The Identification of Dilignols from Dehydrogenation Mixtures of Coniferyl Alcohol and Apocynol [4-(1-Hydroxyethyl)-2-methoxyphenol] by LC-ES-MS/MS

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A LC-ES-MS/MS method for the identification of dilignols formed by the oxidative cross-coupling of coniferyl alcohol and apocynol has been developed. The identification is based on the generation of ammonium adduct ions $[M + NH_4]^+$ by electrospray ionization and thereafter the following fragmentation patterns for the selected precursor ions. Fragmentation of several arylglycerol-β-aryl ether (β-O-4) and phenyl coumarane (β-5) model compounds were studied as a reference.

Keywords: *LC-MS/MS; lignin model compounds; lignols; arylglycerol-β-aryl ethers; electrospray ionization mass spectrometry*

INTRODUCTION

The dehydrogenation theory has formed a basis for the understanding of lignin formation in plant cell biosynthesis since its introduction more than 50 years ago. In the study of the final step in the formation of lignin in cell walls (Syrjänen and Brunow, 1998 and 2000), dehydrogenation of possible precursor model compounds results in complex mixtures of oligomers. The analysis of such mixtures and the reliable identification of individual compounds is a time-consuming task. In recent years combination of LC techniques with mass spectrometers has offered an attractive method for such analysis due to the soft ionization techniques (ES, CI) that allow the study of high molecular weight compounds.

Several examples of successful analyses of lignan and lignin based polyphenolic mixtures by LC-ES/MS has recently been reported (De Angelis et al., 1999; He et al., 1997; Jacobs and Metzler, 1999; Van der Hage and Boon, 1996). The soft ionization method provides a possibility to select conditions in which only a protonated molecular ion $[M + H]^+$, or the NH_4^+, Na^+, or K^+ adduct of the molecule, may be detected. In the instruments providing MS/MS technique it is then possible to selectively investigate the fragmentation of these distinct molecules selected according to the first quadropole information.

In the present paper we report the results of a MS/MS mass spectrometric examination of β-O-4 (**1**−**6**) and β-5 (**7** and **8**) type lignols. Particular attention has been paid to the fragmentation of arylglycerol-β-aryl ether (β-O-4) structures, which are the most abundant structural units in lignin.

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MATERIALS AND METHODS

Materials. Model compounds **1**−**3** were synthesized according to a published method (Sipilä and Syrjänen, 1995), and compound **4** was prepared by oxidative cross-coupling of coniferyl alcohol (**11**) and compound **3** (Syrjänen and Brunow, 1998). The dehydrogenation mixture (contains phenols **5**−**10**, **12,** and 15% oligomers) was obtained by oxidizing enzymatically coniferyl alcohol (**11**) in the presence of apocynol (**12**). Only the phenols **8** and **9** were isolated from the mixture and analyzed separately. The other phenols **5**−**7**, **9, 10,** and **12** were not isolated from the mixture as the composition of the oxidation mixture has been determinated earlier by HPLC, NMR, and MS (Syrjänen and Brunow, 2000).

Analytical Methods. ES-MS/MS (CID) spectra were acquired in the centroid mode using a Micromass Quattro II mass spectrometer (Manchester, U.K.) connected with a Hewlett-Packard 1100 liquid chromatograph. Nitrogen was used as a drying gas and as a nebulizing gas at flow-rates of 250 L/h and of 20 L/h, respectively. Argon was used as a collision gas at a pressure of $1 \cdot 10^{-3}$ mbar. A collision energy of 15 eV, a source temperature of 80 °C, a capillary voltage of 3.0 kV, and a cone voltage of 20 V were applied to produce the spectra at a mass range of 10−700 *m*/*z*. HPLC analysis was carried out on a LiChrospher RP-18 column (4 × 25 mm, 5 μm). The following solvent programming was used: from 25% acetonitrile/75% ammonium acetate (20 mM, pH = 8.54) solvent composition was changed linearly in 6 min to 50%/50%, then in 1 min to 75%/25%, and finally in 3 min to 100% acetonitrile. Injection volume was 20 μL and flow rate 1.0 mL/min; postcolumn splitter 1:10 was used.

