Lignin — distribution, biosynthesis, preparation and structure

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1. Distribution and organization of lignin in the cell walls

The term "lignin" is derived from the Latin word *lignum*, meaning wood. It was introduced by Anselme Payen, in 1838, to represent cellulose-encrusting substances in the lignified plant cell walls (1, 2). Being widely distributed in nature, lignin is the second (after cellulose) most abundant natural polymer in the biosphere and the most abundant aromatic material constituting about 40% of the solar energy in plants. During phylogenetic development of plants lignin had appeared first in *Pteridophytae*. Presently, it is found in higher plants, mosses, and plants of lower taxonomic ranking; neither algae nor liverworts contain this substance. The highest proportions of

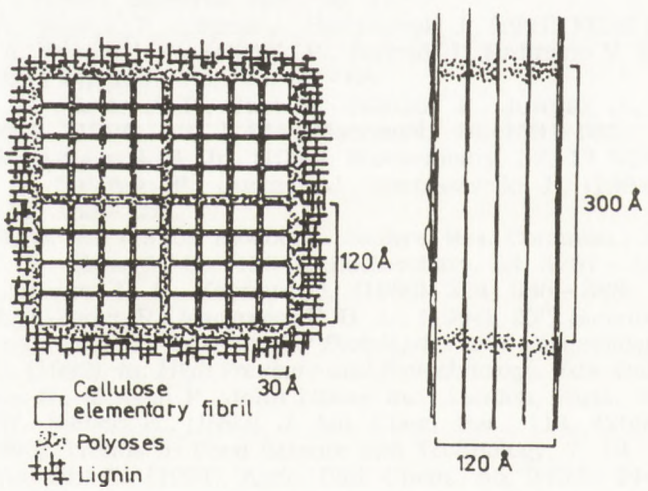


Fig. 1. Model of the ultrastructural organization of the cell wall component of wood (Fengel, 1971) (37).

lignin occur in dead sclerenchymatous cells (in the spaces of inter cellulose micro fibrils in primary and secondary walls) and in vascular parts of the conductive tissue in middle lamellae as a cementing component which connects cells and hardens the cell walls of xylem tissue. In wood, most of the lignin (ca. 80%) is found within the cell walls, where it is interspersed with the hemicelluloses, forming a matrix surrounding cellulose micro fibrils (3,4). Polysaccharide components in micro fibrils are densely packed in layers of lignin, which protects them from the activity of hydrolytic enzymes and other external factors and serves as a stabilizer of the complex structure. One can discern an analogy between steel rods inside a prefabricated element and cellulose fibers in lignin (Fig. 1).

As the plant grows older, the percentage of lignin rises, up to 29% in conifers and 26% in broad-leafed species on a dry matter basis.

2. Biosynthesis

How is lignin synthesized in nature? To elucidate this question two academic trends seem to appear. The first one is based upon the results of long researches of lignin biosynthesis, especially by the group of Freudenberg. This author developed the old Klasons' and Erdmans' free radical theory based on the thesis that lignin is a dehydrogenation polymer (DHP) of coniferyl alcohol or related alcohols. This theory is currently an essential principle of both lignin formation and degradation. The second trend developing the knowledge on the biosynthetic pathways also adapts this knowledge for the better understanding of the lignin chemical structure. After long consequent researches it was possible to establish that lignin is biosynthesized from glucose in the shikimate and phenylalanine-cinnamate pathways via phenylalanine and tyrosine. As the intermediates there appear three precursor alcohols of cinnamate origin, coumaryl, coniferyl and sinapyl (Fig. 2).

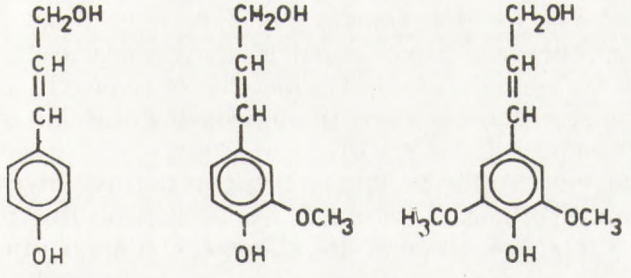


Fig. 2. The precursors of lignin: A. p –hydroxycinnamyl (coumaryl) alcohol; B. 4–hydroxy–3–methoxycinnamyl (coniferyl) alcohol; C. 3,5–dimethoxy–4–hydroxycinnamyl (sinapyl) alcohol (Sarkanen and Ludwig, 1971) (4).

The enzymes most involved in the shikimate phase of biosynthesis are glucose–6–phosphate dehydrogenase (EC 1.1.1.49), phosphogluconate dehydrogenase [decarboxylating] (EC 1.1.1.44), phospho–2–dehydro–3–deoxyheptonate aldolase (EC 4.1.2.15), 3–dehydroquinate synthase (EC 4.6.1.3), 3–dehydroquinate dehydratase (EC 4.2.1.10), shikimate dehydrogenase (EC 1.1.1.25), shikimate kinase (EC 2.7.1.71), 3–phospho–5–enolpyruvylshikimate synthase (non–registered in Enzyme Nomenclature'1984), enolpyruvate transferase (EC 2.5.1.7), chorismate synthase (EC 4.6.1.4), chorismate mutase (EC 5.4.99.5) prephenate dehydratase (EC 4.2.1.51) prephenate dehydrogenase (EC 1.3.1.13), phenylalanine aminotransferase (EC 2.6.1.58), prephenate aminotransferase (non existent in EN' 1984), arogenate dehydrogenase (non–registered in EN'1984) prephenate dehydrogenase (EC 1.3.1.12) tyrosine aminotransferase (EC 2.6.1.5), arogenate dehydratase (non–existent in EN' 1984) and shikimate: NADP oxidoreductase (EC 4.2.1.10). In the cinnamate phase work specific L-phenylalanine ammonia–lyase (EC 4.3.1.5), L–tyrosine phenol–lyase (EC 4.1.99.2), *trans*–cinnamate 4–monooxygenase (cinnamate 4–hydroxylase; EC 1.14.13.11), phenol 2–monooxygenase (phenol hydroxylase; EC 1.14.13.7), p – coumarate–3–hydroxylase (non–registered in EN'1984), ferulate–5–hydrolase (non–registered in EN'1984), catechol methyltransferase (EC 2.1.1.6), 4– coumarate: CoA Ligase (EC 6.2.11.12), cinnamyl–CoA reductase (EC 1.2.1.44) and cinnamyl–alcohol dehydrogenase (EC 1.1.1 195). The biosynthesis starts from glucose (deriving from photosynthesis) which is transformed to shikimic acid. As the first intermediate stage appear condensation of D–erythrose–4–phosphate and phosphoenolpyruvaxe which produces 3–deoxy–2–keto–D–arabinoheptulosonate–7–phosphate. Loss of phosphate and ring closure by this last give 5–dehydroquinate. Then cyclisation product loss of water and produces 5–dehydroshikimate. Two aromatic acids, L–phenylalanine and L–tyrosine, are produced from shikimic acid through shikimic acid 5–phosphate, enolpyruvylshikimate, chorismate and prephenate (by reducible amination of this latter via phenylpyruvate). The aminoacids are deaminated to their corresponding cinnamic acids, which on the way of successive hydroxylation and methylation lead to p-cumaric, caffeic, ferulic, 5–hydroxyferulic and sinapic acids. Finally p–hydroxycinnamic, ferulic and sinapic acids are reduced to corresponding alcohols (Fig. 3).

