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The Incorporation of 3,4-Dichloroaniline, a Pesticide Metabolite, into Dehydrogenation Polymers of Coniferyl Alcohol (DHPs)

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A mechanism by which pesticide metabolites may become incorporated into plant lignins has been studied with the aid of 3,4-dichloroaniline (DCA) and lignin model compounds. DCA was found to react rapidly with a quinone methide representing an intermediate in lignin biosynthesis. The resulting benzylamine group was stable to mild acidolysis under simulated stomach conditions. Addition of DCA to dehydrogenative polymerizations of coniferyl alcohol yielded DHPs containing covalently bonded DCA. NMR spectroscopy showed that the DCA had become bonded to the benzylic position in arylglycerol-β-guaiacyl ether structures. The majority of these structures were phenolic. This observation and the fact that the DCA accumulates in the low molecular weight fraction of the DHP indicates that the DCA functions as a trap for quinone methides and stops the further growth of the polymer.

Chlorinated anilines are released into the environment as primary plant metabolites of acylanilide, phenylurea and carbamate pesticides.[1] Several studies have shown that 3-chloroaniline and 3,4-dichloroaniline are incorporated into lignin both *in vivo* and *in vitro*.[2] Evidence for the formation of covalent bonds has been found from mass spectral and NMR data.[3,4] The structure that has been suggested is a benzylamine that can be formed by addition of the aromatic amine to a quinone methide formed as an intermediate during lignin biosynthesis. Other types of incorporation that have been proposed are bonding to the aromatic ring or merging into the molecular network of lignin without chemical bonding.[4]

In order to study this reaction more closely we have carried out experiments with 3,4-dichloroaniline (DCA) and a model quinone methide, and we have compared the resulting adduct with the adducts formed when coniferyl alcohol (a lignin precursor) is polymerized together with DCA. In order to estimate the animal bioavailability of the bound pesticide residue we have hydrolyzed the chloroaniline compounds under simulated stomach conditions.

Results and discussion

The biosynthesis of lignin macromolecules is initiated by the enzymatic dehydrogenation of *p*-hydroxycinnamyl alcohols yielding resonance-stabilized phenoxy radicals. The combination of these radicals produces dimeric structures, lignols. The growth of the polymer proceeds through the coupling of monomers with these lignols. This type of polymerization is called 'end-wise' polymerization.[5–7] Intermediate quinone methides then react with water, phenolic groups, polysaccharides and possibly also with 'xenobiotics', such as pesticide residues, during biosynthesis in Nature. We found that the quinone methide (**2**) prepared from 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (**1**) reacted rapidly and quantitatively with 3,4-dichloroaniline in dichloromethane to form a benzylamine adduct (**3**) (Fig. 1). The product was found to be a mixture of two diastereomers, with one isomer predominating. This diastereomer was designated the *threo* isomer in analogy with previous studies on the stereochemistry of amine addition to quinone methides.[8]

When coniferyl alcohol was dehydrogenated with H_2O_2 and horseradish peroxidase (HRP) in the presence of 50 mol% DCA, a polymer was formed that contained covalently bound DCA. The dehydrogenation product was divided into two fractions on the basis of solubility in ethyl acetate. The ethyl acetate insoluble fraction (1) and the soluble fraction (2) were formed approximately in the same proportion (w/w) in the case of pure dehydrogenation polymer (DHP). Polymerization in the presence of 3,4-dichloroaniline reduced the amount of fraction 1

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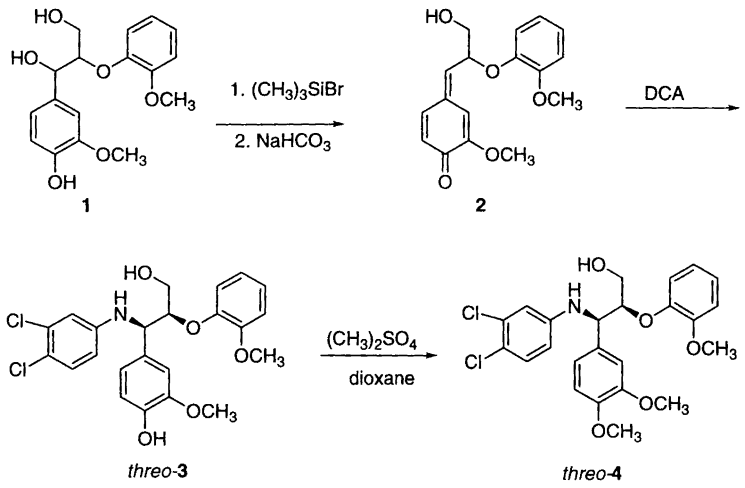


