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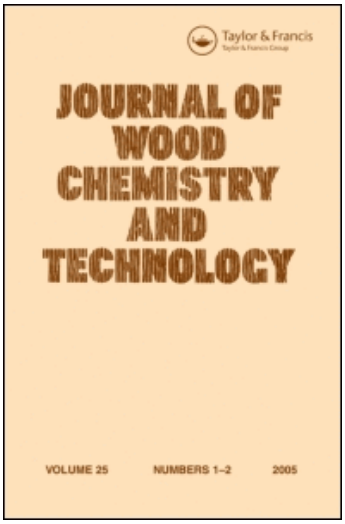

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Chemical Linkage of Pine Polysaccharides to Lignin

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CHEMICAL LINKAGE OF PINE POLYSACCHARIDES TO LIGNIN

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ABSTRACT

Methylation analysis was used to investigate the bonds to lignin of the carbohydrates remaining after enzymatic hydrolysis and alkaline reduction of ball-milled loblolly pine wood and red pine compression wood. The carbohydrates exist as oligomeric chains with degrees of polymerization of 7-14. Approximately one sugar unit per oligomer chain is bonded to lignin. Bonding at C-6 of the hexose units is favored, and the arabinose is bonded exclusively at C-5. Galactan and arabinan are structurally of the 1-4 and 1-5 linked types respectively, characteristic of the so-called "pectic group substances."

INTRODUCTION

The question of the chemical bonds between carbohydrates and lignin in lignocellulosic plant materials is one of the oldest pursued problems of wood chemistry. While at this point there is evidence of more than one bond type, there is no unambiguous proof of any covalent linkage between lignin and carbohydrates in wood.[1,2]

The literature contains few references to applications of polysaccharide structural methods to this problem. Kawamura and Higuchi[3] methylated a xylan-lignin complex and obtained tri-O-methyl-xylose as the only product, implying glycosidic linkage. Freudenberg[4] methylated Bjorkman's lignin and obtained 2,3,6-tri-O-methyl-glucose that he considered to be from a cellulose residue. Neilson

and Richards[5] have recently reported a methylation analysis of a soluble lignin carbohydrate complex isolated from bovine rumen fluids. They concluded that glucose and xylose oligomers were glycosidically linked to lignin since they obtained a preponderance of tri-O-methyl- and tetra-O-methyl-glucose and the corresponding di- and tri-O-methyl-xylose. Ericksson and Lindgren[6] have used mild acid hydrolysis to cleave L-arabinofuranosidic bonds and have concluded that some of the arabinoxylan in spruce is bonded to lignin through the arabinose branch unit. This work was extended to include a "Smith Degradation,"[7] the results of which indicated some lignin bonding at C-2 or C-3 of arabinofuranose as well as ether bonds to each of the hemicellulose sugars.

The present work applies a methylation analysis to the polysaccharide residues still attached to lignin after treatment of ball-milled pine wood with a mixture of cellulolytic enzymes.[8] In this procedure glycosidic bonds to lignin have very likely been hydrolyzed by the enzymes. Ester bonds and possibly ether bonds to the α carbon of free phenolic groups in lignin have been cleaved by a preliminary alkaline borohydride reduction of carbohydrate-reducing end groups. Any carbohydrate linked to lignin with bonds resistant to strong acid hydrolysis, such as carbon to carbon bonds, would not be detected in this analysis.

Interpretation of the results is dependent on detailed knowledge of the structures of the hemicelluloses present in pine. The basic source of this information was the structural data presented by Timell.[9] That review covered the literature to 1964 and no dramatically different structures have been reported since that time.

An end group analysis was performed by deuterium labeling, and the results were used in combination with the methylation analysis to piece together the structures of the polysaccharides and determine the location of their bonds to lignin.

