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Chloroaniline/Lignin Conjugates as Model System for Nonextractable Pesticide Residues in Crop Plants

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In vitro lignins formed by the peroxidase/H_2O_2-mediated polymerization of coniferyl alcohol in the presence of 3,4-dichloroaniline or [^{15}N]aniline were studied by ^{1}H, ^{13}C, and ^{15}N NMR spectroscopy. The anilines were >95% bound to the benzylic α-position of lignin side chains. Mild acid hydrolysis under simulated stomach conditions (0.1 M HCl, 37 °C) was studied as a first estimate of animal bioavailability. The two extremes of facile or slow acid hydrolysis that are known for chloroaniline/lignin complexes could be reproduced by using low or high incorporation ratios of aniline to coniferyl alcohol (10 or 40 mol %, respectively). The case of facile acid hydrolysis and high animal bioavailability may be due to the high mole ratios used and may not be relevant for pesticidal crop plant residue levels of 3,4-dichloroaniline. The latter are typically in the parts per million range. On the basis of ^{15}N NMR spectral fine structure, we propose that the acid-labile linkage may be due to anchimeric assistance in conformers formed at the high aniline molar ratio. The optimized methods presented here allow the use of in vitro lignin copolymers as a reference system for the structural features and the bioavailability of nonextractable pesticide residues in crop plants.

Introduction

A wide range of commercially important herbicides contains chlorinated anilines as structural components, e.g., acylanilide, *N*-phenylurea, and carbamyl derivatives. The free chloroanilines, which are formed as primary metabolites, are conjugated in plants, yielding soluble as well as insoluble (nonextractable) conjugates (*1, 2*). In intact plants and plant cell cultures, the operationally defined lignin fraction has been identified as a major covalent binding site for nonextractable chloroaniline residues (*3*−*5*).

Lignins are formed by an oxidative polymerization of monolignol precursors such as coniferyl alcohol (**1** in Figure 1). The proposed reaction mechanism for lignin biosynthesis involves the formation of a mesomeric monolignol radical that gives rise to achiral and polydisperse polymers with a multitude of binding types. The main interunit linkage present in lignins is the β-aryl ether structure shown in **2** of Figure 1. Pyrolysis/mass spectrometry has indicated a preferential binding of 3-chloroaniline and 3,4-dichloroaniline (DCA) to the α-carbon of lignin side chains (*4*). This position of chloroaniline binding has also been demonstrated by ^{1}H and ^{13}C NMR spectroscopy of in vitro lignins (dehydrogenation polymers, DHP), which were synthesized by peroxidase/H_2O_2-mediated polymerization of coniferyl alcohol in the presence of 4-chloroaniline or DCA (*5*).

The ecotoxicological significance of nonextractable pesticide residues depends on the bioavailability to an animal that ingests it and the potential release of low molecular weight xenobiotic fragments. Bound pesticide residues in plants generally have but low bioavailability in animals (*6, 7*). Feeding of a nonextractable wheat metabolite fraction of [^{14}C] DCA to rats and lambs led to the excretion of 11−20% of the bound xenobiotic in the form of soluble metabolites. Under comparable conditions, lignin DHPs containing 14 or 44 mol % chloroanilines released about 65% of the bound xenobiotic (*8, 9*), indicating that structural differences existed between the wheat and the enzymatic chloroaniline/lignin conjugates. So far, no experimental evidence exists to explain the difference in bioavailability. It has also remained open whether the case of high bioavailability (*8, 9*) is relevant for pesticidal crop plant residue levels of chlorinated anilines.

Here, we present an improved DHP model system that utilizes derivatives of [^{15}N]- and unlabeled aniline derivatives and their characterization by ^{1}H, ^{13}C, and ^{15}N NMR spectroscopy. Mild acid hydrolysis under simulated stomach conditions (0.1 M HCl, 37 °C) is used to mimick the digestion in the animal stomach. Variation of the aniline incorporation rate allowed us to produce and characterize chloroaniline/lignin conjugates with high or low acid sensitivity.

Experimental Section

Chemicals. Coniferyl alcohol was purchased from Fluka (Neu-Ulm, Germany), 3,4-dichloroaniline was from Riedel de Haen (Seelze, Germany), [^{15}N]aniline was from Chemotrade (Leipzig, Germany), and horseradish peroxidase was from Boehringer (Mannheim, Germany). [Ring U-^{14}C]-DCA (Sigma, St. Louis, MO) was purified by HPLC to 99% (*10*). All reagents for NMR spectroscopy were obtained from Merck (Darmstadt, Germany).

