

Oxidative Degradation of Wood. 6. Identification of Carboxylic Aromatic Products of Alkaline Nitrobenzene Oxidation

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Further development of a sequential method for fractionation of alkaline oxidation products is described. The method, as applied to alkaline nitrobenzene oxidation products of protolignin and lignin isolated from kraft black liquor, gives five fractions. Neutrals are first extracted from the alkaline nitrobenzene oxidation mixture. After dimethyl sulfate methylation of phenolic and alcoholic hydroxyl groups, the methyl ethers are separated into carboxy-containing and carboxy-free fractions. These fractions are separated by formation of bisulfite addition compounds and subsequent extraction into aldehyde-containing and aldehyde-free fractions. The two fractions of methyl ethers containing carboxyl groups are subjected to methylation using diazomethane. The resulting methyl ether-methyl esters are separated into individual components and characterized using gas-liquid chromatography. Sufficient amounts of the components were isolated by preparative gas-liquid chromatography for characterization by IR, mass, and NMR spectrometry. Four compounds are reported: vanilloylformic acid, α -hydroxyferulic acid, isohemipinic acid, and metahemipinic acid, which have not been reported by other laboratories as alkaline nitrobenzene oxidation products of lignin. In addition, three acids which are expected, vanillic acid, 5-carboxyvanillin, and dehydrodivanillic acid, were isolated and identified. Aspects concerning the significance of the formation of these products are discussed relative to lignin structure.

In previous communications from this laboratory the use of alkaline nitrobenzene oxidation in the analysis of lignin has been reported. Bicho et al. (1962) described a sequential methylation-extraction procedure for fractionation of and a gas-liquid chromatographic (GLC) system for isolation and analysis of reaction products. Brink et al. (1966) used this procedure and analytical system to characterize the oxidation products that were isolated using oxygen and nitric acid oxidation of protolignin as well as alkaline nitrobenzene oxidation. Brink et al. (1972) described a modified sequential methylation-extraction procedure and method for fractionation of oxidation products. Naveau et al. (1972) applied the modified method in the identification of oxidative products containing carboxy-free aromatic compounds and discussed their formation from protolignin.

In the study reported in this paper, the modified sequential methylation-extraction procedure noted above was applied to the carboxy-containing aromatic compounds (Wu, 1970). The method of sequential separation involves extraction of the aqueous alkaline nitrobenzene reaction mixture with ethyl ether as a first step to remove any neutral components. Components remaining in the ether-extracted, alkaline solution are then subjected to methylation using dimethyl sulfate and sodium hydroxide. Special precautions, described previously (Brink et al., 1972), are taken to maintain pH in the range of 10-11 during the methylation step in order to avoid the formation of artifacts. Groups etherified include phenolic and aliphatic hydroxyls present on moieties produced by the alkaline degradation of lignin. The phenoxy moieties present upon alkaline degradation of softwood lignin are mostly of the guaiacyl type. While still in the aqueous alkaline medium, carboxylate-free methylated ethers are separated from sodium methyl ether carboxylates by extraction with ethyl ether. The extracted alkaline solution is then acidified and again extracted in order to recover

the methyl ether-carboxylic acids. The latter are esterified with diazomethane to form the methyl ether-methyl esters.

The methyl ether-methyl esters are separated by extraction with aqueous sodium bisulfite into carbonyl-containing compounds forming bisulfite addition products and carbonyl-free plus carbonyl-containing compounds not forming bisulfite addition products. This sequential method of fractionation greatly simplified the gas chromatographic analysis. Of nine compounds isolated by preparative GLC from the methyl ether-methyl ester fractions, seven were identified by analytical GLC and spectrometric methods. These compounds, isolated as their methylated derivatives, included three which are expected lignin degradation products. An additional four of these products have not been previously reported from alkaline nitrobenzene oxidation of wood or lignin, except for incomplete evidence we presented on three of these on a preliminary basis (Brink et al., 1972). The other two compounds proved to be secondary reaction products produced in the diazomethane methylation step. Finally, a highly colored compound of unknown structure, presumably produced from nitrobenzene, was found but not further characterized. Possible oxidation pathways to account for the formation of these products will be discussed in a later publication (Wu and Brink, 1977).

RESULTS AND DISCUSSION

Methyl ether-methyl esters from lignin oxidation products were isolated by the preparative GLC technique noted above. Table I lists the identified compounds, their relative retention times, and their yields. The procedure for sequential methylations, bisulfite addition compound formation, and fractionations is shown in Figure 1.

Seven of the compounds isolated have been identified by use of one or more spectrometric methods in conjunction with preparative and analytical GLC. Two additional compounds have been isolated and shown to be artifacts produced in the diazomethane methylation of veratroylformic acid. Table II lists the IR absorption spectra in wave numbers for the isolated compounds. Table III gives some of the results of mass spectrometry.

