A COMPARATIVE INVESTIGATION OF BIOSYNTHETIC LIGNINS OBTAINED FROM CONIFERYL AND p-COUMARYL ALCOHOLS WITH REED AND SPRUCE LIGNINS

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Numerous experiments on nitrobenzene oxidation and ethanolysis have shown that the lignins of gymnospermous plants (conifers), dicotyledonous angiospermous plants (leaf-bearing plants), and monocotyledonous angiospermous plants (herbs and annuals) differ considerably in structure. While the lignins of coniferous species of trees are constructed mainly from guaiacylpropane derivatives, the lignin of leaf-bearing species includes, in addition to these components, a large amount of syringylpropane derivatives. The lignin of annual plants includes derivatives of guaiacyl-, syringyl-, and p-hydroxyphenylpropanes.

These structural differences obviously depend on the nature of the biosynthesis of each type of lignin, which is determined, in its turn, by the phylogenetic and ontogenetic features involved in the process of the evolution of plants. The structural differences of the three types of lignin also exhibit a purely chemical interest in view of the fact that differences in the degree of methoxylation of the aromatic nuclei of the structural constituents of lignin may determine the degree of condensation of the lignins to a considerable extent. Thus, it may be assumed that the lignin of a leaf-bearing tree will be less condensed since there are methoxy groups in positions 3 and 5 of the aromatic nucleus of the syringylpropane structural units and, consequently, these elementary structures are incapable of forming, in the process of the growth of the lignin macromolecules, coumarane structures in which there are C–C bonds involving the C atom in position 5 of the aromatic ring of one phenylpropane unit and the C-2 of the side chain of another, or biphenyl structures of the type of dehydrodivanillin. The lignin of annual plants, on the other hand, should apparently be more condensed as a result of the possibility of the formation of C–C bonds through positions 5 and 3 of the aromatic ring of the p-hydroxyphenylpropane structural elements. The lignin of coniferous species should apparently occupy an intermediate position between these two types of lignins in respect of its degree of condensation. An investigation of the assumed differences was one of the aims of our work. At the present time, thanks to the broad range of investigations performed by C. Freudenberg and his colleagues [1] on the biosynthesis of lignin from coniferyl alcohol in vitro, the main features of the structure and formation of the lignin of coniferous species are known. At the same time, the literature information on the biosynthesis of lignin from p-courmaryl alcohol [2] is sparse and leaves many questions unanswered. In view of this, it appeared of interest to study the dehydropolymer (DHP) synthesized in vitro from p-coumaryl alcohol and to compare its properties with the properties of the dehydropolymer from coniferyl alcohol and the lignin of the reed Phragmatis communis, which we have investigated previously [3]. Consequently, we undertook the in vitro biosynthesis of DHPs separately from coniferyl and p-coumaryl alcohols. The syntheses were effected by Freudenberg's method [4], which he developed for obtaining DPHs by the action of horseradish peroxidase on a solution of coniferyl alcohol in the presence of a dilute solution of hydrogen peroxide at pH 5.5 (citrate buffer).

From coniferyl alcohol the DHP was obtained with a yield of 67.5% and from p-coumaryl alcohol the DHP with a yield of 70.1%, calculated on the amount of cinnamyl alcohol derivative taken.

The biosynthetic lignins consisted of white powders similar to Björkman lignins. The results of their functional composition are given in Table 1 in comparison with those for some samples of Björkman lignin isolated by the mechanical grinding method (MWL: "milled wood lignin"). On comparing the composition of the DPH-1

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TABLE 1. Composition of DHP-1 and DHP-2 in Comparison with Some Types of MWL

