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ORIGINAL ARTICLE

Synthesis of highly deuterated coniferyl alcohol for silencing of NMR signals in the resulting dehydrogenative polymer

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Abstract

To establish a facile methodology for the elucidation of the lignin chain-growth mechanism, the preparation of monolignol that does not show NMR signals in the dehydrogenative polymer (DHP) was attempted. As a monolignol of which aliphatic moieties were deuterated, coniferyl alcohol-d_7 was successfully synthesized from protocatechual-dehyde and malonic acid via the modified Knoevenagel–Doebner reaction and the Luche reduction. The process achieved high to excellent deuteration efficiencies at the aimed positions (i.e., methoxy: > 99%D, α: > 99%D, β: 92%D, and γ: 98%D). DHP was prepared solely from coniferyl alcohol-d_7, and its NMR spectra were compared with those from coniferyl alcohol. The results indicated that: (1) the deuterium atoms at methoxy group, α- and β-positions were highly retained even in the DHP, and their signals were effectively suppressed; (2) a part of the deuterium at γ-position was replaced with H through the reaction; (3) meanwhile, the formation of γ-CH_2 was negligible. This study demonstrated that coniferyl alcohol-d_7 could "silence" the majority of the signals even when converted to DHP. Highly deuterated monolignols can be a unique molecular tool that can differentiate the signals of interest from those derived from monolignols.

Keywords: Coniferyl alcohol, Deuteration, Lignin, Dehydrogenative polymer, HSQC

Introduction

Lignin, one of the major components of wood, is biosynthesized by random radical coupling of coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol, which are termed monolignols. Despite its importance in wood cell walls, the detailed chain-growth mechanism of lignin is poorly understood due to the complicated factors in its process [1–10]. The preparation of dehydrogenative polymer (DHP) is a powerful technique to reproduce the lignin biosynthesis in situ and is an extremely useful bottom-up method to elucidate the chain-growth mechanism of lignin [1, 2, 5–12]. For this purpose, Matsushita et al. prepared DHPs from monolignol dimers

and demonstrated the involvement of radical mediator mechanisms based on their kinetics [11]. Moreover, by preparing DHPs in the presence of lignin–carbohydrate complex (LCC) model compound and monolignols, they carefully elucidated the chain-growth evolving from an LCC substructure [12]. The DHP preparation in the presence of such a precursor (here, we referred to it as "seed compound" to differentiate from "monolignol"s) must be a very promising methodology for clarifying the details of the growth mechanism of lignin, because the seed compounds can be diversified.

Nuclear magnetic resonance (NMR) analysis, mainly heteronuclear single quantum coherence (HSQC), is one of the most straightforward structural analysis methods currently used for lignins and DHPs [13, 14]. The procedure would be much simpler if the above-mentioned DHP strategy was coupled with NMR analyses. However, it would be practically difficult, because the signals

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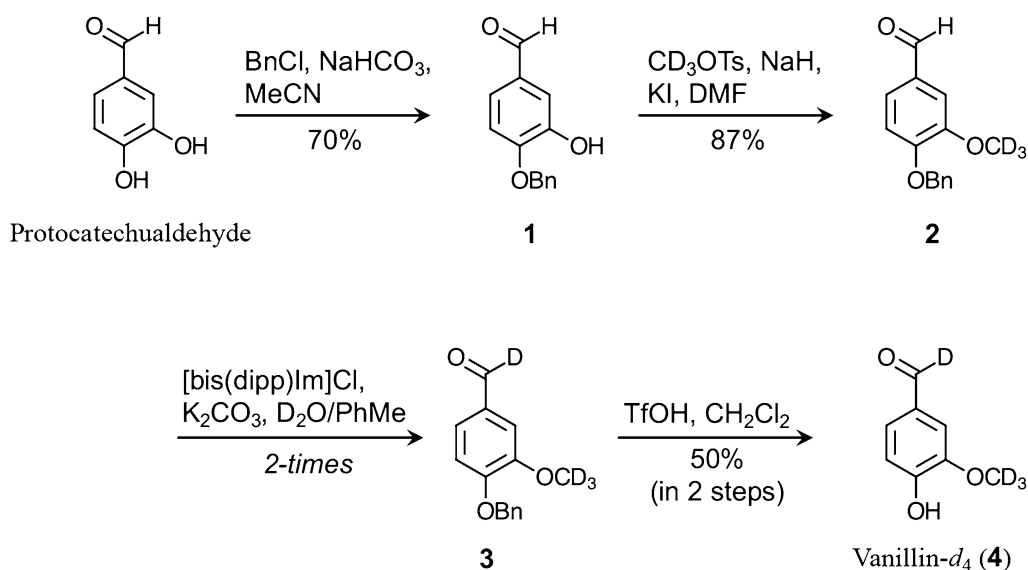
Scheme 1 Synthesis of vanillin-d_4

derived from "monolignol" can disturb the observation of signals of interest.

