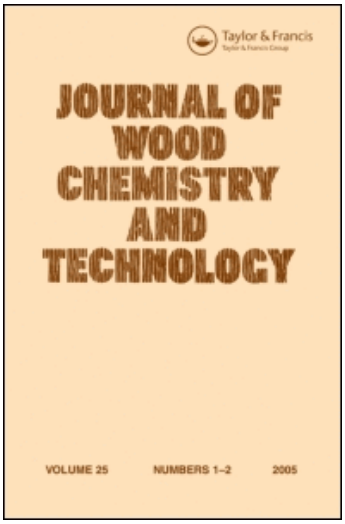

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THE DFRC METHOD FOR LIGNIN ANALYSIS. PART 3. NMR STUDIES

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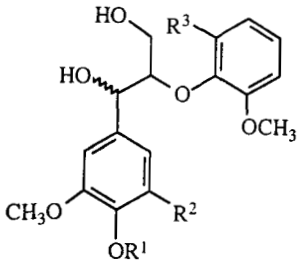
ABSTRACT

Two key reactions in the DFRC method have been examined by NMR. Both acetyl bromide (AcBr) derivatization of lignin and Zn reductive elimination of the β-bromo derivatives from lignin were highly selective and essentially quantitative. Treatment with AcBr in acetic acid efficiently converted β-aryl ether substructures of lignins into β-bromo ethers while γ-hydroxy and phenol groups were acetylated; the following Zn step cleaved brominated β-aryl ethers forming the expected cinnamyl acetates. In view of the high selectivity of AcBr reactions with lignin units and the solubilization of lignocellulosic materials, AcBr derivatization of lignins can be used for NMR characterization of whole lignins.

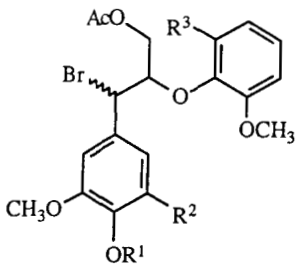
INTRODUCTION

Lignins are complex natural polymers which constitute about 20 to 35% of woody plants. Compared to other cell wall components such as cellulose, lignins are relatively poorly understood with respect to chemistry and structure for several reasons. Firstly, although formed from few monomers, lignins' interunit linkages involve several kinds of covalent bonds, some of which are resistant toward degradation. Secondly, lignins are heterogeneous. Thirdly, the paucity of experimental approaches such as selective and convenient analytical methods, and efficient lignin isolation procedures, remain obstacles to its understanding and utilization.

Lignins are derived from enzyme-mediated dehydrogenative polymerization of three phenylpropanoid monomers, namely *p*-coumaryl, coniferyl, and sinapyl

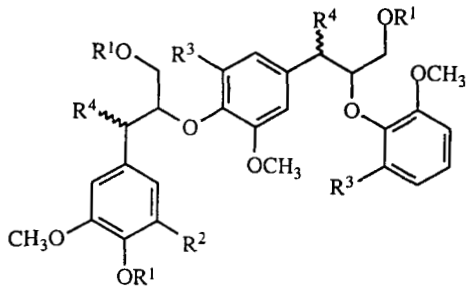


1 $R^2 = R^3 = H$ (**G–G**)
2 $R^2 = H$, $R^3 = OCH_3$ (**G–S**)
3 $R^2 = R^3 = OCH_3$ (**S–S**)
4 $R^2 = OCH_3$, $R^3 = H$ (**S–G**)

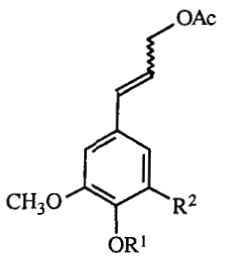


9 $R^2 = R^3 = H$ (**G–G**)
10 $R^2 = H$, $R^3 = OCH_3$ (**G–S**)
11 $R^2 = R^3 = OCH_3$ (**S–S**)
12 $R^2 = OCH_3$, $R^3 = H$ (**S–G**)

a $R^1 = H$
b $R^1 = Ac$
c $R^1 = CH_3$



5 $R^2 = R^3 = H$, $R^4 = OH$ (**G–G–G**)
6 $R^2 = H$, $R^3 = OCH_3$, $R^4 = OH$ (**G–S–S**)
7 $R^2 = R^3 = H$, $R^4 = Br$ (**G–G–G**)
8 $R^2 = H$, $R^3 = OCH_3$, $R^4 = Br$ (**G–S–S**)



13 $R^2 = H$ (**G**)
14 $R^2 = OCH_3$ (**S**)

FIGURE 1. Lignin models and their derivatives from reactions involved in the DFRC method. Convenient syringyl-guaiacyl descriptors are helpful for the following figures; **G** = guaiacyl, **S** = syringyl, $\mathbf{G}_f$ = free-phenolic G unit, $\mathbf{G}_e$ = etherified **G** unit, etc.