The precursor alcohols are often stored in the cambial cells of plants as their β–glucosides, e.g. coniferin, the β–D–glucoside of coniferyl alcohol (Fig. 4).

Before the polymerization reactions the glucosides must be transferred to alcohols by β–glucosidase (EC 3.2.1.21).

How may the polymerization reactions occur in nature? Investigations on the enzymatic lignin synthesis *in vitro* cast some light on this problem. Following Klason (5 – 7) and Erdtmans' (8) suggestion that lignin is probably formed by the polymerization of such compounds as coniferyl alcohol, Freudenberg and coworkers (9 – 12) have performed experiments on lignification by studying the formation of lignin-like products *in vitro* by biosynthesis from coniferyl alcohol. They found that a lignin-like polymer was yielded *in vitro*

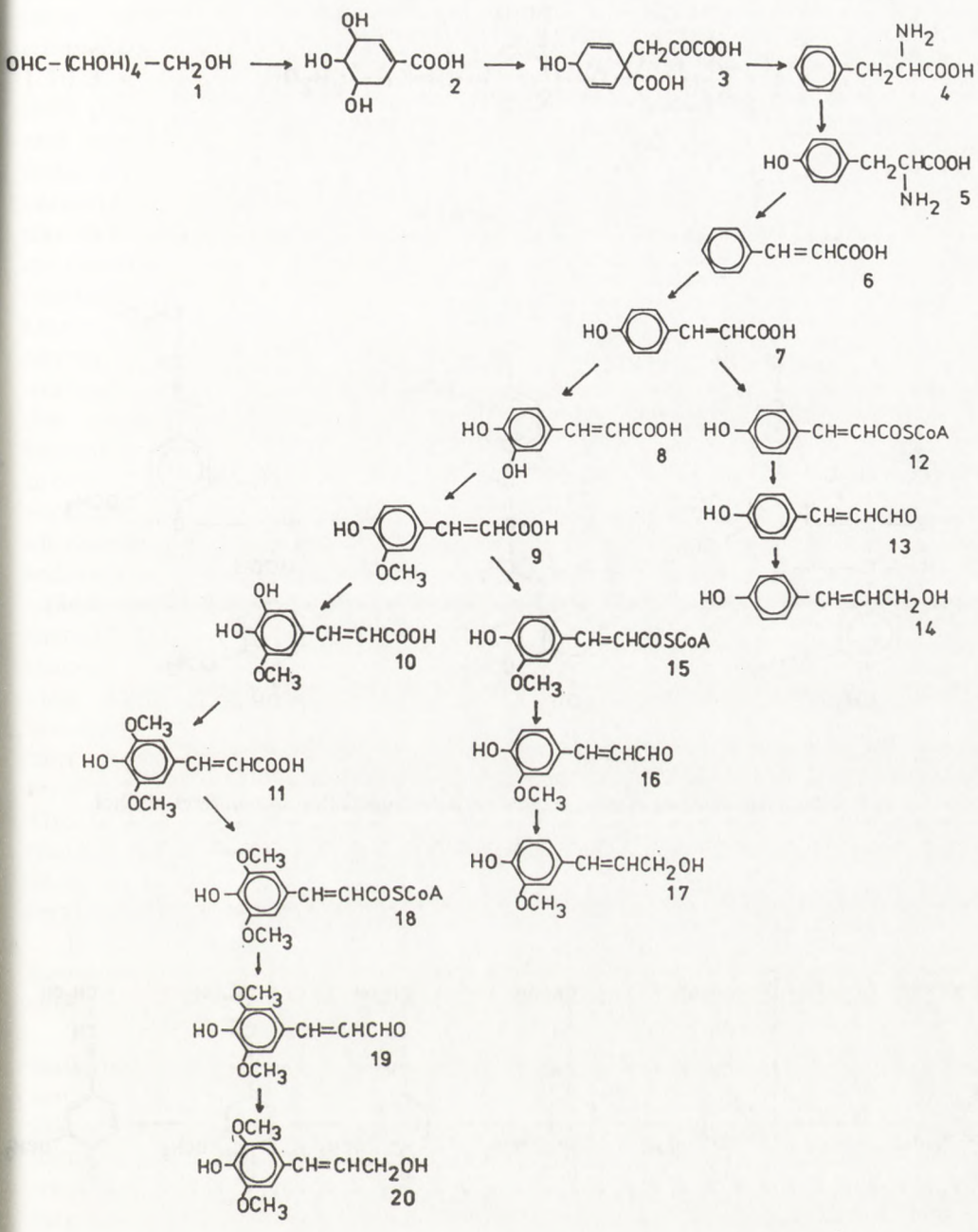


Fig. 3. Shikimate-cinnamate pathway of lignin precursor alcohols biosynthesis (Shimada, 1972) (42).

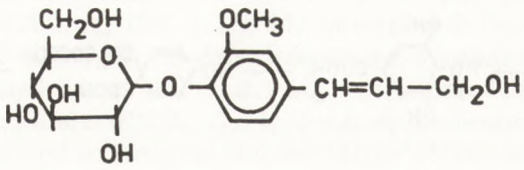


Fig. 4. Coniferin.

