Comprehensive Study on the Chemical Structure of Dioxane Lignin from Plantation *Eucalyptus globulus* Wood

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Results of a comprehensive study on the chemical structure of lignin from plantation *Eucalyptus globulus* Labill are presented. Lignin has been isolated by a modified mild acidolysis method and thoroughly characterized by functional group analysis, by a series of degradation techniques (nitrobenzene oxidation, permanganate oxidation, thioacidolysis, and Py−GC−MS), and ^{1}H and ^{13}C NMR spectroscopy. Plantation *Eucalyptus globulus* lignin was found to be of the S/G type with an extremely high proportion of syringyl (*S*) units (82−86%) and a minor proportion of *p*-hydrophenyl propane (*H*) units (roughly 2−3 mol %). Unknown C-6 substituted and 4-*O*-5′ type syringyl substructures represent about 65% of lignin "condensed" structures. Eucalypt lignin showed high abundance of *β*-*O*-4 (0.56/C_6) structures and units linked by α-*O*-4 bonds (0.23/C_6). The proportion of phenylcoumaran structures was relatively low (0.03/C_6). Different kinds of *β*-*β* substructures (pino-/syringaresinol and isotaxiresinol types) in a total amount of 0.13/C_6 were detected. ESI-MS analysis revealed a wide molecular weight distribution of lignin with the center of gravity of mass distribution around 2500 u.

Keywords: *Lignin; Eucalyptus globulus; functional group analysis; nitrobenzene oxidation; permanganate oxidation; thioacidolysis; analytical pyrolysis; ESIMS; ^{1}H NMR spectroscopy; ^{13}C NMR spectroscopy*

INTRODUCTION

Eucalyptus globulus Labill, a native dicotyledonous tree of southeast Australia, was introduced in the Iberian peninsula by the end of the 19th century, and later in Latin America, where soon it was recognized as a fast-growing tree with potential for pulp and paper production in those regions of the world. Nowadays, extensive short-rotation plantations (about 10-year rotations) of *E. globulus* may be found in Portugal and Spain, representing the major wood source for the production of market hardwood bleached kraft pulp in Europe. In Australia, where eucalypt trees can be found mainly in native forests, there is a recent growing interest in the use of plantation eucalypt (*1*). *E. globulus* represents, within this context, one of the most interesting species among the more than 600 species comprising the genus *Eucalyptus*.

Despite this widespread interest and use of plantation *E. globulus* wood, literature on the detailed chemical and structural analysis of its components is quite scarce and dispersed, and it often refers to mature wood (30 years old and more) from native forests. The chemical composition of mature wood is known to differ significantly from that of young plantation eucalypt, and, hence, some care should be taken when making extrapolations from the chemical features of mature woods to those of young plantation woods. Previous chemical studies on plantation *E. globulus* wood included in-tree and between-tree variations in general chemical composition (*2*, *3*) and its relation with pulp quality (*4*) and kraft pulp yields (*5*). More detailed studies on chemical composition included extractives analysis (*6*−*11*) and neutral and acidic sugars composition (*12*). As far as lignin is concerned, two studies dealing with a brief characterization of isolated lignins from *E. globulus* wood have been previously reported (*13*, *14*). However, the age and origin of the wood were not given. The tree-to-tree variation in the syringyl/guaiacyl ratio of *E. globulus* wood lignin was investigated by Rodrigues et al. (*15*) using analytical pyrolysis.

The presence of polyphenols in eucalypt woods complicates the isolation, quantification (as Klason lignin), and structural analysis of lignins (*16*, *17*). The milled wood lignin (MWL) from both mature (*18*) and plantation (*19*) eucalypt woods was obtained in poor yields and with a high proportion of attached hemicelluloses and tannins which hinder the quantitative analysis of the lignin structural elements. According to our results, the yield of MWL from eucalypt wood varies between 12 and 18%, whereas for the same milling conditions the yield of spruce MWL varies in the range of 30−35%. Good results on the purity of MWL from eucalypt woods were obtained by extraction of sawdust with alkali prior to the milling procedure (*16*). Dioxane lignins are fre-

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quently referred to in structural investigations (*13*, *14*). Our preliminary study on dioxane lignin isolation from *E. globulus* wood showed that even after excessive extraction of sawdust by toluene−ethanol solution a significant part of condensed tannins of catechin and gallocatechin types remained in the wood and affected the structural analysis of lignin (*20*). A mild alkaline extraction prior to wood extraction with dioxane−water solution and some special precautions in the acidolysis procedure allowed a good yield of pure dioxane lignin from *E. globulus* wood with minimal structural changes obtained (*20*). A small proportion of lignin (about 10% of that initially present), however, is eliminated in the alkaline extraction step together with condensed tannins. This alkali-extracted lignin is richer in guaiacyl and *p*-hydroxyphenyl propane units than lignin remaining in the wood (*20*).

In this work we have studied the lignin of short-rotation plantation *E. globulus* isolated from wood previously subjected to a mild alkaline extraction followed by a modified acidic dioxane extraction. Lignin was comprehensively characterized with regard to elementary composition, functional groups, structure, and molecular weight.

MATERIALS AND METHODS

Preparation of Plant Material. 9-Year-old *Eucalyptus globulus* wood used in this study was harvested in an experimental clone plantation located in Quinta de S. Francisco, Aveiro, Portugal belonging to RAIZ − Paper and Forest Research Institute. The trunk of a representative tree of 28 m height (including the tree-top) was divided into 1-m fractions, and the fraction between 5 and 7 m was selected for analysis. The trunk was mechanically transformed into chips (20 mm × 20 mm × 3 mm on average) and milled in a Retsch cross-beater mill SK1 (Haan, Germany), sieved to 40−60 mesh, and air-dried. The extractive-free wood (referred to in the text simply as wood) was obtained by submitting wood sawdust to Soxhlet extraction with ethanol/toluene (1:1, v/v) for 24 h. The lignin content (as Klason lignin) in the wood was 21.8%.

Alkaline Pre-Extraction of Wood Sawdust. The alkaline extraction of extractive-free wood sawdust was performed in nitrogen atmosphere with 0.3% (0.075 mol/L) NaOH solution during 1 h (liquid-to-wood ratio 50:1) under reflux. Extracted wood was thoroughly washed with hot distilled water until the filtrate was neutral, dried at room temperature (under P_2O_5), and used for further analysis.

