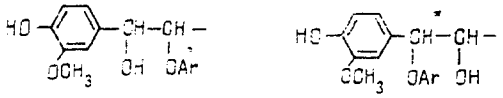


This was shown by resonance signals with the CSs (ppm) 132 (C-1), 112 (C-2 and C-6'), 148-150 (C-3), 142-145 (C-4 and C-4'), 116 (C-5), 120 (C-6) and 153 (C-1'), 103 (C-2'), 150 (C-3'), 109-108 (C-5').

The resonance signals with CSs of 61-60 ppm related to an OCH_3 group (shown by the DEPT method [24]). Such a downfield shift of the signal of an OCH_3 group can be explained by the formation of a C_{ar}—C bond at the C-2 position of the aromatic ring [23].

Thus, the polymer obtained by the action of *C. villosus* on guaiacol (**2**) is formed through C_{ar}—C and C_{ar}—O—C_{ar} bonds realized at the C-5, C-2, C-1 and C-4, C-1 positions of the aromatic rings, respectively.

Under the action of the fungus *C. villosus*, compounds (**13**) and (**14**) underwent similar transformations (with the exception of reactions at C-1). In addition, in the ^{13}C NMR spectrum of polymer (**14'**) resonance signals were observed in the regions of 90-81 and ~70 ppm (C_α, C_β) showing the presence of α- and β-aryl ether bonds [21, 22]:



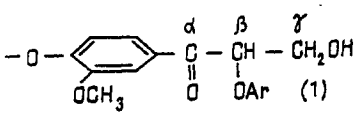
Consequently, during the biological action, addition reactions to the double bond in the side chain of ferulic acid (**4**) take place.

Characteristic for the ^{13}C NMR spectrum of (**14''**) were the same resonance signals as for the spectrum of (**14'**). That is, the action of *P. sanguinea* on substrate (**14**) led to the same consequences as the action of *C. villosus*.

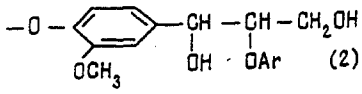
Analysis of the ^{13}C NMR spectrum of (**13''**) showed that the fungus *P. sanguinea* had effected the oxidation of the C_α-atom of the (**13**) side-chain to a C=O group. This was shown unambiguously by resonance signals with CSs of 123-122 ppm, and signals with CSs of 200-197 ppm of C=O groups [21, 22]. Simultaneously with the oxidation of the side-chain the

formation of C_5—O—Ar bonds may take place. This was shown by signals in the following regions of the spectrum of (**13''**) (ppm): 152-150 (C-3, C-5), 143-142 (C-4), 132-130 (C-1), ~109 (C-2) [21, 23].

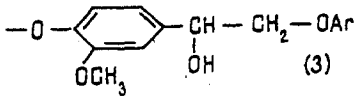
The presence in the spectrum of (**13''**) of resonance signals in the 85-82, 74-72, and 64-63 regions, which are not characteristic for the spectrum of (**13**) can be explained by the formation of structural fragments of the following type [21-23]:



(1)



(2)



(3)

	^{13}C CSs, ppm		
1	195.1	83.2	63.4
2	71.8	82.1	64.0
3	82.0	73.0	

It is interesting that not only the molecules of the initial substrates but also the monomeric units of the already broken down polymer began to participate in the polymerization reactions. Thus, for example, the ferulic acid polymers contained as structural units not only units of the substrate but also vanillin units produced in the biodegradation of the "primary" polymer.

Thus, the basidiomycetes *C. villosus* and *P. sanguinea* are capable of performing oxidative reactions involving the degradation of aromatic compounds. The rate of degradation of veratrylpropane compounds and veratryl-substituted β-O-4 ethers by the fungus *C. villosus* depends on the nature of the substituent in the C_α position of the propane chain and takes place with the the cleavage of the C_α—C_β bond. The fungus *P. sanguinea* includes in its metabolism veratrylmethanes and some veratrylpropnae compounds without cleaving the C_α—C_β bond. The biotransformation by the fungi of substrates containing a phenolic OH group depends on the type of substitution of the aromatic ring and is accompanied by competing polymerization-degradation processes. The polymerization reaction involves not only molecules of the initial substrate but also products of subsequent breakdown of the polymers. The formation of the polymers takes place with the participation of C—C and C—O bonds realized at the expense of the phenolic hydroxyl and the hydrogen at the C-1, C-2, and C-5 atoms of the aromatic rings.