Fig. 1. Preparation of DCA adduct.

and the soluble fraction 2 increased. NMR analysis using HSQC-TOCSY (Fig. 2, Table 1, Scheme 1) showed that fraction 2 contained considerable amounts of DCA bound by a benzylamine bond to a β-O-4 structure. The only other significant change was that the relative amount of 'normal' β-O-4 units without DCA was lower than in a DHP prepared without added DCA. The DCA accumulated in the soluble low molecular weight fraction of the DHP; no NMR signals from bound DCA were detected in the insoluble fraction. One explanation for the low molecular weight of the DCA adduct could be that the addition of DCA caused a 'termination', that is, that the growth of the polymer chain stops after the incorporation of DCA. In order to test this hypothesis

Table 1. Some important C–H connectivities observed in the HSQC-TOCSY spectrum of an acetylated DHP containing DCA. For explanation of symbols, see Scheme 1.

	A	B	C	D	Compound **3**	E
α	[a]	88/5.5	85/4.7	59/4.6	59/4.6	[b]
β	80/4.6	51/3.7	54/3.1	82/4.4	82/4.3	
γ	[a]	65/4.3 and 4.4	72/3.9 and 4.2	62/4.1 and 4.4	63/4.1 and 4.4	65/4.7

[a]Detected only as TOCSY correlations (62/6,1 ppm α and 80/4.1 and 4.3 ppm β). [b]Not visible in this expansion.

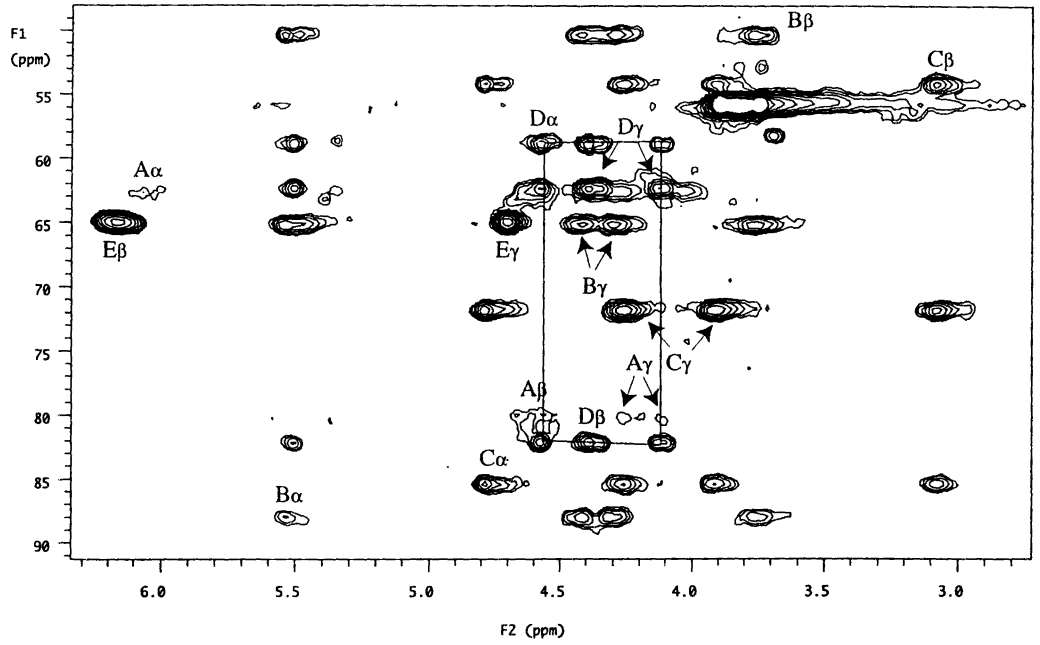
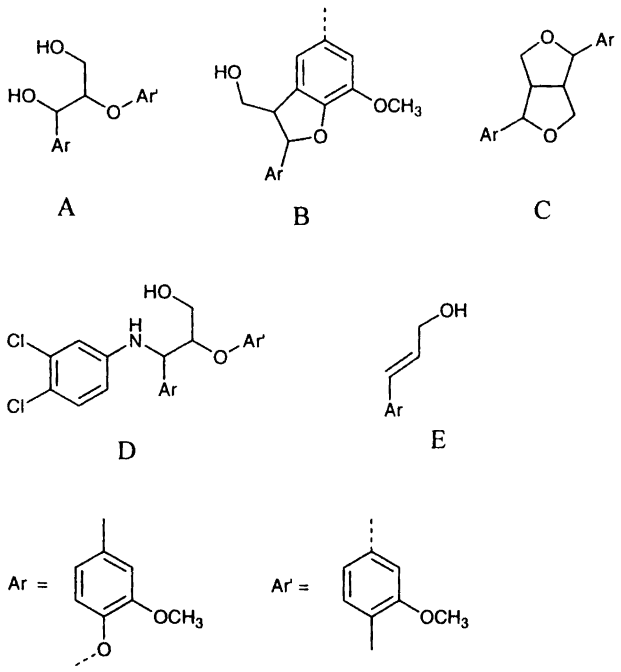