Deuterium (D) is a stable isotope of hydrogen and is one of the most readily available isotopic elements, because it is commercialized in a variety of forms as NMR solvents. Deuterium labeling of compounds is normally used to give an identifier to molecules for their "visibility". The deuterium labeling of monolignols has also been widely applied for this purpose, especially as tracers in the biosynthetic studies of lignin, lignans, or a kind of volatile phytochemicals [15–24]. Another exploit of deuteration is the "invisibility" in NMR analyses. Since the deuteron has a 0.965% relative sensitivity to the proton and requires largely different operating frequencies, it can not be observed under ^{1}H NMR experiments. In addition, the signals of the ^{13}C bound to D are attenuated to negligible levels in ^{13}C and HSQC measurements due to J_{CD} splitting and the lack of the nuclear Overhauser effect. Therefore, if the highly deuterated monolignol was available, it would be useful as a "silent" monolignol in the NMR of the above-mentioned DHP experiments. A similar effort had been previously made by Ralph et al., but it only focused on methoxy groups of monolignols [25]. To enable more detailed and simplified observations, we conceived that a more exhaustive signal suppression, especially on all aliphatic signals, would be required. In this study, we first attempted the synthesis of coniferyl alcohol-d_7, of which aliphatic moieties were highly deuterated. Furthermore, the DHP was prepared solely from coniferyl alcohol-d_7 to validate if the intended "silencing" is really exerted.

Results and discussion

Synthesis of deuterated vanillin

Coniferyl alcohol has been traditionally synthesized via the Knoevenagel–Doebner reaction of vanillin. Adopting the same strategy, this study first synthesized the vanillin, of which methoxy and formyl groups were substituted by deuterium (vanillin-d_4 in Scheme 1). A hydroxy group of protocatechualdehyde was selectively benzylated (**1**), and a CD$_3$ group was introduced (**2**) using (^{2}H$_3$)methyl *p*-toluenesulfonate (CD$_3$OTs) prepared from CD$_3$OD. The H of the formyl group was substituted by deuterium under Geng's umpolung condition with *N*-heterocarbene, in which D$_2$O is used as a deuterium source [26]. The same conversion will also be accomplished via the derivatization to 1,3-dithiane and proton abstraction followed by D-quench, but the protection will have to be reconsidered. The deuteration efficiency in the formyl group was determined to be 98.5%D based on the residual ^{1}H signal on NMR. As an attempt to verify whether an additional treatment can improve the deuteration efficiency, the obtained product was again subjected to the same reaction, and the value was successfully improved to > 99%D (**3**). The following debenzylation was first performed under conventional H$_2$-Pd/C condition in ethyl acetate (EtOAc), but the treatment interestingly reverted 20% of formyl-D to H. The capability of benzyl alcohol oxidation by Pd^{2+} species in the presence of an appropriate oxidant had been reported [27], and the contamination of Pd^{2+} in the commercial Pd/C reagent was experimentally proved [28]. Although the H$_2$-Pd/C is a commonly

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known reductive condition of benzyl carbonyl, this may compete with the oxidation to benzyl carbonyl by Pd^{2+} in the reaction system. Such a mechanism would explain the conversion to ArCHO from ArCDO via ArCDHOH. As an alternative strategy, trifluoromethanesulfonic acid (TfOH), rarely used in debenzylation, was selected as a deprotecting reagent for the reason that its deuterated form (TfOD) was commercially available. Contrary to our anxiety, TfOH itself was highly effective in deprotecting the benzyl group without impairing the deuteration efficiency of the formyl group and without demethylating like other strong Lewis acids. The obtained vanillin-d_4 (**4**) exhibited only aromatic signals on ^{1}H NMR, a proper deuterated ion

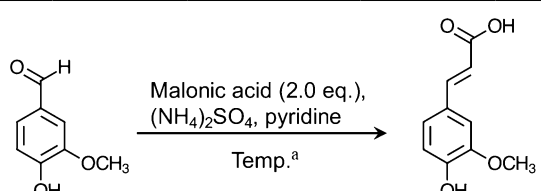peak on mass spectrometry (MS), and the same smell as vanillin.

Synthesis of ferulic acid under aniline-/piperidine-free condition

In the Knoevenagel–Doebner reaction for monolignol syntheses, aniline or piperidine are commonly used as a catalytic reagent. However, it could be easily envisaged that their exchangeable H could impair the deuteration efficiency of the product. Thus, we next attempted the development of the novel Knoevenagel–Doebner condition that can proceed without aniline or piperidine. Ammonium sulfate, of which deuterated form $((ND_4)_2SO_4)$ is commercially available, was selected as an alternative reagent, and the conditions were optimized using undeuterated vanillin and malonic acid. The yields were determined by gas chromatography–mass spectrometry (GC–MS) analyses, and the results are shown in Table 1. In the absence of $(NH_4)_2SO_4$, only 14% of the product, ferulic acid, was detected together with a quantity of unreacted vanillin (Entry 1). The addition of 0.5 eq. $(NH_4)_2SO_4$ evidently promoted the reaction (35%, Entry 2), and the less amount of the pyridine further enhanced the yield (80%, Entry 4). The decreased $(NH_4)_2SO_4$ with increased pyridine did not result in a better result (27%, Entry 3). The optimization of the reaction temperature proved that 70 °C provides a near quantitative reaction (Entries 4–6). The lower temperature might have an effect on preventing the thermal decomposition of malonic acid.
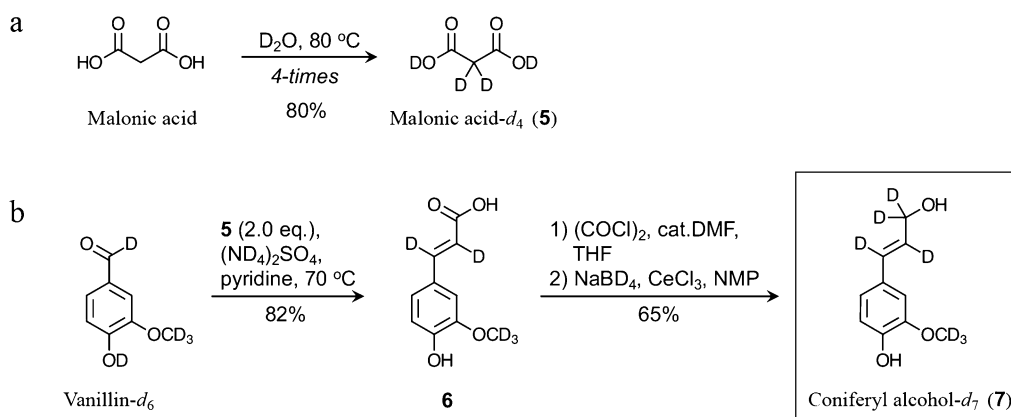
Synthesis of coniferyl alcohol-d_7