EXPERIMENTAL

^{1}H and ^{13}C NMR Spectroscopy. The ^{13}C NMR spectra of the polymeric metabolites and the initial substrates were recorded in dimethyl sulfoxide-d_6 solution containing 0.02 M Cr[acac]$_3$. ^{1}H NMR spectra were recorded in hexamethylphosphorotriamide-d_{18} solution. Bruker WP-200SY spectrometer; working frequencies 200.1 MHz (^{1}H) and 50.3 MHz (^{13}C). Chemical shifts (CSs) of the hydrogen and carbon atoms of model compounds:

^{1}H CSs (in $CDCl_3$ solution) for (**2**): H_{ar}–6.79-6.72, H_{OHphe}–5.40, H_{OCH_3}–3.85 ppm (s.).

^{1}H CSs for (**13**): H_{ar}–6.90-6.60, H_{OHobe}–5.61, H_{OCH_s}–3.87 (s), H_α–4.49 (m), H_β–1.76 (m), H_γ–0.86 (t), H_{OHalc}–1.80 ppm.

^{1}H CSs for (**14**): H_{COOH}–12.10, H_{ar}– 7.50-7.01, H_{OCH_s}–3.81, H_{OHphe}–9.57, H_α–6.78; H_β–6.36 ppm.

^{13}C CSs for (**2**): C-1 — 119.6, C-2–113.4, C-3–148.2, C-4–147.2, C-5–116.1, C-6–121.4, OCH_3–56.0 ppm.

^{13}C CSs for (**13**): C-1–132.8, C-2–110.1, C-3–147.2, C-4–145.1, C-5–115.1, C-6–119.2, C_α–77.2, C_β–29.1, C_γ–10.1, OCH_3–55.8 ppm.

^{13}C CSs for (**14**): C-1–126.8, C-2–112.2, C-3–148.3, C-4–149.4, C-5–116.1, C-6–122.7, C_α–144.5, C_β–116.1, C_γ–168.8 ppm.

Chromato-mass Spectrometry. Mass spectra were recorded on a LKB-2091 instrument, using a capillary column 30 m long with the phase SE-30. The temperature was programmed from 100°C at the rate of 4°C/min. Ionization energy 70 eV.

Cultivation of the Fungi. In this work we used the strain *Coriolus villosus* 0276 obtained from the VNPO [All-Russian Scientific Production Combine] Gidrolizprom collection, and the strain *Phanerochaete sanguinea* (Fr.) Pouz. BKMF-2487D, provided by V. A. Solov'ev, LTA [Kirov Leningrad Institute of Wood Technology] (St. Petersburg). The cultures were maintained on a wort-agar medium. The experiments were conducted in 500-ml Erlenmeyer flasks. Each flask was charged with 50 ml of Kirk's medium [26] with pH 4.0. The substrates were added in amounts of 1.5-2.3 mg in 50 μl of redistilled

dimethylformamide (DMFA) per 50 ml of nutrient medium. Inoculation was done with an agarized block of mycelium having a diameter of 0.5 cm. Each experiment was conducted in duplicate or triplicate. The amount of substrate in the medium without the fungus was monitored simultaneously. The fungus *C. villosus* was grown at 33°C for 10 days, and *P. sanguinea* at 26°C for 30 days. The flasks were flushed with 100% oxygen every two days.