Compounds I, II, IV, VI, IX were identified on the basis of IR spectra and GLC retention times in reference to

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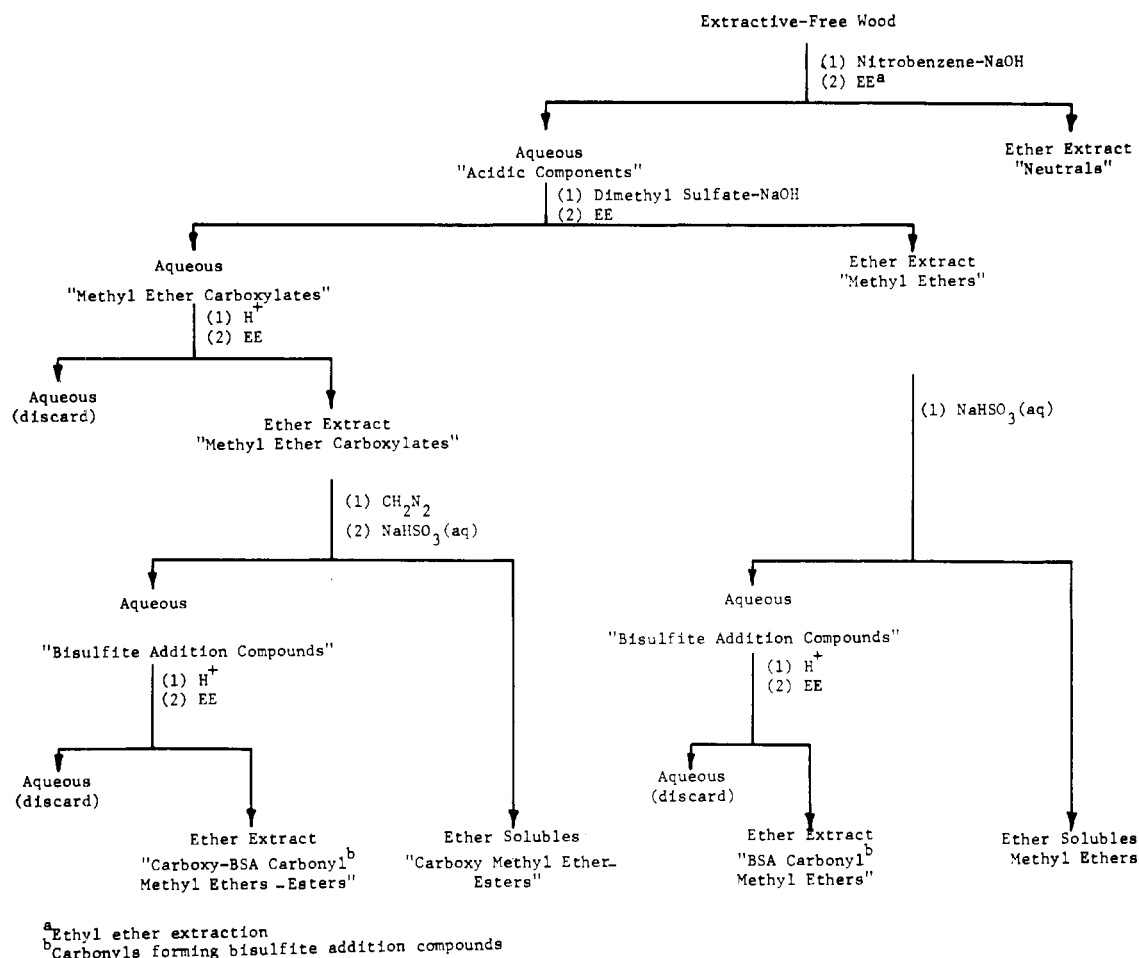


Figure 1. Reaction-extraction sequence for separation of alkaline nitrobenzene oxidation products of lignin.

Table I. Products Obtained by Sequential Methylation of Carboxy-Containing Aromatic Oxidation Products of White Fir Wood^a

		t_R^b	Yield, %	
			A ^c	B ^d
I	Methyl vertrate	1.000	3.78	3.24
II	5-Carbomethoxy-veratraldehyde	1.439	0.80	0.70
III	Methyl veratroylformate	1.612	3.05	2.67
				(5.53) ^e
IV	Dimethyl isohemipinate	1.735	0.60	0.50
V	Artifact of III ^d	1.826	3.06	2.38
VI	Dimethyl metahemipinate	1.918	0.66	0.55
VII	Methyl α ,3,4-trimethoxycinnamate	1.949	0.36	0.30
VIII	Artifact of III ^d	2.071	0.65	0.48
IX	Dimethyl dehydrodivertrate	4.888	0.43	0.37
	Total		11.19	

^a Yields based upon semiquantitative estimation from DC-550 column. ^b Relative retention time; methyl vertrate, 9.8 min elution time. ^c Fully methylated derivative of lignin oxidation product, Klason lignin basis. ^d Calculated as product formed in alkaline nitrobenzene oxidation, Klason lignin basis. ^e Summation of vanilloylformic acid in III + V + VIII.