To practice the devised Knoevenagel–Doebner reaction, malonic acid was also deuterated by treating malonic acid in D_2O at elevated temperature (Scheme 2a). The deuteration efficiencies were estimated on ^{1}H NMR.

Table 1 Optimization of the Knoevenagel–Doebner reaction conditions under $(NH_4)_2SO_4$

Entry	$(NH_4)_2SO_4$ (eq.)	Pyridine (mL)[b]	Temp. (°C)	Yield (%)[c]
1	–	3.0	80	14
2	0.5	3.0	80	35
3	0.1	6.0	80	27
4	0.5	1.0	80	80
5	0.5	1.0	70	99
6	0.5	1.0	90	83

[a] Reaction temperature; [b] Volume to 1 mmol vanillin; [c] GC yield


Scheme 2 Syntheses of malonic acid-d_4 (**a**) and coniferyl alcohol-d_7 (**b**)

Since the single treatment gave only 89.0%D in both the methylene and the carboxy groups, the treatment was repeated four times. While the 2nd treatment gave significantly improved deuteration efficiency (96.2%D), the value hit the ceiling in the 4th treatment (i.e., 97.2%D in the 3rd and 97.7%D in the 4th treatment, respectively).

The resultant malonic acid-d_4 (**5**) and vanillin-d_5 prepared from vanillin-d_4 were treated under the optimized condition in the presence of $(ND_4)_2SO_4$, and the reaction gave ferulic acid-d_5 in an 82% yield (Scheme 2b). The deuteration efficiencies at the α- and β-position of the product were > 99%D and 92%D, respectively. As the same reaction under 5 mol% piperidine instead of $(ND_4)_2SO_4$ resulted in a 33% yield with 86%D at the β-position (data not shown), the present condition was demonstrated to be highly advantageous. The 8% of H incorporated at the β-position was absolutely derived from the residual protium in malonic acid.
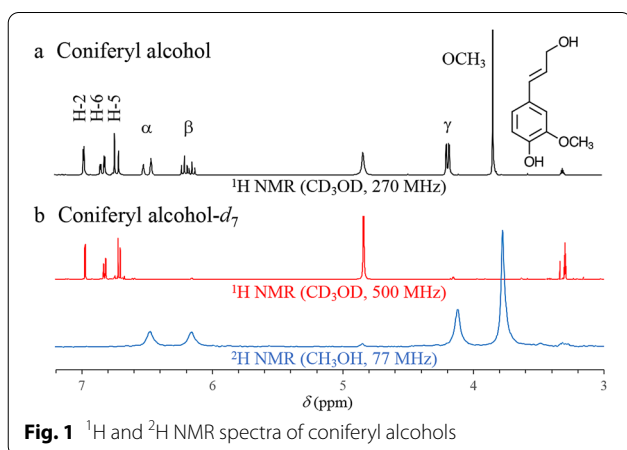
Ferulic acid-d_5 (**6**) was subsequently reduced to coniferyl alcohol-d_7 (**7**) by $NaBD_4$, which is much less costly than $LiAlD_4$ or DIBAL-D, and stably supplied. The conditions were optimized to $NaBD_4$, and the activation with $(COCl)_2$ followed by the Luche reduction in *N*-methylpyrrolidone (NMP) was found to give the best result (Scheme 2b). Briefly, the absence of $CeCl_3$ resulted in the formation of 24% 1,4-reduction product (α,β-dihydroconiferyl alcohol) in addition to 60% target, and the reaction without NMP resulted in 9% of the 1,4-reduction product and 41% of the target. In the activation step, $(COCl)_2$ was most successful compared to 1,1-carbonyldiimidazole (60% yield) or $SOCl_2$ (36% yield), probably due to the difference in reaction residues. The NMR spectra of coniferyl alcohol-d_7 are displayed in Figs. 1 and 2 as a comparison with coniferyl alcohol. In Fig. 1b, aliphatic protons clearly disappeared, and the deuterium signals of corresponding positions could be observed in the ^2H NMR spectrum. The deuterium