RESULTS AND DISCUSSION

The optimization of the ES-MS/MS conditions was performed by the aid of β-O-4 model compounds **1**−**4**. The compounds were dissolved into a mixture of acetonitrile and ammonium acetate buffer and injected separately directly into the ion source. All model compounds gave ammonium adduct ions $[M + NH_4]^+$ with minor fragmentation in the first MS experiment. The ammonium adduct ions from the compounds **1**−**4** were then further fragmented to product ions (Tables 1 and 2).

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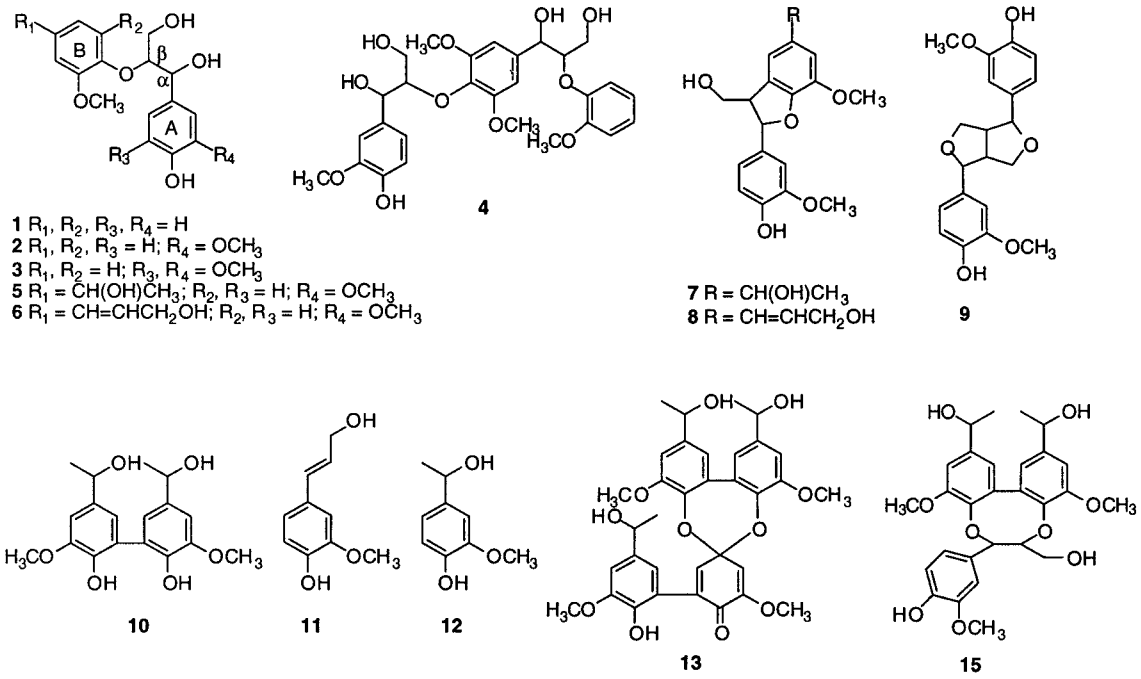


Figure 1. Model compounds **1**−**13** and **15**.

Table 1. m/z Values and Relative Ion Abundance's (%) in ES-MS/MS Spectra of Compounds 1−8

	1	**2**	**3**	**5**	**6**	**4**	**7**	**8**
MW (g/mol)	290	320	350	364	376	546	346	358
$[M + NH_4]^+$	308	338	368	382	394	564	364	376
$[M - NH_4OH]^+$	273(64)	303(50)	333(3)			529(18)	329(7)	
$[M - NH_4OH - H_2O]^+$	255(88)	285(52)	315(17)	329(1)	341(3)	511(2)	311(100)	323(2)
a, e	149(95)	179(37)	209(7)	179(36)	179(78)			
a, c, e		149(7)	179(92)	149(59)	149(23)			
a, g	107(100)	137(100)	167(88)	137(100)	137(100)			
b, f	149(95)	149(7)	149(100)					
d+fragm.in R_1				151(100)	151(48)			
h						349(100)		
i							137(15)	137(100)