corresponding authentic compounds. Three of these compounds, methyl vertrate (I), 5-carbomethoxy-veratraldehyde (II), and dimethyl dehydrodivertrate (IX), are the methylated derivatives of vanillic acid, 5-carboxyvanillin, and dehydrodivanillic acid, respectively. These phenolic acids have been reported in studies of

alkaline nitrobenzene oxidation of wood. The other two compounds identified as dimethyl isohemipinate (IV) and dimethyl metahemipinate (VI) are the methylated derivatives of 5-carboxyvanillic acid and 6-carboxyvanillic acid, respectively. Both of these substituted vanillic acids are known oxidation products of lignin using other than alkaline nitrobenzene.

Compounds III and VII were characterized by GLC and IR, NMR, and mass spectrometry. These products, identified as methyl veratroylformate (III), and methyl 2-methoxy-3-(3,4-dimethoxyphenyl)propenoate (VII) (methyl α ,3,4-trimethoxycinnamate), are the methylated derivatives of vanilloylformic acid and α -hydroxyferulic acid, respectively. In this paper the conventional lignin notation is used in which the carbon adjacent to the aromatic ring is designated as the α carbon (Sarkanen and Ludwig, 1971) only when referring to structures in lignin moieties.

Compound VII was assigned a *trans*-cinnamic acid type structure based on the analytical results noted above as well as on its synthesis from *trans*-ferulic acid (VIIa) by the procedure shown in Figure 2. This compound was also verified by an alternative synthetic method described in the experimental section.

The seven primary products discussed above and shown in Figure 3 are the methylated derivatives of corresponding phenolic acids formed as their respective sodium salts in the alkaline nitrobenzene oxidation of white fir (*Abies concolor*) protolignin and kraft lignin. It is particularly significant that for the first time four of these products, vanilloylformic acid, 5-carboxyvanillic acid, 6-carboxyvanillic acid, and α -hydroxyferulic acid, isolated as the

Table II. IR Absorption Spectra (cm⁻¹) of the Compounds Isolated from Alkaline Nitrobenzene Oxidation of White Fir Wood and from Syntheses

Products isolated ^a	I	II	III	IV	V	VI	VII	VIIId	VIII	IX
Aromatic carboxylate	1710	1705		1718		1710				1720
Aromatic ether	1280	1260		1270		1270				1270
Aromatic ring	1233		1237	1235	1235	1250		1232	1235	1230
Aromatic aldehyde	1015	1000	1020	1060	1025	1024	1025	1020	1028	1020
	1586	1590	1585	1590	1587	1597	1597	1588	1590	1595
	1507		1507	1505	1508	1497	1510	1506	1510	1510
		1695								
		1386								
		1300								
		1200								
Aromatic ketone			1653							
			1281							
Aliphatic carboxylate			1726		1733		1755	1737	1733	
Aliphatic ether			1260		1260		1270	1257	1260	
Methylene							1097	1097		
					2920			2940	2930	
					2840			2820		
					1450			1445	1450	
					756				1350	
Double bond conjugated Alkene trisubstituted							1630			
							1290			

^a Products isolated^b as methyl esters: I, veratrate; II, 5-carbomethoxyveratraldehyde; III, veratroylformate; IV, isohemipinate; V, artifact of compound III; VI, metahemipinate; VII, α ,3,4-trimethoxycinnate; VIIId, methyl- α , β -dimethoxy-(3,4-dimethoxyphenyl)glycerate; VIII, artifact of compound III; IX, dehydrodivertrate. ^b Structures are summarized in Figure 3.

Table III. Mass Spectra (% Relative Intensity) of Some Isolated Compounds

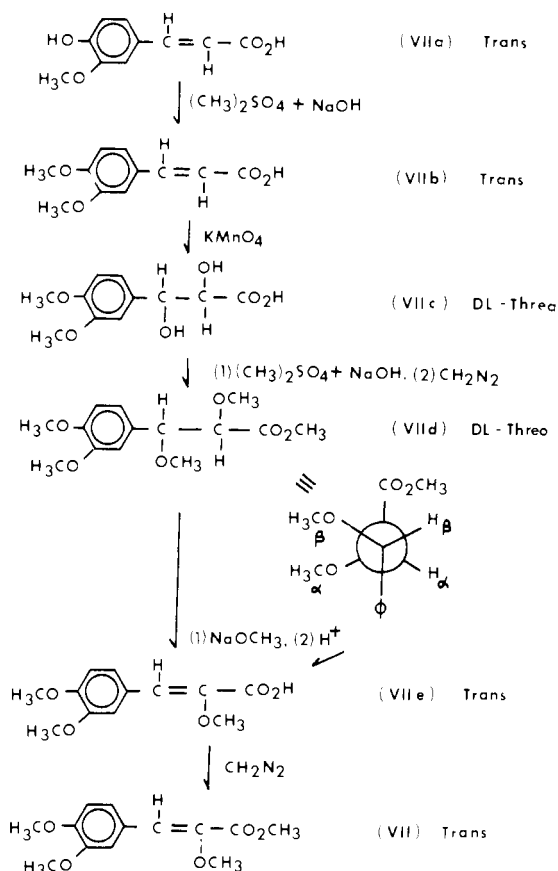
<i>m/e</i>	III	V	VII	VIIId	VIII
284				20 (M ⁺)	
266					89 (M ⁺)
252		100 (M ⁺)	100 (M ⁺)		
237			12		
225				27	
224	22 (M ⁺) ^a				
209			37		8
193		31			22
181			30		
175					18
165	100				
151		81	20	100	100
149	9				
122	18				

