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Evidence for the Biosynthetic Pathway from Sinapic Acid to Syringyl Lignin Using Labeled Sinapic Acid with Stable Isotope at Both Methoxy Groups in *Robinia pseudoacacia* and *Nerium indicum*

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A tracer experiment using synthesized labeled lignin precursors was designed to confirm the actual biosynthetic pathway for syringyl lignin. Tetradeuteroferulic acid-[8-D, 3-OCD_3] and heptadeuterosinapic acid-[8-D, 3,5-OCD_3] were synthesized and fed to shoots of robinia (*Robinia pseudoacacia*) and oleander (*Nerium indicum*) trees. The incorporation of each labeled precursor into lignin was traced by gas chromatography−mass spectrometry. The synthesized sinapic acid, in which both methoxy groups were labeled, was useful in monitoring the conversion of sinapic acid into syringyl lignin. When heptadeuterosinapic acid was fed, syringyl units containing seven deuterium labels were detected. The results of this study support the traditionally accepted pathway that sinapic acid is converted to sinapyl alcohol via sinapoyl-CoA in robinia and oleander.

KEYWORDS: Lignin biosynthesis; heptadeutero sinapic acid-[8-D, 3,5-OCD_3]; tracer experiments; sinapyl alcohol; robinia; oleander; 4-coumarate-CoA ligase (4CL)

INTRODUCTION

Lignin is composed of *p*-hydroxyphenyl-, guaiacyl-, and syringylpropanol monomeric units, which are generated from L-phenylalanine via the phenylpropanoid pathway (**Figure 1**). L-Phenylalanine is converted to *trans*-cinnamic acid and to monolignols: *p*-coumaryl, coniferyl, and sinapyl alcohol. Earlier work (*1*) reported that the conversion of *p*-hydroxyphenyl to guaiacyl and then to syringyl units occurred at the cinnamic acid level. Thus, *p*-coumaric, ferulic, and sinapic acids serve as the precursors of *p*-coumaryl, coniferyl, and sinapyl alcohols, respectively. However, recent work in some angiosperms (*2*, *3*) revealed no activity of 4-coumarate-CoA ligase (4CL) with a sinapic acid substrate. Alternative pathways for the formation of sinapyl alcohol have subsequently been proposed (*4*, *5*). The conversion of guaiacyl to syringyl subunits may occur at the cinnamoyl-CoA level (**10** → **6** → **2** in **Figure 1**) (*6*) or at the cinnamyl alcohol level (**12** → **8** → **4** in **Figure 1**) (*7*, *8*). Alternatively, the conversion may occur at the level of cinnamyl aldehydes (**11** → **7** → **3** in **Figure 1**) (*9*). Recently, a specific enzyme, sinapyl alcohol dehydrogenase (SAD), which catalyzes the reduction of sinapaldehyde to synapyl alcohol (*10*), was identified; this led to the proposal that the syringyl monolignol pathway from coniferaldehyde to sinapyl alcohol occurred via sinapaldehyde.

In general, tracer experiments using isotope-labeled precursors offer a reliable method for studying biosynthetic pathways. In this study, dimethoxy deuterium-labeled syringyl precursors (heptadeuterosinapic acid-[8-D, 3,5-OCD_3]) were used to determine whether sinapic acid is the real precursor of syringyl lignin. The labeled precursors were traced using lignin degradation analysis of newly formed xylem of robinia and oleander. The results suggest that sinapic acid is converted to sinapyl alcohol via sinapoyl-CoA during in vivo lignin biosynthesis in robinia and oleander trees.

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MATERIALS AND METHOD

Synthesis of Labeled Precursors. The sequence of synthesis of heptadeuterosinapic acid from 2,6-dibromophenol is shown in **Figure 2**.