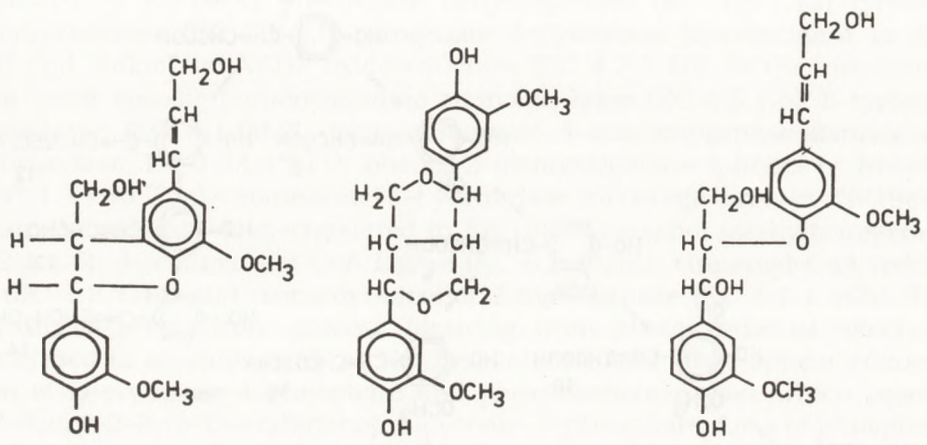


Fig. 5. Dimmers formed during enzymatic dehydrogenation of coniferyl alcohol.

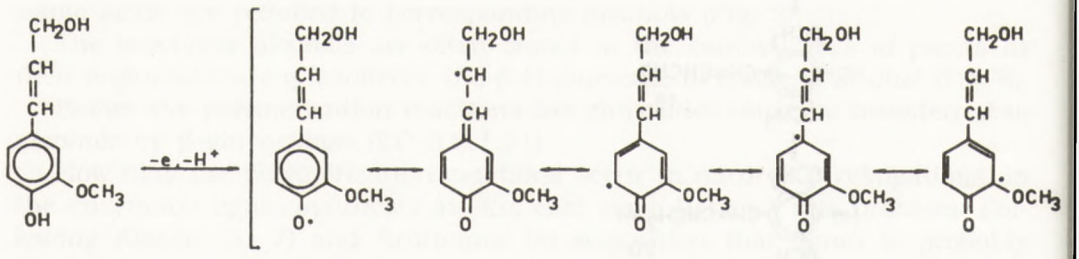


Fig. 6. Phenoxy radicals yielded during enzymatic dehydrogenation of coniferyl alcohol (Glasser, 1980) (39).

during incubation of coniferyl alcohol, under aerobic conditions with fungal (*Psalliota campestris*) laccase (EC 1.10.3.2) or with a horse-radish peroxidase (EC 1.11.1.7) and hydrogen peroxide. A similar enzyme, also capable of causing the formation of a lignin-like polymer from coniferyl alcohol, has been found in cambial cells and cell walls. During incubation, the air or oxygen is passed through the mixture. Within a few hours the originally clear solution becomes turbid, and finally brownish-yellow. It begins to separate and, after a few days, all coniferyl alcohol is converted into an amorphous powder called dehydrogenation polymer (DHP). The mixture is extracted with butanol, dissolving the dimeric and other low-molecular dehydrogenation products. The DHP is filtered off, washed and dried. The butanol extract contains mainly three dimers indicating three ways in which two coniferyl alcohol residues may link together (Fig. 5).

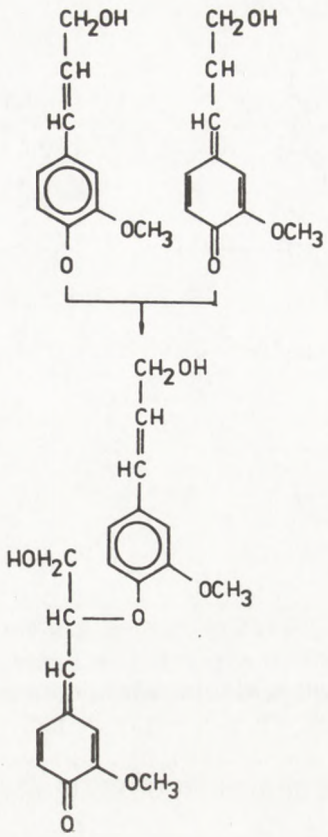


Fig. 7. Formation of quinone methide from coniferyl phenoxy radicals.

How could such dimers be formed? It cannot be studied *in vivo*, but it is known from numerous *in vitro* experiments that the enzymatic dehydrogenation of the p–hydroxycinnamyl alcohols yield free phenoxy radicals (Fig. 6).

The main enzyme working in this process *in vivo* is most probably cell wall peroxidase in combination with hydrogen peroxide as an oxidant. Phenoxy radicals spontaneously condense forming various dimeric structures (dilignols) among which three presented in Fig. 5 are prevalent. Further polymerization involves other radicals formed from phenolic end-groups of dimers which are condensed with each other or with monomeric radical structures forming tri–, tetra–, penta– and oligolignols and also respective free radicals. They finally couple into a heterogeneous, optically inactive, cross-linked and highly polydisperse polymer called lignin.

There also exists a non-radical coupling way, which involves quinone met-

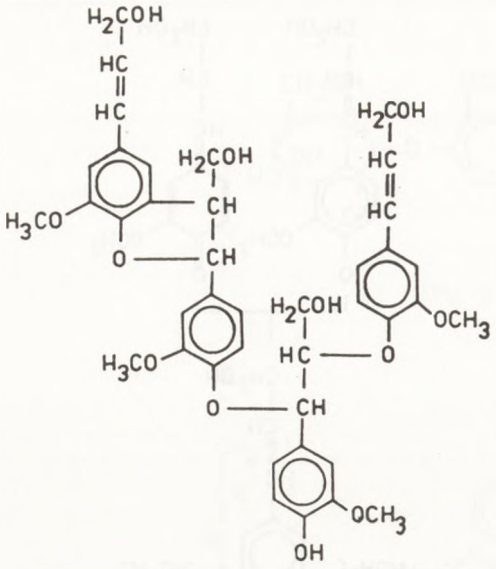


Fig. 8. Tetralignol obtained by coupling of dimeric quinone methide and dehydrodiconiferyl alcohol.

hides. They are formed from some kind of phenoxy radicals. Dimeric quinone methide may serve as an example (Fig. 7). By coupling such dimeric quinone methide with dehydrodiconiferyl alcohol it is possible to obtain tetralignol (Fig. 8). Other quinone methide intermediates may build together with responsible ethers more and more complicated structures and also be partners for the formation of lignin-polysaccharide linkages.