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RESULTS AND DISCUSSION

End Group Analysis

A loblolly pine milled wood enzyme lignin (MWEL) containing 12% residual carbohydrate anhydrides was reduced by sodium borohydride in 0.1M NaOH and reprecipitated. The recovery of reduced MWEL was 90%. This procedure cleaves some alkali sensitive bonds,[8] but the alkali-stable lignin-carbohydrate bonds of prime significance in pulping and bleaching processes are retained. Sugar analyses for the product are presented in Table 1, along with those for a similarly treated MWEL from red pine compression wood. The difference between

TABLE 1
Carbohydrate Composition of Reduced MWEL

	Reducing sugars, paper chromatography		Alditol acetates, gas chromatography	
	% of MWEL as free sugars	Total sugars	% of MWEL as free sugars	Total sugars
	%	Mole %	%	Mole %
Loblolly pine				
Glucose	4.42	33.2	5.42	35.8
Mannose	2.79	21.0	2.92	19.3
Xylose	1.63	14.8	2.25	17.8
Galactose	3.14	23.6	2.99	19.7
Arabinose	0.83	7.4	0.93	7.4
Total	12.81	100.0	14.51	100.0
Red pine compression wood				
Glucose	1.46	11.0	2.31	16.5
Mannose	0.70	5.3	0.76	5.4
Xylose	0.63	5.7	0.57	4.8
Galactose	9.97	75.0	9.96	70.9
Arabinose	0.33	3.0	0.28	2.4
Total	13.09	100.0	13.88	100.0

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the paper chromatographic reducing sugar analysis and the alditol acetate analysis would theoretically be due to reducing end groups initially present in the MWEL and would indicate the apparent chain length for each sugar (assuming no branching or substitution). However, the difference is of the order of magnitude of the reproducibility coefficient of variation for the alditol acetate procedure. Indeed the alditol value for galactose was slightly smaller than the reducing sugar value. A deuterium labeling experiment was performed in aniticipation of obtaining better values for the apparent degree of polymerization ($\overline{DP}$).

The carbohydrate chain length in the alkali-reduced fraction was determined by hydrolyzing the borohydride reduced MWEL followed by conversion of the free sugars to deuterated alditols with sodium borodeuteride. The extent of deuteration was determined by gas chromatography-mass spectrometry of the alditol acetates. The results were compared with fully deuterated standards prepared by reducing a mixture of the five sugars with the same lot of sodium borodeuteride (Table 2). In most cases the same mass fragment can arise from either end of an undeuterated alditol molecule. Thus, after correction for the isotopic abundance of C^{13} the deuterium content is approximately 50% of the total for a given fragment, and a series of fragment pairs separated by one mass unit appear in the spectrum. The fragmentation pattern of alditol acetates has been elucidated[10] and an attempt was made to select fragment pairs with an unambiguous source that were not isomeric with other fragments. The percent of undeuterated alditols is proportional to the number of free reducing end groups present in the carbohydrates remaining in the alkali-stable MWEL fraction. The mean apparent $\overline{DP}$ is an indication of the ratio of nonreducing sugar to reducing sugar present.

It is generally considered that the mass spectra of stereoisomeric alditol acetates are nearly identical[10] and for identification purposes this is true. However, there were marked differences in the deuterium content of certain fragments, e.g., 289-290 of glucitol compared with that from mannitol or galactitol. There

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TABLE 2
Mean Apparent Degree of Polymerization ($\overline{DP}$) of Residual Carbohydrates by Deuterium Labeling

Alditol	Fragment pairs mass No.	Deuterated standards[1/]	Loblolly pine MWEL alditol acetates	Undeuterated	Apparent $\overline{DP}$	Mean apparent $\overline{DP}$
		% D	% D	%		
Mannitol	361-2	50.0	40.6	18.8	5.3	
	289-90	46.9	39.7	15.4	6.5	
	217-8	46.3	39.5	14.6	6.8	
	187-8	49.2	40.7	17.3	5.8	
	175-6	47.5	42.0	11.6	8.6	7
Glucitol	361-2	50.8	47.5	6.5	15.4	
	289-90	30.3	27.3	9.9	10.1	
	217-8	47.5	42.0	11.6	8.6	
	187-8	32.0	30.1	5.9	17.0	
	175-6	46.2	39.8	13.8	7.2	12
Galactitol	361-2	49.6	46.2	6.8	14.7	
	289-90	48.4	43.4	10.2	9.8	
	217-8	48.1	43.7	9.2	10.9	
	187-8	47.3	44.8	5.3	18.9	14
Xylitol	289-90	48.7	42.8	12.1	8.3	
	217-8	46.5	41.7	10.4	9.6	
	187-8	48.2	41.8	13.3	7.5	
	127-8	42.0	36.3	13.6	7.4	8
Arabinitol	289-90	67.7	70.6	[2/] --		
	217-8	42.3	40.1	5.2	19.2	
	187-8	53.9	49.0	9.1	11.0	
	127-8	53.2	48.8	8.3	12.0	14

