Biosynthesis of Liriodendrin by *Liriodendron tulipifera**

Hideto FUJIMOTO** and Takayoshi HIGUCHI**

Abstract——^{14}C-Labeled lignin precursors (L-phenylalanine-U-^{14}C, ferulic acid-2-^{14}C, coniferyl alcohol-2-^{14}C, sinapic acid-2-^{14}C and sinapyl alcohol-2-^{14}C) were administered to young twigs of a yellow poplar (*Liriodendron tulipifera*). Sinapyl alcohol was most effectively incorporated into liriodendrin (dilution value, 6.5～13.5) and syringaresinol (dilution value, 1.1), and followed by L-phenylalanine. However, the incorporation of sinapic acid was rather low and coniferyl alcohol was scarcely incorporated. Liriodendrin isolated as its octaacetate and syringaresinol were all levorotatory. Biogenetic relationship between liriodendrin and syringyl lignin was discussed.

Introduction

It is well known that lignin and its related compounds—degradative products and intermediates of lignin—are optically inactive, in spite of the presence of many asymmetric carbons in the molecules. For example, FREUDENBERG et al. reported that both (±)-syringaresinol and (±)-pinoresinol as intermediates of lignin were obtained in dehydrogenative polymerization of sinapyl and coniferyl alcohols, respectively[1].

On the other hand, optically active lignans, whose constitutional formulae are identical with those of lignin intermediates, have been extracted from many plants. For example, (+)-pinoresinol from the resin of *Pinus* and (+)-syringaresinol from the bark of *Liriodendron*, were reported[2]. Fundamental differences between optically active and inactive dilignols should be derived from their different biosyntheses.

The optical inactivity of lignin is explained as the result of random coupling of phenoxy radicals of hydroxycinnamyl alcohols formed by peroxidase.

On the other hand, the investigation of biosynthesis of lignan is very few and the oxidative coupling of phenylpropanoids as in lignin biosynthesis is speculated to be the most possible pathway. This speculation is based on the fact that almost all lignans are constituted from phenylpropanoids which have hydroxyl groups in *p*-position and are closely related to monolignols.

In this investigation, in order to obtain information on biosynthesis of lignans, the incorporation of ^{14}C-labeled phenylpropanoids by a yellow poplar tree into

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** Division of Lignin Chemistry.

liriodendrin (syringaresinol diglucoside) was examined.

Experimental

1. *Preparation of phenylpropanoids*

L-Phenylalanine-U-^{14}C purchased from J.R.I.A. was diluted with non labeled L-phenylalanine to give suitable radioactivity.

Ferulic acid-2-^{14}C and sinapic acid-2-^{14}C were kindly offered by Mr. NAKAMURA in this laboratory.

Sinapyl alcohol-2-^{14}C (I) was synthesized by the method of KRATZL et al.[3] A drop of 10% HCl was added to sodium malonate-2-^{14}C (about 5 μCi) and the acid solution was dried up at a reduced pressure. 1.2 ml of pyridine, two drops of aniline, 306.3 mg of non labeled malonic acid and 352.0 mg of syringaldehyde were added. The solution was kept overnight at a room temperature and then heated at 60°C for 1 hr. 301.4 mg of malonic acid was added again and after 4 hr, 12 ml of 5% HCl was added. The precipitate of sinapic acid was filtered and dried *in vacuo*. Sinapic acid (362.1 mg) was acetylated for 24 hr in a mixture of 1.2 ml pyridine and 1 ml acetic anhydride. The acetate precipitated into ice water was filtered and dried over silicagel *in vacuo*. The acetyl sinapate (360.4 mg) was dissolved into 6 ml of thionyl chloride and heated at 60~64°C for 2 hr. The reaction mixture was concentrated to dryness *in vacuo*, and the product was recrystallized from benzene. The radioactive acetyl sinapic chloride (319.1 mg) diluted with the cold chloride (1826.1 mg) was dissolved in a mixture of dry benzene and ether with stirring. The solution was cooled at 0°C under nitrogen, and was added $LiAlH_4$ in 55 ml of abs. ether dropwisely for 2 hr. The reaction mixture was kept at 0°C for 2 hr, and then warmed gradually to a room temperature. After 20 hr yellowish precipitate was filtered under a reduced pressure, and added into the well cooled ammonium carbonate solution covered with ether. The aqueous layer was extracted with ether, and the ether solution was dried over sodium sulfate and evaporated. The oily product which did not crystallize from $CHCl_3$-petroleum ether was purified by TLC and analyzed by UV spectrometry.