The synthesis of 2,6-dimethoxyphenol-[2,6-OCD_3] was based on the method of Rao and Stuber (*11*). In a two-neck round-bottom flask fitted with a downward distillation condenser, thermometer, and magnetic stirring bar, freshly cut sodium (1.0 g, 0.043 mol) was dissolved in 20 mL of dry methanol-d_4 (Isotech Inc., Miamisburg, OH). After distillation of 6−7 mL of methanol, a solution of 2,6-dibromophenol (3.0 g, 0.012 mol) and anhydrous copper(II) chloride (1.1 g, 0.008 mol) in 15 mL of dimethylformamide was added. The distillation was continued while the temperature of the oil bath was maintained at 110−115 °C. After 1 h, the reaction mixture was cooled to 100 °C (total methanol distilled = 16 mL), diluted with water (8 mL), then acidified with 6 N hydrochloric acid (15 mL), and extracted with ethyl acetate (2 × 20 mL). The ethyl acetate phase was washed with water (2 × 10 mL), dried with magnesium sulfate, and evaporated in vacuo. The products were redissolved in ethyl acetate, applied to a silica gel column (Wakogel C-200), and eluted with ethyl acetate/hexane (1:4). Fractions containing 2,6-dimethoxyphenol-[2,6-OCD_3] were combined and dried in vacuo to a total yield of 1.29 g (67%).

The synthesis of syringaldehyde-[3,5-OCD_3] was based on the method of Allen and Leubner (*12*). A well-stirred mixture of glycerol

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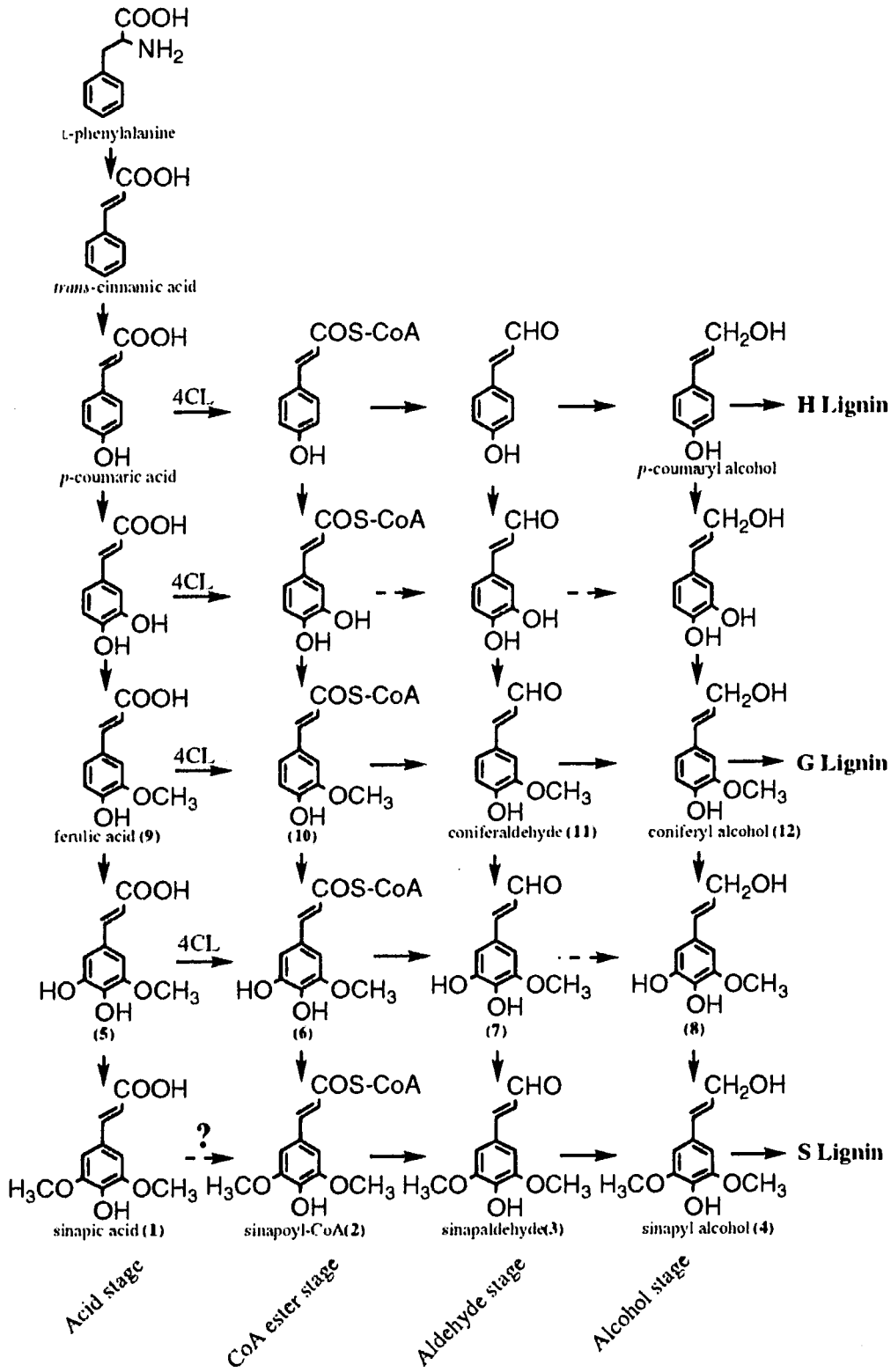