3. Preparation

The main difficulty in the assay of lignin structure is the fact that, as yet, no one has succeeded in separating the total lignin in its original state, from other plant constituents. Strong bonding of lignin with hemicelluloses into a lignincarbohydrate complex considerably hinders isolation of lignin from plant material. The carbohydrate component comprises xylans, mannans, arabans, glucans and galactans (13). Binding of lignin with carbohydrates is mainly of glucoside character, as e.g. in the commonly encountered coniferin (Fig. 4) of wide occurrence in plant kingdom (14). Also ether linkages have been found between alpha carbon of the side chain of the phenylpropane subunit of lignin and the hemicellulose residue (Fig. 9) (15). Because lignin is amorphous, the usual criteria of purity for organic compounds, such as the melting point, cannot be applied. For this reason, the methoxyl content may be considered (purity should increase when methoxyl content grows). Unfortunately this relation does not always occur; it is particularly disturbed when the isolation process is leading in drastic conditions. The increase of methoxyl content does not have to result in better purity of lignin preparation. Either unexpected

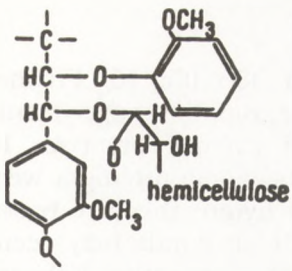


Fig. 9. Ether bond between alpha carbon of the side chain of the phenylpropane subunit of lignin and the hemicellulose residue (Adler, 1952) (15).

loss or an increase of methoxyl content can be observed, which may result from autocondensation or removal of part of lignin during its isolation. Thus, drastic methods of lignin isolation represent now only a historical meaning. From this point of view, two of them are significant: extraction cellulose and hemicellulose from lignified tissue either by treatment with strong (72%) sulfuric acid or with fuming hydrochloric acid. After dillution with water, brown insoluble substance called Klason acid lignin (16) or Willstätter acid lignin (17) remains. Commercially available are lignin preparations isolated from spent liquors of paper industry, like Idulin At produced by West Virginia Pulp and Paper (18) or Peritan Na from Norcen As., Oslo, Norway (19). Although they contain sulphonic groups built in during sulfite processes, these preparations of lignin according to some authors, constitute an adequate model of native lignin (20). Less drastic methods of isolating lignin from plant material consist in applying mild agents for disrupting lignin-hemicellulose linkages. They include either the enzymatic method in which cellulose and hemicellulose hydrolysis is used, or the extraction method. Under the enzymatic method, developed by Nord and Schubert (21), pulverized ligneous material is treated with cellulolytic fungi. The action of cellulases yields "biochemical lignin" devoid of carbohydrates. However, according to more recent findings cellulolytic fungi contain lignin-oxidizing enzymes, including laccase and peroxidase (22 – 24) and for this reason "biochemical lignin" seems not to be identical with the natural substance. The method based on extraction of lignin, using methanol containing 2% anhydrous hydrogen chloride (80 hours with shaking at 90 – 100°C) worked out by Brauns (25) cannot be considered to produce the material identical to the native lignin, as it is in a mixture of lignans (12). More hopeful seem be other extraction methods (13, 26 – 29, 38). They consist in grinding in a ball mill toluene-suspended wood powder. Toluene which diffuses into cells brings about according to Björkman their swelling and wall cracking accompanied by loosening of linkages between lignin and hemicellulose. Lignin is extracted from the ground product with an aqueous solution of dioxan and then precipitated with ethyl ether. On application of Björkman's method it is possible to obtain up to 30% of lignin held in the ligneous tissues. The content of carbohydrates in the preparations remains below 2%. Björkman's preparation is nowadays regarded as the one closest to natural lignin despite its contamination with polysaccharides.

4. Structural models (historical view)

The first lignin model was designed by Adler in 1957 (30) (Fig 10). Further Adler (31) basing mainly on the results of oxidative degradation experiments developed his scheme for spruce lignin (Fig. 11). The scheme comprises 16 phenylpropane units joined with C–O–C or C–C bonds. Some attempts were also aimed at determining the secondary structure of lignin; they are based on the assumption that there exist hydrogen bonds. Such bonds may occur for example between hydroxyl groups and oxygen which forms ether linkages (Fig. 12). In 1964 Freudenberg (32,33) published his own model of lignin structure based on his own dehydrogenative polymerization results as well as on other analytical data available at that time. The model represents 18 prominent C_9–units as a part of a total molecule (in nature over 100; Fig. 13). In 1966, Forss et al. (34) re-evaluated Adler and Freudenberg's lignin theory. They analyzed lignin fragments obtained by decomposition of lignin and fractionated them according to their size. As a result, they presented an opinion that the aromatic components of spruce wood may be divided into two groups, hemilignins (about 6 per cent of the wood) and lignin a high-molecular phenylpropane polymer composed of identical repeating units amounting to about 23 per cent of the wood. A tentative formula of the repeating unit of lignin is shown in Fig. 14. The identical repeating fragments of lignin contain 16 guaiacylpropane and two p–hydroxyphenylpropane units each. The four ether bonds united the repeating lignin units to carbohydrates. In 1974, Nimz (29) and Glassers (35) independently introduced their models of lignin structure based on its nuclear magnetic resonance and computer studies respectively (Figs 15 and 16). Finally, in 1983, Sakakibara (36) published the scheme of lignin structure based on Japanese and other recent results (Fig.17).

5. Details of lignin structure

Analyses of purified lignin preparations have shown that it is a polymer of phenylpropane subunits containing hydroxyl, carbonyl and carboxylic substitutes. The level of functional groups is variable, partly depending on the initial material and on the method of isolation. Ultimate analysis of lignin obtained by Björkman's method from fir wood reveals 83,84% H, 29,68% O, 15,79% OCH_3. Molecular weight of Björkman's lignin is of the order of 11000, this corresponding to about 60 phenylpropane units. The formula of a phenylpropane subunit worked out from the analysis runs is as follows: $C_9H_{8,83}O_{2,37}(CH_3)_{0,96}$ — this in turn corresponding more or less to the coniferyl radical (coniferyl alcohol minus one hydrogen): $C_9H_9O_2CH_3$. Björkman's preparation is nowadays regarded as the one closest to natural lignin despite its contamination with polysaccharides. As a result of recent investigations carried out mainly on application of Björkman's preparation as a model, much has been learned on the lignin macromolecule structure.