1/ Corrected higher mass intensity divided by total for the pair.

2/ See discussion section "End Group Analysis."

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was also considerable difference in the agreement of the results from different fragment pairs depending on the stereoisomer. The fragments from mannitol were in closest agreement with each other. There does seem to be a trend in deviations in that certain fragment pairs, e.g., the apparent $\overline{DP}$ from 187-188 in glucitol and galactitol, give values higher than the mean. In one pair (289-290) the deuterium substitution in arabinitol from loblolly pine MWEL was greater than that from the standards. The reasons for these stereochemically related differences include the appearance of interfering fragments and will have to be investigated for each individual alditol to improve the general utility of the method for end group analysis.

The results for arabinitol could indicate that there are no reducing arabinose end units in the alkali-stable MWEL. However, all five alditols including arabinitol and galactitol were obtained directly from reduced loblolly pine MWEL hydrolyzates after removal of free sugars by oxidation and ion exchange in a manner similar to that reported by Ericksson and Lindgren[6] with reduced spruce MWEL.

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Methylation Analysis

The reduced MWEL was subjected to a methylation analysis as described by Jansson et al.[11] The sample was not completely soluble in DMSO, and three methylations by the Hakomori procedure[12] (dimsyl anion/methyl iodide) were required to give a product whose infrared absorption did not change upon further methylation. Methylated uronic acids undergo β-elimination[11] and are not analyzed by this procedure. Any lignin-uronic acid ester bonds that may have been present initially were presumed to have been saponified by the initial treatment with alkali.

The methylation reaction products were purified by dialysis. The methylated lignin-carbohydrate product precipitated when the reaction mixture was poured into water; however, dissolution and loss through the membrane increased with the number of methylations (possible due to lignin depolymerization and increased water solubilization of the oligosaccharides), and the final recovery

was about 50% of the starting material. The methylated product was enriched in carbohydrate (to 23%); however, the ratios of the individual sugars were similar to that in the starting material. In other experiments, attempted isolation and purification of reaction products by extraction with methylene chloride led to greater fractionation. The gas chromatographable products obtained after hydrolysis, reduction, and acetylation were almost exclusively of carbohydrate origin. The results grouped by individual sugars are given in Table 3.

The preponderance of 2,3,6-tri-O-methyl-hexitols and 2,3-di-O-methyl-xylitol indicates that a majority of the sugar units remaining after enzyme treatment are not bonded to lignin. The quantity of nonreducing end groups (tetra-O-methyl-hexitols and tri-O-methyl-pentitols) corresponds to the quantity of reducing end units determined by the deuterium analysis after allowance for terminal branching units. The penta-O-methyl-hexitol acetates from the reducing end units are highly volatile, and, although detected in the methylation analysis, they were not quantitatively separated or estimated, as discussed later. The overall quantities and ratios of the methylated sugars correspond to the alditol acetate analysis (Table 1). An interesting result is the presence of major quantities of 2,3-di-O-methyl-arabinose and 2,3,6-tri-O-methyl-galactose and the absence of 2,4,6-tri-O-methyl-galactose. This implies that the arabinan and galactan associated with lignin are from the so-called "pectic group substances." This is quite reasonable since Meier[13] found such substances were located in the primary wall of developing tracheids, and they would be expected to be intimately associated with middle lamella lignin. Also, compression wood galactan is the only other known 1→4 linked galactan in pine, and compression wood is highly lignified. The appearance of these hemicelluloses is not due to the inability of the enzyme to hydrolyze these links. In preparing a similar enzyme lignin, J. M. Harkin (unpublished results, FPL, 1970) tested the digestibility with pectinase and found that no additional sugars were released. The third member