Isolation of Lignin. Lignin was isolated by acidolysis from alkali pre-extracted sawdust in a nitrogen atmosphere by the dioxane method, adapted from a previously published procedure (*21*), with some modifications, as follows:

Sawdust (25 g, dried under P_2O_5) was placed in a 2-L three-necked flask fitted with a reflux condenser, nitrogen bubbler, and a dropping funnel. The solvent, 250 mL of dioxane/water (9:1, v/v) mixture containing 1.82 g of hydrogen chloride equivalent to 0.2 M, was added slowly from the funnel. The reaction mixture, under nitrogen, was heated with a heating mantle and refluxed at 90−95 °C for a period of 40 min. Then, the mixture was allowed to cool in a nitrogen atmosphere to around 50 °C. The liquid phase was decanted and the solid residue was subjected to the next extraction with 200 mL of the acidic dioxane/water solution for a period of 30 min, as described above. Two more extractions were effected in the same manner. The last (fourth) extraction was performed without addition of hydrochloric acid in the dioxane/water mixture. Each portion of extract was concentrated separately (200 mL to around 40 mL) and then the concentrates were combined and lignin was precipitated by addition of the dioxane solution in cold water (about 1600 mL). Filtered isolated lignin was extracted with diethyl ether (100 mL), then washed with water, and freeze-dried. The amount of lignin extracted represents about 80% of lignin in the alkali pre-extracted wood (based on Klason lignin).

Chemical Analysis. Methoxyl groups in lignin were analyzed by gas chromatography using a modified Zeisel method (*22*), whereas phenolic hydroxyls were analyzed by aminolysis (*23*), and total hydroxyl groups were analyzed by acetylation (*23*). Carbonyl groups were determined by oximation in the presence of triethanolamine, and carboxyl groups were determined by chemisorption method with calcium acetate (*23*).

Analyses by Chemical/Physical Degradation Techniques. A lignin sample was subjected to a series of analyses using chemical degradation techniques: nitrobenzene oxidation (*24*), permanganate oxidation (*25*), thioacidolysis (*26*), and pyrolysis−gas chromatography−mass spectrometry (*27*). All analyses were carried out according to the previously published standard protocols (*24*−*27*).

Analysis by ^{1}H NMR. The ^{1}H NMR spectrum of the acetylated lignin in chloroform ($CDCl_3$) solution (2% concentration) was obtained using a Bruker AMX 300 spectrometer (Strasbourg, France), operating at 300 MHz, at room temperature. The acquisition parameters used were as follows: 12.2 μs pulse width (90°); 2 s relaxation delay; and 300 scans.

Analysis by ^{13}C NMR. The ^{13}C NMR spectrum was recorded on a Bruker AMX 300 spectrometer operating at 75.5 MHz. Lignin samples were dissolved in DMSO-d_6 (ca. 23% concentration); the mixture was placed into 10-mm-diameter tubes and the spectra were recorded at 318 K with TMS as internal reference. The inverse gated decoupling sequence, which allows quantitative analysis and comparison of signal intensities, was used with the following parameters: 90° pulse angle; 12 s relaxation delay; 16 K data points; and 18000 scans. The ^{13}C DEPT NMR subspectrum was acquired with a θ = 135°, a one bond heteronuclear coupling constant ($^1J_{C-H}$) of 150 Hz, using a 23% lignin solution in 5-mm-diameter tubes. The HETCOR spectrum was acquired with the same lignin solution in DMSO-d_6 used for the acquisition of the quantitative ^{13}C NMR spectrum and using the Bruker pulse program HXCO. Data were collected in a 2048 × 256 matrix with a 10-ppm spectral width in F_1 (^{1}H) and 150-ppm in F_2 (^{13}C), and then processed in a 2048 × 1048 matrix. This experiment was optimized for one bond heteronuclear coupling constant ($^1J_{C-H}$) of 146 Hz.

Vapor Pressure Osmometry (VPO) Analysis. The number-average molecular weight (M_n) of lignin (nonacetylated) was obtained by VPO in 2-methoxyethanol (60 °C) using a Knauer Vapor Osmometer and benzil as a calibration standard.

Electrospray Ionization Mass Spectrometry (ESI-MS) Analysis. Negative-mode ESI mass spectra were acquired with a VG AutoSpecQ (VG Analytical Manchester, UK). The instrument of EBEqQ geometry was equipped with a Micromass ESI source. Procedures for sample preparation and calibration of the instrument were the same as those described previously (*28*).

RESULTS AND DISCUSSION

Functional Groups Analysis. The *E. globulus* dioxane lignin was subjected to analysis of the principal functional groups. Amounts of functional groups were calculated per phenylpropane unit (C_9). Results are summarized in Table 1.

The lignin sample showed a high content of methoxyl groups (164/100 C_9). This is a particular characteristic for lignins from *Eucalyptus* woods having methoxyl group contents remarkably higher than those found for other hardwood lignins (*29*).

Obviously, acidolysis reactions are responsible for the amount of phenolic hydroxyl groups (29 OH_{ph}/100 C_9) in eucalypt dioxane lignin that exceeds the values reported on MWL from hardwoods (22−26 OH_{ph}/100 C_9)

Table 1. Analysis of Functional Groups in *E. Globulus* Dioxane Lignin[a]

functional group	abundance (/100 C_9)
OCH_3	164
total OH	117 (121)
alcohol OH total	88 (91)
primary OH	68
secondary β-OH	4
benzylic OH	16
phenolic OH	29 (30)
total carbonyl	24
CHO in benzaldehyde type structures	(3)
CHO in cinnamaldehyde type structures	(4)
CHO nonconjugated	(2)
Cα=O/Cβ=O	15
carboxyl	4

[a] Data on functional group analytes obtained by ^{1}H NMR of acetylated sample ($CDCl_3$) shown in parentheses; formula based on C_9 ($C_9H_{8.55}O_{2.73}(OCH_3)_{1.64}$) was calculated from elemental analysis data (C, 60.8%; H, 6.4%) and methoxyl group content (23.8%).

(*30*). Thus, approximately a third of the structural units of *E. globulus* dioxane lignin contain phenolic hydroxyl groups.