Fig. 2. Side chain region of a HSQC-TOCSY spectrum of acetylated ethyl acetate soluble fraction of DHP prepared with addition of DCA. Some important correlation signals are marked with symbols (see Scheme 1) and the signals from the β-O-4 units bonded to DCA are connected with straight lines.



Scheme 1.

we methylated the phenolic hydroxy group of model compound **3** to obtain a model for an etherified DCA-containing unit. Comparison of the NMR signals for the etherified (**3**) and the phenolic (**4**) model compound with the spectrum of the DHP–DCA adduct suggested that most of the units bearing a DCA-group were indeed phenolic, thus lending support to the 'termination' hypothesis.

Lignin is believed to be indigestible by animals. It has been claimed previously that the animal bioavailability of lignin bound pesticide residues depends on the method used to prepare the copolymerization compound of 3,4-dichloroaniline. It has been found that acid-labile linkages of dichloroaniline occur more in enzymatically prepared lignin than in wheat plants.[9] We have tested both our dimeric adduct and the DHP adduct using the simulated stomach conditions described in Ref. 9. This mild acidolysis did not release any DCA from either of our products.

Experimental

General. ^{1}H, ^{13}C NMR spectra (samples in $CDCl_3$) were recorded on a Varian Gemini 2000 200 MHz spectrometer. HSQC-TOCSY data were acquired on a Varian Inova 300 MHz spectrometer. Chemical shifts were measured relative to solvent signals at 7.26 ppm (^{1}H) and 76.9 ppm (^{13}C). HPLC separations were carried out on an HP 1090 liquid chromatograph equipped with a Hypersil ODS 5 μ 200 × 2.1 mm column and UV detector. Methanol–water solvent mixtures were used as the eluent. A high resolution mass spectrum was obtained with an MS VG AutoSpecQ and a normal spectrum with an MS Jeol JMS-SX 102 at 70 eV potential and are presented as *m/z*. TLC plates (Silica gel 60 F_{254}) and Silica gel 60 (230–400 mesh ASTM) used in flash chromatography (column diameter 3 cm, packing 15 cm) were purchased from Merck. 4′-Hydroxy-3′-methoxy acetophenone (Aldrich) and vanillin (Merck) were used as starting materials. Horseradish peroxidase 450 U mg^{-1} was obtained from Serva and 3,4-dichloroaniline from Fluka. Acetylations were performed with a mixture of acetic anhydride and pyridine (1 : 1). Dioxane was distilled before it was used for the preparation of DHP.