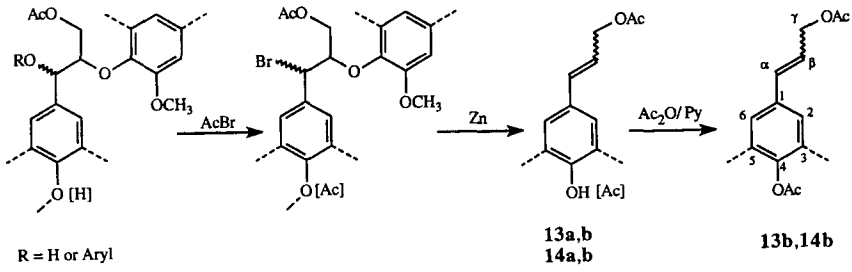


FIGURE 2. Reactions involved in the DFRC degradation of models and lignins.

alcohols. Their structural units are linked to each other somewhat randomly by several kinds of ether and carbon-carbon bonds.[1-3] The most frequent interunit linkages are arylglycerol-β-aryl (β-O-4) ethers, e.g. **1-4** (Figure 1). Others include β-5, β-1, β-β, 5-5, and 5-O-4 linkages that are more resistant toward degradation. Because of the dominance of β-aryl ethers in lignins, their cleavage has been studied extensively. It has long been one of the most important research targets to find mild, selective and efficient methods for cleaving β-O-4 ethers. Such cleavage is a key either for an efficient degradation of polymeric lignins during chemical pulping or for analysis of various linkages present in lignins.

Recently, we developed the DFRC method,[4-8] a simple and powerful method which selectively and efficiently cleaves α-ether and β-ether linkages and allows quantitative analysis of lignin structural units involved in uncondensed structures (Figure 2). In this work we report results obtained by using NMR techniques with lignin models and isolated lignins (MWLs) to show the high selectivity and high yield conversions involved in the method, and to better understand those reactions involved in the DFRC method. We also provide a convenient method for applying solution-state NMR spectroscopy to the entire lignin fraction.

EXPERIMENTAL

Materials and Reagents

All materials and reagents used in this study have been described in previous papers.[7,8] The AcBr stock solution is 8% (v/v) AcBr in acetic acid.

Lignin Models

Dimers **1-4**[9] and trimers **5-6**[10] were prepared by standard methods.

Isolated Lignins

Lignins were isolated essentially via Björkman's procedures[11] as described previously.[12,13]

The DFRC Procedures for Degradation of Models and Lignins

a) Bromination: The lignin model (~20 mg) or the isolated lignin (100 mg) was dissolved in AcBr stock solution (3 mL for models, 15 mL for lignins) in a 10 or 25 mL round bottom flask. The solution was kept in a 50 °C oil bath for 2 h with gentle stirring. Then the volatile solvent and reagent were removed by rotary evaporation at <40 °C with several additions of acetone. The residue was used for NMR analysis.

b) Zn reductive elimination: The residue was dissolved in dioxane/acetic acid/water (5/4/1 by volume, 3 mL for models or 15 mL for lignins).To the well stirred solution was added 50 mg or 100 mg of Zn dust. The mixture was continuously stirred for 30 min. Standard extraction into methylene chloride gave the degradation product.

c) Acetylation: Degradation products were acetylated with 2 mL of Ac_2O/pyridine (1:1) for 40 min.

NMR Spectra

^1H and ^{13}C NMR spectra of lignin model and lignin degradation products were recorded under standard small-molecule conditions on a Bruker AMX-360 instrument in acetone-d_6; the central solvent peaks were used as internal reference (^1H, 2.04 ppm; ^{13}C, 29.80 ppm). Assignments were made by the usual compliment of 1D and 2D NMR experiments.