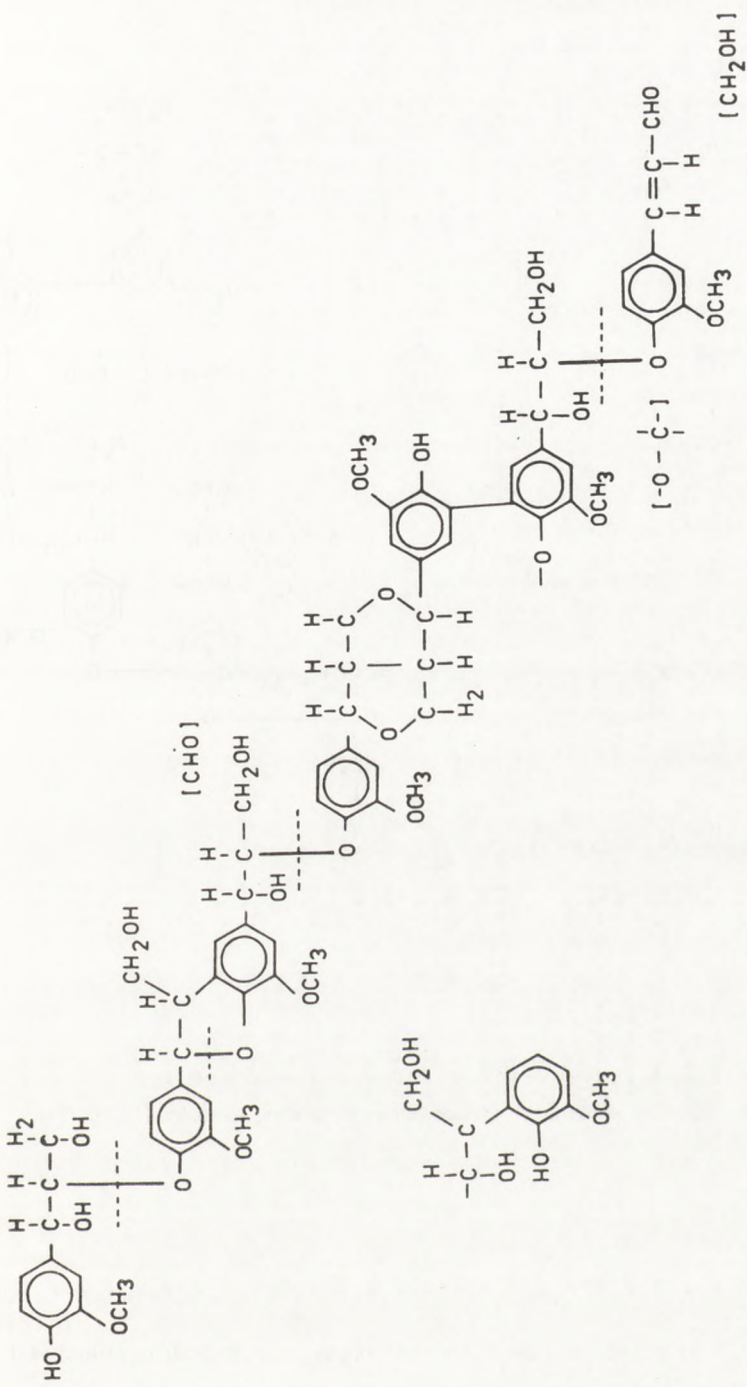


Fig. 10. The first model of lignin structure (Adler, 1957) (30).

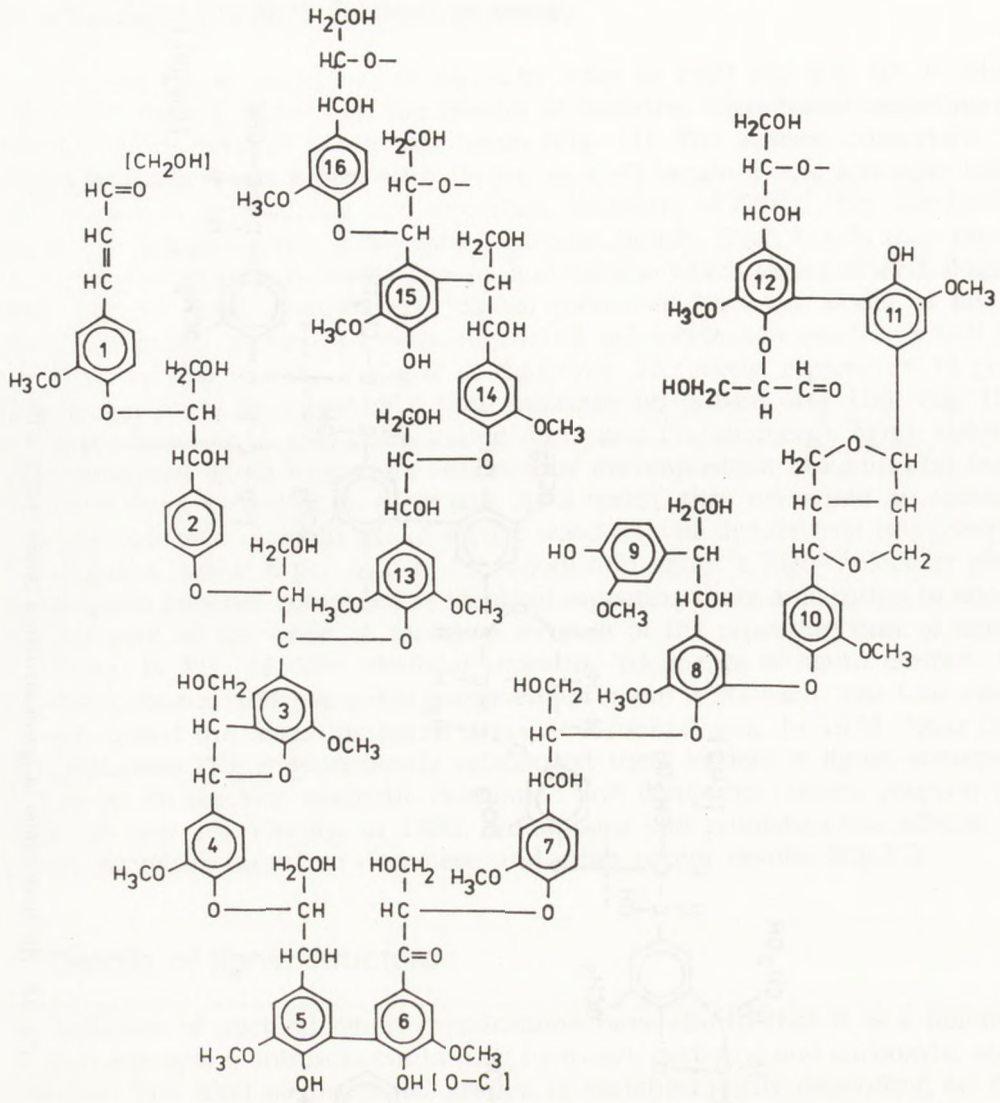


Fig. 11. The scheme of spruce lignin according to Adler (1977) (31).

Fig. 13. Structural scheme of lignin according to Freudenberg (1964) (41).