Table 2. m/z Values and Relative Ion Abundance (%) in ES-MS/MS Spectra of Compounds 1−8 and 10

compd	*m*/*z* values and relative ion abundance's (%) of other product ions not mentioned in Table 1
1	123(4), 131(18), 137(26), 161(2), 179(27), 223(2), 243(64)
2	161(56), 162(8), 253(3), 273(31)
3	137(3), 161(14), 191(53), 192(22), 283(5), 303(34)
5	161(52), 162(24), 193(3), 241(3), 253(3), 273(25), 285(14), 297(12), 299(3), 311(2)
6	119(28), 131(13), 147(43), 161(64), 162(40), 163(15), 175(56), 187(21), 193(13), 205(35), 217(4), 251(3), 279(49), 309(19), 311(4), 323(2)
4	163(2), 167(5), 179(2), 183(30), 197(3), 225(25), 301(3), 331(9), 333(7), 481(1)
7	149(4), 175(4), 193(8), 251(4), 267(2), 279(22), 299(27)
8	149(8), 161(25), 162(5), 175(48), 187(17), 193(8), 199(6), 247(5), 251(6), 279(10), 291(2), 311(11), 329(2)
10	299(100), 239(1)

After optimization, the system was tested with the dehydrogenation mixture. The mixture consisted of monomeric and dimeric phenols and 15% of oligomers. The LC column was connected, and the mixture was analyzed. HPLC chromatogram of the mixture, the peak assignments, and the *m*/*z* value of ammonium adduct ions for each HPLC peak are shown in Figure 2. Apocynol (**12**) and compound **5** were eluted together; other components were eluted separately. In the MS spectrum of the mixture, ammonium adduct ions $[M + NH_4]^+$ from all dimers (**5**−**8** and **10**) except **9** were observed. In addition to the known phenols **5**−**10** and

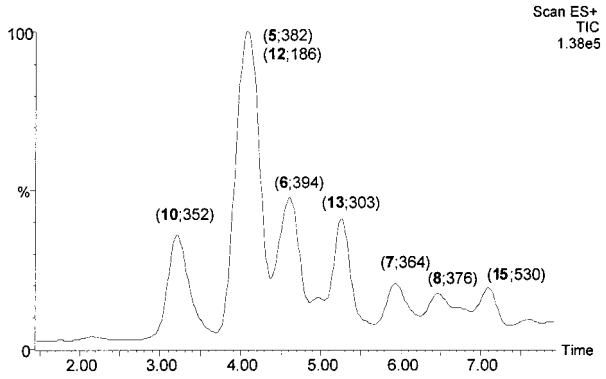
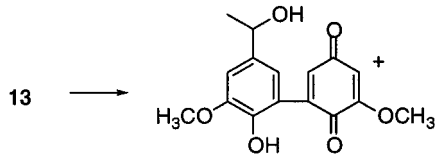


Figure 2. The HPLC chromatogram of the dehydrogenation mixture. Compound numbers and *m*/*z* values of ammonium adduct ions for each peak are given above the corresponding peak. For details of the HPLC analysis see Materials and Methods.

12 two other mass peaks (*m*/*z* 303 and *m*/*z* 530) were detected. The formation of the peaks could not be explained by the fragmentation of the known components of the mixture. We suggest that the peak *m*/*z* 303 is formed by the fragmentation of dioxepin **13**. The cleavage of ether linkages between the two dehydrodiapocynol moieties may yield the *para* quinone fragment ion **14** (Figure 3). Comparable structure was formed when similar spiro ketal was treated with mineral acids (Pew and Connors, 1969). The peak with *m*/*z* value of



m/z 303

14

Figure 3. Fragment ion *m/z* 303.

530 probably originates from the ammonium adduct ion of dibenzodioxocin **15**. Dioxepin **13** and dibenzodioxocin **15** are possible dehydrogenation products of coniferyl alcohol and apocynol (Pew and Connors, 1969; Karhunen et al., 1985). We did not observe these product earlier because of different chromatographic conditions (Syrjänen and Brunow, 2000).

The ammonium adduct ions obtained in the first MS sector were then fragmented to the product ions. The MS/MS spectra obtained in the second MS sector were compared to reveal general fragmentation patterns for β-O-4 and β-5 lignols.

Fragmentation of β-O-4 Model Compounds 1−6. Compounds **1**−**3** gave very similar fragmentation, and model compounds **5** and **6** also followed the same fragmentation pattern as **1**−**3.** However, more fragments were observed because of the extra side chains of compounds **5** and **6** (Figure 4, Tables 1 and 2). Due to the lack of MS/MS/MS information of the fragments, the peak assignments in this paper must be considered tentative.