Synthesis of Model Compounds. *erythro*-1-(4-Hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol (**2a**) was synthesized according to Nakatsubo et al. (*11*). 3-(4-Hydroxy-3-methoxyphenyl)-3-(3,4-dichlorophenylamino)-2-(2-methoxyphenoxy)propan-1-ol (**2b**) and [^{15}N]-3-(4-hydroxy-3-methoxyphenyl)-3-phenylamino-2-(2-methoxyphenoxy)-propan-1-ol (**2c**) were synthesized as follows. In a 100-mL two neck round-bottom flask, 0.1 mmol of **2a** in 40 mL of dichloromethane was placed under a nitrogen atmosphere. At room temperature, 1 mmol of trimethylsilyl bromide was added within 1 min to the stirred solution with a syringe through a septum, and the reaction was allowed to proceed for 4 min to yield the α-brominated product. The reaction mixture was washed twice with 20 mL of saturated NaCl. The organic phase containing the quinone methide was dried over 3 g of Na_2SO_4 for 30 min, and the solvent was removed by a stream of dry nitrogen at 0 °C. Chloroform (20 mL) was added to the residue, and Na_2SO_4 was removed by filtration. To the organic solution, 0.2 mmol of 3,4-dichloroaniline or [^{15}N]aniline was added during a 20-min period under a nitrogen atmosphere for the synthesis of **2b** and **2c**, respectively. The mixture was incubated for 2 h at 22 °C. An aliquot was examined for product purity by analytical HPLC

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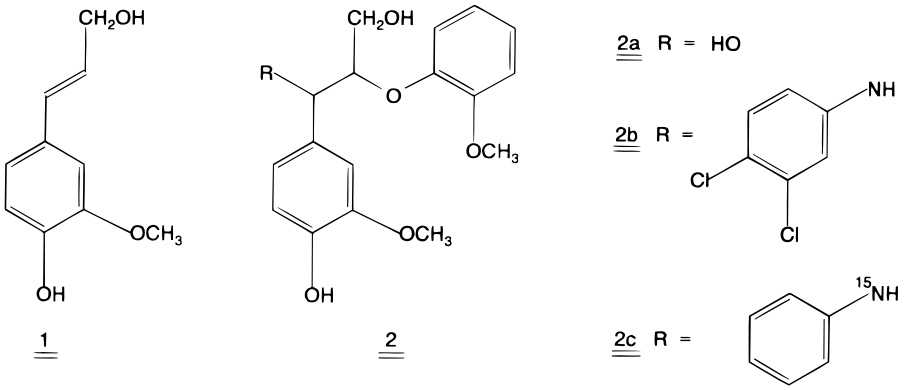

FIGURE 1. Structures of the lignin precursor, coniferyl alcohol (1), of the β-aryl ether side chain structure of lignins (2), and of model compounds without xenobiotic (2a) or containing 3,4-dichloroaniline (2b) or [^{15}N]aniline (2c) in benzylamine linkages.

(Millenium system, Waters, Eschborn, Germany); column: Vydac (4.6 mm × 25 cm, particle size 5 μm), Separations Group, Hesperia, CA; gradient: 0.05% H_3PO_4 in water (5 min), followed by a linear gradient from 0 to 100% acetonitrile in water (45 min) with a solvent flow of 1 mL min^{-1}; eluting compounds were detected with a diode array detector (Waters, Eschborn, Germany) between 200 and 450 nm. Two main products were detected and separated by semi-preparative HPLC (Millenium system, Waters, Eschborn, Germany); column: Nucleosil C-18 (10 mm × 25 cm, particle size 5 μm), Knauer, Berlin, Germany; gradient: 50% acetonitrile in water (5 min), followed by a linear gradient to 100% acetonitrile (25 min) with a solvent flow of 3 mL min^{-1} (diode array detection between 200 and 450 nm). The purity of the homogeneous compounds was assessed by diode array HPLC (analytical method as above. R_t: **2a**, 19.2 min; **2b**, 28.9 min (threo isomer) and 29.2 min (erythro isomer); **2c**, 25.9 min (threo isomer) and 26.8 min (erythro isomer). The identity of the compounds was assured by UV, IR, and NMR spectroscopy (only NMR data are shown in this report; for details see ref *10*).

Synthesis of Dehydrogenation Polymers (DHPs). All syntheses were carried out in 0.1 M sodium phosphate (NaP_i) buffer (pH 6.5) under a nitrogen atmosphere at room temperature (*10*). To a stirred solution of 2000 U of peroxidase in 100 mL of NaP_i, separate solutions of H_2O_2 (2.5 mmol in 75 mL of NaP_i) and of coniferyl alcohol (2.0 mmol in 75 mL of NaP_i) were added without an additional component to yield DHP 1. Aniline substrates (DCA; 2.0 or 0.2 mmol in 75 mL of NaP_i to yield DHP 2 and DHP 4, respectively) or [^{15}N]aniline (2.0. or 0.2 mmol in 75 mL of NaP_i to yield DHP 3 and DHP 5, respectively) were also employed. All solutions were added at rates of 20 mL h^{-1}. A [^{14}C]-3,4-DCA tracer was included in some of the 3,4-DCA experiments (*10*). The total reaction time was 5 h. The formed DHPs were pelleted by centrifugation (25 min, 15000*g*); washed with 20 mL of 1% (w/v) SDS, 20 mL of 1 M NaCl, and 3 × 20 mL of H_2O; lyophylized; and stored at −20 °C until further analysis. Polymer yields with regard to incorporated coniferyl alcohol were always >90%.