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TABLE 3
Methylation Products From Residual Polysaccharides in Reduced Loblolly Pine MWEL By Gas Chromatography

Partially methylated alditol acetates	ECNSS-M			SP-1000	
	t_{rel}	Mole %	t_{rel} (Ref. 11)	t_{rel}	t_{rel} (Ref. 11)
Mannitol					
2,3,4,6-tetra-O-me	1.00	1a/ (2.7)	1.00	1.00	1.00
2,3,6-tri-O-me	2.21	11.4	2.20	1.72	1.79
2,6-di-O-me	3.75	0.2	3.35	2.55	2.65
3,6-di-O-me	4.28	0.1	4.15	2.90	2.96
2,3-di-O-me	4.69	2.6	4.38	3.23	3.29
Glucitol					
2,3,4,6-tetra-O-me	1.00	1a/ (3.0)	1.00	1.00	1.00
2,4,6-tri-O-me	1.98	0.4	1.95	1.72	1.72
2,3,6-tri-O-me	2.58	1b/ (24.8)	2.50	1.89	1.94
3,6-di-O-me	4.28	1b/ (0.1)	4.40	2.90	2.94
2,3-di-O-me	5.39	2.1	5.39	3.50	3.50
Glucitol hexaacetate	13.00	2.9	3/--	7.69	3/--
Galactitol					
2,3,4,6-tetra-O-me	1.24	3.2	1.25	1.14	1.14
2,4,6-tri-O-me	2/--		2.28	2/--	1.94
2,3,6-tri-O-me	2.5	1b/ (13.8)	2.42	1.83	1.86
2,3,4-tri-O-me	3.42	0.9	3.41	2.37	2.42
3,6-di-O-me	(4.28)	1b/ (0.1)	4.35	3.02	3/--
2,3-di-O-me	5.77	2.1	5.68	3.74	3.66
2,4-di-O-me	6.50	1.2	6:35	4.31	4.19
Xylitol					
2,3,4-tri-O-me	0.65	1.8	0.68	0.76	0.67
2,3-di-O-me	1.53	8.6	1.54	1.24	1.23
2-O-me	2.97	1c/ (2.4)	2.92	2.04	2.04
3-O-me	2.97	1c/ (.9)	2.92	2.04	2.02
Arabinitol					
2,3,5-tri-O-me	0.46	1.7	0.48	0.66	0.54
2,3,4-tri-O-me	0.82	0.1	0.73	2/--	3/--
2,3-di-O-me	1.33	2.8	3/--	1.16	1.15
Others					
1,2(3),4-tri-O-me tetratol				0.47	3/--
Glycerol triacetate				0.51	3/--
1,2,3,5,6-penta-O-me hexitol				0.59	3/--
1,2,3,5,6-penta-O-me hexitol				0.61	3/--

1/ Area values in parentheses are overlapping peaks whose individual areas were measured by other methods: (a) Reducing group analysis, (b) other gas chromatography, and (c) mass spectrometry.
2/ Not observed.
3/ Not reported in literature.

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of the pectic group, pectin itself, may be linked to lignin through the uronic acid substituents and thus lost by saponification.

The large quantity of 1→4 linked glucose is also interesting. The amount is far in excess of that required by pine glucomannan. This implies a 1→4 glucan such as starch or cellulose. Starch has been found in sapwood epithelial cells. Although in the past lignin has been considered most likely to be bonded to hemicelluloses, covalent bonds to cellulose are a real possibility; the present analysis cannot distinguish between cellulose and starch. Another unexpected finding was the appearance of glucitol hexaacetate and only the glucitol hexaacetate as about 3% of the total sugar quantity. This observation was confirmed repeatedly from various samples. While this could imply bonding at carbons 2, 3, and 6, a more reasonable interpretation is that these are occluded or microcrystalline fragments of cellulose that are inaccessible to the enzyme and to the methylating reagents. The association with lignin may be chemical or purely physical.