^a (M⁺) = parent ion.

methylated derivatives III, IV, VI, and VII, respectively, were found as products of this reaction.

Reactions are discussed below whereby each of the phenolic acids, identified in this study as its methylated derivative, could be formed from characteristic moieties which have been proposed as a part of the structure of protolignin.

Vanillic Acid. Vanillic acid, identified as methyl veratrate (I), is expected as a product of the nitrobenzene oxidation of lignins (Freudenberg et al., 1940; Pearl, 1950; Leopold, 1952). Based on studies of model compounds, vanillin has been reported to be essentially stable to oxidation by alkaline nitrobenzene (Kavanagh and Pepper, 1955; Roadhouse and MacDougall, 1956). Thus, it is not a significant precursor of vanillic acid. Model compounds, with the exception of those having a ketol structure, gave low yields of vanillic acid (Leopold, 1952; Pew, 1955). Accordingly, vanillic acid must be formed from lignin structures and not as a secondary oxidation product of vanillin. The possibility that vanillic acid was derived from intermediates containing functional groups, such as a ketol, could be explained by nucleophilic attack of the hydroxyl

**Figure 2. Synthesis of *trans*-methyl α ,3,4-trimethoxycinnamate.**

ion on the keto carbon, followed by a reverse Claisen condensation. This hypothesis is in agreement with the observation that vanillic acid has also been found as a product of alkaline hydrolysis of lignin (Ishihara and Kondo, 1950; Pearl et al., 1957; Sobolev and Schuerch, 1958). During oxidation, hydrolysis with the formation of

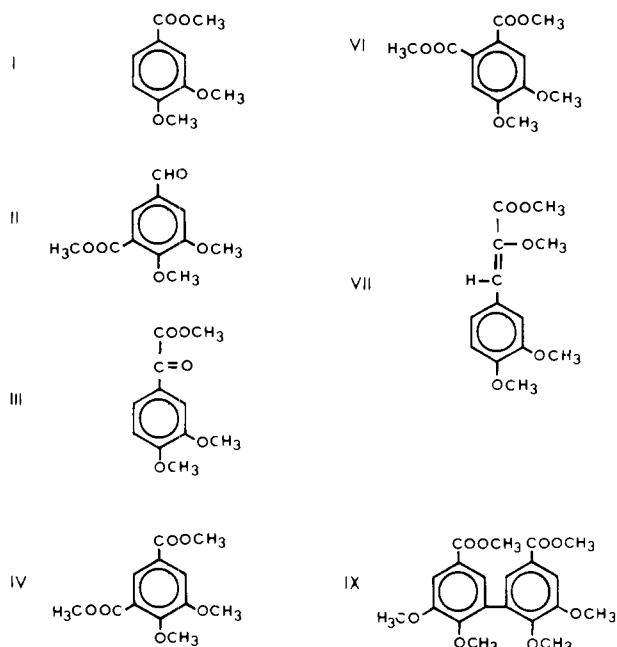


Figure 3. Summary of structures of products isolated.

vanillic acid could be promoted by formation of new structures which could undergo a reverse Claisen condensation. Structures such as vanilloylhydroxyacetic acid, α -ketopropioguaiacone, and vanilloylacetic acid are considered to be potential vanillic acid forming intermediates. It is known upon alkaline hydrolysis that benzoylacetic acid gives the hydrolytic cleavage product of a reverse Claisen condensation, benzoic acid, and the decarboxylation product, acetophenone (Levine and Hauser, 1944).

5-Carboxyvanillin. 5-Carboxyvanillin, identified as 5-carbomethoxyveratraldehyde (II), has been reported as a product of alkaline nitrobenzene oxidation of lignins (Freudenberg et al., 1940; Pew, 1955) and is generally considered to originate from the lignin moiety through oxidation. In studies of the oxidation of model compounds, it has been produced from 4,6-disubstituted guaiacols in yields of 6–26%. It is pertinent to note that a 0.3% yield of 5-carboxyvanillin was found in the alkaline nitrobenzene oxidation of eugenol, thereby indicating that condensation and subsequent oxidation may occur at the 6 position of 4-substituted guaiacols (Pew, 1955). Decarboxylation of this compound to give vanillin (Leopold, 1950; Freudenberg and Plankenhorn, 1947) may also occur to a small extent.