Fig. 2 ^{13}C NMR spectra of coniferyl alcohols
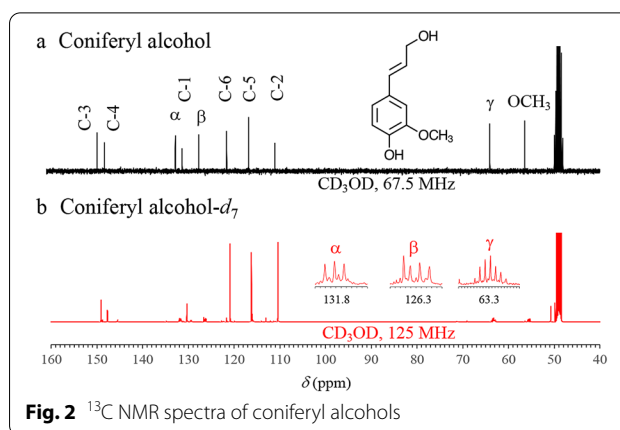
efficiency at γ-position was 98%D, and the undesired 1,4-reduction product was not detected. The comparison of ^{13}C NMR spectra also ensured the structure of coniferyl alcohol-d_7 (Fig. 2). The signals of the deuterated carbons were significantly attenuated, which was especially evident in Cβ: The Cβ-D triplet (126.4 ppm) is weaker than that of the residual Cβ-H singlet (126.7 ppm) despite the large difference in the presence (92% vs. 8%). As a result, highly deuterated coniferyl alcohol (methoxy: > 99%D, α: > 99%D, β: 92%D, and γ: 98%D) was successfully synthesized by modifying conventional strategy and using commercially accessible deuterium sources. The present study first achieved the deuteration of all aliphatic positions, including the new α- and β-positions, while the deuteration of monolignols had been conducted mainly on their methoxy and/or γ-positions [12, 15, 16, 19–25, 29] and sometimes on the 2, 5, and 6 positions [11, 17, 18] to date.

Preparation and NMR analyses of deuterated DHP

To examine whether the NMR signals are really "silenced", DHP was prepared from coniferyl alcohol-d_7 (DHP-*d*) and compared with that from coniferyl alcohol (DHP-*h*). Both reactions were performed by adding horseradish peroxidase and H_2O_2 buffer solutions to the monolignol solutions in an aqueous (H_2O) buffer. The yields were essentially the same (DHP-*h*: 56.8 mg DHP-*d*: 63.1 mg from *ca*. 100 mg starting materials), which reflects the comparable reactivity of deuterated monolignol. The exchangeable protons of the products were replaced with D using CD_3OD, and the products were analyzed on ^1H and edited HSQC NMR (Fig. 3). The panels Fig. 3a, b are the spectra of DHP-*h*, and c–f are those of DHP-*d*. In the panels, Fig. 3a–d, the same vertical magnifications were used for ^1H NMR, and the same thresholds were applied for HSQC spectra. The spectra in panels e and f were "deeper" slices of c and d. Here, in-phase signals

Fig. 1 ^1H and ^2H NMR spectra of coniferyl alcohols

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are shown in blue and anti-phase signals in red. The typical cross-peaks for lignin substructures, namely, aryl ethers (A and A'), β–β (B), and β-5 (C), were observed in the aliphatic region of DHP-*h* (Fig. 3b). A cross-peak at 3.1/56 ppm, designated "U" in Fig. 3b, is a specific signal arisen in the present condition and can be possibly derived from β-1 substructure, though it was difficult to conclude clearly due to the lack of enough examples for this structure. By contrast to Fig. 3b, hardly any ^{1}H and HSQC signals are observed in Fig. 3d, except those of residual water (3.42 ppm) and cinnamyl alcohol-γ (CAγ, 4.07 ppm). By lowering the slice to the level at which excessive aromatic signals were rendered (Fig. 3e), a part of the aliphatic signals became observable in the HSQC (Fig. 3f). A subtle difference in the aromatic region (6.5–7.5 ppm) of ^{1}H NMR spectra will stem from the minor signals that resonate at δH 6.60–6.85/δC 122–130 (unrendered in Fig. 3a), which will probably be related signals to β-proton of cinnamaldehyde. The intensities of cross-peaks Aβ, A'β, Bβ, and Cβ in Fig. 3f were approximately 4, 7, 15, and 17% of those in Fig. 3b, respectively. The increments in Bβ and Cβ from the original 8% β-H may suggest a partial exchange from β-D to H, but it would be more reasonable to consider these differences within the margin of error, given the inexplicable decrements in Aβ and A'β. The same silencing was likewise observed in the unidentified signal (U in Fig. 3b). Rather, the reversions to H were obvious at the γ-positions. This is typically seen in the signals, CAγ (4.07 ppm in Fig. 3d) and cinnamaldehyde-H (8.8–9.1 ppm, not displayed) in ^{1}H NMR. The intensities of the corresponding cross-peaks in DHP-*d*, calculated with the cross-peak of H-6/C-6 as a reference, were 13% and 49% of those in DHP-*h*, respectively. In addition, all the cross-peaks of γ-positions, including CAγ, appeared as in-phase signals and no anti-phase signals were observed in Fig. 3f. These results indicated that a part of D at γ-position was replaced by H, but this reversion was limited only to the formation of γ-CDH, without γ-CH$_2$. This type of substitution had already been reported in the tracer feeding experiments on *Eucalyptus camaldulensis* or *Ginkgo biloba* L. [15, 16]. Our results newly demonstrated that the same substitution could occur in a more simplified redox system. With respect to the α-positions, no signals were detectable even in the lower slice (Fig. 3f). This result was very surprising for us, because there had