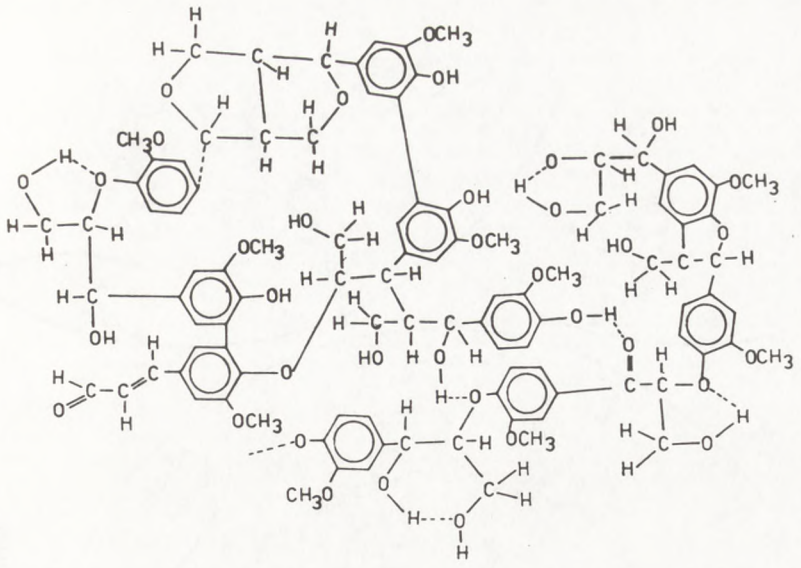
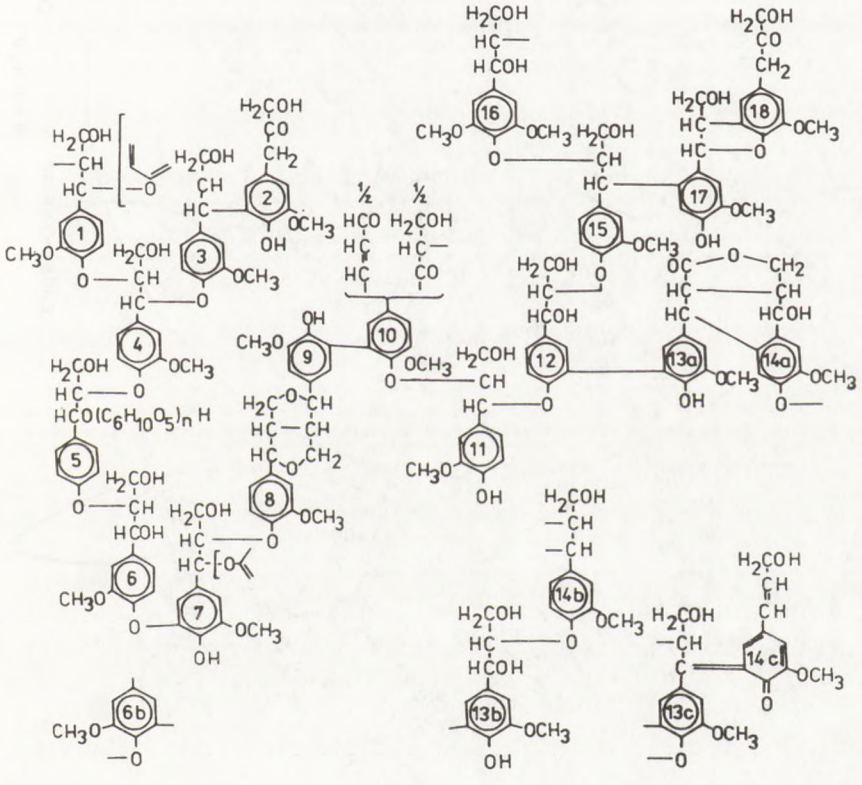


Fig.12. Lignin structure showing hydrogen bonds (Brauns, 1962) (40).