Syringyl and *p*-coumaryl model compounds (**3** and **1**) followed the same fragmentation patterns as guaiacyl models (**2**, **5**, and **6**), but intensities of the product ions were different. A fragmentation (a, g), elimination of NH_4OH, and cleavage of the Cα-Cβ bond yielded the base peak for compounds **1**, **2**, **5**, and **6**. The fragmentation (a, g) gives a resonance stabilized benzylic cation which explains the intensity of this fragmentation. For syringyl compound **3**, the fragment ion (a, g) was also intense, but the base peak was *m/z* 149 which is apparently the fragmentation (b, f). Also *p*-coumaryl model (**1**) gave the fragment ion at *m/z* 149 with high intensity. Peaks *m/z* 137, *m/z* 149, *m/z* 161, and *m/z* 179 were found from all product ion spectra. A product ion *m/z* 151 was found from spectra of models **5** and **6**, and it was the base peak for compound **5** (Figure 4). All mass spectra in this publication were given for *erythro* compounds. For model compounds **5** and **6** also the *threo* isomers were studied, but only small intensity differences between the mass spectrum of *erythro* and *threo* isomers were found. The base peak for trimer **4** was *m/z* 349 (fragmentation h, Figure 5). Other product ions of the model **4** are listed in Tables 1 and 2.

Fragmentation of β-5 Model Compounds 7 and 8. The oxidation mixture studied contains two different β-5 dimers. The HPLC peak giving *m/z* 364 comes from compound **7**. However ammonium adduct ion *m/z* 376 can, in theory, originate from compound **8** or **9**. Interestingly, only one peak in HPLC chromatogram gave

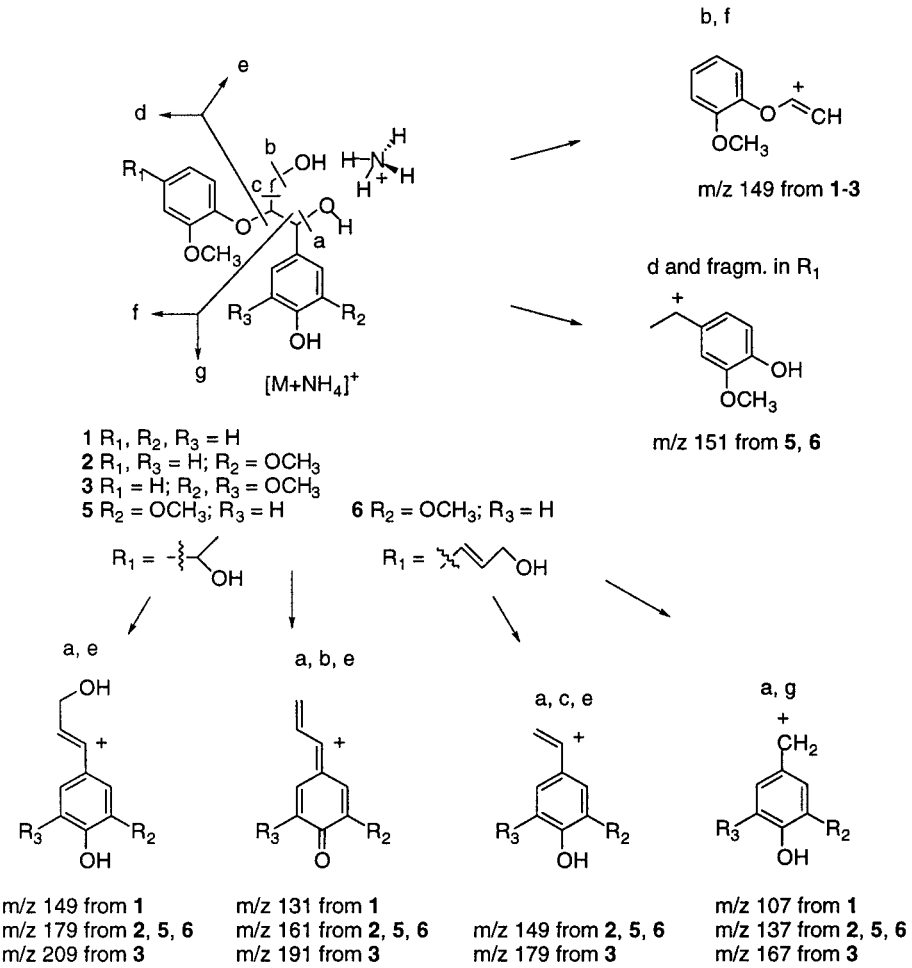


Figure 4. Fragmentation of β-O-4 model compounds **1**−**3**, **5**, and **6.**

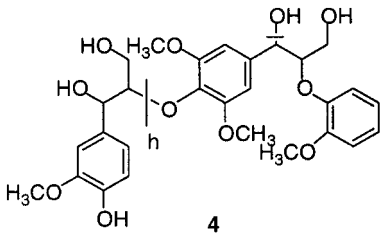


Figure 5. Fragmentation of compound **4**.