been concern that the reactive benzyl-D might be easily substituted for H. Totally, the deuterium atoms at α- and β-positions were highly retained in the resultant DHP, even in the system filled with a vast amount of H. Regarding the γ-position, partial substitution to H was shown to occur, but the anti-phase signals in HSQC were effectively silenced due to the negligible formation of γ-CH$_2$. Therefore, the deuteration of γ-position will effectively work if the purpose is to observe CH$_2$ signals, such as sugar–CH$_2$ in the seed structure.

Conclusions

Toward the development of the molecular tool that enables facile analyses of lignin formation reaction, the preparation of highly deuterated monolignol was undertaken, and coniferyl alcohol-d_7 of which aliphatic moieties were highly deuterated (methoxy: > 99%D, α: > 99%D, β: 92%D, and γ: 98%D) was successfully synthesized. Our synthetic strategy will be applicable for the site-selective and multiple deuterations, including those of other monolignols. The DHP obtained from coniferyl alcohol-d_7 showed highly "silenced" ^{1}H and HSQC NMR spectra without accompanying significant reversion to protium at α- and β-positions. On the other hand, a part of deuterium at γ-position was substituted for H through the reaction. Thus, the deuteration at γ-position can be optional, whereas it still has an advantage that γ-CH$_2$ signals are effectively silenced in HSQC. Our study has demonstrated that DHP preparation with deuterated monolignols can provide extremely simplified NMR spectra, which can be a powerful methodology for analyzing the structural alteration of the undeuterated seed structures.

Materials and methods

Unless otherwise stated, all commercially available chemicals were used without further purification. Tetrahydrofuran (THF) was distilled from benzophenone ketyl radical under argon. Thin layer and column chromatography were performed with Silica Gel 70F$_{254}$ Wako (Fujifilm Wako Pure Chemicals Co. Ltd., Osaka, Japan) and Silica Gel 60N –spherical, neutral– (Kanto Chemicals Co., Tokyo, Japan), respectively. NMR spectra were measured on a JEOL JMN EX-270 (JEOL, Tokyo, Japan) or Bruker AVANCE Neo (Bruker, Billerica, MA, USA) spectrometer. Chemical shifts are reported in ppm (δ-scale) using tetramethylsilane (CDCl$_3$) or solvent peaks (CD$_3$OD or

(See figure on next page.)
Fig. 3 ^{1}H and HSQC NMR spectra of DHP (**a** aromatic region; **b**: aliphatic region), and DHP-*d* (**c** and **e** aromatic region; **d** and **f** aliphatic region) in DMSO-*d$_6$*. The panels **e** and **f** are slices of **c** and **d** with a lower threshold, respectively. The dashed squares are ×3 magnification of those areas. All ^{1}H NMR spectra are shown in the same magnification normalized with the solvent peaks at 2.49 ppm. The blue cross-peaks are in-phase signals, whereas the red peaks are anti-phase signals. Symbols in the HSQC spectra represent the assignments to respective substructures (A: β-*O*-4; A': β-*O*-4/α-*O*-4; B: β-5; C: β–β; CA: cinnamyl alcohol), and subscripts (α, β and γ) indicate their position in the aliphatic chains. The symbol U indicates an unidentified signal