High-performance Liquid Chromatography. The model compounds were synthesized by methods described in the literature [27]. Samples with a volume of 200 μl were taken every day and centrifuged, and the supernatants were analyzed by HPLC on a Milikhrom-1 chromatograph using a column filled with the sorbent Nucleosil 5-C_{18}. The efficiency of the column (for naphthalene) was 4.5 thousand theoretical plates. The volume of sample introduced into the column was 20 μl. Elution was conducted in the isocratic regime (40% solution of methanol in 0.01 M phosphate buffer, pH 4.3) or stepwise with the successive use of methanol solutions of increasing concentration (from 10 to 70%). The rate of feed of eluent was 100 μl/min. Detection was carried out at λ 280 nm. The metabolites were identified by comparing their retention times and the spectral ratios A_{260}/A_{210} and A_{280}/A_{210} with the analogous indices for standard specimens, and also by the method of adding a standard specimen to the sample under investigation. The amounts of the compounds in the samples were calculated from the areas of the chromatographic peaks in comparison with the areas of the peaks of samples with known concentrations.

Gel Chromatography. The culture liquid (1 ml) was evaporated in a current of nitrogen, and the polymer was dissolved in DMFA and analyzed on a Milikhrom-4 chromatograph, using a 4 × 200 mm column filled with the sorbent Separon Hema S-100 (15 μm) (Czechslovakia). The efficiency of the column was 1500 t.p., the eluent being abs. DMFA with the addition of 0.03 M H_3PO_4 and 0.03 M LiBr. The rate of flow of eluent was 20 ml/min, and the volume of sample added, 10—20 μl. Detection was carried out at wavelengths of 280 and 350 nm. The column was calibrated on toluene and lignin. The weight-average ($\bar{M}_w$) and number-average ($\bar{M}_n$) molecular masses were calculated from the chromatogram by the method of [28]. The authors thank S. G. D'yachkob for performing the calculations.

Preparative Isolation of the Polymers. The fungi were grown under the conditions given above with a substrate concentration of 80 mg/liter. The culture liquid (1 liter) was acidified to pH 2 and extracted with chloroform, and the extract was evaporated in a rotary evaporator and analyzed by NMR.

Preparative Isolation of Muconic Acid, a Product of the Biodegradation of Pyrocatechol. The fungus *P. sanguinea* was grown under the conditions given above at a substrate concentration of 40 mg/liter for 17 days. The mycelium was filtered off, and the filtrate was acidified to pH 2 and extracted with a fourfold volume of diethyl ether. To eliminate the fatty acids formed during the incubation process as metabolites of the fungus, the ethereal extract, evaporated to 150 ml, was treated three times with 5% $NaHCO_3$ solution. The aqueous solution, containing sodium salts of the acids, was acidified to pH 5 and extracted with a fourfold volume of chloroform. The desired product remained in the $NaHCO_3$ solution. This solution was acidified to pH 1 and extracted with diethyl ether, and the extract was dried over anhydrous Na_2SO_4 and evaporated to dryness.

Methylation of Muconic Acid. A solution of the product in 5 ml of abs. methanol was heated on the water bath under reflux for 6 h, and gaseous HCl was slowly bubbled in. Completeness of methylation was checked by HPLC. The reaction mixture was diluted with a fivefold volume of 10% NaCl solution and extracted three times with diethyl ether. The extract was dried over anhydrous Na_2SO_4 and evaporated to dryness. The methyl ester was analyzed by mass spectrometry.

Mass spectrum of (**22**), m/z (%): 170 (M^+7), 139 (15), 123 (8), 111 (100), 79 (11), 59 (10), 51 (10).

REFERENCES

1. V. Shubert, The Biochemistry of Lignin [in Russian], Les. Prom-st', Moscow (1968).
2. K. V. Sarkanen and C. H. Ludwig, Lignins: Occurrence, Formation, Structure, and Reactions, Wiley-Interscience, New York (1971).
3. T. K. Kirk and M. Shimada, in: Biosynthesis and Biodegradation of Wood Components, T. Higuchi (ed.), San Diego (1985), Ch. 21, p. 579.
4. T. K. Kirk and F. Natsubo, Biochem. Biophys. Acta, **756**, 376 (1983).
5. T. Umezawa, F. Nakatsubo, and T. Higuchi, Agric. Biol. Chem., **47**, 2677 (1983).
6. M. S. A. Leisola, B. Schmidt, U. Thanei-Wyss, and A. Fiechter, FEBS Lett., **189**, 267 (1985).
7. T. Higuchi, Wood Res., 58 (1986).