1-(4-Hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (**1**) was prepared by using a modified version of a previously published[10] synthesis. 4′-Hydroxy-3′-methoxyacetophenone (46.4 g, 0.28 mol, Aldrich) was dissolved in ethanol (130 ml). Benzyl chloride (33.0 ml, 0.29 mol) and anhydrous potassium carbonate (38.0 g) were added and the mixture was refluxed for 4 h. The warm reaction solution was poured into 0.5 M sodium hydroxide (900 ml). The resulting precipitate was suction filtered and washed with cold water. The crude product was recrystallized from ethanol. Yield 52.4 g, 0.20 mol (73%), m.p. 87–88 °C (lit.[11] 86 °C). The benzylated acetophenone derivative (37.6 g, 0.15 mol) was dissolved in ethanol (850 ml) with warming. When the solution had cooled to room temperature, bromine (8.5 ml, 0.17 mol) was added while a stream of nitrogen was bubbled through the solution. After about 2 h a white crystalline precipitate had formed. The solution was cooled and the product collected by filtration. Yield 40.2 g, 0.12 mol (80%), m.p. 104.5–106 °C (lit.[11] 103 °C). The reaction with guaiacol (yield 83%) and with formaldehyde (yield 91%) to give 3-hydroxy-2-(2-methoxyphenoxy)-1-[3-methoxy-4-(phenylmethoxy)phenyl]-1-propanone were carried out according to Ref. 12. The carbonyl group was reduced with $NaBH_4$ in 1,4-dioxane and the benzyl group was removed by catalytic hydrogenation in ethanol.[13] The product, an oil, was a mixture of *erythro* and *threo* isomers and was used without purification for the preparation of the quinone methide.

Quinone methide (**2**). Compound **1** (0.97 g, 3.0 mmol) was treated with bromotrimethylsilane (1.20 ml, 9.0 mmol) in dry dichloromethane under a stream of nitrogen. After 4 min the mixture was shaken with saturated sodium bicarbonate as described in the procedure of Ralph and Young[8] and the solution used for the reaction with dichloroaniline.

3-(3,4-Dichloroanilino)-3-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propanol (**3**), *(3,4-dichloroaniline adduct).* 3,4-Dichloroaniline (0.81 g, 5.0 mmol) in a small amount of dichloromethane was added to the solution of freshly prepared quinone methide (0.90 g, 3.0 mmol) in dry dichloromethane (30 ml) under nitrogen. The reaction mixture was stirred at room temperature for 1 h. Residual dichloroaniline was removed from the

mixture by extraction with four 30 ml portions of 1 M hydrochloric acid. The organic layer was washed once with 30 ml portions of saturated sodium bicarbonate and saturated sodium chloride. The organic phase was dried with anhydrous sodium sulfate and evaporated to give a yellowish solid in 80% yield (1.11 g, 2.4 mmol).[8] Purification of the crude product was carried out by column chromatography. The pure product was separated by elution with ethyl acetate–toluene (0.7:1.0 v/v). The fractions with the desired product were combined and evaporated to give a white solid that decomposed without melting. NMR shifts for acetylated adduct: ^{1}H NMR: δ 2.05 (3 H, s, $OCOCH_3$ aliph.), 2.29 (3 H, s, $OCOCH_3$ arom.), 3.76 (3 H, s, OCH_3), 3.90 (3 H, s, OCH_3), 4.08 (2 H, m, Hα *threo*), 4.38 (1 H, m, Hβ *threo*), 4.59 (1 H, t, Hγ *threo*), 5.50 (d, NH *erythro*), 5.62 (d, NH *threo*), 6.33 (dd, H6′ *erythro*), 6.39 (1 H, dd, H6′ *threo*), 6.61 (d, H2′ *erythro*), 6.65 (1 H, d, H2′ *threo*), 7.08 (d, H5′ *erythro*), 7.11 (1 H, d, H5′ *threo*), 6.84–7.04 (ArH). ^{13}C NMR: 20.7 ($OCOCH_3$), 55.7 (OCH_3), 59.1 (Cγ), 62.3 (Cα), 82.2 (Cβ), 110.9 (C2), 112.2 (C3″), 113.2 (C6′), 115.0 (C2′), 119.4 (C5), 120.2 (C4′), 120.4 (C6″), 121.2 (C5″), 122.9 (C6), 124.2 (C4″), 130.4 (C5′), 132.5 (C3′), 138.4 (C1), 139.5 (C4), 146.3 (C1′), 147.0 (C1″), 150.8 (C2″), 151.4 (C3), 168.7 ($OCOCH_3$ arom.), 170.4 ($OCOCH_3$ aliph.), Normal MS: the molecular ion was weak, the main fragments were *m/z* (int. %): 50 (6), 63 (9), 81 (14), 99 (11), 109 (32), 124 (37), 149 (6), 161 (100), 236 (3), 272 (5), 296 (17), 328 (3), 358 (6). High resolution MS (*m/z* 250–500): 272 (74), 296 (100), 319 (7), 331 (13), 343 (8), 381 (10), 393 (6), 431 (7). HRMS: 463.094917. Calc. for $C_{23}H_{23}Cl_2NO_5$: 463.095329.