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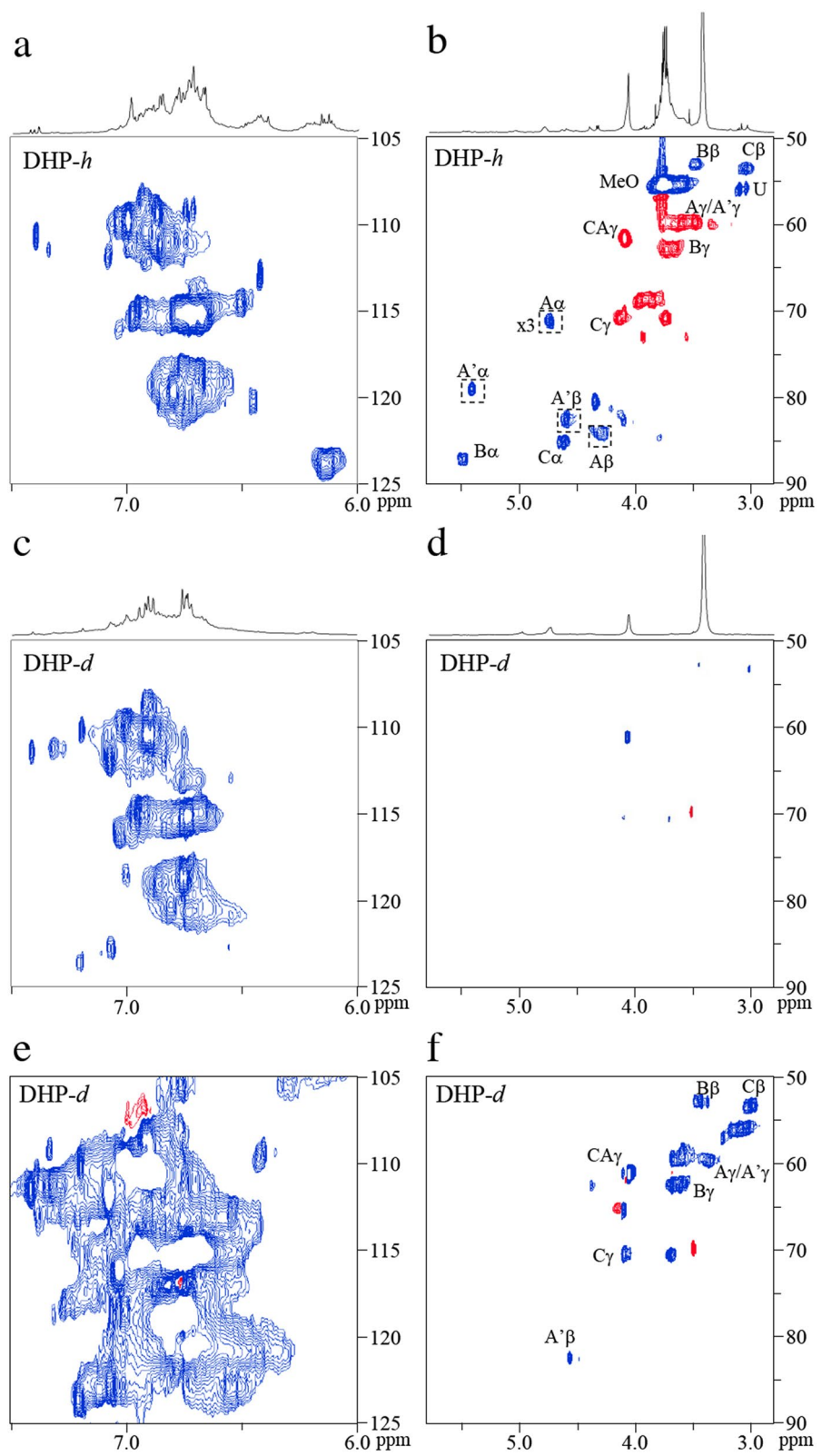
Fig. 3 (See legend on previous page.)

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dimethyl sulfoxide (DMSO)-d_6) as the internal standards. Coupling constants (*J*) are given in Hz. Mass spectra were acquired with FI mode on a JEOL JMS-T100GCV equipment. All MS and NMR spectra on AVANCE Neo were measured at the GC–MS and NMR Laboratory, Faculty of Agriculture, Hokkaido University.

4-(Benzyloxy)-3-hydroxybenzaldehyde (1)

To a solution of 3,4-dihydroxybenzaldehyde (7.37 g, 53.4 mmol) in 100 mL of MeCN, NaHCO$_3$ (5.83 g, 1.3 eq.) was added at room temperature. The suspension was allowed to stir at 60 °C for 1 h, and 6.77 g of benzyl chloride (BnCl, 1.0 eq.) was added. The reaction was continued at 80 °C overnight. The mixture was acidified with 1 M aqueous HCl and washed with EtOAc (×3). The combined organic layer was neutralized with brine and dried over anhydrous Na$_2$SO$_4$. After removal of the solvent, the crude product was purified by silica gel column chromatography (hexane then EtOAc:hexane = 1:2). After evaporation of the combined target fractions, the product was recrystallized from EtOAc (×3) to give a total of 8.50 g of the 4-*O*-benzylated product as a pale-yellow solid (70%).

4-(Benzyloxy)-3-(^{2}H$_3$)methoxybenzaldehyde (2)

Sodium hydride (5.6 g, *ca*.127 mmol) was added to a THF (100 mL) solution of CD$_3$OD (3.24 g, 90 mmol) portionwise at 0 °C. After gas generation stopped, 20.6 g of TsCl (1.2 eq.) was added at the same temperature. After the reaction was completed, 100 mL of Et$_2$O and cold water were added to the mixture. The water layer was recovered and again washed with Et$_2$O (×2). The combined organic layer was washed with aqueous sat.NaHCO$_3$ and brine. The solution was dried over anhydrous Na$_2$SO$_4$, and the solvent was removed in vacuo to afford 19.1 g of (^{2}H$_3$) methyl *p*-toluenesulfonate as a mixture with mineral oil.