NMR Spectroscopy. The NMR spectra were acquired at 303 K with a Bruker AC 400 spectrometer operating at 400.13 MHz proton frequency. Dimethyl sulfoxide-d_6 was used as solvent and internal reference (2.49/39.00 ppm). ^{15}N NMR spectra were referenced to internal nitromethane (0.00 ppm), and methanol was an internal reference for ^{1}H/^{13}C. Proton and HH−COSY spectra were acquired with an inverse 5-mm broadband probe (90°: 8 μs), while ^{13}C and ^{15}N (90°: 7.5 and 13.5 μs) as well as two-dimensional CH- and NH-XHCORR (^1J: 150, 80 Hz) and COLOC [^nJ: 10, 7.5 (CH), 4 (NH)] spectra were obtained with a broadband 5 mm probe (90°: ^{13}C, 8 μs; ^{15}N, 13.5 μs) using Bruker standard software. Proton NOE difference spectra were recorded with 90° pulses to avoid SPT effects. For semiquantitative ^{15}N NMR spectra of DHPs, inverse gated decoupling with a relaxation delay of 45 s was used; ^{15}N-refocused-INEPT spectra were recorded with a 90° ^{1}H purge pulse at the end of the t_2 delay. Typical conditions for 2D NMR spectra: magnitude HH−COSY: 1K data points in F2 and 256 increments in F1; phase-sensitive (TPPI) HH−COSY: 2K data points in F2 and up to 1K increments in F1 corresponding to a digital resolution in F2 of 1.5 Hz; heteronuclear correlation spectra: 4K data points in F2 and 256 increments in F1.

Mild Acid Hydrolysis. A threo/erythro mixture of compound **2b** (threo/erythro ratio: 85:15) was incubated in 100 μL of 0.1 M HCl at 37 °C. Hydrolysis was terminated by addition of 110 μL of 0.1 M NaOH, followed by extraction with 200 μL of ethyl acetate. The decrease of the amount of **2b** [R_t, 28.9 min (threo isomer); R_t, 29.2 min (erythro isomer)] and the increase of DCA (R_t, 22.6 min) and **2** (R_t, 19.2 min) were followed by diode-array RP-HPLC analysis and used to quantify the hydrolysis rate (internal standard: 2,4-dichloroaniline; R_t, 25.8 min). Alternatively, hydrolysis was followed by ^{1}H NMR spectroscopy without an extraction step. DHP 3 and DHP 5 were incubated in an NMR tube in DMSO-d_6/D_2O (3:1, v/v) acidified with HCl to pH 1.0 at 37 °C. The protonated [^{15}N]aniline accumulating during hydrolysis was quantified by ^{1}H signal integration (H-2, H-6: 7.34 ppm; H-4: 7.40 ppm; H-3, H-5: 7.45 ppm); all signals assigned to protonated [^{15}N] aniline had higher ppm values than copolymer signals (details see ref *10*). In addition, DHP hydrolysis was followed by ^{15}N NMR analysis. DHP 3 and DHP 5 were treated as described above. The changes in ^{15}N signal intensity of the resonances at −313.7 and −317.3 ppm (bound [^{15}N]aniline; threo and erythro isomer, respectively) and at −323.7 ppm (free [^{15}N]aniline) were used to quantify the reaction.

Results and Discussion

Synthesis and Characterization of Model Compounds. The present investigation on the binding type of chloroanilines in DHPs employs NMR spectra of model compounds related to the lignin β-aryl ether subunit (**2**), which is the predominant linkage found in native lignins. The anilines were attached to the α-position by a biomimetic mechanism involving a quinone methide intermediate resulting in the formation of benzylamines. **2b** and **2c** (Figure 1) have two optical centers, corresponding to a mixture of threo (RR/SS) and erythro (RS/SR) forms. Accordingly, two main diastereomeric reaction products were obtained with 3,4-dichloroaniline (DCA) or [^{15}N]aniline as substrates as determined by HPLC analysis. The substances were purified by semipreparative HPLC and subjected to NMR spectroscopy. By comparison of these data with literature data (*12*−*15*), the presence of a threo:

TABLE 1. ^{1}H and ^{13}C NMR Data of Model Substances for the Binding Type of Chloroanilines in Dehydrogenation Polymers