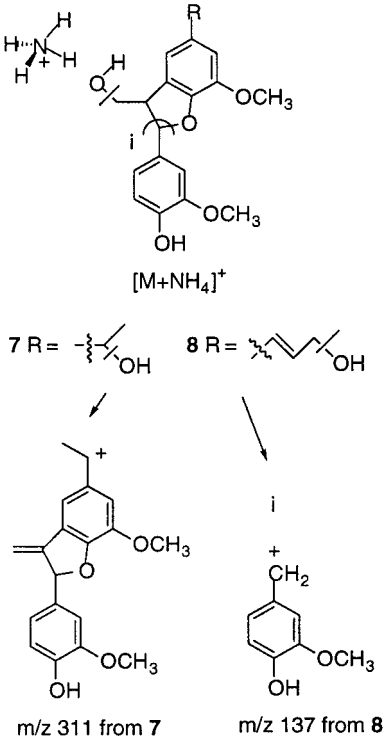


Figure 6. Main fragments of β-5 model compounds **7** and **8.**

that adduct ion. To determine the identity of the *m/z* 376 peak, previously isolated compound **8** was analyzed separately, and the MS/MS spectra of the isolated pure compound **8** and the *m/z* 376 peak were compared. The MS/MS spectra were similar. Fragmentation of model compounds **7** and **8** is summarized in Figure 6 and in Tables 1 and 2. The compound **8** gave *m/z* 137 (fragmentation i) as the base peak like β-O-4 dimers. Model compound **7** also gave a product ion at *m/z* 137, but the base peak was *m/z* 311 which corresponds to $[M - NH_4\text{-}OH - H_2O]^+$ fragmentation. Product ions *m/z* 149, *m/z* 175, *m/z* 193, *m/z* 279, and *m/z* 311 were found from both product ion spectra. Peak *m/z* 161 was found from the product ion spectrum of model **8** and from spectra of β-O-4 dimers but not from the product ion spectrum of compound **7**. It apparently comes from the coniferyl alcohol part of the dimer **8** (see Figure 4). Peak *m/z* 179 which was found from all spectra of β-O-4 model compounds was not found from the spectra of model compounds **7** and **8**.

Fragmentation patterns that we have observed for β-O-4 and β-5 type compounds are well in accordance with earlier results obtained by other ionization techniques (Houghton, 1985; Ito et al., 1994; van der Hage et al., 1995; Yoshikawa et al., 1995). No ES-MS spectra for model compounds, used in this study, have been published, but EI-MS and CI-MS spectral information for nonacetylated models is available [**2** (Kovácik et al., 1980), **3** (Pardini et al., 1991; van der Hage et al., 1995), **5** (Syrjänen and Brunow, 2000), **6** (Nakatsubo and Higuchi, 1980; Miki et al., 1980), and **8** (Kovácik and Skamla, 1969; Hirai et al., 1994)].

Fragmentation of Model Compounds 9 and 10. Pinoresinol (**9**) did not give an ammonium adduct ion with this method. We tried to inject pure **9** directly to the ion source to get a reference mass spectrum but without success. The 5−5 dimer from apocynol (**10**) gave two product ions. The base peak was $[M - NH_4OH - H_2O]^+$ at *m/z* 299 and also elimination of both OCH_3 groups (*m/z* 239) was detected.

As a summary, LC-ES-MS/MS provides a convenient tool for the characterization of the complex mixtures of lignols. The method is suitable for the detection of arylglycerol-β-aryl ether and phenyl coumarane type structures but, unfortunately, is not unambigious, as shown by the failure of pinoresinol to form any ions in the MS experiment. For the analysis, no derivation is needed, products can be analyzed directly from the oxidation mixture with reverse phase LC-ES-MS/MS, and baseline separation of the peaks in HPLC is not necessary.

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