RESULTS AND DISCUSSION

Reactions of lignins with AcBr

The DFRC degradation method includes two key steps: a) Solubilization of lignin

by bromination and acetylation with AcBr, b) reductive cleavage with Zn dust (Figure 2).[4-6] It is essential for a quantitative analytical method that every reaction involved is close to quantitative so that results from the method are reliable.

The first step of the DFRC method involves AcBr treatment under mild conditions. Reactions of some lignin model compounds with AcBr have been reported. Nimz found aryl ether cleavage, bromination and acetylation of a phenylcoumaran lignin model compound.[14] Iiyama[15] found that AcBr, with addition of small amounts of perchloric acid in acetic acid, effected acetylation, bromination and β-ether cleavage on lignin models. We have shown[16] with guaiacyl lignin models that, under mild conditions, AcBr cleanly effected three reactions on β-aryl ether substructures: a) acetylation of γ-hydroxyl and phenolic hydroxyl groups, b) Bromination of α-hydroxyl groups, and c) cleavage and bromination of α-aryl ethers, Figure 2 . Here we can see that when syringyl β-aryl ether or mixed β-ether models were subjected to AcBr treatment under similar conditions such reactions were as clean as in the case of guaiacyl models (Figure 3). All bromo derivatives of β-ether models used for NMR characterization were total crude products from AcBr treatment so that the NMR spectra shown here diagnostically reflect the nature of AcBr reactions with substrates. In the same way we will later show by ^{13}C NMR the reactions of AcBr with isolated lignins. As shown in Figure 3 conversions from starting β-O-4 models **1-4** to their corresponding α-bromo acetates **9-12** are essentially quantitative. ^{13}C-NMR spectra of those bromo derivatives were fully assigned through 2D HMQC and HMBC experiments; their partial data are listed in Table 1.

Since polymeric lignins are much more complex than model compounds, it is never clear whether reactions effective on models will work on real world lignins until they are tested and carefully evaluated. Thus two isolated lignins from loblolly pine and kenaf were used in this work. ^{13}C NMR Spectra of the AcBr treated lignins are shown in Figure 4. For comparison, spectra of AcBr treated derivatives from two syringyl β-ether dimers and one guaiacyl β-ether trimer are also shown. From Figure 4, it is obvious that AcBr affected aryl β-ether substructures of lignins in the same way as it did in β-ether models because dominant peaks in spectra of lignin derivatives completely match those in spectra of the corresponding model derivatives. In other words, the AcBr derivatization step on β-ether substructures of lignins was clean and almost quantitative. This observation hints that AcBr derivatization may

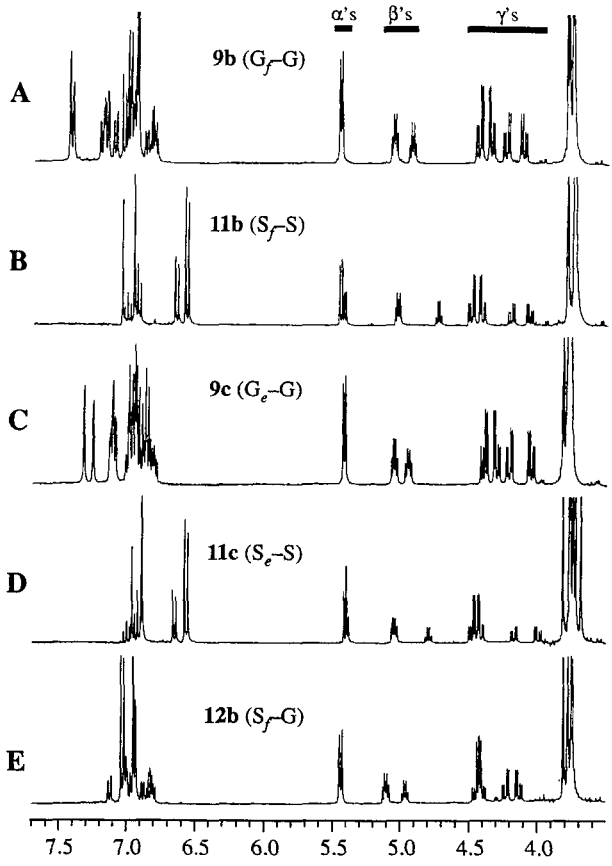


FIGURE 3. ^{1}H NMR spectra of derivatives from AcBr treatment of β-ether dimers. A) **9b**; B) **11b**; C) **9c**; D) **11c**, E) **12b** (see Figures 1 and 2).

be used in NMR characterization of whole lignin fractions and possible in whole plant cell walls; in addition to the clean reactions noted here, AcBr is well known for its ability to fully solubilize lignocellulosics. Reactions of AcBr with substructures other than β-ethers remain to be assessed, however.