	^{1}H NMR data						^{13}C NMR data			
		chemical shift (ppm)			coupling const. (Hz)			chemical shift (ppm)		
compd	assignment	(threo)	(erythro)	splitting[a] (intergral)	(threo)	(erythro)	assignment	(threo)	(erythro)	cross-peaks in ^{1}H,^{13}C COSY, long-range couplings (^{1}H,^{13}C-COLOC)
2a	H-2		7.03	d (1 H)		2.0	C-1		133.28	^2J (H-2), ^2J (H-α), ^3J (H-5), ^3J (OH-α)
	H-5		6.69	d (1 H)		8.0	C-2		111.30	^1J (H-2), ^3J (H-6), ^3J (H-α)
	H-6		6.79	dd (1 H)		8.0, 2.0	C-3		146.99	^3J (H-2), ^3J (OCH_3-3), ^3J (H-5)
	OCH_3-3		3.66	s (3 H)			C-4		145.47	^3J (H-2), ^3J (H-6)
	OH-4		8.70	s (1 H)			C-5		114.60	^1J (H-5), ^2J (H-6)
	H-3′		6.90	dd (1 H)		7.4, 2.1	C-6		119.56	^1J (H-6), ^2J (H-5), ^3J (H-2), ^3J (H-α)
	H-4′		6.84	dd (1 H)		7.4, 2.1	OCH_3-3		55.47	^1J (methyl H atoms)
	H-5′		6.80	m (1 H)			C-1′		148.13	^2J (H-6′), ^4J (H-4′)
	H-6′		6.98	dd (1 H)		7.5, 2.1	C-2′		149.81	^2J (H-3′), ^3J (OCH_3-2′), ^3J (H-4′), ^3J (H-6′)
	OCH_3-2′		3.65	s (3 H)			C-3′		112.75	^1J (H-3′), ^2J (H-4′)
	H-α		4.73	dd (1 H)		4.7	C-4′		121.02	^1J (H-4′), ^2J (H-3′), ^3J (H-6′)
	H-β		4.31	m (1 H)		4.7	C-5′		120.68	^1J (H-5′), ^2J (H-6′), ^3J (H-3′)
	H-γ		3.61	m (2 H)		11.6, 4.7	C-6′		116.00	^1J (H-6′), ^2J (H-5′)
	OH-α		5.27	d (1 H)		4.7	OCH_3-2′		55.63	^1J (methyl H atoms)
	OH-γ		4.58	t (1 H)		4.7	C-α		71.71	^1J (H-α), ^2J (OH-α), ^4J (H-2)
							C-β		83.79	^1J (H-β), ^3J (OH-β), ^3J (OH-α)
							C-γ		60.15	^1J (H-γ)
2b[b]	H-2	7.28	7.26	d (1 H)	2.3	2.4	C-1	138.50	138.30	^2J (H-2), ^2J (H-6)
	H-5	7.00	6.99	d (1 H)	8.2	8.3	C-2	112.05	112.25	^1J (H-2)
	H-6	6.79	6.79	dd (1 H)	8.2, 2.3	8.3, 2.4	C-3	150.61	150.63	^2J (H-2)
	OCH_3-3	3.70	3.71	s (3 H)			C-4	138.30	138.32	^3J (H-2), ^3J (H-6)
	H-3′	6.95	6.94	dd (1 H)	8.0, 1.8	7.9, 1.9	C-5	122.39	122.47	^1J (H-5)
	H-4′	6.86	6.85	dd (1 H)	8.1, 2.0	7.9, 1.9	C-6	119.45	119.52	^1J (H-6), ^3J (H-2)
	H-5′	6.80	6.78	m (1 H)			OCH_3-3	55.49	55.69	^1J (methyl H atoms)
	H-6′	6.93	6.91	dd (1 H)	8.2, 2.0	8.3, 2.1	C-1′	147.19	147.37	
	OCH_3-2′	3.73	3.72	s (1 H)			C-2′	150.28	150.09	^2J (H-3′)
	H-2″	6.88	6.86	d (1 H)	2.7	2.7	C-3′	112.69	112.81	^1J (H-3′)
	H-5″	7.20	7.19	d (1 H)	8.8	8.9	C-4′	124.38	124.27	^1J (H-4′)
	H-6″	6.64	6.61	dd (1 H)	8.8, 2.7	8.9, 2.7	C-5′	122.64	122.79	^1J (H-5′)
	H-α	4.83	4.83	d (1 H)	5.4	5.6	C-6′	120.58	120.73	^1J (H-6′), ^2J (H-5′)
	H-β	4.66	4.72	m (1 H)			OCH_3-2′	55.70	55.64	^1J (methyl H atoms)
	H-γ	4.05 (γ_1)	4.15 (γ_1)	dd (1 H)	11.3, 6.0		C-1″	147.86	147.94	^2J (H-2″), ^3J (H-5″)
		4.16 (γ_2)	4.27 (γ_2)	dd (1 H)	11.3, 4.1		C-2″	113.86	113.69	^1J (H-2″)
							C-3″	131.04	131.18	^1J (H-3″) ^2J (H-2″), ^3J (H-5″)
							C-4″	116.95	116.61	^3J (H-2″), ^3J (H-6″)
							C-5″	130.30	130.32	^1J (H-5″)
							C-6″	113.24	113.41	^1J (H-6″), ^3J (H-2″)
							C-α	57.34	57.27	^1J (H-α), ^3J (H-β)
							C-β	80.64	80.82	$^1J^{(H-\beta)}$, ^3J (H-α), ^3J (H-γ_1), ^3J (H-γ_2)
							C-γ	63.15	63.17	^1J (H-γ), ^3J (H-β)
2c	H-2	6.98	7.07	m (1 H)			C-1	131.98	130.71	(only COSY data available)
	H-5	6.63	6.64	d (1 H)	8.0	8.1	C-2	111.53	112.35	^1J (H-2)
	H-6	6.79	6.80	d (1 H)	8.0		C-3	147.29	147.14	
	OCH_3-3	3.78	3.79	s (3 H)			C-4	114.97	145.39	
	H-3′	6.97	6.97	d (1 H)	8.1		C-5	114.97	114.76	^1J (H-5)
	H-4′	6.89	6.89	dd (1 H)	7.6, 1.7		C-6	119.71	119.72	^1J (H-6)
	H-5′	6.78	6.81	m (1 H)			OCH_3-3	55.65	55.71	^1J (methyl H atoms)
	H-6′	7.01	7.01	d (1 H)	7.9	7.9	C-1′	147.66	147.90	
	OCH_3-2′	3.67	3.72	s (3 H)			C-2′	150.08	150.17	
	H-2″,H-6″	6.55	6.53	d (2 H)	8.4	8.4	C-3′	112.65	112.68	^1J (H-3′)
	H-3″,H-5″	6.98	6.98	m (2 H)			C-4′	121.96	121.95	^1J (H-4′)
	H-4″	6.47	6.36	t (1 H)	7.2	7.2	C-5′	120.68	120.79	^1J (H-5′)
	H-α	4.62	4.59	d (1 H)	4.0	4.2	C-6′	117.23	117.55	^1J (H-6′)
	H-β	4.30	4.41	m (1 H)			OCH_3-2′	55.47	55.46	^1J (methyl H atoms)
	H-γ	3.39 (γ_1)	3.45 (γ_1)	m (1 H)			C-1″	147.92	147.61	
		3.63 (γ_2)	3.54 (γ_2)	m (1 H)			C-2″,C-6″	113.10	113.89	^1J (H-2″), ^1J (H-6″)
							C-3″,C-5″	128.64	128.74	^1J (H-3″), ^1J (H-5″)
							C-4″	116.00	114.70	^1J (H-4″)
							C-α	56.65	57.05	^1J (H-α)
							C-β	84.32	83.55	^1J (H-β)
							C-γ	59.87	60.21	^1J (H-γ)