The amount of benzyl alcohol groups was determined based on the increase of methoxyl group content after lignin treatment in absolute methanol (*23*) in the presence of *p*-toluenesulfonic acid as catalyst. The proportion of primary hydroxyl groups (presumably attached to Cγ in the propane chain) was determined by reaction with phthalic anhydride in benzene solution (*23*). The number of hydroxyl groups attached to Cβ (about 4 OH/C_9) was calculated by difference from the total amount of hydroxyl groups in the lignin determined by acetylation and the quantity of benzyl alcohol, phenolic groups, and primary hydroxyls (Table 1).

The content of carbonyl groups in the analyte (24 CO/100 C_9) is higher than that reported for MWL samples from different woods (14−18 CO/100 C_9) (*30*) and can be explained by the undesirable formation of Hibbert's ketones during acidolysis.

The ^{1}H NMR spectrum (Figure 1) allows the abundance of different kinds of aldehyde groups to be determined by integration of resonances from specific formyl protons in the lignin. The integration of signals of the formyl protons at 9.55−9.75 ppm gives the contents of aldehyde groups linked at Cγ and conjugated with vinylic moieties (in cinnamaldehyde type units). The integration of formyl proton signals at 9.75−10.0 ppm gives the quantity of aldehyde groups in benzaldehyde type units (*31*). The resonances detected at 9.30−9.55 ppm can be assigned to formyl protons in nonconjugated aldehyde groups. Such groups appear at Cβ during lignin acidolysis as a result of partial degradation of β-*O*-4 linkages (*32*). Results of hydroxyl group analysis obtained by wet chemistry methods are in good agreement with data obtained by ^{1}H NMR of the acetylated sample using the signal of methoxyl groups as an internal standard (Table 1). This fact suggests that data from wet chemical analysis and ^{1}H NMR spectroscopy are coherent and can be combined to get new additional information. Thus, the content of ketone groups may be estimated as the difference between the total amounts of carbonyl groups determined by oxidation and the contents of aldehyde groups detected by ^{1}H NMR (9 HCO/100 C_9). This approach gives a value of 15 CO/100 C9 ((24 CO − 9 CO)/100 C_9). It must be noted that the calculated total amount of ketone groups should also include a small proportion (1−2/100 C_9) of quinone groups normally present in MWL and dioxane lignins (*23*). Results of analysis of carbonyl groups are summarized in Table 1.

The content of carboxyl groups (4 COOH/100 C9) in isolated lignin is very similar to that found in the literature for dioxane and MWL preparations from different woods (*23*, *30*). These data do not confirm the COOH contents that were previously reported for dioxane (*13*) and Brauns lignins (*14*) from *E. globulus* wood (13−17 COOH/100 C9).

Determination of Molecular Weight. The molecular weight determination was done by a traditional method in lignin chemistry, vapor pressure osmometry (VPO) in 2-methoxyethanol, as well as by the more advanced electrospray ionization coupled with mass spectrometry (ESI-MS) technique. The eucalypt dioxane lignin ESI-MS spectrum shows the mass distribution possessing a maximum at about *m*/*z* 2400−2600, indicating the predominance of dodecamers in the analyte (2500/M_{ppu}) (spectrum is not shown). The average molecular mass of the lignin, obtained as the center of gravity on the mass distribution curve, is in good agreement with the number-average molecular weight determined by VPO (M_n = 2180 u).

The composition of the low-molecular-weight fraction is of particular interest because it represents relatively simple native lignin fragments or degradation products that can be studied in detail. This low-molecular-weight fraction of eucalypt dioxane lignin was obtained by preparative size-exclusion chromatography (700 × 25 mm column packed with Sephadex G-25) using 0.05 M NaOH solution as eluent and subjected to ESI-MS analysis in the negative ion mode. This fraction, which was not eliminated by diethyl ether extraction (see Materials and Methods), represents 4% of the total amount of lignin and is composed essentially of monomeric and dimeric structural units released in the acidolytic isolation procedure (Figure 2). The signals at *m*/*z* 151, 165, and 195 were assigned to vanillin, acetoguaiacone, and acetosyringone, respectively. These compounds were also detected in the diethyl ether extract from the isolated lignin as well. Different dimeric/trimeric substructures are represented by the set of peaks at *m*/*z* 250−500 (Figure 2). The identification of lignin oligomer structures is difficult because of scarce information available on ESI-MS/MS patterns for this kind of compounds. The compound represented by a characteristic peak at *m*/*z* 339 was assigned to the disaccharide composed by residues of 4-*O*-methyl-D-glucuronic acid and D-xylose (Glc*p*A-Xyl*p*). This disaccharide was previously found in the sample of kraft eucalypt lignin (*33*). Thus, the low-molecular-weight fraction of dioxane eucalypt lignin is contaminated with oligosaccharides.

Nitrobenzene Oxidation. The ratio between the main structural units in lignin was estimated based on analysis of nitrobenzene oxidation products (Table 2). The data clearly show that eucalypt lignin is essentially a S/G type lignin with a minor proportion of *p*-hydroxyphenyl propane (H) units. The low yield of *p*-hydroxybenzaldehyde can be explained partially by poor conversion of H-type lignin structures in the nitrobenzene oxidation analysis (*24*). The total yield of nitrobenzene oxidation products (37.5%) is lower than the yield reported for other hardwoods (42−50%; *24*). This fact