Figure 1. Overview of the phenylpropanoid pathway.

(7.4 mL) and boric acid (2.16 g, 0.035 mol), in a three-neck round-bottom flask fitted with a condenser, thermometer, and stirring apparatus, was dehydrated by heating in an oil bath to exactly 170 °C. This temperature was maintained for 30 min and then allowed to drop. When the temperature reached 150 °C, a mixture of 2,6-dimethoxyphenol-[2,6-OCD_3] (1.6 g, 0.01 mol) and hexamethylenetetramine (1.54 g, 0.011 mol) was added as rapidly as possible through the neck holding the thermometer. The temperature dropped to ~125 °C. Heating was rapid initially but was slowed when the temperature reached 145 °C and then stopped at 148 °C. The temperature was maintained at 150−160 °C for ~6 min. At the end of this reaction time the mixture was cooled to 110 °C as rapidly as possible, and concentrated sulfuric acid (1.84 mL) in water (6.2 mL) was added to the reaction mixture. After 1 h of stirring, the mixture was cooled to 25 °C in an ice bath. The boric acid, which separated from the solution, was removed by filtration and washed with 4 mL of water. The filtrate and washings were combined and extracted with chloroform (3 × 10 mL). The chloroform solution was evaporated in vacuo. The products were redissolved in ethyl acetate, applied to a silica gel column (Wakogel C-200), and eluted with ethyl acetate/hexane (1:1), and the fractions containing syringaldehyde-[3,5-OCD_3] were combined and dried in vacuo to a total yield of 0.68 g (40.5%).

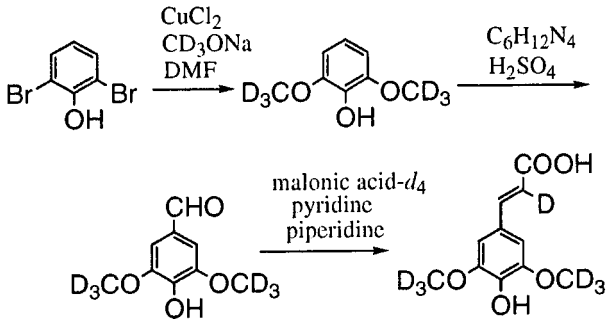

Figure 2. Synthesis of heptadeuterosinapic acid-[8-D, $(-OCD_3)_2$] from 2,6-dibromophenol.

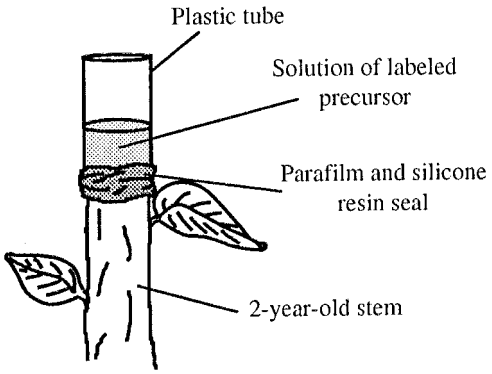

Figure 3. Administration of labeled precursor to living trees of robinia and oleander.