To the solution of **1** (8.35 g, 36.6 mmol) in *N*,*N*-dimethylformamide (DMF, 80 mL), NaH (1.76 g, *ca.* 44 mmol) was added portionwise at room temperature. The crude (^{2}H$_3$)methyl *p*-toluenesulfonate (8.31 g) and KI (600 mg, 0.1 eq.) were added to the mixture, which was stirred at room temperature. After the overnight reaction, the mixture was partitioned between water and EtOAc (×3). The combined organic layer was washed with water and brine and dried over anhydrous Na$_2$SO$_4$. After removal of the solvent by evaporation, the product was purified by silica gel column chromatography (EtOAc:hexane = 1:1), giving 7.83 g of the product (87%) as a pale yellow solid.^{1}H NMR (CDCl$_3$, 500 MHz): δ 5.24 (2H, s, CH$_2$), 6.98 (1H, d, *J* = 8.0 Hz, H-5), 7.30–7.46 (7H, m, H-2, H-6 and benzyl aromatic), 9. 83 (1H, s, formyl); ^{13}C NMR (CDCl$_3$, 125 MHz): δ 55.2 (OCD$_3$, m, *J* = 22 Hz), 70.8 (CH$_2$), 109.3 (C-2), 112.4 (C-5), 126.5 (C-6), 127.1 (benzyl),

128.1 (benzyl), 128.7 (benzyl), 130.2 (C-1), 136.0 (benzyl), 150.0 (C-3), 153.5 (C-4), 190.8 (formyl). HR-FI-MS: *m/z* = 245.11312 [M]$^+$ calcd. for C$_{15}$^{1}H$_{11}$^{2}H$_3$O$_3$, found 245.11358.

4-Hydroxy-3-(^{2}H$_3$)methoxybenzene(^{2}H)carbaldehyde (4, Vanillin-*d*$_4$)

Compound **2** (5.20 g, 21.2 mmol) was dissolved in 16.6 mL of toluene. To the solution, 40 mL of D$_2$O, 2.94 g of K$_2$CO$_3$ (1.0 eq.) and 897 mg of 1,3-bis(2,5-diisopropylphenyl)imidazolium chloride ([bis(dipp)Im]Cl, 0.1 eq.) were added. The reaction was allowed to stir at 40 °C overnight and then partitioned with EtOAc (×2). The water layer was acidified with 1 M aqueous HCl and washed with EtOAc. The combined organic layer was washed with 1 M aqueous HCl, aqueous sat.NaHCO$_3$ and brine. The solution was dried over anhydrous Na$_2$SO$_4$, and the solvent was removed in vacuo. The purification by flash silica gel column chromatography eluting with EtOAc:hexane = 1:2 afforded 5.29 g of crude product, of which deuteration efficiency was 98.5%D at the formyl group. This could be estimated by the comparison with an integration value of H-5 (7.10 ppm in ^{1}H NMR). The same treatment was repeated for the recovered product with half amounts of the reagents, which gave 5.18 g of product with > 99%D of deuteration efficiency in the formyl group.

The crude product was dissolved in 50 mL of CH$_2$Cl$_2$, and 2.0 mL of TfOH (22.7 mmol) was added dropwise to the solution at − 20 °C. After the completion of the reaction, 2.3 mL of 10 M aqueous NaOH was added to the mixture. The mixture was partitioned between 1 M aqueous HCl and EtOAc (×3). The combined organic layer was neutralized with brine and dried over anhydrous Na$_2$SO$_4$. After removal of the solvent, the crude product was purified with flash silica gel column chromatography eluting with EtOAc:hexane = 1:2 to give 1.65 g of vanillin-*d*$_4$ (50% from **2**). ^{1}H NMR (CDCl$_3$, 500 MHz): δ 7.04 (1H, d, *J* = 8.4 Hz, H-5), 7.42–7.45 (2H, m, H-2 and H-6); ^{13}C NMR (CDCl$_3$, 125 MHz): δ 55.2 (OCD$_3$, m, *J* = 22 Hz), 108.8 (C-2), 114.3 (C-5), 127.4 (C-6), 129.7 (t, *J* = 3 Hz, C-1), 147.1 (C-3), 151.7 (C-4), 190.6 (t, *J* = 27 Hz, formyl). HR–FI-MS: *m/z* = 156.07167 [M]$^+$ calcd. for C$_8$^{1}H$_4$^{2}H$_4$O$_3$, found 156.07245.

(2,2-^{2}H$_2$)Propane(^{2}H$_2$)dioic acid (Malonic acid-*d*$_4$, 5)

Malonic acid (2.08 g, 20.0 mmol) was dissolved in 6.0 mL of D$_2$O, and the solution was allowed to stir overnight at 80 °C. The solution was frozen, and the solvent was removed by freeze-drying. Deuteration efficiency was evaluated by ^{1}H NMR: Forty-two point zero milligrams of the product were dissolved in 0.45 mL of DMSO-*d*$_6$ containing 2.2 mg of benzaldehyde as an internal

standard. The integration values of CH_2 at 3.22 ppm and COOH at 12.57 ppm were compared with those of intact malonic acid, respectively. The treatment and analysis were repeated four times, and 1.73 g of white solid was obtained (80%).

GC–MS analyses for the optimization of reaction conditions

A portion of the reaction mixture equivalent to 1.0 mg of the starting material was sampled and diluted with 800 μL of EtOAc. The resultant solution was mixed with 200 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide and filtered with a 0.45 μm PTFE membrane filter. The sample was allowed to stand for 1 h at room temperature. One microliter of each solution was analyzed on GC-2010/QP-2010 system (Shimadzu, Kyoto, Japan) under the following conditions: Column: BPX5 30 m, φ0.25 mm, df 1.0 μm (SGE Analytical Science, Melbourne, VIC, Australia); Injection unit: 250 °C; Column oven: 80 °C for 3.0 min, 40 °C/min for 6.0 min then 320 °C for 4.0 min; Carrier gas control: constant linear velocity (40.0 cm/s); Carrier gas: helium; Interface: 250 °C; Ion source: 250 °C. Total ion chromatograms (TIC) were obtained, and the yields were calculated simply based on the area of each compound per total of areas. Retention times of trimethylsilyl-derivatized compounds were as follows: Vanillin: 8.17 min; α, β-dihydroconiferyl alcohol: 8.81 min; Coniferyl alcohol: 9.22 min; Ferulic acid: 9.73 min.