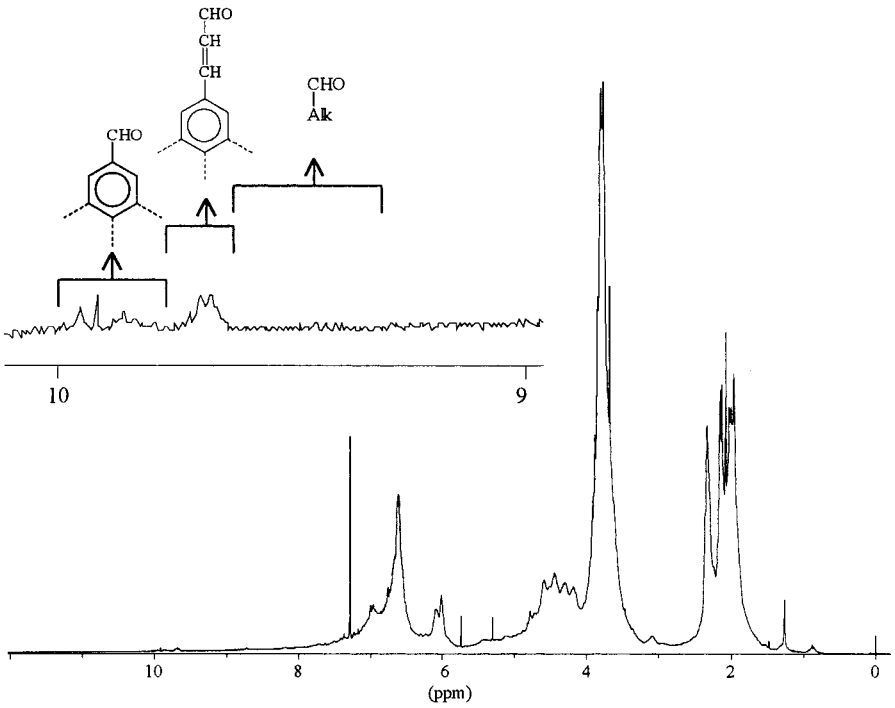


Figure 1. ^{1}H NMR spectrum ($CDCl_3$) of acetylated eucalypt lignin. Expanded range from 9 to 10 ppm shows resonances of formyl protons.

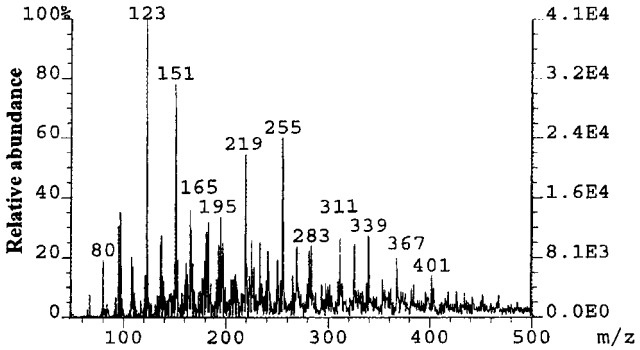


Figure 2. ESI-MS spectrum (negative ion mode) of the low-molecular-weight fraction from *E. globulus* dioxane lignin with expanded mass range from *m/z* 50 to 500.

reflects a high abundance of "condensed" structures in the eucalypt lignin that are not accessible for the analysis.

The alkaline pre-extraction of eucalypt sawdust prior to extraction with dioxane−water mixture (Materials and Methods section) did not significantly influence the S/G ratio (Table 2). The yield of nitrobenzene oxidation products from lignin obtained without alkaline pre-extraction is lower than that from lignin obtained after the alkali pre-extraction procedure. Such a difference can be explained by the presence of tannins in the former sample (*20*). Results on the S/G ratio are very close to those previously reported for *E. globulus* dioxane lignin obtained by the same analytical method (*13*).

Analysis by Pyrolysis−GC−MS. Analytical pyrolysis combined with gas chromatography and mass spectrometry (Py−GC−MS) is a suitable method for estimating the ratio of different structural units in lignins (*27*). This technique was applied to complement the nitrobenzene oxidation analysis. The S:G:H ratio 84.5:15:0.5 obtained, based on the analysis of the pyrolysis products, is consistent with nitrobenzene oxidation data (Table 2). The relatively low yield of pyrolysis products derived from *p*-hydroxyphenyl structures can tentatively be explained by its high condensation in the lignin sample.

Permanganate Oxidation Analysis. The technique of permanganate oxidation provides structural information about both "condensed" and "noncondensed" phenolic units of lignin. Thus, about 30% of eucalypt dioxane lignin structural units are involved in this analysis (Table 1). The identified methylated products formed in the oxidation of lignin with potassium permanganate and their relative abundances are presented in Table 3.

p-Hydroxybenzoic acid (**1**), vanillic acid (**2**), and syringic acid (**3**) methyl esters are derived from uncondensed *p*-hydroxyphenyl, guaiacyl, and syringyl structural units, respectively (*25*). The isohemipinic acid methyl ester (**4**) originates primarily from phenylcoumaran-type structures. The dicarboxylic acid methyl esters **5** and **6** can be tentatively assigned to dilignol structures linked by α-6 and *β*-*β* linkages (isotaxiresinol-type structures) previously suggested by Nimz (*34*) as being present in beech lignin. Also, structures **5** and **6** can be assigned to phenylisochroman type structures linked by *β*-6 and *γ*-*O*-α linkages, as previously reported by Sudo and Pepper (*35*) for aspen lignin. However, these tentative assignments should be clarified by other techniques, and they will be subjected to future investigations.

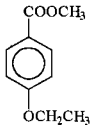
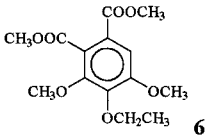
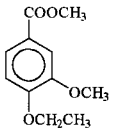
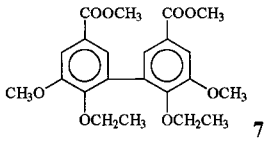
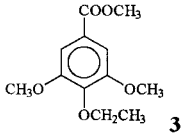
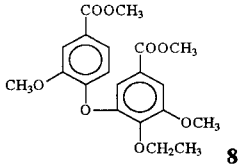
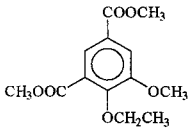
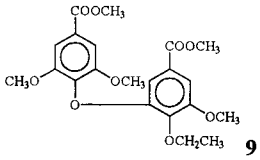
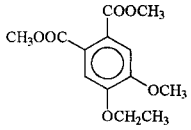
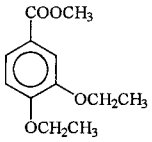
It could be expected that part of the products **4**−**6** derived from lignin "condensed" structures would be formed during the acidolytic isolation procedure. However, this contribution of acid-catalyzed condensation reactions to the relative abundance of products **4**−**6** in the dioxane lignin is not of great importance because similar results on permanganate oxidation were obtained using eucalypt wood sawdust (*36*).