[a] s, singlet; d, doublet; dd, doublet of doublets; m, multiplet; t, triplet. [b] Acetylated, acetyl group signals not listed.

erythro ratio of 85:15 was determined for both **2b** and **2c**. The most informative techniques for the characterization of the model compounds were two-dimensional NMR experiments involving ^{1}H,^{1}H and ^{13}C,^{1}H correlation spectroscopy, which allowed the assignment of all resonances, as summarized in Table 1.

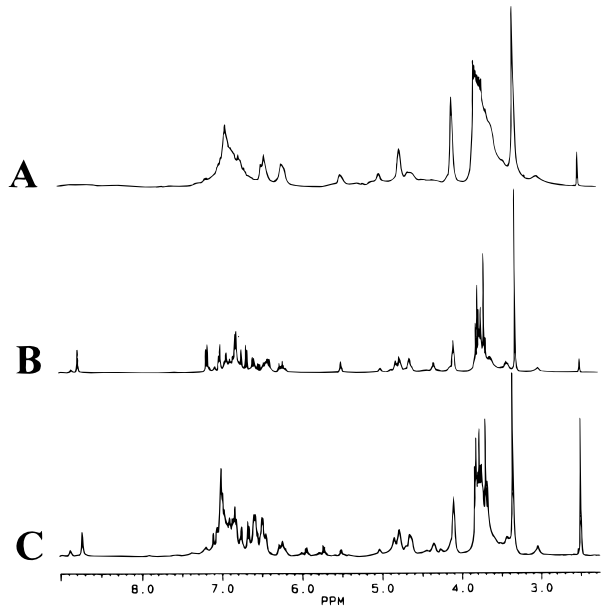

FIGURE 2. ^{1}H NMR spectra of dehydrogenation polymers (DHPs) in dimethyl sulfoxide-d_6 at 303 K; A, reference lignin (DHP 1); B, DCA/lignin copolymer (DHP 2); C, [^{15}N]aniline/lignin copolymer (DHP 3).

Synthesis and General Characterization of Chloroaniline/Lignin Conjugates. The in vitro polymerization of coniferyl alcohol by horseradish peroxidase/H_2O_2 in the presence or absence of aniline cosubstrates was carried out by modification of the previously described procedure (*5*). A reference DHP (DHP 1) was synthesized from coniferyl alcohol **1** alone. Aniline/lignin conjugates were prepared from **1** plus DCA (DHP 2, employed molar ratio of **1** to DCA = 1:1); DHP 4 (employed molar ratio of **1** to DCA = 10:1) or **1** plus [^{15}N]aniline (DHP 3, employed molar ratio of **1** to [^{15}N]aniline = 1:1); DHP 5 (employed molar ratio of **1** to [^{15}N]aniline = 10:1). The insoluble reaction products were washed with strong detergent and repeatedly with water.

High-performance size-exclusion chromatography (HPSEC) was performed in dimethyl formamide using polystyrene molecular weight standards (*10*). The DHP copolymers were found to be polydisperse with a broad maximum at 8−11 kDa. Absorbance at 280 nm and incorporated radioactivity showed coelution at a constant ratio over the entire molecular weight range (*10*).