Heptadeuterosinapic acid-[8-D, 3,5-OCD_3] was synthesized by reacting hexadeuterosyringaldehyde-[3,5-OCD_3] with malonic acid-d_4 (99%; Isotech Inc.). The melting point of heptadeuterosinapic acid was 193 °C. ^{1}H NMR (Jemini-2000; JEOL, Tokyo, Japan) in acetone-d_6 with tetramethylsilane (TMS) as the internal standard resulted in chemical shifts of δ 6.40 (1H, d, J = 15.9 Hz, C_8H), 7.01 (2H, s, Ar−H), and 7.58 (1H, m, C_7H). Heptadeuterosinapic acid was trimethylsilylated in pyridine and *N,O*-bis(trimethylsilyl)trifluoroacetamide. MS (Mstation JMS-700; JEOL) of trimethylsilylated heptadeuterosinapic acid resulted in peaks at *m/z* (relative intensity) of 375 (100), 374 (41), 360 (47), 342 (86), 341 (37), 327 (20), 253 (14), and 73 (42).

Trideuterovanillin-[3-OCD_3] was synthesized from 3,4-dihydroxybenzaldehyde according to the method of Umezawa et al. (*13*).

Tetradeuteroferulic acid-[8-D, 3-OCD_3] was synthesized by reacting trideuterovanillin-[3-OCD_3] with malonic acid-d_4. The melting point of tetradeuteroferulic acid was 173 °C. ^{1}H NMR in acetone-d_6 with TMS as the internal standard resulted in chemical shifts of δ 6.37 (1H, d, J = 15.9 Hz, C_8H), 6.87 (1H, d, J = 8.4 Hz, Ar−H), 7.12 (1H, d, J = 9.0 Hz, Ar−H), 7.32 (1H, s, Ar−H), and 7.6 (1H, m, C_7H). Tetradeuteroferulic acid was trimethylsilylated in pyridine and *N,O*-bis(trimethylsilyl)trifluoroacetamide. MS of trimethylsilylated tetradeuteroferulic acid resulted in peaks at *m/z* (relative intensity) of 342 (100), 341 (37), 327 (45), 309 (40), 294 (18), 253 (33), and 73 (45).

The methoxy groups of both precursors (heptadeuterosinapic acid and tetradeuteroferulic acid) were completely labeled with deuterium. About 60−70% of the 8-hydrogens in the side chain were substituted with deuterium.

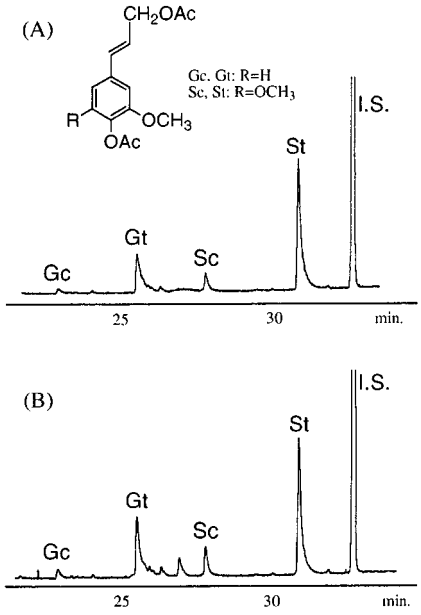

Figure 5. Gas chromatograms of DFRC monomers derived from the differentiating xylem of robinia (A) and oleander (B). G_c, *cis*-4-acetoxyconiferyl acetate; G_t, *trans*-4-acetoxyconiferyl acetate; S_c, *cis*-4-acetoxysinapyl acetate; S_t, *cis*-4-acetoxysinapyl acetate; I.S., internal standard (docosane).