○ Sinapyl alcohol (I) (597 mg), total activity 1.15 μCi, specific activity 0.407 μCi/mM.

○ Sinapyl alcohol-2-^{14}C and coniferyl alcohol-2-^{14}C used in the second feeding experiment were prepared by the same procedure.

○ Sinapyl alcohol (II) (1.73 g), total activity 12.64 μCi, specific activity 1.54 μCi/mM.

○ Coniferyl alcohol (1.78 g), total activity 11.65 μCi, specific activity 1.18 μCi/mM.

2. *Feeding experiment*

a) *Preliminary experiment*

Young twigs (shoots) of a yellow poplar tree grown in the campus of the Wood Research Institute, Kyoto University, were collected in August, 1974. Non labeled sinapyl alcohol (207.6 mg) and coniferyl alcohol (200.4 mg) were dissolved in each 50 ml of 0.025 M phosphate buffer (pH 7.0) and fed to 30.4 g and 30.2 g of sliced bark tissues of the shoots. The absorption of the alcohols by the tissues was accomplished by evacuation in a vacuum desiccator. After standing for 10 hr at 26°C, the tissue was homogenized in hot EtOH and the ethanolic extract was treated as shown in Fig. 1.

Yield of liriodendrin octaacetate

Control	50.8 mg
Coniferyl alcohol fed	42.8 mg
Sinapyl alcohol fed	59.4 mg

b) *Experiment with radioactive sinapyl alcohol (I)*

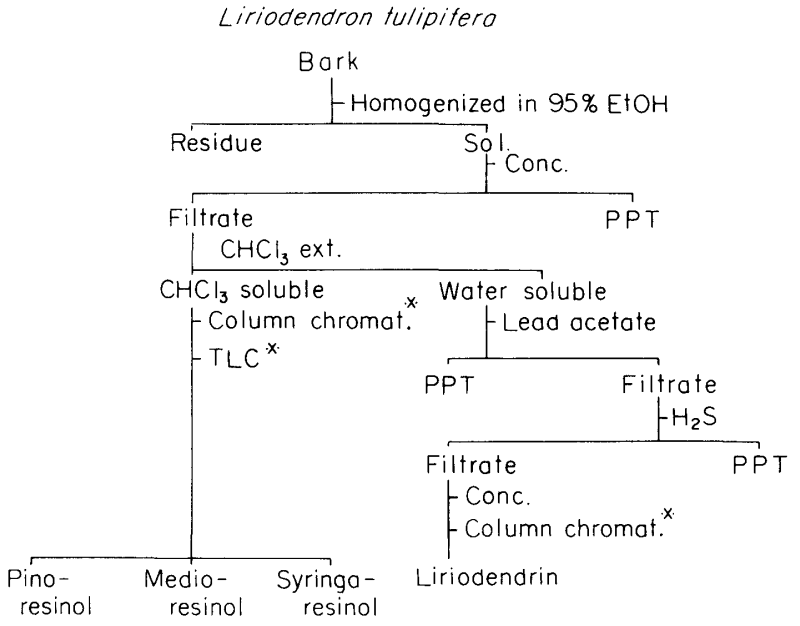
A young twig (shoot) of the same yellow poplar in experiment a) was collected in September, 1974. ^{14}C-Labeled sinapyl alcohol (I) (594.3 mg, 0.47 μCi/mM) was dissolved in 100 ml of distilled water and administered to the twig from the cut end. After absorption of the fed solution, the plant was allowed to metabolize for two days at a room temperature, and then the bark was peeled and homogenized in 95% EtOH. The ethanolic extract was treated by the same procedure shown in Fig. 1, and syringaresinol was isolated from $CHCl_3$ layer.

c) *Experiment with radioactive sinapyl alcohol (II) and other phenylpropanoids*

Young twigs (shoots) of the same yellow poplar tree were collected in June, 1975, and the following compounds were administered; L-phenylalanine-U-^{14}C, ferulic acid-2-^{14}C, coniferyl alcohol-2-^{14}C, sinapic acid-2-^{14}C, and sinapyl alcohol-2-^{14}C. All the compounds fed were dissolved in 200 ml of water respectively. Ferulic and sinapic acids were fed as their sodium salts. The fed plants were kept under illumination in a draft chamber, and each twig sucked up the solution almost in a day. Then, distilled water was added and the plants were allowed to metabolize for 3 days. The barks were peeled and treated by a similar procedure described above.