Comparing peaks in the spectra of AcBr treated lignins with data listed in Table 1, it is not difficult to assign those which arise from β-aryl ether substructures. It is also possible to partially distinguish between peaks from etherified (internal) units

TABLE 1. ^{13}C NMR Chemical Shifts of Selected Carbons in Compounds **9-14**

	isomer	α	β	γ	A1	A2	A3	A4	A5	A6
9b	*erythro*	52.72	81.29	64.26	137.94	114.32	141.05	151.67	123.18	122.15
	threo	54.96	81.02	63.96	138.40	113.91	141.08	152.13	123.55	121.61
9c	*erythro*	53.95	81.33	64.45	131.51	113.65	150.70	150.05	111.85	122.51
	threo	55.94	81.46	64.01	132.00	113.16	150.75	150.20	112.18	121.83
10b	*erythro*	53.60	81.34	64.79	138.43	114.41	140.85	151.74	122.99	122.17
	threo	54.89	81.45	64.50	138.36	114.45	140.88	151.74	123.14	121.89
10c	*erythro*	54.78	81.38	64.96	132.02	113.56	150.50	149.82	111.83	122.54
	threo	55.68	81.85	64.32	132.40	113.67	150.54	149.87	112.07	122.06
11b	*erythro*	53.97	81.27	65.00	137.88	106.71	152.63	129.67	152.62	106.71
	threo	55.33	81.42	64.46	138.32	106.77	152.70	129.72	152.70	106.77
11c	*erythro*	54.43	81.19	65.13	134.98	107.44	153.73	136.61	153.73	107.44
	threo	55.67	81.66	64.34	135.36	107.40	153.91	136.25	153.91	107.40
12b	*erythro*	52.92	81.12	64.38	137.40	106.77	152.73	129.69	152.73	106.77
	threo	55.39	80.77	63.89	137.91	106.23	152.97	129.73	152.97	106.23
13a	*trans*	134.87	121.49	65.58	128.04	110.16	148.48	147.75	115.90	121.15
13b	*trans*	133.61	124.87	65.13	136.25	111.11	152.41	140.76	119.91	123.74
	cis	132.79	127.30	61.67	135.80	113.81	152.10	140.31	121.80	123.58
13c	*trans*	134.56	122.24	65.48	130.31	110.36	150.49	150.57	112.57	120.77
	cis	133.30	122.24	61.88	129.90	112.49	150.08	150.01	113.47	125.34
14a	*trans*	135.10	121.78	65.53	128.12	105.07	148.77	137.20	148.77	105.07
14b	*trans*	133.98	124.92	65.11	135.60	104.02	153.26	129.48	153.26	104.02
	cis	133.39	125.95	61.65	135.15	106.35	153.00	129.08	153.00	106.23
14c	*trans*	134.50	123.83	65.27	132.95	104.94	154.49	139.37	154.49	104.94
	cis	133.71	126.40	61.78	132.52	107.21	154.22	138.88	154.22	107.21

and unetherified (terminal) units, and even structural isomers although the ratio between isomers are no longer the same as in original lignins because bromination is not stereoselective or stereospecific.[16]

Lignins have been treated with halogen-containing reagents such as HI,[17,18] iodotrimethylsilane,[19-21] acetyl iodide,[17] pivaloyl iodide,[22] and bromotrimethylsilane.[23] All such treatments were found to give rise to halogen-containing derivatives; α-iodination on lignin side-chains was gerenally found when iodo-reagents were used. Large changes in chemical shifts of α-carbon signals were found when α-iodination occurred on the lignin side-chains.[24] In AcBr treatment, α-bromination also brought about upfield shifts of 20-22 ppm in the α-carbons, and by 2-3 ppm in β-carbons. But unlike treatment with iodo-reagents which cleaved β-ether linkages, mild AcBr treatment did not result in cleavage of such linkages. Another unique advantage is that bromo-derivatives from lignins are stable enough to be useful for NMR experiments and suitable for the following step of the DFRC method. We will demonstrate this later. Dry HI treatment of lignin may result in extensive cleavage of β-ethers; however the resulting iodo-derivatives were not stable enough to make routine quantitation possible,[18] and may dimerize again leading to unpredictable products.[22]