Several products were detected in small amounts in the early portion of the chromatogram. These products, largely emanating from reducing end units and identified by mass spectrometry, would be very volatile, and their recovery is not quantitative under the conditions used in this analysis. The 4-O-acetyl-1,2,3,5,6-penta-O-methyl-hexitols arise from the reducing end of 1→4 linked hexosans. The retention time at t_{rel} 0.61 is the same as that observed for the corresponding glucitol derivative. The 1,2(3),4-tri-O-methyl-tetratol evidently exists as a reducing end group. It is not likely to be an artifact of either the enzymatic or chemical reactions but may have been created during ball-milling. Both possible isomeric tetratols were detected after hydrolysis of reduced milled wood enzyme lignin and conversion of all sugars to alditol acetates. Glycerol triacetate may arise from a carbohydrate or lignin fragment.

Hemicellulose Structure

Assuming that the structures of the hemicelluloses are the

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same as those previously characterized from delignified pine holocelluloses, the results for each of the wood sugars can be combined and the hemicellulose structure assembled (Table 4). All values are given as percent of total methylated sugars, so the extent of bonding per oligosaccharide is obtained by dividing the amount of substituted sugar by the total given for the polymer. For example, if all of the mannose comes from galactoglucomannan, the backbone $\overline{DP}$ is 7. If the ratio of mannose to glucose is the reported 2.7:1[14] and the galactose (3.7% of the hemicellulose)[9] is proportionately

TABLE 4
Hemicellulose Structures from the Methylation Analysis of Loblolly Pine MWEL

	Substitution			Composition	Degree of polymerization
	By galactose	By arabinose	By lignin		
				Mole % of total sugars	
Galactoglucomannan				19.7 mannose	7
1 → 4 glucose-mannose backbone				7.2 glucose	
1 → 6 branching by terminal galactose units				0.9 galactose	
Substitution at C-6 of					
Mannose	1/ 0.66	2/ --	1.9		
Glucose	0.24	--	0.76		
Substitution at C-6 of galactose	--	--	Possible		
Substitution at C-2 and C-3 of mannose and glucose	--	--	0.4		
Glucan					
1 → 4 glucose				29.1 glucose	13
Substitution at C-6	--	--	1.2		
Galactan					
1 → 4 galactose				20.1 galactose	14
Substitution at C-6	Possible	--	1.2		
Substitution at C-2	--	--	0.1		
Arabinogalactan				2.9 galactose	?
1 → 3, 1 → 6 galactose				0.3 arabinose	
1 → 6 branches of arabinose or 1 → 5 arabinose					
Substitution of galactose at C-6	0.9	0.3	0.3		
Substitution of side chain galactose	--	--	0.9		
Substitution of side chain arabinose	--	--	Likely		
Arabinoxylan					
1 → 4 xylose				15.6 xylose	8
1 → 3 branching by terminal arabinose				1.5 arabinose	
Substitution of xylose at C-3	--	1.4	1.0		
Substitution of xylose at C-2	--	--	0.9		
Arabinan				3.2 arabinose	(8)
1 → 5 arabinose					
Substitution at C-5	--	--	0.4		

1/ All values are % of total sugars.
2/ -- No substitution.

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distributed at C-6 of the backbone units, the excess 2,3-di-O-methyl-mannose is bonded to lignin through C-6. A proportionate amount of lignin bonding can be assigned to 2,3-di-O-methyl-glucose. Trace amounts of substitution at C-2 and C-3 were observed. The galactose side chain may be unsubstituted (2,3,4,6-tetra-O-methyl) or substituted at C-6 by lignin (2,3,4-tri-O-methyl).