3. *Isolation of liriodendrin, syringaresinol and pinoresinol*

As shown in Fig. 1, the peeled bark was homogenized in 95% EtOH with a ULTRA-TURRAX-homogenizer, and the homogenate was filtered under a reduced pressure. The filtrate was concentrated to 20~30 ml to give an oily substance. The concentrated solution was extracted with hexane, and the aqueous layer was then extracted with $CHCl_3$. The $CHCl_3$ layer contained syringaresinol, medioresinol



* : See the experimental section

Fig. 1. Separation and isolation of resinols and their glucosides.

and pinoresinol[4], and the aqueous layer contained their glucosides. The $CHCl_3$ layer was applied to silicagel column chromatography and eluted with MeOH-$CHCl_3$ (5 : 95). The resinol fraction which was detected on TLC by a purple coloration with vanillin-sulfuric acid reagent was then applied to silicagel preparative TLC, developed with EtOAc-cyclohexane (1 : 1). Crystalline syringaresinol was isolated from only sinapyl alcohol (I) fed plant. In other cases, any of syringaresinol, pinoresinol and medioresinol could not be isolated. Then, authentic pinoresinol and syringaresinol prepared by dehydrogenative polymerization of corresponding alcohols were added to the respective fractions. The radioactive products were then isolated by preparative TLC, and purified by recrystallization from EtOH. Accordingly the optical activity and dilution value of these resinols, except of the syringaresinol from sinapyl alcohol (I) fed twigs, could not be determined.

To the aqueous layer, which contains liriodendrin, an excess of saturated basic lead acetate solution was added. Yellowish precipitate was filtered off with the aid of Hyflo-super-cell, under a reduced pressure. The excess of the lead was precipitated with H_2S, the solution was filtered with the aid of Hyflo-super-cell, and the filtrate was concentrated *in vacuo*. The crystalline liriodendrin could not be obtained and then, the aqueous solution was submitted to sephadex column chromatography (G-15) eluted with water, cellulose column chromatography (Whatmann CF-11), the upper layer of BuOH-AcOH-H_2O (4 : 1 : 5), and silicagel column chromatography (Wakogel), MeOH-$CHCl_3$ (20 : 80), successively. By these procedures liriodendrin

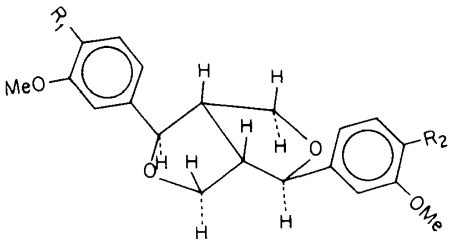
was obtained as crystal from MeOH. The crystals were filtered and acetylated by the following procedure; about 15~20 mg of liriodendrin was suspended in a mixture of 0.1 ml of pyridine and 0.1 ml of acetic anhydride and heated at 95°C for 45 min. The reaction mixture was cooled at a room temperature and allowed to react overnight. The solution was evaporated and the residue was decolored with active charcoal in hot MeOH, and recrystallized from 95% EtOH.

Radioactivity and optical activity of the compounds were measured by a Beckman LS-100 liquid scintillation counter and a Parkin-Elmer polarimeter model 241, respectively.

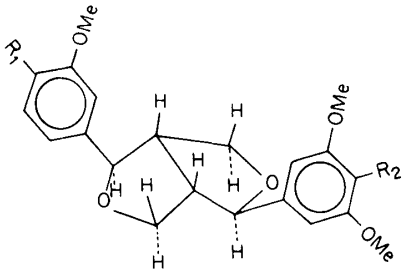
Results

Preliminary feeding experiment of non labeled sinapyl alcohol and coniferyl alcohol showed no significant increase in amount of liriodendrin octaacetate obtained from fed plants. The differences in amount between fed and control plants were about 10 to 20%, which seems to be within an experimental error after purification by preparative TLC and repeated recrystallization.