Sample	% C	% H	% O	% OCH_3	Composition of the structural C_6-C_3 unit	Mol. wt. of a C_9 unit	Composition of the initial alcohol	Loss of H per C_9 unit in biosynthesis (moles)	Increase in the amount of H_2O per C_9 unit
DHP-1	65,10	6,23	28,67	16,81	$C_9H_{7,88}O_2(H_2O)_{0,30}(OCH_3)_1$	184,28	$C_9H_{10}O_2$ (OCH_3 replaced by H)	10—7,88=2,12	0,30
DHP-2	70,22	5,86	23,92	0	$C_9H_{8,31}O_2(H_2O)_{0,30}$	153,71	$C_9H_{10}O_2$	10—8,31=1,69	0,30
DHP from coniferyl alcohol, (Freundenberg, 1963) [4])	—	—	—	—	$C_9H_{7,14}O_2(H_2O)_{0,38}(OCH_3)_1$	185	$C_9H_{10}O_2$(OCH_3 replaced by H)	10—7,14=2,86	0,38
Spruce MWL (Freudenberg, 1964 [7, 11])	65,03	5,83	29,14	15,53	$C_9H_{7,12}O_2(H_2O)_{0,40}(OCH_3)_{0,92}$	182,84	The same	9,08—7,12= =1,96	0,40
Spruce MWL (Mikhailov and Shorygina [12])	62,38	6,17	31,45	15,03	$C_9H_{7,47}O_2(H_2O)_{0,74}(OCH_3)_{0,96}$	191,55	$C_9H_{10}O_2$ (OCH_3 replaced by H)	9,04—7,47= =1,57	0,74
MWL (Phragmatis communis (Sydkov and Shorygina [3])	61,01	6,23	32,76	17,59	$C_9H_{7,15}O_2(H_2O)_{0,94}(OCH_3)_{1,11}$	198,8	$C_9H_{10}O_2$(OCH_3 replaced by H)	8,89—7,15= =1,74	0,94

from coniferyl alcohol with the composition of the DHP-2 from p-coumaryl alcohol obtained under similar conditions accurately corresponding to those of Freudenberg, it can be seen that the loss of hydrogen on biochemical synthesis is greater in the first case (2.12 atoms per C_9 unit) than in the second (1.69 atom per C_9 unit). However, the increase in the amount of water was the same in both cases, amounting to 0.3 mole. If the four principles of the growth of lignin polymolecules put forward by Freudenberg [5] – continuing dehydrogenation, the addition of phenols to quinone methides, the polymerization of the quinone methides, and the rearrangement of the γ-aryl ethers – are adopted, it is obvious that the difference in the loss of hydrogen in the synthesis of the DHPs from coniferyl and p-coumaryl alcohols must be due to some redistribution of the intensities of the individual processes. It must also be mentioned that the DHP-1 obtained by Freudenberg had a larger increase in the amount of water (0.38 mole per C_9 unit) than the DHP-1 that we isolated (0.3 mole per C_9 unit) (see Table 1).

The number of phenolic OH groups in the DHP-1 that we prepared was 0.05 mole per structural unit lower than for the spruce MWL obtained by N. P. Mikhailov in the laboratory of the Institute of Organic Chemistry. In order to determine the phenolic OH groups, we used the chemosorption method in Enkvist's variant [6] (Table 2). For a comparative determination of the degree of condensation of the lignin by C–C bonds we used primarily decomposition with metallic sodium in liquid ammonia.

A. F. Semechkina and N. N. Shorygina [7] have shown that the yield of ether-soluble phenols after the reaction of lignin with a solution of metallic sodium in liquid ammonia under standard conditions is determined by the structure of the lignin. The yield of ether-soluble phenols from the lignins of leaf-bearing species is higher than from the lignins of coniferous species. The yield of phenols also depends on the conditions under which the lignin was isolated from the plant tissues. Natural lignin (not isolated from wood) gives a higher yield of soluble phenols. MWL occupies the second place with respect to the yield of these phenols. The lowest yield of phenols is obtained from the lignins after acid treatments, and among these lignins hydrolysis lignin occupies the last position.

We have studied the decomposition by metallic sodium in liquid ammonia [7] of four samples of lignin: DHP-1, DHP-2, spruce MWL, and the

TABLE 2. Content of Phenolic OH Groups in Lignin Samples

Sample	Chemosorption method [6]	
	%	moles of phenolic OH groups per C_9 unit
DHP-1	2,20	0,24
DHP-2	2,02	0,22
Spruce MWL	2.58	0,29
MWL (Phragmatis communis	3,25	0,38

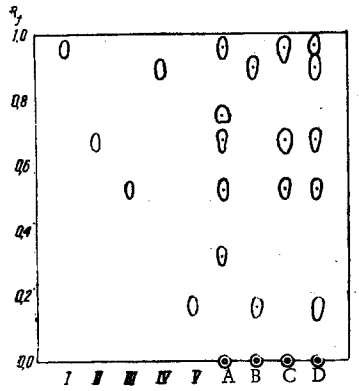

Fig. 1. Chromatogram of monomeric phenols resulting from the decomposition of lignins with metallic sodium in liquid ammonia: I) dihydroeugenol; II) 1-guaiacylpropan-1-ol: III) 3-guaiacylpropan-1-ol; IV) 1-(4-hydroxyphenyl)propane; V) 1-(4-hydroxyphenyl)propan-1-ol. A) Mixture of phenols from DHP-1; B) mixture of phenols from DHP-2; C) mixture of phenols from spruce MWL; D) mixture of phenols from reed MWL. The spots with R_f 0.31 (violet) and 0.75 (pink) have not been identified.