Chemical shifts of β- and γ-carbons from different kinds of β-aryl ether structures are very similar (Table 1) so that peaks around 81 ppm (β-carbons) and 64 ppm (γ-carbons) are relatively sharp. Because of the dominance of β-ethers in lignins, these resonances are relatively intense (Figure 4). However, chemical shifts for α-carbons vary over a relatively broad range from 53 to 56 ppm, dependent on their stereochemistry and the nature of the 4-O- substituent (4-O-etherified or 4-OH), especially for guaiacyl units (Table 1 and Figure 4). It is therefore possible to estimate the ratio between 4-O-etherified and 4-OH guaiacyl units connected by aryl β-ethers in lignins. Thus about 60% of β-ether units are etherified and 40% are free phenolic in this pine lignin (Figure 4) (Lu, unpublished data). It is not as easy to provide such an estimation for syringyl units. However, it is not difficult from the ^{13}C NMR spectrum of AcBr treated kenaf lignin (Figure 4) to estimate the ratio of syringyl to guaiacyl units connected by β-ether linkages by comparing the area of peaks around 107 ppm and peaks around 114 ppm. Thus it is obvious that kenaf lignin has dominant β-ether units with very high syringyl:guaiacyl ratio.[13,25]

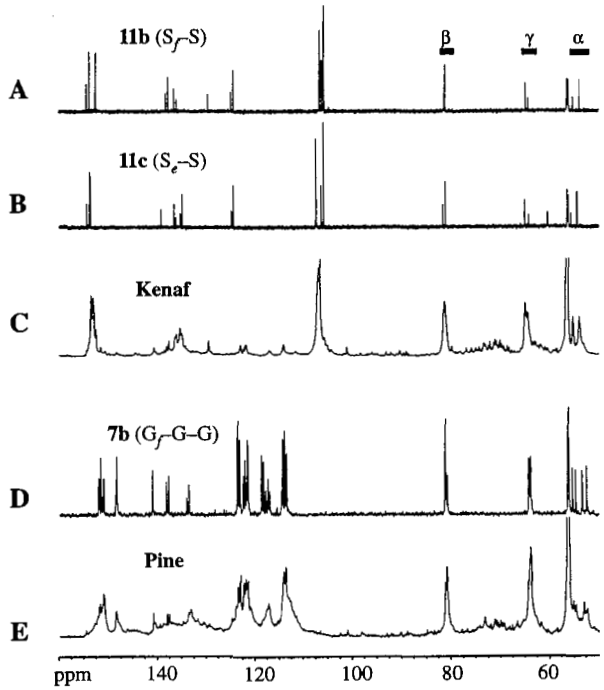


FIGURE 4. ^{13}C NMR spectra of compounds **11b,c** (A, B), **7b** (D) and AcBr treated kenaf (C) and pine (E) lignins.

In addition to peaks of dominant β-ether substructures, there were still some unidentified peaks in the range of 65 to 80 ppm in ^{13}C NMR spectra of AcBr treated lignins. They may come from lignin's other structures and contaminant carbohydrates. It will be important to investigate reactions of AcBr with substructures other than β-ethers and with carbohydrates in order to fully understand the DFRC method and the effects of AcBr on plant cell walls. Such studies are in progress in our laboratory.

Reductive Elimination of β-Bromo Ethers

The establishment of the DFRC method for lignin analysis resulted from the recognition that AcBr under mild conditions has produced lignin derivatives (β-