3-(3,4-Dichloroanilino)-3-(3,4-dimethoxyphenoxy)-2-(2-methoxyphenoxy)propanol (**4**). Methylation of the phenolic hydroxy group of the 3,4-dichloroaniline adduct was carried out with dimethyl sulfate in dioxane–water.[14] After acetylation the product was purified by flash chromatography. ^{1}H NMR: δ 2.08 (3 H, s, $OCOCH_3$ aliph.), 3.82 (3 H, s, OCH_3), 3.85 (3 H, s, OCH_3), 3.94 (3 H, s, OCH_3), 4.00 (2 H, m, Hα *threo*), 4.36 (1 H, m, Hβ *threo*), 4.55 (1 H, t, Hγ *threo*), 5.49 (1 H, d, NH *erythro*), 5.65 (1 H, d, NH *threo*), 6.36 (1 H, dd, H6′ *erythro*), 6.41 (1 H, dd, H6′ *threo*), 6.61 (d, H2′ *erythro*), 6.65 (1 H, d, H2′ *threo*), 7.08 (1 H, d, H5′ *erythro*), 7.10 (1 H, d, H5′ *threo*), 6.79–7.04 (ArH). ^{13}C NMR: 20.5 ($OCOCH_3$ aliph.), 55.5 (OCH_3), 55.7 (OCH_3), 59.0 (Cγ), 62.2 (Cα), 82.5 (Cβ), 109.8 (C2), 111.2 (C5), 112.1 (C3″), 113.3 (C6′), 115.0 (C2′), 119.5 (C6), 120.0 (C4′), 120.5 (C6″), 121.1 C5″), 124.2 (C4″), 130.3 (C5′), 131.7 (C1), 132.5 (C3′), 146.3 (C1′), 147.2 (C1″), 148.6 (C4), 149.1 (C3) 150.8 (C2″), 170.5 ($OCOCH_3$ aliph.).

Ferulic acid. Vanillin (30.4 g, 0.20 mol) and malonic acid (31.2 g, 0.30 mol) were dissolved in pyridine (57 ml) and piperidine (3.5 ml). The reaction mixture was stirred at 40–44 °C for four days. Ferulic acid solidified when the slightly cooled reaction solution was poured slowly into a mixture of ice (117 g), water (200 ml) and concentrated sulfuric acid (60 ml). The pale yellow precipitate was isolated by suction filtration and the solid was washed with ice–water. The yield of dry ferulic acid was 80% (31.0 g, 0.16 mol).[15]

Methyl ferulate. Ferulic acid (10.0 g, 0.05 mol) was esterified using methanol (83 ml) and concentrated sulfuric acid (1.1 ml) according to the literature method. The product was obtained as a syrupy, yellowish liquid in 80% yield (8.3 g, 0.04 mol).[15]

4-(3-Hydroxy-1-propenyl)-2-methoxyphenol (coniferyl alcohol) was prepared by DIBAL-H reduction of methyl ferulate in toluene.[16] The solid product was obtained in 98% yield (1.83 g, 0.01 mol). The product was acetylated to aid NMR spectroscopy. ^{1}H NMR: δ 2.10 (3 H, s, $OCOCH_3$ aliph.), 2.30 (3 H, s, $OCOCH_3$ arom.), 3.83 (3 H, s, OCH_3), 4.71 (2 H, d, Hγ), 6.24 (1 H, m, Hβ), 6.61 (1 H, d, Hα, 6.97 (3 H, m, ArH). ^{13}C NMR: 20.3 ($OCOCH_3$ aliph.), 20.6 ($OCOCH_3$ arom.), 55.4 (OCH_3), 64,5 (Cγ), 109.8 (C2), 119.0 (Cβ), 122.5 (C6), 123.2 (C5), 133.2 (Cα), 134.9 (C4), 139.2 (C1), 150.8 (C3), 168.7 ($OCOCH_3$ arom.), 170.5 ($OCOCH_3$ aliph.).