Incorporation rates of the various anilines were determined through radioactivity content of a DHP synthesized with a [^{14}C]DCA tracer, integration of ^{1}H NMR spectra, and elemental analysis of DHPs 2−5. The molar ratios of coniferyl alcohol to aniline found were 2.6 ± 0.1:1 for DHP 2 and DHP 3 and 10.5 ± 0.5:1 for DHP 4 and DHP 5. These values corresponded to 40 mol % aniline in DHP 2 and DHP 3 and 10 mol % aniline in DHP 4 and DHP 5, respectively.

Spectral Assignments. The ^{1}H NMR spectra of DHPs 1−3 are shown in Figure 2. A broad multiplet assigned to H-γ appeared at 3.61−3.80 ppm. A further common feature was the presence of olefinic propenyl side chains with typical signals at 6.46 ppm (H-α, d, J = 16 Hz), 6.20 ppm (H-β, m), and 4.12 ppm (H-γ). The signal at 4.12 ppm was significantly decreased in DHP 2 and DHP 3. The H-α and H-β resonances in DHP 1 appeared at 4.70 and 4.32 ppm, respectively. DHP 2 and DHP 3 showed an additional signal at 4.63 ppm, which was assigned to H-α in a benzylamine bonding by comparison with the model substances. The ^{1}H- and ^{13}C NMR spectra of DHP 2 and DHP 3 showed a better resolution of signals than DHP 1, allowing the assignment of the signals in the lignin conjugate spectra attributed to the incorporated anilines by means of two-dimensional NMR techniques (DHP 2: H-6, 6.52 ppm; H-2, 6.59 ppm; H-5, 7.18 ppm; DHP 3: H-4, 6.48 ppm; H-2, H-6, 6.57 ppm; H-3, H-5, 6.99 ppm; see Table 2).

The ^{13}C NMR spectra of DHPs 1−3 are shown in Figure 3. Lignin resonances of DHP 1 (Table 2) were readily assigned (*10*) by reference to the extensive ^{13}C NMR data in the literature (*12*−*15*). Spectral features related to the incorporation of anilines were of special interest. Therefore, only resonances related to the covalent binding of anilines are discussed here. In comparison to the ^{13}C NMR spectra of DHP 1, a remarkable sharpening of nearly all resonances was observed with DHP 2 and DHP 3**,** and a number of new signals appeared. On the basis of the model compound spectra (Table 1) and 2D NMR spectroscopy [HH−COSY, CH−COSY, CH−COLOC; NH−COLOC], resonances of DCA in DHP 2 [113.3 ppm (C-6), 113.8 ppm (C-2), 116.5 ppm (C-4), 130.9 ppm (C-5), 131.0 ppm (C-3), 150.0 ppm (C-1)], and [^{15}N]aniline in DHP 3 [113.1 ppm (C-2, C-6), 115.0 ppm (C-4), 128.7 ppm (C-3, C-5), 148.0 ppm (C-1)] were assigned (Table 2). The most striking new spectral features of the aniline/lignine copolymers concerned the side chain signals. Concomitant with a decrease of the signal intensity of C-α (71.2 ppm) in the copolymer spectra, distinctive new resonances were detected at 56.7 and 84.3 ppm. The ^{1}H,^{13}C−COSY as well as the dublet splitting at 56.7 ppm ($^1J_{(NC)}$ = 10 Hz) clearly indicated that these signals were caused by C-α and C-β, respectively. Comparison of these chemical shifts with the model substance data (Table 1) provided unambiguous evidence for the benzylamine binding of the anilines (Table 2).

The ^{15}N NMR spectra of DHP 3 and DHP 5 (Figure 4) revealed two signal groups of exactly the same chemical shift values as observed with model compound **2c** corresponding to threo (−317.3 ppm) and erythro (−313.7 ppm) isomers in the ratio of 85:15. These peaks exhibited a fine structure that depended on the aniline molar ratio (Figure 4C). We propose preferred conformations of the substituents at the benzylic carbon in DHPs at higher proportions of incorporated aniline and deduce the existence of different stable conformations from this fine structure of the ^{15}N NMR resonances in the spectra of DHP 3 and DHP 5 (cf. Figure 1). The signal pattern of INVGATE and ^{1}J(NH)-INEPT and ^{1}J(NH)-DEPT-90 ^{15}N NMR spectra of DHP 3 and DHP 5 are identical, and this indicates that all resonances originate from singly protonated nitrogen atoms. Considering the uniformity of influences of the molecular environment on the magnetic shielding constant in ^{13}C and ^{15}N NMR of unprotonated aromatic amines, one would expect a corresponding fine structure in the ^{13}C NMR spectra of DHP 3 and DHP 5 if the fine structure is caused by long-range effects originating from differences in the major interunit linkages within the DHPs. However, this was not observed in any of the 1D and 2D proton and carbon NMR spectra.