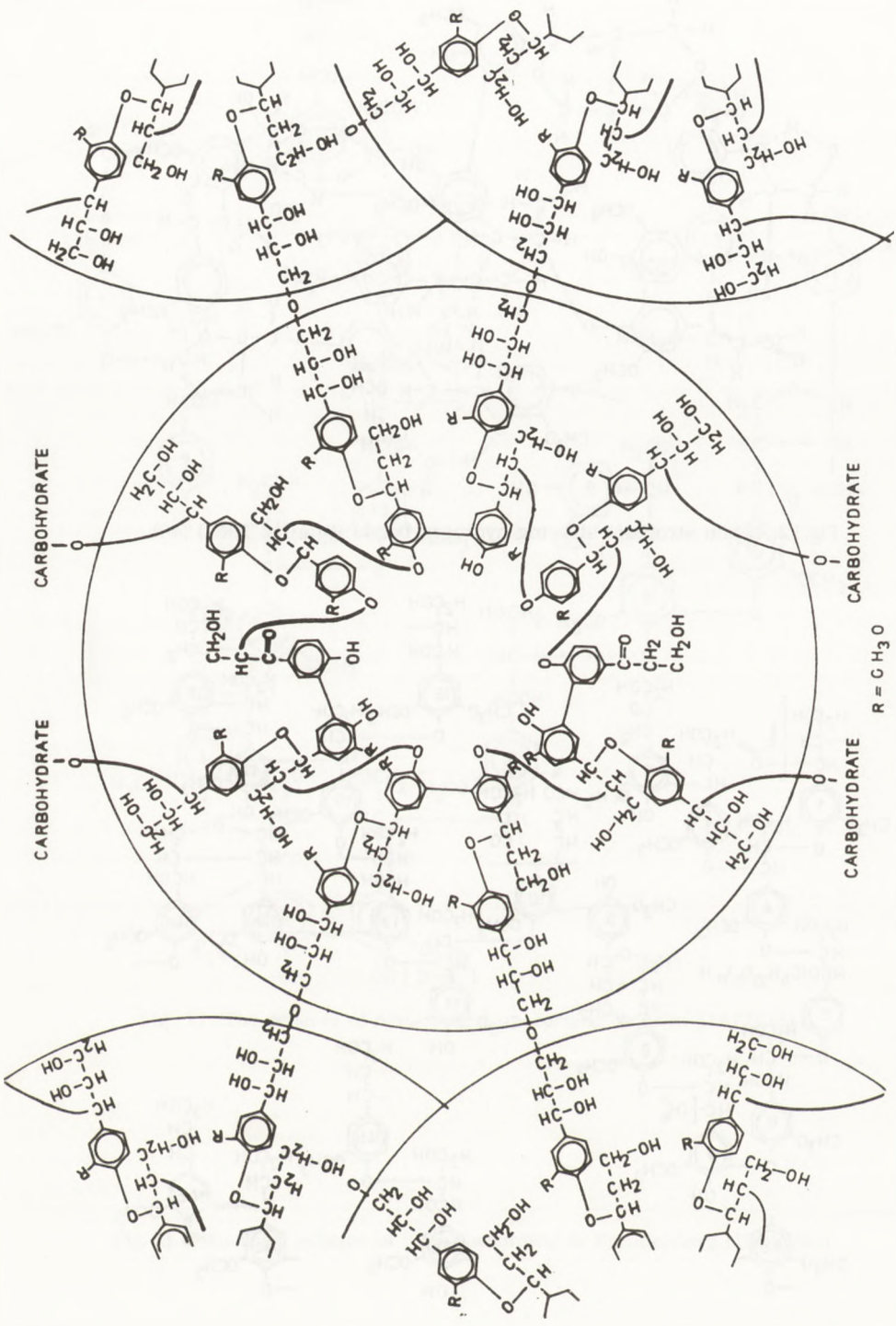


Fig.14. The repeating units of spruce lignin according to Forss and coworkers (1966) (34).

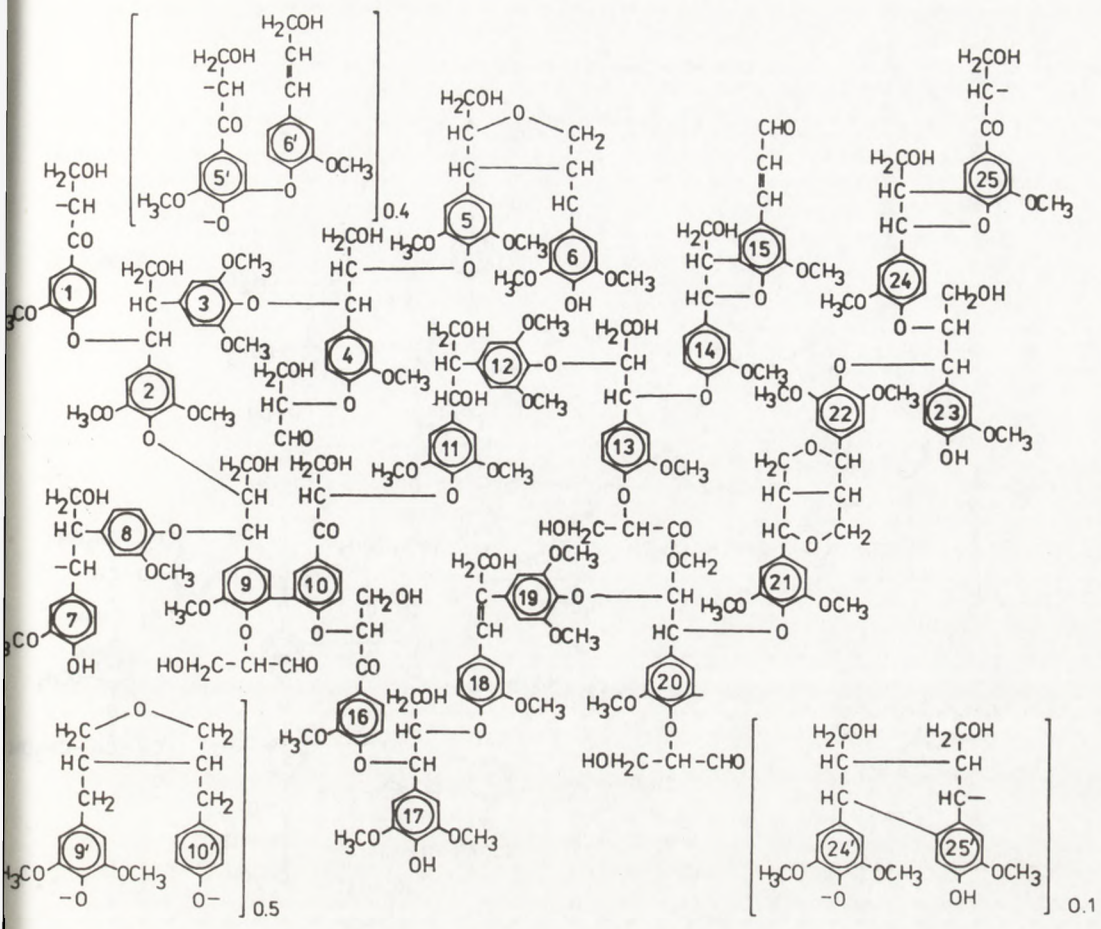


Fig.15. Structure of beech-wood lignin proposed from degradative studies and 13C–NMR spectroscopy by Nimz (1974) (29).

It contains at least 12 various linkages connecting aromatic nuclei. The dominant is guaiacylglycerol–β–aryl ether substructure (β–O–4 type linkage, 40 – 60% in lignin macromolecule, appearing between aromatic units 1 – 2, 6 – 7, 8 – 9, 20 – 21 and 24 – 27), second in abundance is phenylcoumaran type (10% between units 4 – 5, 21 – 22 and 25 – 26), third–diarylpropane and diphenyl (5 – 10%, 27 – 28 and 9 – 10 respectively), fourth–diphenyl ether (5%, 17 – 23) and fifth–pinoresinol (< 5%, 7 – 8). The diarylpropane linkage (β–1 type) has served as a model in various transformation studies. The major features of lignin structure are now well understood, although some details are still being clarified (Fig. 17).