Excess glucose is assigned to glucan. The proportionate amount from the deuterium analysis indicates a $\overline{DP}$ of 13. The quantity of nonreducing end is consistent with this finding. The glucitol hexaacetate is assumed to arise from center chain units. Thus, it appears, but is not conclusively shown, that lignin is bonded to some cellulose fragments. The majority of galactose comes from a 1→4 linked galactan. The bonding is almost exclusively at the C-6 position with a trace detected at C-2. The smaller amount of arabinogalactan has a substitution pattern characteristic of that polymer. However, there does seem to be an excess of C-6 substitution that is assigned to lignin. Lignin bonding to the galactose and arabinose side chains at C-6 and C-5, respectively, is likely.

The xylan polymer has more C-3 substitution than normal for a softwood arabinoxylan, and there is more 2-O-methyl-xylose than terminal arabinose units. Therefore, lignin must be bonded to the C-3 position of xylopyranose or the C-5 position of the arabinofuranose substituent or both.

It is not necessary to invoke an arabinan structure to explain the methylation results providing the reducing units are explained as monomers linked C-5 to lignin; however, the amount of arabinose and the preponderance of "pectic group type" galactan and the methylation results are consistent with the 1→5 linked arabinan chain. Only C-5 substitution was detected, although total absence of other substitution has not been rigorously proven.

There are other possible interpretations of the results, but the analysis presented is consistent with the present data and information in the literature. For the most part, at least one bond to lignin per oligomer can be rationalized; and with the

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exception of arabinose, the bonding sites to carbohydrates are mixed. The major exception to one bond per chain is the glucan. The origin of the glucitol hexaacetate is not clear. Presumably it is from microcrystalline or otherwise inaccessible cellulose. If these are considered complete chains, the $\overline{DP}$ of the methylated glucan is decreased to more closely approximate one substituent per chain. Another possible explanation would be a bond to lignin that was not hydrolyzed by acid. A second, extended hydrolysis failed to release any further sugar, but the possibility is being tested further. Some less likely interpretations of the overall results include lignin units dispersed between 1→4 links and the possibility that the associated polymers are of unique structures not yet characterized from loblolly pine.

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Compression Wood

Red pine compression wood enzyme lignin was reduced and methylated in the same manner as the loblolly pine. This material contained 16% carbohydrates and was highly enriched in galactose (70% of the total sugars). The results (Table 5) fully support the observations with loblolly pine enzyme lignin. The galactan is of the 1→4 linked type, and the majority of the units are unbonded. The average $\overline{DP}$ is 13 (as it was in loblolly pine MWEL) and the partially methylated units that indicate bonding to lignin amount to about one per chain. The preponderance of the 2,3-di-O-methyl derivative indicates lignin bonding is almost exclusively at C-6. The presence of 2,3,4-tri-O-methyl- and 2,4-di-O-methyl-galactitol indicates that some 1→3,1→6 linked arabinogalactan is also present.

The appearance of glucitol hexaacetate and no galactitol hexaacetate supports the presumption that its source is inaccessible cellulose and not a general, incomplete methylation.

Summary

Although the assumptions necessitated by the complexity of the hemicellulosic structures preclude a definitive interpretation, the major conclusions are convincingly supported. The sugar units

TABLE 5
Methylated Sugars From Reduced Red Pine Compression Wood MWEL By Gas Chromatography[1/]

Partially methylated alditol acetate	ECNSS-M		SP-1000
	t_{rel}	Mole % of total carbohydrate	t_{rel}
Mannitol			
2,3,4,6-tetra-O-me	1.00	[2a/] (0.8)	1.00
2,3,6-tri-O-me	2.18	2.0	1.88
2,6-di-O-me	3.56	[2b/] (0.3)	2.71
3,6-di-O-me	4.26	0.5	3.04
2,3-di-O-me	4.67	0.5	3.21
Glucitol			
2,3,4,6-tetra-O-me	1.00	[2a/] (0.9)	1.00
2,4,6-tri-O-me	1.93	0.7	1.63
2,3,6-tri-O-me	2.5	[2b/] (10)	1.97
2,3-di-O-me	5.39	0.4	3.50
Glucitol hexaacetate	13.30	2.2	8.19
Galactitol			
2,3,4,6-tetra-O-me	1.24	5.7	1.14
2,3,6-tri-O-me	2.42	[2b/] 62	1.94
2,3,4-tri-O-me	3.42	0.5	2.38
2,6-di-O-me	3.56	[2b/] (0.6)	2.71
2,3-di-O-me	5.82	5.5	3.80
2,4-di-O-me	6.35	0.4	4.29
Xylitol			
2,3,4-tri-O-me	0.67	0.7	0.76
2,3-di-O-me	1.49	1.8	1.24
2-O-me	2.87	[2c/] [2c/] (0.75)	2.07
3-O-me	2.87	[2c/] (0.25)	2.07
Arabinitol			
2,3,5-tri-O-me	0.49	0.4	[3/] --
2,3-di-O-me	1.24	[2b/] (1)	[4/] --