DHP. Coniferyl alcohol (0.45 g, 2.50 mmol) was dissolved in distilled 1,4-dioxane (5.8 ml). The solution was injected into a 105 ml portion of de-aerated disodium hydrogen phosphate buffer (0.01 M, pH 6.50) with stirring under argon. Hydrogen peroxide (0.25 ml of 9.90 M H_2O_2, 2.50 mmol) in a small amount of buffer was added to a second 105 ml portion of de-aerated phosphate buffer under argon. Polymerization was carried out by adding simultaneously both solutions of reagents to a third 52 ml portion of de-aerated buffer solution, which contained horseradish peroxidase (1.5 mg of activity 450 U mg^{-1}), using peristaltic pumps over a period of 20 h. The reaction vessel was protected from light and the reaction was stirred at room temperature under argon. More enzyme catalyst (0.7 mg) was added during the course of the reaction. When the addition was complete, stirring was continued for 8 h. Aqueous sodium chloride (4 g in 20 ml) was added to the reaction mixture and the DHP was recovered by extraction with one 50 ml portion and with three 30 ml portions of ethyl acetate with efficient magnetic stirring. The insoluble product from the first extraction was collected from the surface of the organic layer and dried under vacuum. The yield of brown DHP powder, fraction 1, was 42% (0.19 g) of the starting material (0.45 g). The liquid phases were poured into a separatory funnel and the layers were separated. The combined ethyl acetate extracts were evaporated to give a pale brown solid, fraction 2, in 49% yield (0.22 g).[17]

Copolymerization with 3,4-dichloroaniline. Coniferyl alcohol (0.45 g, 2.50 mmol) and 3,4-dichloroaniline (0.20 g, 1.23 mmol) were dissolved in distilled 1,4-dioxane

(3.8+2.0 ml). The solutions were injected into a 105 ml portion of de-aerated disodium hydrogen phosphate buffer (0.01 M, pH 6.50) with stirring under argon. The oxidant hydrogen peroxide (0.25 ml of 9.90 M H_2O_2, 2.50 mmol) in a small amount of buffer was added to a second 105 ml of de-aerated phosphate buffer under argon. Polymerization was carried out by adding simultaneously both the solutions of reagents to a third 52 ml portion of de-aerated buffer solution of HRP (1.5 mg of activity 450 U mg^{-1}) as described in the case of DHP. The reaction and the isolation of the product were carried out as in the synthesis of DHP. The yield of insoluble brown powder (fraction 1) was 14% (0.09 g) of the starting materials (0.65 g). The combined ethyl acetate extracts were evaporated to give a pale brown solid (fraction 2) in 62% yield (0.40 g).[1,3,4,18]

Hydrolysis of 3,4-dichloroaniline adduct. Purified 3,4-dichloroaniline adduct (12.5 mg) was diluted with 1,4-dioxane to 25 ml. Exactly 2.0 ml aliquots were transferred to ten reaction containers and 0.5 ml of 0.1 M hydrochloric acid was added to the sample solutions (dioxane–0.1 M HCl 8:2 v/v). Hydrolysis was continued stirring at 37 C for 5, 10, 15, 30, 60, 90, 120, 180, 240 and 300 min. At the end of the reaction periods the samples were transferred to an ice bath and the pH was adjusted to slightly alkaline with 0.1 M sodium hydroxide. The volumes of the mixtures were checked. The samples were de-aerated and filtered before HPLC analysis.[9]

Hydrolysis of the copolymerization product of 3,4-dichloroaniline. Polymer (71.0 mg) was mixed with 0.1 M HCl (3 ml) and hydrolyzed with stirring at 37 °C for 6 and 24 h. The mixtures were transferred to an ice bath and neutralized with 0.1 M NaOH. The water was evaporated off, after which the samples were acetylated and dissolved in $CDCl_3$ and the solution was centrifuged before NMR measurement.

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