5-Carboxyvanillic Acid. 5-Carboxyvanillic acid, identified as dimethyl isohemipinate (IV), has not previously been reported as a product of alkaline nitrobenzene oxidation of lignins or model compounds. It has been reported as a product of potassium permanganate oxidation of lignins (Freudenberg and Muller, 1938; Richtzenhain, 1950a,b,c). Whether 5-carboxyvanillin will be oxidized by alkaline nitrobenzene to give 5-carboxyvanillic acid is not known. However, as previously noted, vanillin is not oxidized, excepting in very low yields, to vanillic acid under the conditions of alkaline nitrobenzene oxidation. Furthermore, due to the presence of the deactivating carboxyl group, it may be predicted that 5-carboxyvanillin would exhibit even greater resistance to oxidation to 5-carboxyvanillic acid than vanillin to vanillic acid. The formation of this compound, using more drastic permanganate oxidation, supports the concept that β -5, α -5, or 5-5' linkages exist in the lignin moiety. However, as previously discussed (Naveau et al., 1972), a portion of this product could be produced by condensation with

formaldehyde to give the hydroxymethyl group. The latter would then be oxidized to form the carboxyl group in the 5 position.

Vanilloylformic Acid. Vanilloylformic acid has been identified as methyl veratroylformate (III). Also two artifacts, V and VIII, have been isolated and shown to be derived from III. It is pertinent that vanilloylformic acid, represented by these three products, was the most abundant carboxy-containing compound formed in alkaline nitrobenzene oxidation of protolignin. Similar results were also obtained in the isolation of the alkaline nitrobenzene oxidation products from the lignin isolated from kraft black liquor as well as the lignin remaining in kraft pulp (Wu, 1970).

Veratroylformic acid has been isolated as a product of permanganate oxidation of cuoxam lignin that had been hydrolyzed with alkali and then methylated with dimethyl sulfate, followed by diazomethane (Freudenberg et al., 1936). Also, vanilloylformic acid has been isolated as a product of alkaline hydrolysis of unoxidized lignosulfonates (Pearl and Dickey, 1952) or by cupric oxide oxidation of lignosulfonates (Glennie et al., 1955). This keto acid has also been isolated by alkaline permanganate oxidation. Larsson and Miksche (1969) reported a yield of 2.1% based upon a methylated Bjorkman lignin.

Nitrobenzene oxidation of model compounds, such as α -ketopropioguaiacone, guaiacylaceton, and acetoguaiacone, gave only 2.5–11% of vanilloylformic acid (Kratzl, 1957; Kratzl et al., 1960). By the same oxidation acetoveratrone gave a 90% yield of vanilloylformic acid and only 4.25% vanillin and 0.5% vanillic acid. Decarboxylation of this acid to vanillin and oxidation of a minor portion of the vanillin to vanillic acid were proposed to account for these products. Although vanilloylformic acid has not been previously isolated from protolignin, it is pertinent to note that Kratzl considered it to be one of the most important intermediates in forming vanillin, the major product of an air oxidation procedure. The large amounts of this keto acid found in this study could indicate that the techniques of isolation previously reported in the literature concerning nitrobenzene oxidations of lignins were more drastic than the one we have used.

The facile side reactions of veratroylformic acid with diazomethane to form secondary reaction products are discussed later.

6-Carboxyvanillic Acid. 6-Carboxyvanillic acid, identified as dimethyl metahepinate (VI), was isolated in a yield of 0.66%. Previous to our studies, this compound was isolated only as a product of potassium permanganate oxidation. Although hydrochloric acid lignin, when subjected to alkaline permanganate oxidation, gave 1% of this acid (Richtzenhain, 1950a,b,c), lignins not previously subjected to condensation by acid treatment have only given trace amounts.

It has been postulated that this compound is a secondary reaction product produced by condensation at the 6 position of a guaiacylpropyl unit with formaldehyde (Freudenberg and Bittner, 1953) or with a propyl side chain of a neighboring guaiacylpropyl unit (Richtzenhain, 1950c). Oxidation of the condensed unit then gives the observed acid. Under the influence of mineral acid, condensation at the 6 position is favored and such structures could give the corresponding acid on alkaline oxidation. Previous work (Richtzenhain, 1950a,b,c) had indicated that protolignin and thiolignin which had been exposed to acid treatment failed to yield this compound. Under conditions used in our work, a distinction cannot be made between a mechanism involving a condensation

in the 6 position, followed by oxidation to a carboxylic acid or the natural occurrence of such an oxidizable linkage in protolignin to account for the presence of this compound.

Dehydrodivanillic Acid. Dehydrodivanillic acid, identified as dimethyl dehydrodivertrate (IX), is a known oxidation product obtained in nitrobenzene oxidation of lignin (Pearl, 1950). The postulation that the biphenyl linkage (5-5') occurs in lignin is supported by the formation of this compound as well as dehydrodivanillin reported in a previous work (Brink et al., 1972).