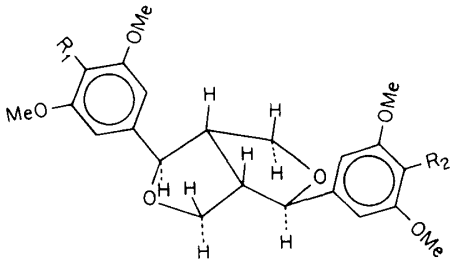
Then ^{14}C-labeled sinapyl alcohol was fed to a young twig of a yellow poplar



R_1 , R_2 = H : (+)-Pinoresinol
R_1=H, R_2= Glu : Pinoresinol monoglucoside*
R_1 , R_2= Glu : Pinoresinol diglucoside*



R_1 , R_2 = H : (+)-Medioresinol
R_1=H, R_2= Glu) : Medioresinol monoglucoside*
R_1=Glu, R_2=H)
R_1 , R_2 = Glu : Medioresinol diglucoside*
* : Not isolated



R_1 , R_2 = H (+)-Syringaresinol
R_1=H, R_2= Glu : Syringaresinol monoglucoside
R_1 , R_2=Glu : Syringaresinol diglucoside (Liriodendrin)

Fig. 2. Resinols and their glucosides in yellow poplar.

tree and examined whether or not the alcohol is true precursor of liriodendrin. The radioactivity of the compounds and dose for the plant are shown in Table. 1. The weight of whole twigs, to which each phenylpropanoid was administered, and of the bark are shown in Table 2. The incorporation of sinapyl alcohol is shown in Table 3. It seems important that syringaresinol, as a free form, which was never obtained from control plant, was isolated in this experiment. Dilution value of

Table 1. Radioactivity of the compounds administered.

	Compounds administered				
	L-Phenyl-alanine	Ferulic acid	Coniferyl alcohol	Sinapic acid	Sinapyl alcohol (II)
Weight (mg)	566.0	517.0	222.1	516.2	522.2
Specific activity (μCi/mM)	0.140	0.972	1.180	0.906	1.538
Total activity (μCi)	0.481	2.119	1.456	1.802	3.823

Table 2. Fresh weight of twigs of a yellow poplar tree.

	Compound administered					
	L-Phenyl-alanine	Ferulic acid	Coniferyl alcohol	Sinapic acid	Sinapyl alcohol (II)	Control
Whole twig (g)	390.4	356.0	360.0	343.2	381.4	470.0
Bark (g)	80.4	65.0	61.0	78.2	81.4	70.0
Xylem and leaves (g)	310.0	295.0	295.0	265.0	300.0	400.0

Table 3. Incorporation of sinapyl alcohol (I).

	Compound administered	Product	
	^{14}C-Sinapyl alcohol (I)	Syringaresinol	Liriodendrin octaacetate
Dose (μmole/g fresh weight)	36.2	——	——
Yield (mg)	——	34.9	7.3
Total activity (μCi)	1.15	0.0615	0.0004
Specific activity (μCi/mM)	0.407	0.370	0.0579
Dilution value	——	1.05	6.52

Table 4. Utilization of phenylpropanpids by a yellow poplar tree.

Compound administered	Chloroform soluble (%)	PPT by lead acetate (%)	Liriodendrin (%)	Water soluble (%)	Total activity (%)
L-Phenylalanine-U-^{14}C	0.08	3.34	0.02	3.71	7.15
Ferulic acid-2-^{14}C	0.18	1.07	0.01	0.51	1.77
Coniferyl alcohol-2-^{14}C	0.08	0.11	0.00	0.44	0.63
Sinapic acid-2-^{14}C	0.49	2.29	0.00	0.48	3.26
Sinapyl alcohol-2-^{14}C (II)	0.31	0.52	0.08	0.74	1.64

the syringaresinol and liriodendrin octaacetate were only 1.05 and 6.52 respectively. Thus, the incorporation of sinapyl alcohol into syringaresinol and liriodendrin was found to be very good, and it was confirmed that sinapyl alcohol is an immediate

Table 5. Incorporation of various phenylpropanoids into liriodendrin.

Compound administered	Dose (μmole/g fresh wt.)	Specific activity (μCi/mmole)	Liridendrin octaacetate		
			Yield (mg)	Specific activity (μCi/mmole)	Dilution value
L-Phenylalanine-U-^{14}C	8.79	0.140	27.5	0.00363	38.6
Ferulic acid-2-^{14}C	6.06	0.971	12.1	0.00927	104.8
Coniferyl alcohol-2-^{14}C	3.46	1.180	22.7	0.00026	4540.3
Sinapic acid-2-^{14}C	5.63	0.906	30.2	0.00271	334.5
Sinapyl alcohol-2-^{14}C	6.54	1.540	27.9	0.11333	13.6

Table 6. Incorporation of phenylpropanoids into the resinols.