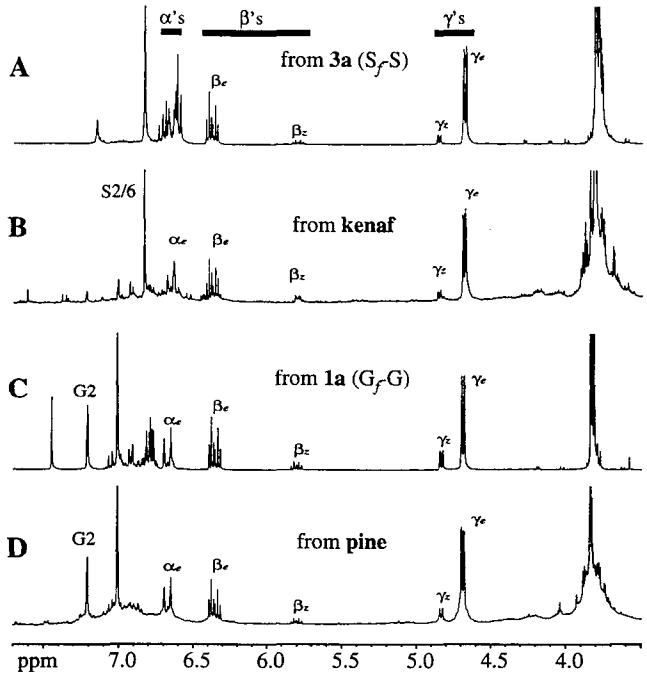


FIGURE 5. ^{1}H NMR spectra of DFRC monomers released from **3a** (A), **1a** (C), kenaf lignin (B) and pine lignin (D).

bromo ethers) which are ideally set up for reductive elimination. Reductive eliminations of β-bromo ethers with metals such as magnesium, zinc and metal complexes such as chromium(II)-ethylenediamine have been used in organic synthesis although the mechanisms have not been well understood. Ethylenes have been produced in quantitative yields from bromoethers by elimination of the bromo- and alkoxy- or aryloxy-groups through the use of Cr(II)en complex in DMF,[26,27] and alkenes were obtained in yields from good to excellent from β-bromoethyl alkyl ethers with Zn in isopropyl or *t*-butyl alcohols.[28-30]

When β-bromo aryl ether derivatives from AcBr treatment of lignin models were reacted with Zn in dioxane/acetic acid/water solution for 30 minutes, the corresponding elimination products were cleanly formed. Figure 5 shows the ^{1}H

NMR spectra of the crude products resulting from AcBr treatment followed by Zn reductive elimination. It is apparent from Figure 5 that those elimination products are the expected substituted cinnamyl acetates by comparing with NMR data of authentic compounds (Table 1) which were synthesized according to previously described procedures.[31] That about 5-8% of the *cis*-products were also formed indicates that the Zn step cannot be a stereospecific concerted process and must involve carbanion or radical-ion intermediates.[32] The NMR spectra (Figure 5) of the entire product from the full DFRC method also indicated that the expected 4-hydroxycinnamyl acetates were cleanly formed from typical β-ether models. The ^{13}C NMR data of those products are listed in Table 1.

Also shown in Figure 5 are spectra of DFRC final products from isolated kenaf and pine lignins (Figure 5B and 5D). These spectra clearly show that the expected monomeric products, cinnamyl acetates, are dominant in DFRC final products; they are accompanied by other minor products (minor monomers, dimers and oligomers) which could not be identified here, although dimeric DFRC products (about 5% of the lignin from pine) have now been isolated and identified.[33] As shown by ^{13}C NMR (Figure 4C), kenaf lignin has very high content of syringyl units connected by β-ether linkages. After DFRC degradation, kenaf lignin produced products with a high content of sinapyl acetates which are clearly shown in the aromatic range in Figure 5B. Signals from γ-protons of coniferyl acetate and sinapyl acetate are too close to differentiate.[34] The signal from aromatic 2/6-protons in sinapyl acetate appears at 6.84 ppm whereas signals of aromatic 2-protons in coniferyl acetate are at 7.21 ppm.[34] Integrating these peaks provides an estimate of the ratio of sinapyl acetate to coniferyl acetate in the DFRC product mixture. In the case of kenaf lignin this β-ether 'S/G' ratio is 5.5, consistent with our GC results.[5]

In this work two trimers **5a** and **6a**, more representative of lignin than dimers, were used. The ^{1}H NMR spectra of DFRC products (before and after final acetylation) from trimer **6a** are shown in Figure 6. Thus 4-acetoxyconiferyl acetate **13b** and sinapyl γ-acetate **14a** (Figure 6A) were cleanly produced after the Zn reductive elimination step from β-bromo ether trimer **8b** (formed from AcBr treatment of trimer **6a**) . In the same way, the corresponding products **13a** and **13b** resulted from trimer **5a** (NMR spectrum not shown here). Note that from the guaiacyl-syringyl-syringyl trimer **6a**, the initially free phenolic unit, i.e. the guaiacyl one, led to phenolic acetate **13b** and the originally etherified unit, i.e. the syringyl one, produced free