α -Hydroxyferulic Acid. α -Hydroxyferulic acid, identified as methyl α ,3,4-trimethoxycinnamate (VII), has not been reported previously as an oxidation product of lignin. It may arise from dehydration of the guaiacylglyceric acid or by formation of 2-hydroxy-3-(4-hydroxy-3-methoxyphenyl)propen-1-ol (α -hydroxyconiferyl alcohol), followed by its oxidation to α -hydroxyferulic acid. Guaiacylglycerol may be obtained by hydrolysis of the guaiacylglycerol- β -guaiacyl ether moiety in lignin. Both guaiacylglycerol and a trimeric structure similar to guaiacylglycerol- β -guaiacyl ether have been reported as products in lignin hydrolysis (Bjorkman, 1954; Sano and Sakakibara, 1970; Nimz, 1965, 1967).

Discovery of the presence of this compound supports the earlier hypotheses proposed by Hibbert (Mitchell and Hibbert, 1944; Hibbert, 1942; Fisher et al., 1944) that lignin is composed of hydroxyconiferyl alcohol type structures. Upon alcoholysis these give the observed Hibbert's ketones. Also, it has been postulated (Kratz, 1949) that α -hydroxyconiferyl alcohol could be obtained through dehydration of guaiacylglycerol.

Finding α -hydroxyferulic acid as a product of alkaline nitrobenzene oxidation is pertinent since the 3-carbon side chain structure, which is universally accepted as the structure in protolignin, is preserved. This compound may serve as an important intermediate in the oxidation of lignin in that it could give the α - β diketo acid by further oxidation. The diketo acid can then be decarboxylated and oxidized to give the observed vanilloylformic acid. Alternatively, the diketo acid may undergo alkaline hydrolysis to form vanillic acid or guaiacol, or it may form vanillin through decarboxylation (Wu and Brink, 1977).

Artifacts of Veratroylformic Acid. Compounds V and VIII were shown to be secondary reaction products formed from diazomethane methylation of veratroylformic acid (III, acid form). These products were not present in the methylation mixture containing methyl veratroylformate (III) formed by sulfuric acid catalyzed reaction with methanol. It was observed that during the methylation of veratroylformic acid, diazomethane in excess of the amount required to form the ester reacted further with methyl veratroylformate (III) to give V as the major product and VII as a minor component. A yield of V in excess of 95% was observed on prolonged methylation of III with diazomethane. This indicated that the ester form rather than the free acid form was involved in the secondary reaction.

Compound V is a colorless liquid in contrast to III which is a yellow solid. The characteristic IR absorption bands due to carbonyl in III were not present in V or VIII; hence, the reactions by which these artifacts were formed from diazomethane must involve the carbonyl group. Also, permanganate oxidation of V gave only veratric acid. This indicated that the aromatic nucleus in III was intact after the secondary reactions had taken place. In Table II absorption bands due to the methylene group are shown in the IR spectrum of V but not in the spectrum of III. Addition of two methylene groups to III was indicated by

the data from the mass spectra given in Table III. Data obtained using NMR are given in the Experimental Section. Based on the data summarized above, a structure cannot be unambiguously assigned.

In consideration of very similar IR spectra (Table II) and a possible difference in molecular weight of 14 (Table III), it is suggested that VIII is a homologue of V. The amount of fraction VIII isolated was insufficient to permit more complete analysis. Clearly, both V and VIII have been established as artifacts of III. Rigorous proof of their structures is subsidiary to the objectives of this paper and for this reason has not been further pursued herein. However, based on these results, the amount of vanilloylformic acid consumed in forming these two artifacts has been calculated and included as a part of the yield of this product in Table I.

EXPERIMENTAL SECTION

The procedure for alkaline nitrobenzene oxidation of white fir (*Abies concolor* Lindl.) and the methylation, gas chromatographic, and spectrometric methods used have been fully described previously (Wu, 1970; Brink et al., 1972; Naveau et al., 1972).

Reference Compounds. Veratric acid was available commercially and was used without further purification. Methyl vertrate was prepared from veratric acid by diazomethane (CH_2N_2) methylation, followed by recrystallization from methanol-water.

5-Carboxyvanillin, isohemipinic acid, and dehydrodivanillic acid were available from earlier work (Kane and Brink, 1955). 5-Carboxyvanillin was prepared by a method similar to Profft and Smirnow (1965) from *o*-vanillic acid and hexamethylenetetramine, mp 262–265 °C (lit. 255–256 °C). Methylation with dimethyl sulfate (Me_2SO_4), followed by CH_2N_2 gave 5-carbomethoxyveratraldehyde as colorless crystals, mp 70–71 °C. 5-Carboxyvanillic acid was prepared in 75% yield by silver oxide oxidation of 5-carboxyvanillin using the method of Pearl (1952). The product was isolated as a white solid, mp 283–284 °C (lit. 283–284 °C). Methylation using Me_2SO_4 followed by CH_2N_2 gave dimethyl isohemipinate as colorless crystals, mp 57–58 °C (lit. 57–58 °C). Dehydrodivanillic acid was prepared by the method of Elbs and Lerch (1916) and methylated using Me_2SO_4 , followed by CH_2N_2 yielding dimethyl dehydrodivertrate as colorless needles, mp 128–130 °C (lit. 129–130 °C).