	Pinoresinol (%)	Syringaresinol (%)
L-Phenylalanine	0.0062	0.0108
Ferulic acid	0.0025	0.0056
Coniferyl alcohol	0.0047	0.0021
Sinapic acid	0.0055	0.0099
Sinapyl alcohol (II)	0.0008	0.0068

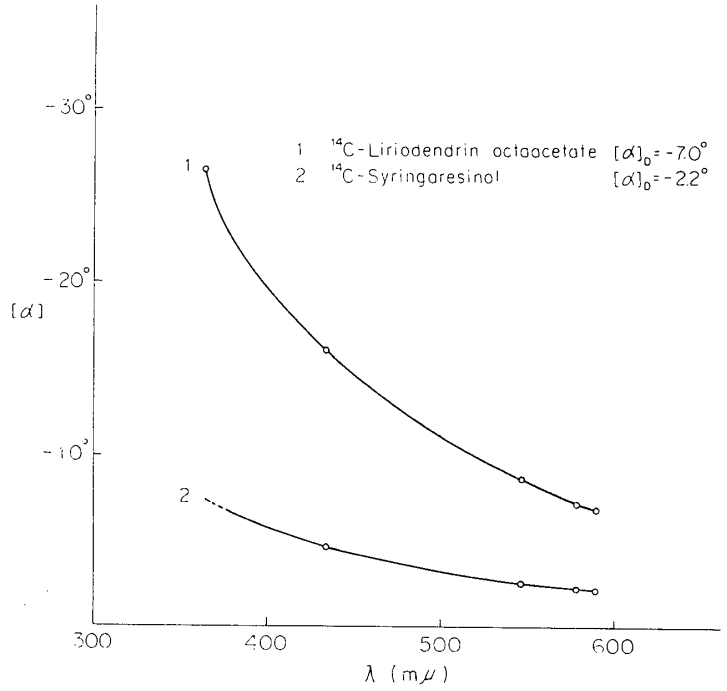


Fig. 3. Optical rotation of ^{14}C-liriodendrin octaacetate and ^{14}C-syringaresinol.

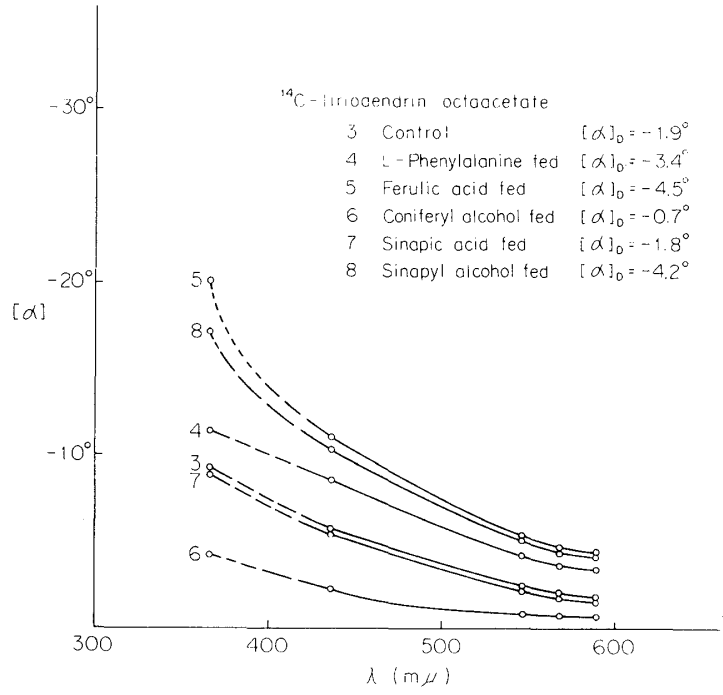


Fig. 4. Optical rotation of ^{14}C-liriodendrin octaacetate.

intermediate of syringaresinol and liriodendrin.

The incorporation of other lignin precursors in the second experiment is shown in Table 4~6. Again, sinapyl alcohol (II) was most effectively incorporated into liriodendrin, and the dilution value was 13.6. But in this experiment, syringaresinol. was not isolated and the incorporation of the alcohol was rather lower than that of sinapyl alcohol (I), which was fed in a large amount in the first experiment. L-Phenylalanine was incorporated into liriodendrin remarkably next to sinapyl alcohol. On the other hand, the incorporation of sinapic acid was rather low and coniferyl alcohol was scarcely incorporated. The optical activities of syringaresinol and liriodendrin octaacetate were slightly levorotatory, which are different from naturally occurring ones in a yellow poplar tree as shown in Fig. 3 and 4.