Plant Material and Administration of Precursors. As shown in **Figure 3**, the upper parts of 2-year-old shoots of robinia (*Robinia pseudoacacia* L.) and oleander (*Nerium indicum* Mill.) trees that were growing on the campus of Nagoya University were removed in July. A small well was made at the top of the perpendicular stem, and the well was filled with 50 mL (robinia) or 25 mL (oleander) of a 4 mM aqueous solution of the precursor in a 4:6 (v/v) mixture of 66.7 mM KH_2PO_4 and 66.7 mM Na_2HPO_4 (pH 7.1) (**Figure 3**). After 4 weeks, each shoot was harvested and put into an 80% ethanol solution to remove extractives. Then, it was trimmed with a blade to a small block containing the cambium zone and differentiating xylem (longitudinal direction = 15 mm, tangential direction = 5 mm, radial direction = 5−10 mm). The block was frozen and fixed with ice on the freezing stage of a sliding microtome. One hundred micrometer thick tangential sections were cut successively from the surface of the bark-free xylem with a sliding microtome, as described previously (*14*).

Analysis of Lignin. The derivatization followed by reductive cleavage (DFRC) method of Lu and Ralph (*15*) was used to degrade the lignin. Acetylated derivatives were analyzed by GC-MS. Mass spectra were recorded at 70 eV with an Mstation JMS 700 mass spectrometer, coupled to an HP 6890 gas chromatograph (Hewlett-Packard, Wilmington, Del., USA) with a fused silica capillary column (TC1, 30 m × 0.25 mm i.d.; GL Sciences Inc., Tokyo, Japan). The sample (3 μL) was injected at 220 °C. The temperature was programmed to increase from 150 to 300 °C at 3 °C/ min, and the carrier gas was helium.

RESULTS AND DISCUSSION

A tracer experiment, using a stable isotope-labeled precursor, heptadeuterosinapic acid, was designed to study the pathway

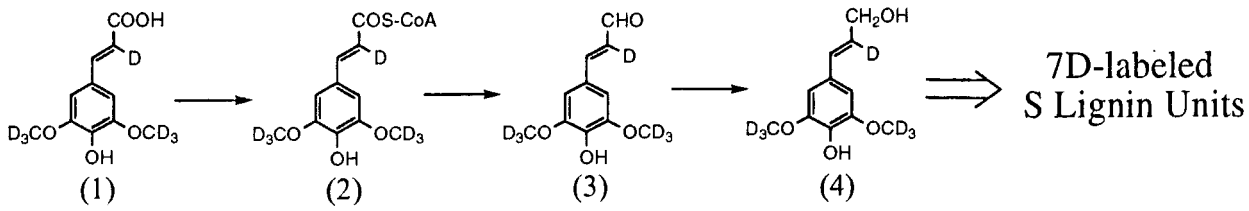

Figure 4. Possible pathways from heptadeuterosinapic acid-[8-D, $(-OCD_3)_2$] (**1**) to seven-deuterium (7D)-labeled syringyl lignin.