MWL of Phragmatis communis. The maximum yield of ether-soluble phenols was obtained from DHP-1 (25%). Then follows spruce MWL (20%). The reed MWL gave a 16.6% yield of phenols and DHP-2 a 15% yield. The facts given above permit the assumption that a greater content of p-coumaryl structures in the lignin leads to a higher degree of its condensation.

The chromatographic separation of the phenols from the four samples of lignin mentioned showed that, apart from DHP-2, they form dihydroeugenol, 1-guaiacylpropan-1-ol, and 3-guaiacylpropan-1-ol. Of the phenols from DHP-2, 1-(4-hydroxyphenyl)propane and 1-(4-hydroxyphenyl)propan-1-ol have been identified. On a chromatogram, DHP-1 yielded two spots, one of which was not obtained from any lignin studied by us previously (Fig. 1).

To confirm and supplement the chemical results, we studied the IR and UV spectra of the samples obtained. It is known that broad and, frequently, overlapping absorption bands are characteristic for the IR spectra of lignin and its derivatives. In view of this, the interpretation of the spectra is frequently difficult. The IR spectra of the four samples studied have some similarity. This is due to the fact that there are common structural elements in the molecules of all the samples studied. The spectra showed strong and broad bands at 3400 cm^{-1} (OH group), 3000, ~1600, and 1520 cm^{-1}, etc. (benzene ring), and 2940, 1465, and 1428 cm^{-1} (vibrations of CH of alkyl radicals). The band at about 1600 cm^{-1} due to the C = C stretching vibrations of a benzene ring is strong in the spectra of all four samples. In DHP-2 it is a doublet which, according to the literature [8], is characteristic for the lignin of monocotyledons.

However, the IR spectra of the samples considered differ significantly in the region of the nonplanar CH deformation vibrations of the benzene ring (700-900 cm^{-1} region), the values of which depend on the position and number of the substituents in the ring. In the spectrum of DHP-2 (Fig. 2), there is only one strong band at 840 cm^{-1} in this region. If the results of the investigations of Kawamura and Higushi [8] are followed, it may be assumed that the molecule of DHP-2 contains mainly 1,4-disubstituted benzene rings. Furthermore, according to Bolker and Sommerville [9], the presence of a band at 835 cm^{-1}, which appears in the IR spectra of the lignins of leaf-bearing species, shows the presence of 1,2,3,5-tetrasubstitution in the ring. Thus, the question of the number and positions of the substituents in the aromatic part of the molecule of DHP-2 is still obscure. In the case of the other lignins studied, DHP-1 and spruce MWL, the usual bands for guaiacyl lignins characteristic of a 1,3,4-trisubstituted benzene ring (860 and 828 cm^{-1} and 868 and 827 cm^{-1}, respectively) are found in this part of the spectrum. A band at 1150 cm^{-1}, which is also characteristic of a 1,3,4-trisubstituted benzene nucleus [8], is likewise found in the spectra of spruce MWL and DHP-1. It is absent from that of DHP-2.

The IR spectra of reed MWL and DHP-2, unlike the spectra of the coniferyl lignins, have no band at 1090 cm^{-1} (this band is ascribed by Kawamura and Higushi [8] to the vibrations of aliphatic ether linkages or to secondary OH groups).

The spectra of the lignins studied also differ at 1275 and 1220 cm^{-1} where the absorption bands corresponding to $C_{ar} - O$ and OH groups are located. While in the spectra of spruce MWL and DHP-1 the