At lower molar aniline ratios, the ^{15}N NMR signal splitting was much less marked (Figure 4B). The ^{1}H,^{15}N−COLOC spectrum of DHP 3 showed cross-peaks of −317.3 to 4.63 ppm (threo) and of −313.7 to 4.61 ppm (erythro), indicating a geminal NH-α coupling (data not shown). The proposed binding of chloroanilines to aromatic guaiacyl rings (*9*) was not observed. An azomethine binding of DCA or [^{15}N]aniline to C-γ of cinnamyl aldehyde side chains was likewise not detected by any of the NMR techniques applied. Azomethine model compounds were highly unstable in aqueous buffer (data not shown). Thus, the benzylamine attachment was the only binding type of anilines detected.

Hydrolytic Stability. The structural analyses were complemented by reactivity studies. The hydrolysis kinetics of the benzylamine bond at pH 1.0 and 37 °C (simulated stomach conditions; *9*) was investigated by three independent methods

TABLE 2. Assignment of NMR Signals in In Vitro Synthesized Insoluble DCA/Lignin Conjugates Using Model Substances[a]

assignment	δ in 2a	δ in 2b	δ in 2c	δ in DHP 1	δ in DHP 2	δ in DHP 3
H-α	4.73	4.63	4.61	4.70	4.73; *4.63*	4.73; *4.63*
H-β	4.31	4.31	4.30	4.10	4.32 (m)	4.30 (m)
C-α [^1J(NC)]	71.7	57.0	56.7 (d) (9.9 Hz)	71.2	71.3; *56.7*	71.0; *56.7* (10 Hz)
C-β	84.8	80.6	83.6	84.9	84.9; *84.1*	84.9; *84.2*
^{15}N[aniline]			−317.2 (threo) −313.6 (erythro)			−317.3 (threo) −313.7 (threo)

[a] The chemical shift values of additionally appearing signals in DHP 2 and DHP 3, when compared to DHP 1, are italic (d, dublet; m, multiplet). All spectra were determined in DMSO-d_6. Shift values are given in ppm units.

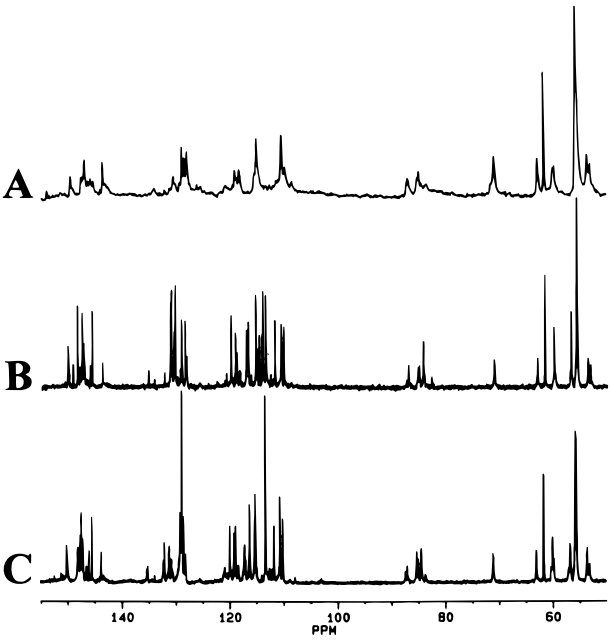

FIGURE 3. ^{13}C NMR spectra of dehydrogenation polymers (DHPs) in dimethyl sulfoxide-d_6 at 303 K; A, reference lignin (DHP 1); B, DCA/lignin copolymer (DHP 2); C, [^{15}N]aniline/lignin copolymer (DHP 3).

for the following materials: DCA in **2b** and [^{15}N]aniline in DHP 3 and DHP 5. RP-HPLC analysis of the hydrolysis of **2b** resulted in the accumulation of DCA and **2a**, which were quantified by diode array detection with 2,4-dichloroaniline as an internal standard. **2b** hydrolyzed linearly with a rate of 7% per day (Figure 5). For a ^{1}H NMR analysis of hydrolysis, DHP 3 and DHP 5 were incubated in DMSO-d_6/D_2O (3:1) in the NMR spectrometer (pH 1.0, 37 °C), and the liberated protonated anilines were quantified by signal integration. The hydrolysis kinetics of DHP 3 (40 mol % [^{15}N]anilines) displayed the characteristics of a nonlinear first-order reaction with a rapid release of [^{15}N]aniline within the first 10 min (16% initial release) followed by a significantly slower hydrolysis (to 20% DCA after 3 h and 25% DCA after 15 h). [^{15}N]Aniline release from DHP 5 (10 mol % [^{15}N]aniline) was much slower (6−7% after 3 h, 15% after 15 h) and reproduced quite well the hydrolysis kinetics of nonextractable DCA conjugates in wheat cell walls (*9*). As a third method, ^{15}N NMR spectroscopy of DHP 3 and DHP 5 was utilized to directly observe the hydrolytic liberation of [^{15}N]aniline in the NMR tube (same conditions as described above). The integrals of the signals at −313.7 and −317.3 ppm (bound [^{15}N]aniline) and at −323.7 ppm (free [^{15}N]aniline) were used to quantify the reaction. The low sensitivity of ^{15}N NMR spectroscopy allowed integration only every 20 min with DHP 3 and every 4 h with DHP 5 (corresponding to 30 and 320 accumulated spectra, respectively). Increased broadening of the NMR signals also impeded an exact quantification. Still, the aniline released after 3 h was reproducibly determined as 30% from DHP 3 and 15% from DHP 5 in accordance with the hydrolysis characteristics obtained by ^{1}H NMR spectroscopy.