The permanganate oxidation product **7** is derived from 5 to 5' biphenyl type structures, while products **8** and **9** originate from 4-*O*-5'diaryl ether type substruc-

Table 2. Results on Nitrobenzene Oxidation of *E. Globulus* Dioxane Lignin

	oxidation products (mol. %)				
isolation method	*p*-hydroxybenzaldehyde	vanillin	syringaldehyde	yield, % Klason lignin	molar ratio S:G
with alkali pre-extraction	trace	14	86	37.5	86:14
no alkali pre-extraction	trace	18	82	33	82:18

Table 3. Permanganate Oxidation Products of *Eucalyptus Globulus* Dioxane Lignin

Oxidation product (carboxylic acid methyl ester)	Frequency of occurrence (mol. %)	Oxidation product (carboxylic acid methyl ester)	Frequency of occurrence (mol. %)
 **1**	3	 **6**	7
 **2**	18	 **7**	2
 **3**	57	 **8**	1
 **4**	2	 **9**	7
 **5**	3	 **10**	0

tures. The product **10**, normally appearing in the analysis of eucalypt lignins contaminated by tannins, was not detected, confirming the high purity of the sample obtained (Table 3).

It may be assumed, on the basis of the relative abundance of permanganate oxidation products, that the proportion of "uncondensed"/"condensed" units in lignin is about 78/22. The main contribution to the condensed part of lignin (about 65%) is inserted by syringyl units linked by 4-*O*-5' bonds and carbon−carbon linkages in the position 6 of phenolic unit as revealed from the abundance of product **6**.

The molar ratio of S:G:H units determined by permanganate oxidation technique is about 68:29:3. These data indicate that the proportion of phenolic units of guaiacyl type is higher than the proportion of phenolic units of syringyl type because in nitrobenzene oxidation half-abundance of guaiacyl units was detected (Table 2).

Analysis by Thioacidolysis. This technique was utilized in the lignin investigation to estimate the proportions of different structural units linked by ether linkages (essentially of alkyl-*O*-aryl type). Data on thioacidolysis analysis are presented in Table 4. The S:G ratio obtained (82:18) was very close to that previously found by nitrobenzene oxidation (86:14). Enol ether type structures giving products **B** and **G** (Table 4) can

Table 4. Degradation Products (as TMS derivatives) Derived from *E. Globulus* Dioxane Lignin during Thioacidolysis (*μ*mol/g)

	thioacidolysis product[a]	abundance (*μ*mol/g)
A	G-CH(SEt)$_2$	8
B	G-CH$_2$−CH(SEt)$_2$	24
C	G-CH(SEt)$_2$−CH$_3$	6
D	G-CH(SEt)-CH(SEt)-CH$_2$Set	262
E	S−CH(SEt)$_2$	22
F	S−COOH	13
G	S−CH$_2$−CH(SEt)$_2$	7
H	S−CH(SEt)$_2$−CH$_3$	88
I	S−CH(SEt)=CHSEt	8
J	S−CH(SEt)-CH=CHSEt	4
K	S−CH(SEt)-CH(SEt)-CTH$_2$SEt	1195
	total yield, *μ*mol/g	1635
	S:G ratio	82:18

[a] Guaiacyl and syringyl aromatic groups are designated as G and S, respectively.

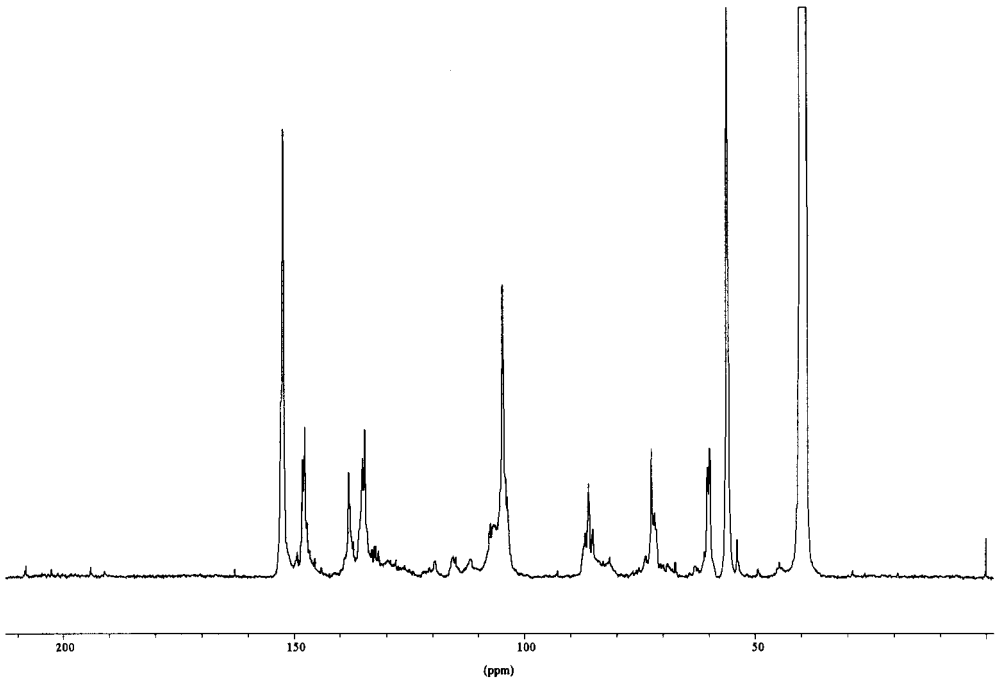


Figure 3. Quantitative ^{13}C NMR spectrum of *E. globulus* dioxane lignin (DMSO-d_6).

originate from dioxane lignin or be formed during thioacidolysis analysis (*26*). Thus, it is clearly impossible to ascertain which proportion of enol ether structures belonged to original lignin and what amounts were generated in the thioacidolysis procedure. In any case, the total amounts of products **B** and **G** were insignificant: less than 2% of the other thioacidolysis products.

Eucalypt dioxane lignin showed a relatively high yield of product **H** after thioacidolysis (Table 4). This product, which is normally absent in the analysis of MWLs (*26*), presumably arises from acetosyringone-type structures formed during acidolysis as a result of cleavage of *β-O*-4 structures. Acetoguaiacone and acetosyringone are typical reaction products of lignin acidolysis (*32*). Thus, the ratio of abundance of products **C** + **H** and **D** + **K** (Table 4) can give an idea of the molar proportion of intact *β-O*-4 structures and those degraded to Hibbert ketones attached to the lignin network. This value is estimated to be 6:94. Some small proportion of Hibbert ketones is present in the form of monomeric phenolic compounds (acetoguaiacone and acetosyringone), as revealed by the ESIMS analysis.

No degradation products derived from *p*-hydroxyphenyl type structures were found. This feature can be assigned to the low yield of H-type lignin structures in the thioacidolysis, as well as to the possible occurrence of condensation reactions (*26*).