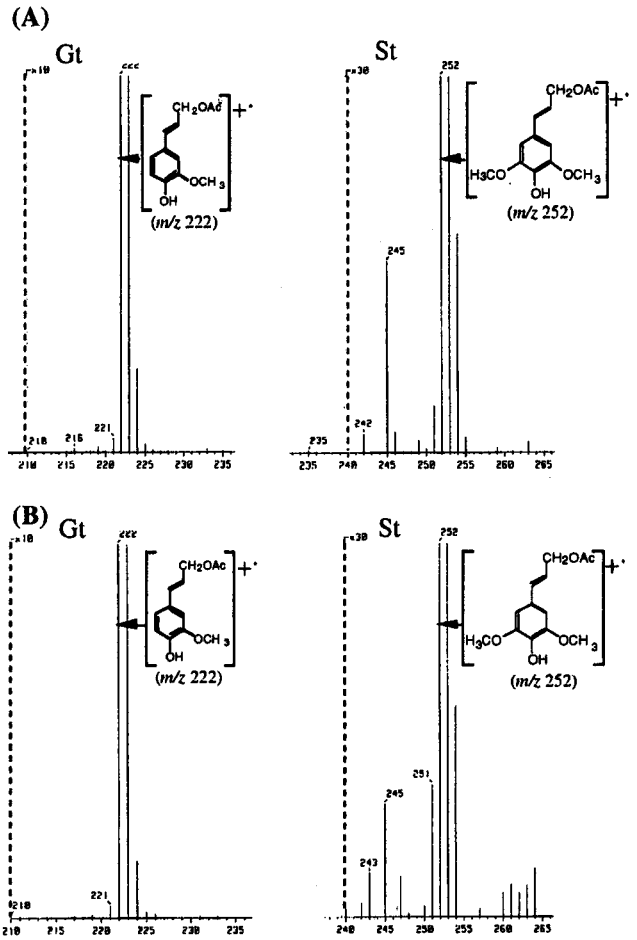

Figure 6. Partial EI mass spectra of DFRC products (G_t and S_t) from robinia (A) and oleander (B) fed nonlabeled ferulic acid as a control. The intensities (*Y* axis) were extended by 10- or 30-fold to compare with those of labeled peaks (**Figures 7** and **8**).

Table 1. S/G Ratios in DFRC Monomers (G and S) from Robinia and Oleander[a]

	robinia			oleander		
	normal	fed with FA[b]	fed with SA[c]	normal	fed with FA[b]	fed with SA[c]
S/G ratio	3.50	3.70	3.10	3.63	4.40	3.65

[a] The sections used for these measurements were between 500 and 600 mm from the cambium. [b] Fed with labeled ferulic acid. [c] Fed with labeled sinapic acid.

from sinapic acid to sinapyl alcohol via sinapoyl-CoA. Sinapic acid, labeled at both methoxy groups, is an excellent study tool because the amount of deuterium in the degradation products of newly formed lignin in a living tree can be counted directly using GC-MS or nuclear magnetic resonance. Labeled sinapic acid, heptadeuterosinapic acid-[8-D, 3,5-OCD_3], was synthesized in three steps from 2,6-dibromophenol (**Figure 2**), and labeled ferulic acid, tetradeuteroferulic acid-[8-D, 3-OCD_3], was synthesized as previously described. Methoxy groups of both sinapic and ferulic acid were completely labeled with deuterium; however, only 60−70% of their side chains' 8-hydrogens were deuterium-substituted. This loss of deuterium was caused by the substitution of 2-deuterium in labeled malonic acid for hydrogen in the reaction medium before condensation. The substitution of the 2-deuterium for hydrogen in labeled malonic acid occurred easily in pyridine, as determined by ^{1}H NMR (data not shown).

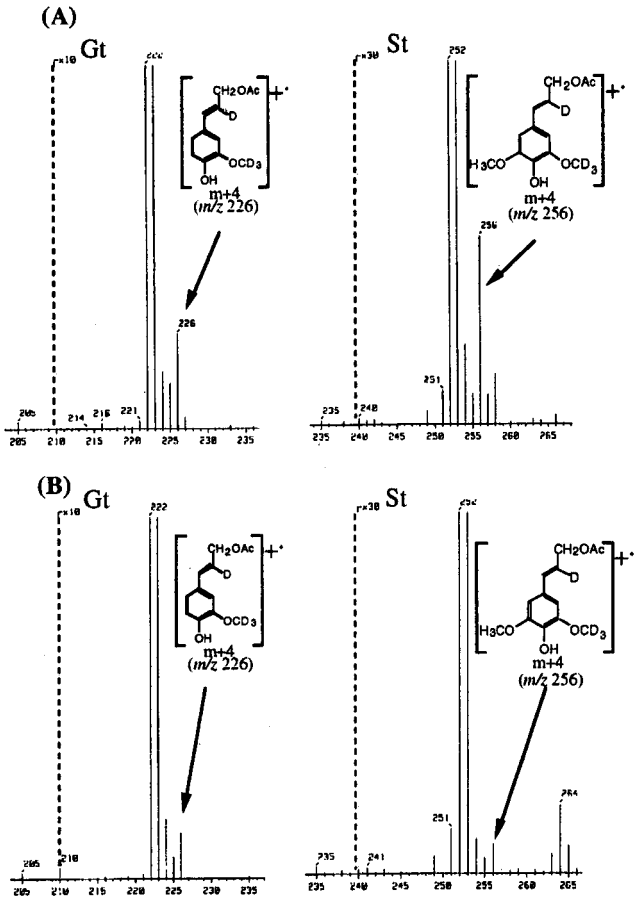

Figure 7. Partial EI mass spectra of DFRC products (G_t and S_t) from robinia (A) and oleander (B) fed tetradeuteroferulic acid-[8-D, $-OCD_3$].