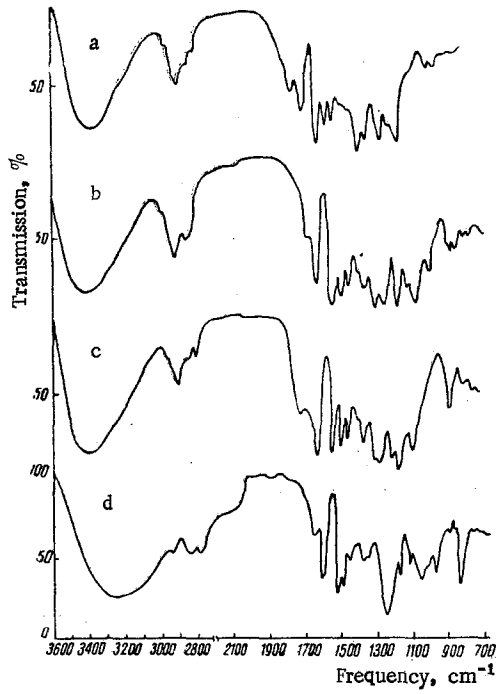

Fig. 2. IR spectrum of spruce MWL (a); DHP-1 (b); Phragmatis communis MWL (c); and DHP-2 (d).

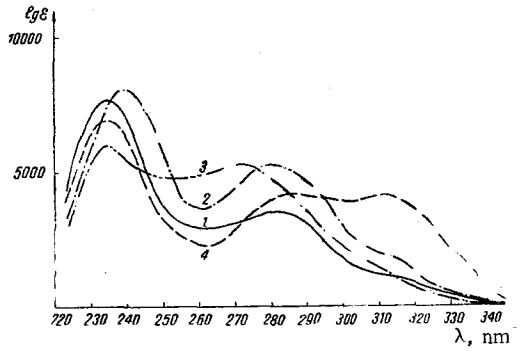

Fig. 3. UV spectra of spruce MWL (1), DHP-1 (2), DHP-2 (3) and reed MWL (4).

1275 cm^{-1} band is stronger than the 1220 cm^{-1} band (the differences in the intensities of the bands being less in the spectrum of DHP-1), in the spectrum of reed MWL the ratio of the intensities of the bands is reversed. This is in harmony with what is stated by Kawamura and Higushi [8].

The spectrum of DHP-2 differs from the others. In the 1200-1300 cm^{-1} region it has only one strong band – at 1247 cm^{-1}. In the spectrum of DHP-2 a band appears at 1496 cm^{-1} which is not included in the spectra of the other samples studied and which, according to Kolboe and Ellefsen [10] can be assigned to a coumarane system.

In the spectrum of DHP-2, as in that of spruce MWL and DHP-1, there is a band at 1660 cm^{-1} characteristic for a C = O group conjugated with an aromatic nucleus.

Consequently, the main differences between the IR spectra of DHP-2 and those of the other samples are due to differences in the substitution of the aromatic nuclei and also in the nature and number of ether linkages and OH groups.

The UV spectra (Fig. 3) show, in agreement with the results of our study of the IR spectra, the structures of the aromatic parts of the molecules of the spruce MWL and DHP-1 are very similar but they differ somewhat from the structure of reed MWL and considerably more from the structure of the DHP-2 molecule.

EXPERIMENTAL

The IR spectra were taken on a UR-10 instrument (samples molded into tablets with KBr), and the UV spectra on a Unicam SP-700 instrument.

Biosynthetic Lignins

Coniferin was isolated from the cambial layer (sap) of a pine (15-20 years) freshly felled at the end of May. After repeated recrystallization from water, mp 174-178° C. Yield 60 g.

Coniferyl alcohol was obtained by the cleavage of the coniferin with β-glucosidase [13]. Mp 73-74° C (benzene). About 10 g of crystalline coniferyl alcohol precipitated.

Emulsin (β-glucosidase) was isolated from sweet almonds [14]. Yield 13.48 g.

Peroxidase was obtained from horseradish by Wilsätter's method [14]. About 3 g of peroxidase was prepared from 5 kg of digested fresh horseradish. The activity of the peroxidase was determined by Boyarkin's method on a FÉK-56 [photoelectric colorimeter] in comparison with the activity of crystalline peroxidase. The activity of the horseradish peroxidase was 0.62 of a relative unit.

Preparation of DHP-1 from Coniferyl Alcohol [4]. With stirring, a solution of 0.8 g of coniferyl alcohol in 5 ml of acetone and 400 ml of water was added by drops from one funnel and simultaneously 400 ml of a freshly prepared 0.025% of H_2O_2 was added by drops from a second funnel to a solution of 400 mg of peroxidase (from horseradish) in a mixture of citrate buffer (15 ml, pH 5.5) and 400 ml of water at 20° C.