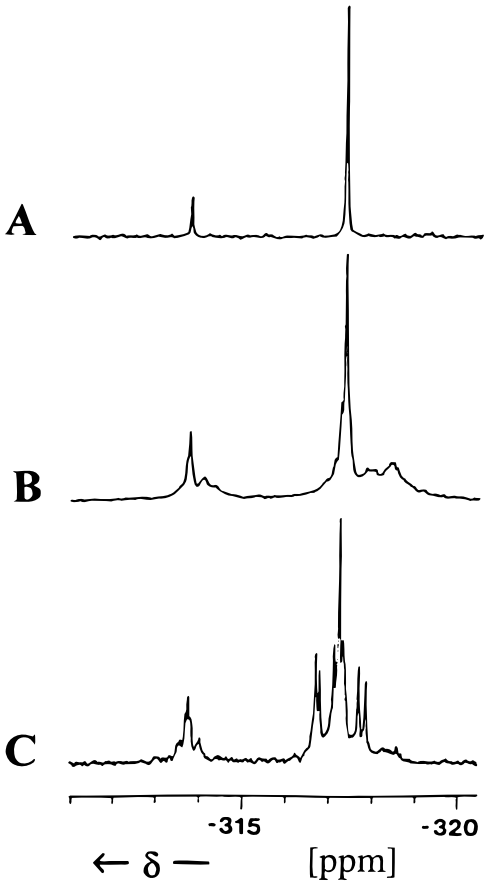

FIGURE 4. ^{15}N NMR spectra of (A) model compound 2c of Figure 1, (B) DHP 5, and (C) DHP 3. The smaller peak to the left corresponds to the erythro configuration, and the main peak to the right corresponds to the threo configuration.

The faster hydrolysis of bound anilines in DHP 2 or DHP 3 (40 mol % anilines) as compared to DHP 4 or DHP 5 (10 mol % anilines) appeared to be related to anchimeric assistance. Neighboring phenyl ether groups could be responsible because the only significant spectral difference between the two DHP types was the multiplet structure of Figure 4C. Anchimeric effects are frequently utilized for the depolymerization of lignins in wood pulping (*16*). In conclusion, we present here the first unequivocal data on the binding type of anilines in DHPs. Acid lability, which is associated with high animal bioavailability (*8, 9*), was only observed at 40 mol % aniline. This value is close to the mole ratios of 14% and 44% employed in the animal experiments (*8*) but far above the parts per million level that is typical for pesticidal crop plant residues of chlorinated anilines. Therefore, the previous experiments showing high animal bio-

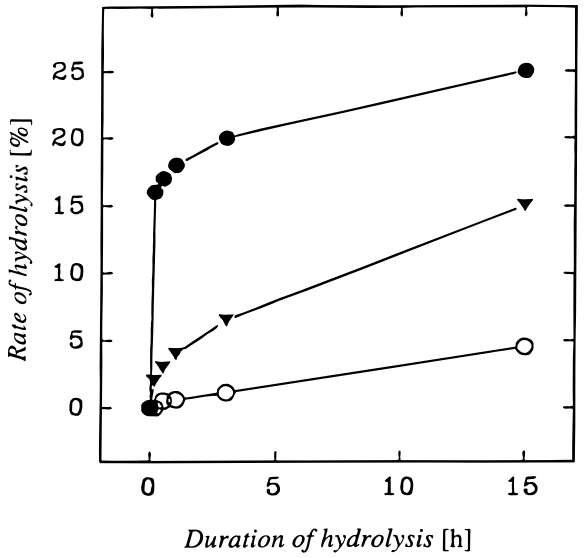

FIGURE 5. Mild acid hydrolysis (0.1 M HCl, 37 °C) of enzymatically prepared aniline/lignin conjugates (DHPs) and of model substance 2b of Figure 1. Hydrolysis (% of initial) is plotted versus time (h). The release of DCA from 2b (○) was quantified by HPLC analysis. The release of DCA or [^{15}N]aniline from DHP 2 and DHP 3 (●) and from DHP 4 and DHP 5 (▼) was determined by quantitative ^{1}H NMR spectroscopy.

availability (*8*, *9*) may not be relevant for actual pesticidal field residue levels.

In conjunction with previous studies using a native nonextractable wheat cell wall residue (*9*), the present study shows that defined DHPs can serve as simple model systems for nonextractable pesticide residues of crop plants. The methods presented here may help to reduce the number of experiments on animals required in registration procedures for new pesticides and other industrial chemicals.

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