Lignin Analysis by ^{13}C NMR Spectroscopy. The structural composition of eucalypt dioxane lignin has been elucidated following previously described methodology for quantitative ^{13}C NMR spectroscopy (*37*). The quantitative ^{13}C NMR spectrum of eucalypt dioxane lignin is presented in Figure 3. The carbon assignments for lignin structural elements have been previously described (*37*−*41*).

An additional confirmation of signal assignments in principal lignin structures was done based on the HETCOR spectrum (Figure 4). This spectrum was interpreted using established carbon−proton correlations (*41*−*46*). The assignments for carbon−proton cross-peaks in lignin phenylpropane units (C_9) linked by *β-O*-4, *β-β*, and *β*-5 bonds are presented in Table 5. The clear detection of carbon atoms in the side chain of *β*-1 substructures was difficult, because of its low abundance in lignin. For the same reason it was impossible to detect a cross-peak corresponding to the C*γ* atom in the cinnamyl alcohol type structures, despite the existence of a characteristic signal at 61.8 ppm (*38, 45*) in the ^{13}C NMR spectrum (Figure 3).

The quantification of *β-O*-4 bonds in lignin structures without ($0.52/C_6$) and with ($0.04/C_6$) a Cα=O group in the propane chain per aromatic group C_6 (integrated intensity of signals at 103−156 ppm) was done based on C*γ* resonances at 59.3−60.8 and 62.5−63.8 ppm, respectively (Table 6). However, the real abundance of *β-O*-4 structures in in situ eucalypt lignin is higher than $0.56/C_6$ because a portion of them are degraded during the isolation procedure. As estimated on the basis of results of thioacidolysis, approximately 6% of *β-O*-4 bonds are broken during the acidolytic lignin isolation to form the acetoguaiacone and acetosyringone type structures. Thus, the natural abundance of *β-O*-4 bonds in eucalypt lignin is expected to be about $0.59/C_6$. This value is similar to that reported previously for birch MWL (*47*) and much higher than the value reported for *Eucalyptus grandis* MWL (0.47/C9) by Piló-Veloso (*48*).

The amount of *β-β* and *β*-5 bonds per C_6 was calculated by integration of the C*β* resonance at 51.0−53.8 ppm in these structures (Table 6). A clear assignment of ^{13}C NMR signals from *β-β* and *β*-5 structures is difficult. This was achieved by combination of ^{13}C and ^{1}H spectroscopy data and is discussed later.

Only a small quantity of cinnamyl alcohol structures ($0.02/C_6$) was detected in eucalypt lignin (Table 6). This value was calculated based on the C*γ* resonance at 61.8 ppm and is very close to those normally reported for hardwood lignins (*30, 47, 48,*).

Signals for lignin at around 45 ppm in DMSO-d_6 solution (or at around 47 ppm in acetone-d_6 solution) were previously assigned in oleander lignin to the C*β* atom in tetrahydrofuran (THF) type structures (*49*). The spectrum of eucalypt lignin shows two partially resolved

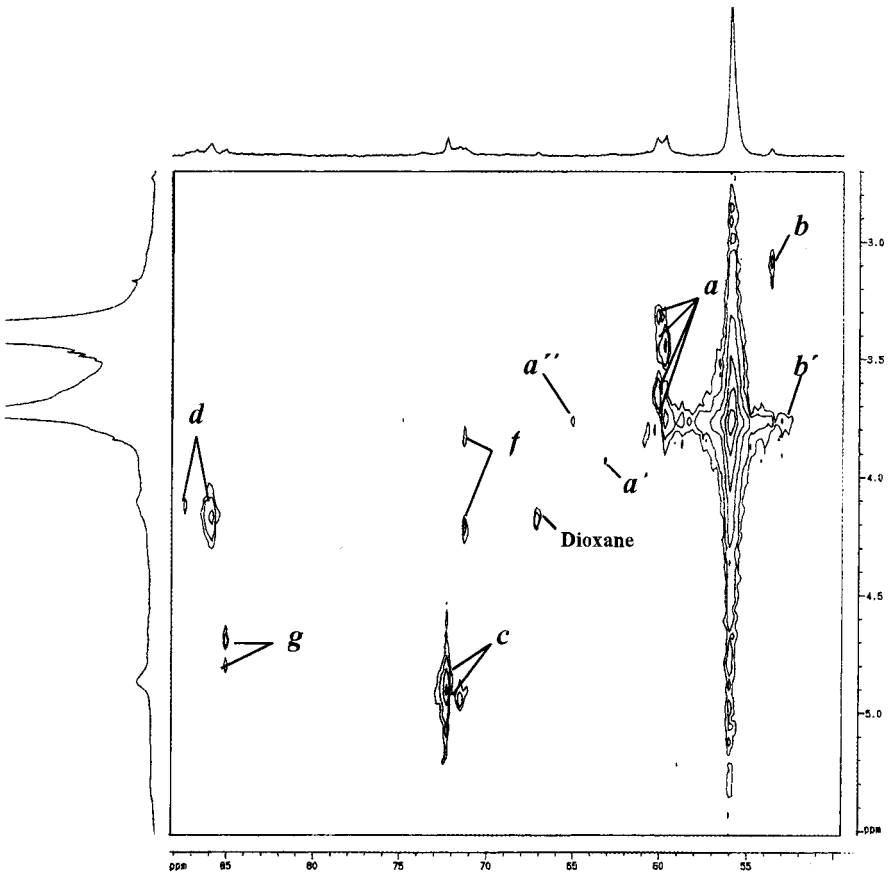


Figure 4. Aliphatic side-chain region of HETCOR spectrum of *E. globulus* dioxane lignin.