Each solution was added over a day at the rate of approximately 3 ml per hour. During the first day the reaction mixture became turbid. Stirring was continued overnight. On the following day, another 300 mg of peroxidase in 13 ml of buffer solution was added, and the addition of the same solution was continued at the same rate. The solution was stirred all night. On the third day, yet another 250 mg of peroxidase in 13 ml of buffer solution and, by drops, 420 mg of coniferyl alcohol in 210 ml of water and 210 ml of the H_2O_2 solution were added. Then, over 3 days, 2 liters of H_2O_2 of the same concentration and, each morning, 150 mg of fresh enzyme and 10 ml of buffer solution were added.

The concentration of the dilute H_2O_2 solution, prepared not more than a week beforehand, was determined iodometrically. The fine suspension of biosynthetic lignin was evaporated in vacuum to a volume of 150 ml. After this, the precipitate was centrifuged off and left to the following day in beakers under a layer of water (30 ml). On the following day, the precipitate was again centrifuged off and it was dried in a vacuum desiccator over P_2O_5.

The preparation obtained (DHP-1) was dissolved in a small amount of dioxane–water (9:1). The solution was filtered through a No. 4 glass filter. After some hours, with stirring water was added by drops to the DHP-1 solution to bring about precipitation. The liquid was centrifuged off. The residue was dried first at the ordinary temperature and then at 56° C over P_2O_5 in a vacuum pistol for 3 days. This gave 0.82 g of dry DHP-1. The yield was 67.5% on the initial alcohol.

Found %: C 65.10; H 6.23; OCH_3 16.81.

p-Coumaric acid (16 g) was obtained from p-hydroxybenzaldehyde [16] with mp 205-206° C (water). The yield was 25-30% of the initial p-hydroxybenzaldehyde. On acetylation, the acetate of p-coumaric acid with mp 202-206° C was formed in quantitative yield. The methyl ester of acetyl-p-coumaric acid was obtained by methylating the acetate with diazomethane, mp 81-82° C.

p-Coumaryl alcohol (2.74 g) was obtained by Freudenberg's method [4], which was developed for the preparation of coniferyl alcohol. Yield 35% of the initial ester, mp 120-121° C.

DPH-2 from p-Coumaryl Alcohol. DHP-2 was obtained in the same way as DHP-1 [4]. From 1.2 g of p-coumaryl alcohol, 0.81 g of dry DHP-2 was obtained, i.e., 70.1% on the initial alcohol.

Found %: C 70.22; H 5.86.

Decomposition of DHP-1 and DHP-2 by Metallic Sodium in Liquid Ammonia. Weighed samples of DHP (0.2 g) were suspended in liquid ammonia, and metallic sodium (80% of the weight of DHP taken) was gradually added. After the blue solution had become completely decolorized (with a reaction time of 2 days), the ammonia was evaporated off to dryness and the reaction mixture was freed from the last traces of ammonia by the prolonged passage of dry oxygen-free nitrogen. The pulverulent mass remaining in the reaction vessel was treated with water-saturated ether, and then a small amount of water (50 ml) was added to the reaction mixture. A current of CO_2 was passed into the aqueous alkaline solution, giving a precipitate of phenols. The mixture was extracted with ether. The weight of ether-soluble phenols was 0.05 g (25% of the initial sample) for DHP-1 and 0.03 g (15%) for DHP-2. Yield 15%.

Paper Chromatography. An ethanolic solution of the phenols under investigation was deposited on a 15 × 30 cm sheet of paper (Whatman No. 1) at a distance of 4 cm from one edge. The chromatograms were run for 4-6 h with benzene–petroleum ether–water (1:1:1) by the descending method. The spots were revealed with diazotized sulfanilamide in butanol. After drying, the chromatograms were sprayed with a half-saturated solution of sodium carbonate. The phenols appeared in the form of colored spots.

SUMMARY

1. Biosynthetic lignins have been obtianed by Freudenberg's method from coniferyl and p-coumaryl alcohols.

2. In its elementary and functional composition, DHP-1 is similar to the corresponding material obtained by Freudenberg.

3. For DHP-1 the yield of ether-soluble phenols is considerably higher than for DHP-2, which may indicate a greater degree of condensation of the latter as compared with the former.

4. Reductive decomposition by a solution of metallic sodium in liquid ammonia has shown that the phenols obtained from reed lignin have a complex composition.

5. A comparison of the IR and UV spectra of the biosynthetic lignins obtained and of reed and spruce MWLs has been performed.

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