Metahemipinic acid, prepared by hot permanganate oxidation of *m*-meconine, gave colorless crystals on recrystallization, mp 200–202 °C (lit. 202 °C). On CH_2N_2 methylation dimethyl metahemipinate was recovered as a colorless liquid. *m*-Meconine (4,5-dimethoxyphthalide) was prepared by the method of Edwards et al. (1925) from veratric acid and formaldehyde as colorless needles, mp 159–161 °C (lit. 155–157 °C).

Methyl veratroylformate, prepared by CH_2N_2 methylation of veratroylformic acid, yielded yellow crystals, mp 58–60 °C, on recrystallization: NMR (CCl_4) ArH, δ 7.62 (1 H, d, $J = 8$ Hz), 7.53 (1 H, s, overlays doublet), 6.89 (1 H, d, $J = 8$ Hz), ArOCH_3 and COOCH_3 , 3.92 (9 H, s, broad). Compounds related to veratroylformic acid were described by Glennie et al. (1955). The synthetic procedure shown in Figure 3 was used to synthesize *trans*-methyl α ,3,4-trimethoxycinnamate (VII). The starting compound, *trans*-ferulic acid (VIIa), was obtained commercially. On *O*-methylation with dimethyl sulfate *trans*-dimethoxycinnamic acid (VIIb) was obtained. By mild oxidation of VIIb with potassium permanganate in aqueous solution containing an excess of sodium hydroxide (Wiberg and Saegebarth, 1957), two hydroxyl groups were

added to the double bond in a cis manner to yield DL-threo-3,4-dimethoxyphenylglyceric acid (VIIc), mp 80–84 °C. After cooling the product gave a second melting point at 128 °C. Cis addition has been reported by Riiber (1915), Riiber and Berner (1917), and English and Gregory (1947) in the preparation of substituted phenylglyceric acids by alkaline permanganate oxidation of substituted cinnamic acids.

Upon methylation of compound (VIIc), with dimethyl sulfate and then diazomethane, methyl α,β -dimethyl- β -(3,4-dimethoxyphenyl)glycerate (VIIId) was formed: NMR (CCl₄) ArH, δ 6.77 (3 H, s, broad), β -CH, 4.28 (1 H, d, J = 6.5 Hz), ArOCH₃, 3.81 (6 H, s), α -CH, 3.71 (1 H, d, overlays ArOCH₃), COOCH₃, 3.349 (3 H, s), β -OCH₃, 3.33 (3 H, s), β -OCH₃, 3.21 (3 H, s). A smaller amount of methyl α -methyl- β -(3,4-dimethoxyphenyl)glycerate was also formed as a product of incomplete methylation. Upon refluxing (VII) with sodium methoxide in methanol, an E2 (concerted) elimination of α -hydrogen and β -methoxyl (or β -hydroxyl) took place and gave the observed *trans*- α -methoxycinnamic acid. Finally, diazomethane methylation of the *trans* acid gave the *trans*- α,β,γ -trimethoxycinnamate (VII): NMR (CCl₄) ArH, δ 7.24 (1 H, dd, J = 9 Hz, J = 2 Hz), 6.87 (1 H, s, overlays doublet), 6.82 (1 H, d, J = 9 Hz), ArCH=C, 7.45 (1 H, d, J = 2 Hz), ArOCH₃, 3.85 (6 H, s), COOCH₃, 3.80 (3 H, s), α -OCH₃, 3.76 (3 H, s). Elimination of the more acidic α -hydrogen is favored over that of the less acidic β -hydrogen in structure (VIIId) (or the corresponding monoether). The product (VII) was isolated and purified by preparative GLC.

An alternative synthesis was also used to prepare compound VII. A solution of methyl methoxyacetate in benzene was added dropwise to a solution of veratraldehyde and methanol free sodium methoxide in benzene at 0 to 5 °C over a period of 1 h. The reaction mixture was acidified after 2 h with 10% aqueous sulfuric acid and extracted with ether. The organic phase was washed with aqueous sodium bicarbonate, water, aqueous sodium bisulfite, and finally water. After drying the ether solution with anhydrous sodium sulfate, the solvent was evaporated and the resulting solids were redissolved in dry ether. Compound VII was then isolated using preparative GLC. The IR and NMR spectra of the products isolated using the two methods of synthesis were identical.