Table 5. $^{13}C/^{1}H$ Chemical Shifts in *E. globulus* Dioxane Lignin as Revealed from HETCOR Spectrum (Figure 4)

cross-peak designation	$\delta\ ^{13}C/^{1}H$ in lignin (ppm)	assignments
a	59.3−60.7/3.30−3.75	Cγ-Hγ in β-O-4 structures (*erythro* and *threo*)
a′	63.2/3.98	Cγ-Hγ in β-O-4 structures containing CαO
a″	64.8/3.78	Cγ-Hγ in β-5 structures
b	53.7/3.10	Cβ-Hβ in β-β structures
b′	53.2/3.76	Cβ-Hβ in β-5 structures
c	71.7−72.3/4.92−5.05	Cα-Hα in β-O-4 structures (*erythro* and *threo*)
d	85.9, 87.5/4.11−4.18	Cβ-Hβ in β-O-4 structures (*erythro* and *threo*)
f	71.3/3.85, 4.26	Cγ-Hγ in β-β structures
g	85.0/4.70−4.82	Cα-Hα in β-β structures

Table 6. Quantitative Estimation of Different Structural Elements in *E. globulus* Dioxane Lignin from ^{13}C NMR Spectrum (Figure 3)

structural element	number/aromatic group C_6
β-β + β-5 structures	0.13
isotaxiresinol type structures	0.03
cinnamyl alcohol structures	0.02
β-*O*-4 structures without Cα=O	0.52
β-*O*-4 structures with Cα=O	0.04
Ar-O	2.54
Ar-C	1.29
Ar-H	2.17
S:G:H ratio	84:14:2
CHO in benzaldehyde-type structures	0.02
CHO in cinnamaldehyde-type structures	0.03
CO	0.15

signals at 44.7 and 44.2 ppm (Figure 3). The origin of the resonance at 44.7 ppm, which shows as a negative signal in the ^{13}C DEPT-135 spectrum (see Supporting Information), is not clear. The resonance at 44.2 ppm can be assigned to the CβH group in β-β substructures, because in the ^{13}C DEPT-135 spectrum a positive signal at 44.2 ppm was observed. The resonance at 49.2 ppm, which shows as a positive signal in the ^{13}C DEPT-135 spectrum, can be tentatively assigned to CαH in isotaxiresinol type (α-6 + β-β) structural units according to ^{13}C NMR database for lignin model compounds (*41*). These structures were suggested previously for beech lignin (*34*) and may be formed during the acidolytic isolation procedure (*18*). Signals of Cβ atoms in isotaxiresinol type structures are expected to be at around 44 ppm (*41*), i.e., in the same region as the resonance at 44.2 ppm. Considering that the intensity of signals at 49.2 and 44.2 ppm is similar, they were tentatively assigned to Cα and Cβ atoms respectively in isotaxiresinol type structures. The amount of isotaxiresinol structures was estimated to be about 0.03/C_6 (Table 6).

The region of tertiary aromatic carbons in the ^{13}C NMR spectrum was defined based on the ^{13}C DEPT CH (θ = 135°) spectrum. The number of tertiary aromatic carbons of syringyl propane units (*S*) were estimated in the spectrum region 103−110 ppm and those of guaiacyl propane units (*G*) were estimated in the region 110−123 ppm. The contribution of tertiary aromatic

carbons from *p*-hydroxyphenyl propane (H) units (namely from C-3 and C-5) is negligible because less than 3 mol % of these structural units was detected by degradation techniques. This fact allows the estimation of the S/G ratio based on spectroscopic data [S/G = (integral at 103−110 ppm)/(integral at 110−123 ppm) * 3/2] as previously reported (*50*). The number of H units was estimated from resonances at 160.2 and 162.2 ppm, assigned to C-4 in the corresponding structures (*40*). Thus, the S/G/H ratio obtained was 84/14/2 (Table 6), which is in good agreement with the results obtained by chemical degradation techniques.

The ^{13}C NMR spectrum region at 123−137 ppm was integrated and attributed to quaternary carbons in aromatic nuclei linked with a carbon atom of another substructure (Ar−C). The total amount of Ar−C bonds was estimated to be 1.29/C_6. In other words, 29% of the structural units have more than one Ar−C bond. This value is about twice that estimated based on permanganate oxidation analysis. Such inaccuracy is due to the uncounted contribution of Cα and Cβ atoms in cinnamyl alcohol structures, Cβ in cinnamaldehyde structures, and C-2,6 in H units situated in the region of 123−137 ppm. The correction of the abundance of Ar−C linked structures, based on known amounts of cinnamyl alcohol, cinnamaldehyde, and H-type structures (Table 6), gave a value of 1.18/C_6.

^{13}C NMR spectroscopy gives a sufficiently reliable picture on the distribution of carbonyl groups in lignin (*50*). The amounts of aldehyde groups were estimated by integrating the spectrum region at 191−192 ppm (CHO groups in benzaldehyde type structures) and at 193.5−194.5 ppm (CHO groups in cinnamaldehyde type structures). The semiquantitative estimation of ketone groups was based on the integral at 195−210 ppm. Results on the quantification of different carbonyl groups are shown in Table 6. It is noteworthy that the amount of each particular carbonyl group per C_6 calculated from the ^{13}C NMR spectrum is very similar to that obtained by wet chemistry methods per phenylpropane unit (C_9) (Table 1). The quantity of *β-O*-4 structures containing CαO (0.04/C_6) and the estimated amount of CαO groups at 195−198 ppm (0.06/C_6) are very similar for absolute values. The signals from CO groups in acetoguaiacone/acetosyringone structures (about 0.03/C_6) may also contribute to the spectrum region 195−198 ppm. This means that most of the ketone groups are localized at the Cβ position of the propane chain (0.15 − 0.04 − 0.03 = 0.08/C_6). Apparently, a significant part of these CβO groups was formed during the lignin isolation.

Lignin Analysis by ^{1}H NMR Spectroscopy. ^{1}H NMR spectroscopy was used to confirm quantitative estimations for some structural elements done by ^{13}C NMR and to obtain additional structural information on lignin. All calculations were made per one C_9 using the resonance of methoxyl protons as an internal standard. The ^{1}H NMR spectrum of eucalypt lignin is presented in Figure 1.

The abundance of *β-O*-4 structures was estimated based on the resonance of Hα at 5.80−6.25 ppm (*31*, *42*, *51*). The resonances of Hα in *β*-1 structures may also appear in the same spectral range (*51*). However, because of the low abundance of *β*-1 structures, their contribution can be ignored. The value obtained for the content of *β-O*-4 structures without CαO (0.52/C_9) equals that determined by ^{13}C NMR spectroscopy per C_6 (0.52/C_6). The amount of *β*−*β* structures, 0.10/C_6 (Table 6), was calculated based on the Hβ resonance at 3.00−3.15 ppm (*22*). Assuming that the numbers of structural elements in the lignin calculated per C_6 and C_9 are not so different, the amount of *β*-5 structures can be roughly estimated as the difference between the sum of *β*-*β* and *β*-5 structures determined by ^{13}C NMR (Table 6) and the amount of *β*-*β* structures determined using data of ^{1}H NMR. Such estimation gives a value of 0.03/C_6. Thus, the abundance of *β*-5 structures in dioxane lignin from *E. globulus* wood is two times lower than in MWL (0.06/C_6) from *Betula verrucosa* wood (*47*) and about three times lower than in MWL (0.08/C_6) from *E. grandis* wood (*48*).