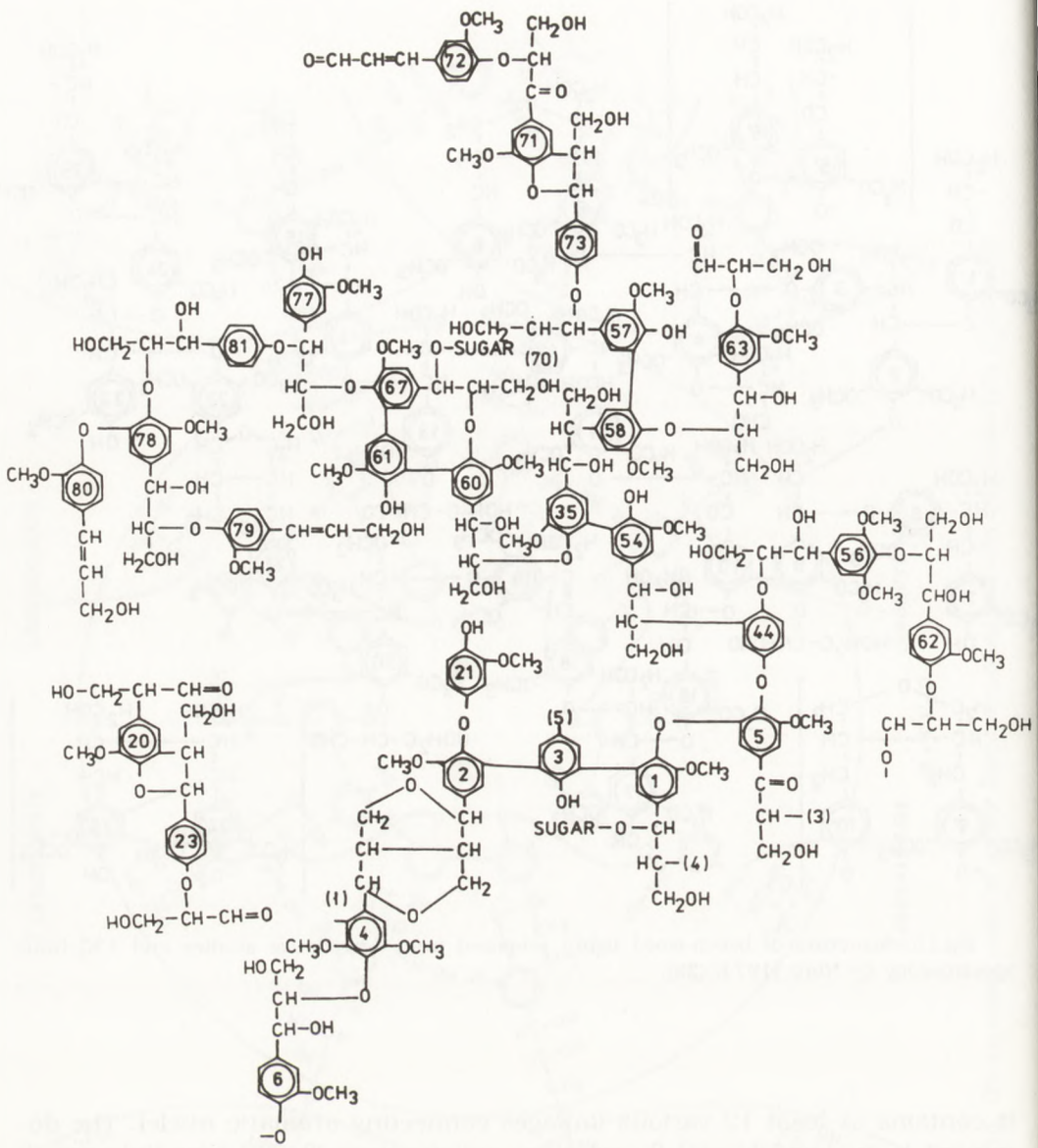


Fig.16. Fragment of simulated by computer softwood lignin model (Glasser and Glasser, 1974) (35).

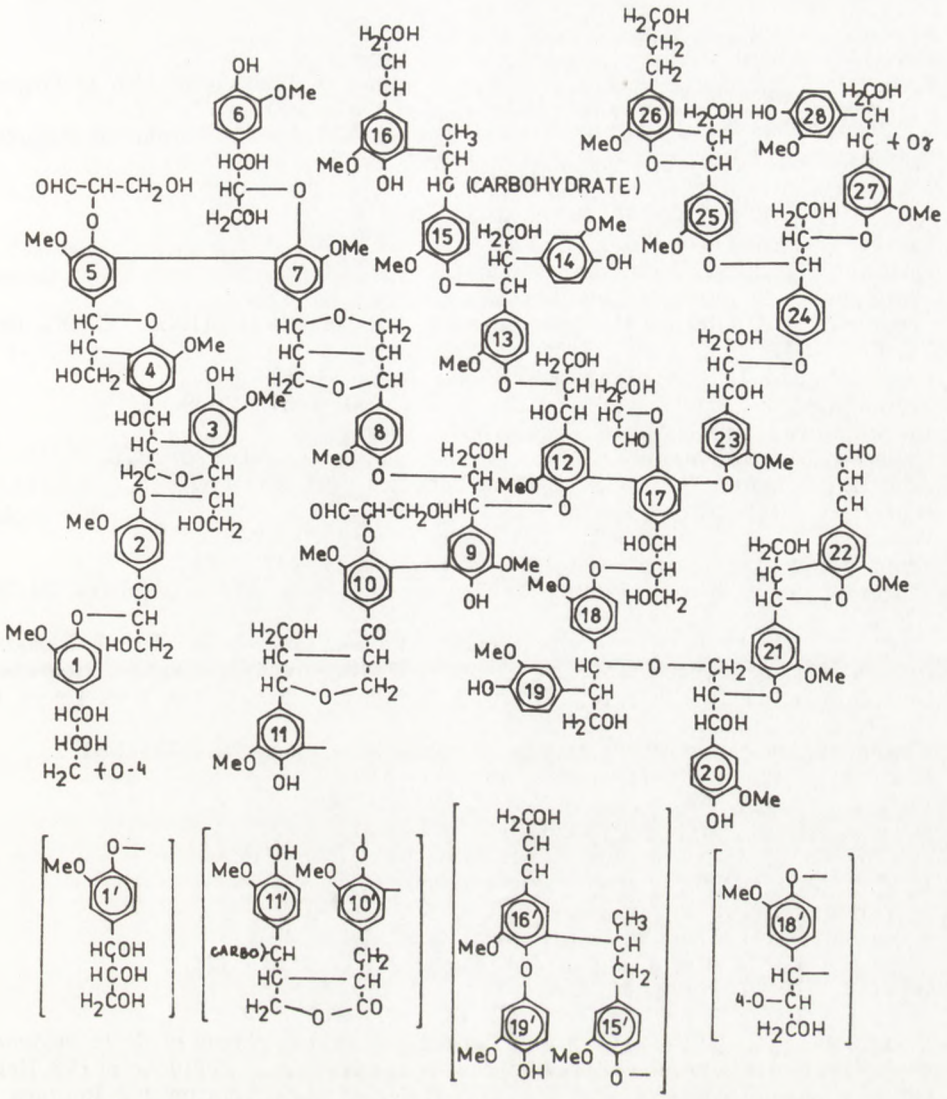


Fig.17. Structural model of softwood lignin (Sakakibara, 1983) (36).

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Lignina — występowanie, biosynteza, wyodrębnianie i struktura

Streszczenie

Przedstawiono w ujęciu historycznym rozwój badań nad ustalaniem struktury ligniny, jej biosyntezą, metodami wyodrębniania oraz rozmieszczeniem w ścianie komórkowej.

Key words:
lignin, biosynthesis, structure models, preparation, distribution, laccase, peroxidase.

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