1/ See reference values of Table 3.

2/ Area values in parentheses are overlapping peaks whose individual areas were measured by other methods: (a) reducing group analysis, (b) other gas chromatography, and (c) mass spectrometry.

3/ Eluting with solvent peak.

4/ Overlaps 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl galactitol.

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remaining attached to lignin after methylation of alkaline-borohydride-reduced loblolly pine MWEL exist as fragments of known pine polysaccharide chain structures. Additional bonded units that were assigned to lignin were detected on the magnitude of one per chain. The major lignin bonding involves the primary hydroxyl positions although occasional random bonding to secondary hydroxyl positions is indicated. Galactose and arabinose linkages were of a nature characteristic of pectic group or compression wood galactans and arabinans. The results with compression wood ball-milled enzyme lignin support each of the above conclusions.

EXPERIMENTAL

The preparation of loblolly pine milled wood enzyme lignin has been described by Obst.[8] A 1-gm quantity was reduced with 100 mg of $NaBH_4$ in 20 ml of 0.1M NaOH at room temperature for 3 hours. The reduced lignin was precipitated with acetic acid to pH 4.3, centrifuged, washed with dilute acetic acid, and freeze dried.

The reduced enzyme lignin (100 mg) was subjected to a methylation analysis as described by Jansson et al.[11] Three applications of the Hakomori[12] method were required to give a product whose infrared spectra (KBr) was not changed by further methylation. After each methylation the product was isolated by dialysis in Spectrapor 6 tubing with a nominal 1,000 MW cutoff. After the first methylation, recovery was nearly quantitative, but losses occurred upon subsequent methylations and the final yield was 52 mg. Myo-inositol (0.3 mg) was added as an internal standard, and 36.2 mg of methylated product were hydrolyzed with 1.0 ml of 72% H_2SO_4 followed by dilution to 3% and secondary hydrolysis at 120° C for 1 hour. The partially methylated sugars were then converted to the alditol acetates and analyzed. The internal standard area was 4% of the total sugar area.

Gas chromatography was performed at 170° C on a 9-ft nickel 1/8-in. OD column packed with 5% ECNSS-M on Gas Chrom Q, 60/100 mesh. Gas chromatography-mass spectrometry was performed by Raltech

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Scientific Services, Inc., Madison, Wis., with a 20M SP-1000 glass capillary WCOT column in a Finnigan 4021-T instrument at 180° and 200° C. Relative retention times were interpolated between 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol and 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-glucitol and compared with literature values. A relative response factor of 1.0 was assumed for detection of the partially methylated alditol acetates with flame ionization.[15]

Sugar analyses were performed by the paper chromatographic method of Saeman et al.[16] and by gas chromatography of the alditol acetates on the ECNSS-M column using myo-inositol hexaacetate as the internal standard.

Reducing end groups were estimated by hydrolyzing the $NaBH_4$ reduced enzyme lignin and converting the reducing sugars to alditols with $NaBD_4$. The deuterium content of the individual alditols was determined by gas chromatography-mass spectrometry of the alditol acetates on a 6 ft by 2 mm ID glass column of 3% OV225 on chromosorb WHP 100/200 mesh at 190° C. Analyses were performed by Raltech.

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