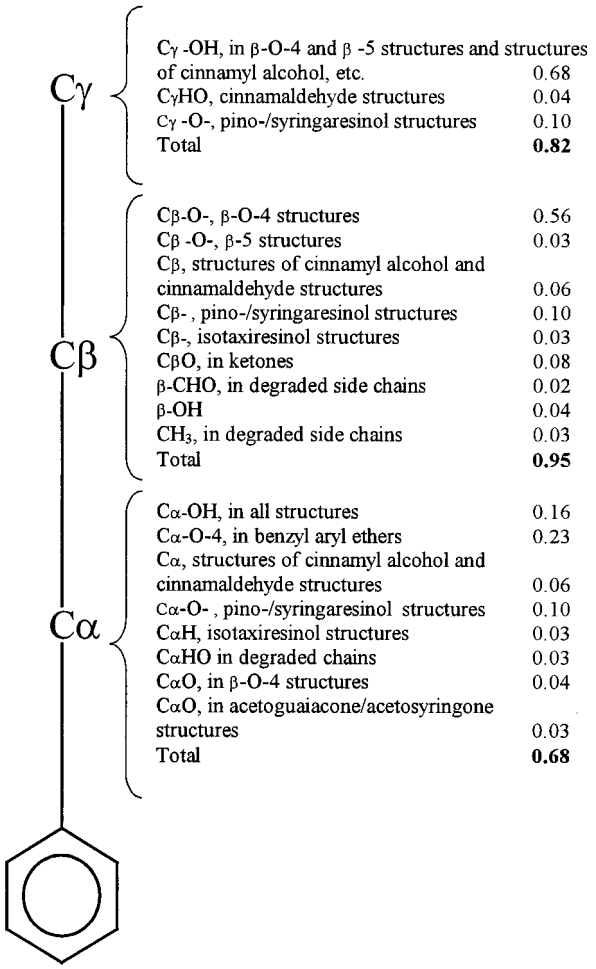


Figure 5. Rydholm diagram illustrating the distribution of functional groups per phenylpropane unit in *E. globulus* dioxane lignin.

As follows from experiments on a synthetic lignin, dehydropolymerizate (DHP), the Hα resonance in noncyclic benzyl aryl ethers is observed in the spectrum region of 5.20−5.50 ppm and observed in *β*-5 structures at 5.30−5.70 ppm (*31*, *43*, *52*). Such an approach allows estimation of the number of benzyl aryl ethers per one C_9, which was about 0.23 (Table 4). Because the number of cyclic α-*O*-4 moieties in phenylcoumaran structures is about 0.03/C_6, as discussed above, the number of noncyclic benzyl aryl ethers should be about 0.20 /C_6 (0.23/C_6 − 0.03/C_6).

The ^{1}H NMR spectrum shows several signals in the range 0.8−1.6 ppm assigned to CH_2 or CH_3 groups in aliphatic chains (total amount of 0.11/C_9). The strong resonance at 1.25 ppm is probably due to CH_2 groups

in the aliphatic moieties. In the HETCOR spectrum (data not shown in Figure 4) the correlation between the carbon resonance at 28.9 ppm and the proton resonance at 1.25 ppm was observed. Moreover, the ^{13}C DEPT CH ($\theta = 135°$) spectrum shows a negative signal at 28.9 ppm, confirming the assignment to methylene groups.

Distribution of Functional Moieties in the Side Chains of Eucalypt Lignin. The total distribution diagram of the functional groups in the side chains of eucalypt dioxane lignin is presented in Figure 5 which summarizes chemical analysis data (Table 1) and structural information obtained by spectroscopic techniques (Table 6). All the results in Figure 5 are presented per C_9. Some of the structural information that was impossible to obtain from chemical analyses and ^1H NMR spectroscopy is from database obtained from ^{13}C NMR spectra, supposing that the number of structural elements per C_9 and C_6 groups should be similar.

The best balance for different structural elements attached to the side chain was achieved in the case of Cβ atom (0.95 substitutions per C_9 are identified from 1.00 theoretically predicted). Thus, the total amount of the remaining nonidentified inter-unit bonds (β-1, β-5, β-6 type structures, etc.) and functional groups at Cβ atom should be rather small. The low amount of identified substitutions at Cγ atom ($0.82/C_9$) can be explained, partially, by the elimination of certain amounts of CγH_2OH groups in the acidolytic isolation procedure (at a minimum $0.05/C_9$, Figure 5). The contribution of nonidentified structures, such as α-*O*-γ in phenylisochroman structures and others, is also possible. The series of signals at around 167 and 171 ppm (roughly about 0.02 carbons per C_6) can be tentatively assigned to γ-COOH groups in cinnamic acid and 1-phenylpropanoic acid type structures, respectively. The series of carbon resonances at 12−26 ppm that correlated with proton signals at 0.5−1.6 ppm in the HETCOR spectrum can be assigned to CγH_3 groups.

The strongest discrepancy was observed at the Cα atom in the propane chain (Figure 5). This fact, at least partially, is due to the poor knowledge about lignin structures containing CαH_2 groups. Part of these signals overlaps at 35−42 ppm with solvent (DMSO-d_6). This spectral region should contain signals from Cα of THF, phenylisochroman, β-1, β-6, and other structures. In addition, it should be noted that eucalypt lignin showed significant amounts (about 10 mol %) of condensed lignin units in the position 6 of the aromatic group (Table 4). Part of these units (almost one-third) were attributed to isotaxiresinol structures. The rest of the condensed phenylpropane units are of unknown structures and may include undetermined substitutions in the Cα atom.

Supporting Information Available: Table containing data on phenolic products obtained from analytic pyrolysis of *E. globulus* dioxane lignin. Figure containing the DEPT CH (θ =135) spectrum of *E. globulus* dioxane lignin. This material is available free of charge via the Internet at http://pubs.acs.org.

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