Discussion

The biogenetic pathway of lignin is outlined as follows.

L-Phenylalanine → → Ferulic acid → → Sinapic acid

↓ ↓

↓ ↓

Coniferyl alcohol Sinapyl alcohol

↓ ↓

Guaiacyl lignin Syringyl lignin

In this experiment, sinapyl alcohol which is an immediate precursor of syringyl lignin was most effectively incorporated into liriodendrin and syringaresinol. Then

it is clear that sinapyl alcohol is also a precursor of liriodendrin and syringaresinol. However, it is notable that all liriodendrin isolated as its octaacetate from this feeding experiment was levorotatory (Fig. 3, 4), whereas liriodendrin octaacetate obtained from a large scale extraction of old bark was dextrorotatory ($[\alpha]_D$+4.61°). The difference in optical activity of the liriodendrin and its acetate in both cases may be partly ascribed to the age of the bark of yellow poplar tree as found in the separate experimet. That is, the $[\alpha]_D$ of liriodendrin from a young twig (about 1～2 year old) was +3.86° in pyridine, while the $[\alpha]_D$ from an old bark (about 8～10 year old) was +8.93° in pyridine. Therefore it may be conceivable that two enantiomers are formed in a yellow poplar tree and the ratio of the amount of (+)-and (−)-liriodendrins differs between tissues. In the old bark, the ratio of (+)-isomer may be predominant. This may be the reason why the isolated liriodendrin as its octaacetate from very young twigs used in this feeding experiment was levorotatory.

In order to investigate the influence of acetylation conditions on optical rotation, liriodendrin of the same sample was acetylated by the following three different conditions; (1) liriodendrin was suspended in a mixture of pyridine and acetic anhydride (1 : 1) and allowed to react at a room temperature with stirring for 24 hr. (2) Liriodendrin was suspended in the same mixture and heated at 95°C for 45 min. and then allowed to react for 24 hr. at a room temperature the same condition adopted in this experiment. (3) liriodendrin was dissolved in the same mixture and heated at 95～97°C for 24 hr. The $[\alpha]_D$'s of liriodendrin octaacetate were 4.74°, 4.61° and 0.15° in $CHCl_3$ respectively. Except for the most drastic condition (3), the $[\alpha]_D$ is practically the same. It can therfore be presumed that the acetylation condition adopted in this feeding experiment is not drastic and that the negative value of the liriodendrin octaacetate is not ascribed to the acetylation conditions.

Syringaresinol was isolated from only the plant to which sinapyl alcohol (I) was fed in a large amount, and the dilution value of the resinol was remarkably low. However the optical rotation of the syringaresinol was again slightly levorotatory. Since the occurrence of (+)-syringaresinol ($[\alpha]_D$+18.96) is known in a yellow poplar tree[4], the formation of the radioactive (−)-or racemic syringaresinol may be ascribed to an anomalous metabolism derived from a large supply of sinapyl alcohol. Because it is well known that sinapyl alcohol gives (±)-syringaresinol by peroxidase and H_2O_2 as an intermediate of syringyl lignin.[5] This assumption was supported by the fact that the yield of syringaresinol decreased remarkably with the low dose of sinapyl alcohol as shown in Table 6. Therefore, the occurrence of (−)-syringaresinol in the first experiment may be explained as follows; (±) syringaresinol was formed as in lignin biosynthesis by random coupling of phenoxy radicals of

sinapyl alcohol which was fed in a large amount. (±)-syringaresinol is mainly incorporated to syringyl lignin but (+)-syringaresinol in the racemic compound would be partly consumed to form its glusocide, and then (−)-syringaresinol would be remained.

Next to sinapyl alcohol, L-phenylalanine was efficiently incorporated into liriodendrin. However, the dilution value of sinapic acid was much higher than that of L-phenylalanine. This findings may be explained by participation of multienzyme system in the biosynthesis of sinapyl alcohol. In multienzyme system it is often found that an initial substrate is more effectively incorporated than other intermediates[6]. Thus the result in the present investigation indicates that the biogenetic pathways of liriodendrin and syringyl lignin are common up to sinapyl alcohol as outlined, and the alcohol is incorporated into syringyl lignin dehydrogenatively by peroxidase but partly into (+)-syringaresinol and its glucoside stereospecifically by a special enzyme.

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