Labeled lignin was analyzed by the DFRC method, which is well suited for tracer analysis, because it selectivity cleaves *β-O*-4 ether-linked structures in lignin to form C_6-C_3 monomers, retaining the labeled *β*-deuterium of the side chains. Interpretation of the mass spectral data is facilitated by the fact that the acetylated DFRC monomers are composed of C, H, and O atoms, which each have fairly rare naturally occurring isotopes. Thioacidolysis cleaves at the same linkages as DFRC but yields a base peak of C_6-C_1 structures on electron ionization mass spectrometry (EI-MS). The products of thioacidolysis contain S and Si atoms after trimethylsilylation for gas chromatography−mass spectrometry (GC-MS), and the presence of naturally occurring isotopes of S and Si make it more difficult to distinguish the D-enriched peak from the naturally occurring isotopic peaks in EI-MS analysis of the derivative compounds.

As shown in **Figure 4**, heptadeuterosinapic acid (**1**) was administered to living trees and incorporated into lignin. If the deuterium-labeled methoxy group of heptadeuterosinapic acid was retained during incorporation into lignin, seven deuterium (7D)-labeled syringyl units should be detected by GC-MS. This would confirm the existence of a route from sinapic acid to sinapyl alcohol via sinapoyl-CoA (**1** → **2** → **3** → **4** in **Figure 4**) and would support the traditionally accepted pathway in which sinapic acid is the precursor of syringyl lignin (*1*). If one deuterium-labeled methoxy group of heptadeuterosinapic acid was released by demethylation or demethoxylation, four deuterium (4D)-labeled syringyl units could be detected (**5**−**12** in **Figure 1**). If sinapic acid is not a precursor of syringyl lignin, however, no labeled syringyl lignin should be detected.

The gas chromatograms and mass spectra of guaiacylpropane (G) and syringylpropane (S) monomers derived by DFRC from

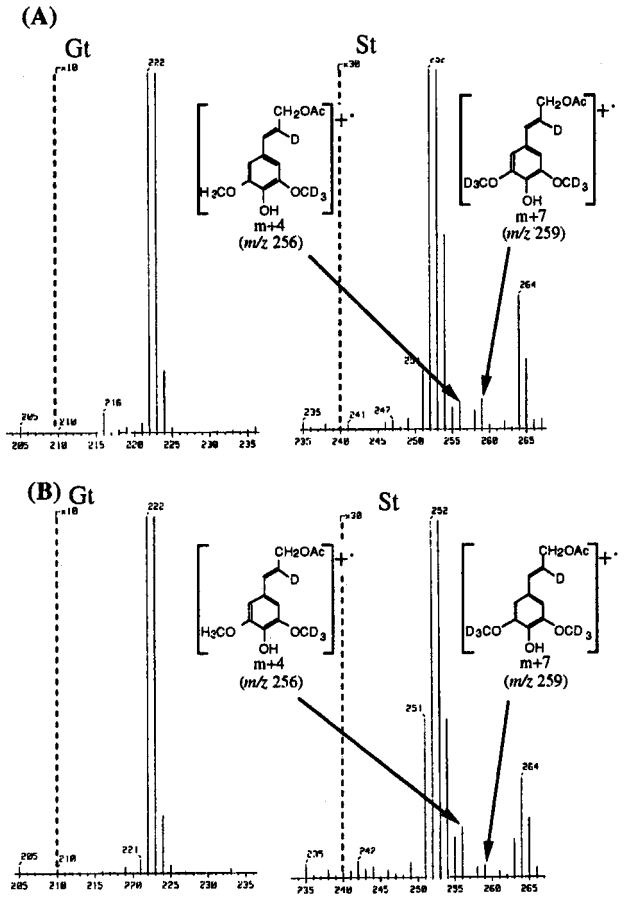

Figure 8. Partial EI mass spectra of DFRC products (G_t and S_t) from robinia (A) and oleander (B) fed heptadeuterosinapic acid-[8-D, 3,5-$OCD_3)_2$].