The NMR of compound V, the more abundant artifact of veratroylformic acid, indicated the presence of: (CCl₄ solvent) ArH, δ 6.73 (3 H, s, broad); ArOCH₃ plus 2 H, 3.80 (8 H, s, broad); COOCH₃, 3.68 (3 H, s); unspecified, 3.10 (2 H, d, J = 3 Hz), 2.93 (1 H, d, J = 6 Hz), 2.55 (1 H, d, J = 6 Hz).

CONCLUSIONS

An efficient, rapid, reaction-extraction sequence has been developed for the separation of the alkaline nitrobenzene oxidation products of proto- and derived lignins into five fractions that can then be cleanly fractionated into components by gas-liquid chromatography. First, the aromatic products are separated into carboxy-containing and carboxy-free methyl ethers after methylation with dimethyl sulfate. Each of these fractions can then be separated into aldehyde- (and certain ketone-) containing and aldehyde-free methyl ethers using bisulfite. Using this procedure to examine the carboxy-containing methyl ethers, four previously unreported carboxy-containing products of alkaline nitrobenzene oxidation were isolated from both protolignin and kraft lignin of white fir.

Two of these unreported acids, α -hydroxyferulic acid and vanilloylformic acid, identified as the methylated derivatives, are considered especially significant as in-

termediate oxidative products of lignin. The yield of veratroylformate was almost twice that of veratrate (vanillic acid). Thus, this keto acid has been shown to be the major acidic product of alkaline nitrobenzene oxidation. The isolation of α -hydroxyferulic acid, although in small yield, is especially significant as an intermediate in the oxidative degradation of lignin since it represents the retention of the three-carbon side chain structure of lignin. Oxidative procedures previously described in the literature, with the exceptions noted, have degraded the alkyl substituents of the aromatic moieties of lignin to carboxy or formyl groups only. Several pertinent literature references have been given relative to such degradation products. Thus, it appears that the alkaline nitrobenzene oxidation we have used has provided milder conditions than heretofore reported since some carboxylic acids isolated have retained side chains of two- and three-carbon atoms. Possible pathways for oxidation of lignin structures are postulated (Wu, 1970), in part, based on the isolation of these products.

The other two carboxy-containing derivatives, 5-carboxyvanillic acid and 6-carboxyvanillic acid, isolated as the dimethyl esters of isohemipinic and metahemipinic acids, respectively, have been reported previously as alkaline nitrobenzene oxidation products. Their formation is consistent with the evidence that the C-5 and C-6 positions in the aromatic structures are involved in linkages between moieties of protolignin. However, the possibility cannot be dismissed that some portion of these products is formed by condensation and subsequent oxidation in the isolation procedures used.

The isolation of the methylated analogues of vanillic acid, 5-carboxyvanillin and dehydrodivanillic acid, commonly reported products of alkaline nitrobenzene oxidation of lignin, was expected. The yield of vanillic acid was appreciably lower than anticipated because of the formation of the intermediate oxidation product, vanilloylformic acid.

Veratroylformic acid reacted with diazomethane to give secondary reaction products as well as the methyl ester in the final methylation step. These products have been characterized using spectrometric methods of analysis. The amount of vanilloylformic acid represented by these artifacts has been estimated as a part of the yield of this acid.

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Structure and Function of Dioxygenases. One Approach to Lignin Degradation

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The evolution of biosynthetic pathways leading to the formation of those structural natural products found in higher plants occurred in an oxygen-rich environment. Mixed function oxygenases evolved and functioned in catalyzing the insertion of molecular oxygen into basic structural polymers such as lignin, as well as natural products such as flavanoids and alkaloids. In addition to the critical role played by molecular oxygen in biosynthesis, oxidative degradative pathways evolved for the return of these natural products to the carbon cycle in the form of carbon dioxide. Oxidative degradation of natural products occurs by oxygen insertion reactions which are catalyzed by the mixed function oxygenases and by the dioxygenases. Reaction mechanisms for the mixed function oxygenases are now quite well understood, and a number of chemical model systems have been designed which catalyze such oxidation reactions. However, at the present time we do not have enough pertinent information on the mechanism of action of dioxygenases. This report provides some information to allow for the design of chemical model systems which mimic dioxygenase activity.

The appearance of molecular oxygen in the biosphere had an enormous influence on the evolution of electron-transfer enzymes. For example, the iron complex heme, which initially may have evolved to facilitate the reduction

of soluble inorganic ions such as sulfate and nitrate, adapted to function in the reduction of oxygen to water. The latter reaction clearly evolved as a detoxification mechanism since "activated" molecular oxygen is exceedingly toxic to the anaerobic bacteria. With the onset of aerobic systems superoxide dismutase evolved as a defense mechanism to reduce superoxide anion (O_2^-) toxicity (Dagley, 1976; McCord et al., 1971). Anaerobic bacteria do not contain superoxide dismutase and therefore these organisms die when exposed to an environment which contains oxygen. Clearly the ability to cope with molecular oxygen became increasingly important in the evolution of higher forms of life.

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