8. N. Duran, J. Rodriguez, A. Ferraz, and V. Campos, Biotechnol. Lett., **9**, 357 (1987).
9. L. A. Golovleva, N. M. Myasoedova, B. P. Baskunov, and V. I. Shevchenko, Mikrobiologiya, **58**, No. 2, 256 (1989).
10. G. P. Aleksandrova, S. A. Medvedeva, V. A. Babkin, V. A. Solov'ev, O. N. Malysheva, and S. Z. Ivanova, Khim. Drev., No. 6, 77 (1989).
11. V. A. Babkin, V. A. Solov'ev, G. P. Aleksandrova, S. A. Medvedeva, and O. N. Malyshev, Method of Bleaching Sulfate Cellulose [in Russian], Byul. Izobret., No. 19, 121 (1991).
12. M. I. Buryachenkov, A. N. Zakazov, I. V. Volchatova, G. P. Aleksandrova, S. A. Medvedeva, and V. A. Babkin, in: Abstracts of Lectures at a Conference on the Complex Utilization of Wood Raw Material and Methods of Solving Ecological Problems in Pulp and Paper Factories [in Russian], Bratsk (1991), p. 4.
13. S. A. Medvedeva, I. V. Volchatova, S. Z. Ivanova, N. F. Aprelkova, V. A. Khutoryanskii, I. A. Antipova, S. G. Seredkina, and V. A. Babkin, Khim. Drev., No. 3, 76 (1991).
14. S. A. Medvedeva, I. V. Volchatova, S. G. Seredkina, I. A. Belousova, I. A. Antipova, and V. A. Babkin, in: 7th International Symposium on Wood and Pulp Chemistry, Beijing (1993).
15. M. Tien and T. K. Kirk, Proc. Natl. Acad. Sci. USA, **81**, 2280 (1984).
16. I. V. Volchatova, S. A. Medvedeva, and V. A. Babkin, Biokhimiya, **59**, No. 6, 797 (1994).
17. L. V. Vasil'eva, I. V. Shchukin, and V. A. Babkin, Élektrokhimiya, **29**, No. 4, 481 (1993).
18. K. E. Hammel, M. Tien, B. Kalyanaraman and T. K. Kirk, J. Biol. Chem., **260**, 8348 (1985).
19. T. K. Kirk and R. L. Farrell, Annu. Rev. Microbiol., **41**, 465 (1987).
20. T. K. Kirk, M. Tien, S. Groan, et al., in: Materials of the IIIrd International Conference on Biotechnlogy in the Pulp and Paper Industry, Stockholm (1986), p. 5.
21. H. D. Ludemann and H. Nimz, Makromol. Chem., **175**, 2393 (1974).
22. K. P. Kringstad and R. Morek, Holzforschung, **37**, 237 (1983).
23. A. Parta and P. K. Mukhopadhyay, J. Indian Chem. Soc., **60**, 265 (1983).
24. C.-L. Chen and D. Robert, Methods Enzymol., **161**, 137 (1988)
25. L. V. Kanitskaya, An Investigation of Multicomponent Systems of Natural Origin by Quantitative NMR Spectroscopy [in Russian], Dissertation for ... Candidate of Chemical Sciences, Irkutsk (1990).
26. T. K. Kirk, W. J. Connors, L. F. Lorenz, and J. I. Zeikus, Arch Microbiol, **113**, 277 (1978).
27. S. A. Medvedeva, V. A. Khutoryanskii, S. Z. Ivanova, L. N. Spiridonova, V. A. Babkin, and N. I. Baram, Khim. Drev., No. 3, 72 (1990).
28. L. N. Shatenshtein, Yu. P. Vyrskii, N. A. Gravlikov, P. P. Alikhanov, K. N. Zhdanova, and A. L. Izpolenikov, Determination of the Molecular Weights of Polymers [in Russian], Moscow—Leningrad (1964).