the differentiating xylem of robinia and oleander fed nonlabeled ferulic acid are shown in **Figures 5** and **6** and were used as control profiles. Each of the monomers occurred in cis and trans isomeric forms. The trans isomers, *trans*-4-acetoxyconiferyl acetate and *trans*-4-acetoxysinapyl acetate, were used to estimate the incorporation of D from the precursors (**Figure 5**). The molecular ion peaks for G and S monomers are at *m*/*z* 264 and 294, respectively. In general, aromatic acetates easily lose a ketene group (*m* 42). For G and S monomers, this loss results in base peaks at *m*/*z* 222 and 252, respectively (M − 42) (**Figure 6**). **Table 1** shows the S/G ratio of the lignin fed with labeled ferulic acid or sinapic acid and normal lignin calculated from yields of DFRC monomers. The S/G ratios of newly formed lignin after feeding were similar to those of normal lignin.

Partial mass spectra of DFRC products from the differentiating xylem of robinia and oleander fed tetradeuteroferulic acid or heptadeuterosinapic acid are shown in **Figures 7** and **8**, respectively. When fed tetradeuteroferulic acid, 4D-labeled guaiacyl (*m* + 4, *m*/*z* 226) and 4D-labeled syringyl (*m* + 4, *m*/*z* 256) monomers were observed (**Figure 7**), indicating that ferulic acid was converted into guaiacyl and syringyl lignin monomers (coniferyl alcohol and sinapyl alcohol, respectively). When fed heptadeuterosinapic acid, no D-enriched peaks were present in guaiacyl lignin monomers, but 4D (*m* + 4, *m*/*z* 256) and 7D (*m* + 7, *m*/*z* 259) labels were detected in syringyl monomers (**Figure 8**). The observation of 7D-labeled syringylpropane units suggested that sinapic acid was converted into syringyl lignin without loss of labeled methyl groups (**1** → **2** → **3** → **4** in **Figures 1** and **4**). This result confirms that sinapic acid is converted into sinapyl alcohol via sinapoyl-CoA, as previously proposed (*1*). The observation of 4D-labeled syringylpropane units indicated that sinapic acid is converted into syringyl lignin with the release of a single methyl group, suggesting that sinapic acid may be demethylated or demethoxylated during syringyl lignin biosynthesis. If the sinapic acid was converted to ferulate, it should be incorporated into 4D-labeled guaiacylpropane lignin, but this was not detected. Perhaps sinapic acid is demethylated to form 5-hydroxyferulate but is not dehydroxylated to form ferulate; this hypothesis requires further clarification. According to Ishihara and Miyazaki (*16*), laccase from *Coriolus versucolor* can demethylate methoxylated phenols. It is not known whether laccase from woody plants can also catalyze this demethylation.

Biosynthesis of a lignin precursor has generated considerable interest. The formation of sinapyl alcohol has been a point of some confusion, although most recent studies agree that no 4CL activity occurs with a sinapic acid substrate (*2*, *3*, *17*). A recent report of work in aspen describes the isolation of sinapyl alcohol dehydrogenase (SAD), which catalyzed the conversion of sinapylaldehyde from coniferylaldehyde to sinapyl alcohol (*10*). In contrast, our study shows that sinapylaldehyde is generated from sinapic acid via sinapoyl-CoA in robinia and oleander trees. The combination of tracer experiments and enzyme assays will contribute to a complete understanding of the mechanism of lignin biosynthesis.

ABBREVIATIONS USED

4CL, 4-coumarate-CoA ligase; DFRC, derivatization followed by reductive cleavage; GC-MS, gas chromatography−mass spectrometry; EI-MS, electron ionization mass spectrometry.

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