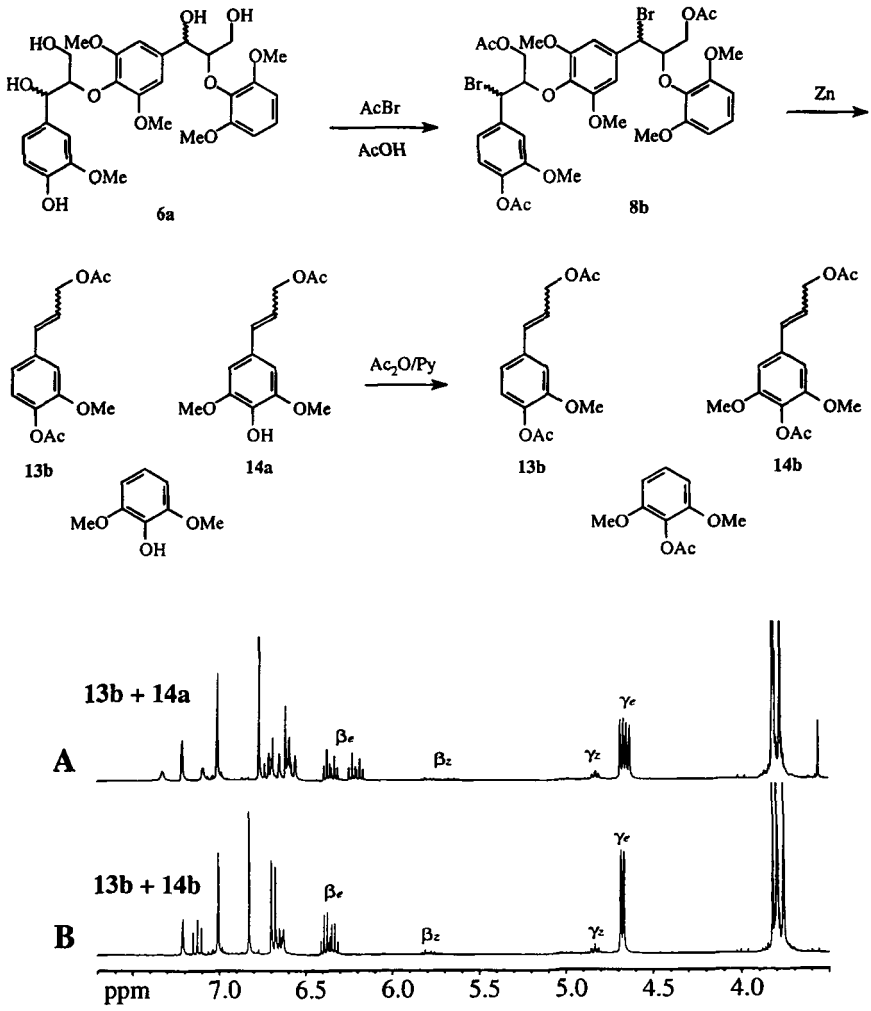


FIGURE 6. DFRC reactions for trimer **6a** showing how free-phenolic and acetylated products arise, and ^{1}H NMR spectra of released monomers A) after the Zn step and B) after acetylation.

phenolic component **14a**. Therefore one more acetylation step after Zn reductive elimination is necessary to obtain the simplest and most easily quantifiable mixture and spectrum (Figure 6B). Differentiating free phenolic and etherified releasable β-ether units is possible by the DFRC method if either no final derivatization is used or if another appropriate final derivatization is chosen instead of acetylation. Availability of this data without the requirement for pre-methylating the sample may become an attractive asset of the DFRC method.

CONCLUSIONS

The two step DFRC method for lignin analysis proved to involve very clean reactions for β-aryl ether substructures. ^{13}C-NMR spectra of AcBr treated lignins confirmed that β-ether structures in lignin were selectively converted to expected β-bromo ether derivatives. Solubilization of lignocellulosic material and clean derivatization of lignin by AcBr provides a convenient system to examine whole lignins by NMR. The following Zn reductive elimination under DFRC conditions was an equally clean reaction, producing cinnamyl acetates as the only monomeric products from arylglycerol-β-aryl (β-O-4) ethers. The NMR observations on each step of the DFRC method concur with previous GC results.

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