(*E*)-3-[4-Hydroxy-3-(²H₃)methoxyphenyl](2,3-²H₂) prop-2-enoic acid (6, Ferulic acid-*d*₅)

Prior to the reaction, the phenolic H of **4** was exchanged for D by repeating the addition and evaporation of CD_3OD three times to obtain vanillin-*d*₅.

To 2.4 mL of pyridine, 168 mg of $(ND_4)_2SO_4$ (0.5 eq.) and 520 mg of malonic acid-*d*₄ were added, and which was stirred for 10 min at room temperature. To the mixture was added 377.2 mg of vanilline-*d*₅ (2.40 mmol) and the reaction was allowed to stir at 70 °C overnight. Pyridine was azeotropically removed with toluene, and the residue was dissolved in a small quantity of acetic acid (AcOH)/methanol (MeOH). The mixture was subjected to silica gel column chromatography ($CHCl_3$, then MeOH:$CHCl_3$ = 10:90). The evaporation of the target fractions gave 391.4 mg of white solid (82%). ^{1}H NMR (CD_3OD, 500 MHz): δ 6.29 (0.08H, s, H-*β*), 6.80 (1H, d, *J* = 8.2 Hz, H-5), 7.04 (1H, dd, *J* = 8.2 and 2.0 Hz, H-6), 7.15 (1H, d, *J* = 2.0 Hz, H-2); ^{13}C NMR (CD_3OD, 125 MHz): δ 55.6 (OCD_3, m, *J* = 22 Hz), 111.6 (C-2), 115.5 (t, *J* = 24 Hz, Cβ-D), 115.8 (Cβ-H), 116.4 (C-5), 123.9 (C-6), 127.7 (C-1), 146.5 (t, *J* = 24 Hz, Cα), 149.3 (C-4), 150.5 (C-3), 171.0 (carbonyl). HR–FI–MS:

$m/z = 199.08929$ [M]$^+$ calcd. for $C_{10}{}^1H_5{}^2H_5O_4$, found 199.08870.

4-[(*E*)-3-Hydroxy(1,2,3,3-²H₄)prop-1-enyl]-2-(²H₃) methoxyphenol (7, Coniferyl alcohol-*d*₇)

Ferulic acid-*d*₅ (347.3 mg, 1.74 mmol) was dissolved in 5.0 mL of THF. To the solution, one drop of anhydrous DMF and 150 μL of $(COCl)_2$ (1.0 eq.) were added in this order at 0 °C. Anhydrous $CeCl_3$ (429 mg, 1.0 eq.) was suspended in 10 mL of NMP, which was sonicated for 15 min, and 146 mg of $NaBD_4$ (2.0 eq.) was added to the suspension. At −20 °C, the acid chloride solution was added dropwise to the reductant suspension. The reaction was then allowed to stir overnight at room temperature. The solvents were removed under reduced pressure, and the resultant residue was resuspended in MeOH containing AcOH (minimum quantity for neutralization). The crude product was directly subjected to silica gel column chromatography ($CHCl_3$, then MeOH:$CHCl_3$ = 10:90). Careful evaporation of the target fractions gave 224.5 mg of coniferyl alcohol-*d*₇ (65%) as a mixture with 6% NMP. ^{1}H NMR (CD_3OD, 500 MHz): δ 6.16 (0.08H, s, H-*β*), 6.72 (1H, d, *J* = 8.0 Hz, H-5), 6.83 (1H, dd, *J* = 8.0 and 2.0 Hz, H-6), 6.98 (1H, d, *J* = 2.0 Hz, H-2); ^{13}C NMR (CD_3OD, 125 MHz): δ 55.5 (m, *J* = 22 Hz, OCD_3), 63.3 (quintet, *J* = 21 Hz, Cγ), 110.5 (C-2), 116.3 (C-5), 120.9 (C-6), 126.4 (t, *J* = 23 Hz, Cβ-D), 126.7 (Cβ-H), 130.4 (C-1), 131.9 (t, *J* = 23 Hz, Cα), 147.8 (C-4), 149.2 (C-3). HR–FI–MS: $m/z = 187.12333$ [M]$^+$ calcd. for $C_{10}{}^1H_5{}^2H_7O_3$, found 187.12258.

DHP preparation

Monolignols (coniferyl alcohol: 100.2 mg; coniferyl alcohol-*d*₇: 107.1 mg as a mixture with NMP) were separately dissolved in 1.0 mL of acetone, and to the solutions were added 49 mL of phosphate-buffered saline (PBS, pH 6.1, 50 mM phosphate). The solutions containing 0.5 mg of horseradish peroxidase (Fujifilm Wako Pure Chemicals Co. Ltd., Osaka, Japan) in 25 mL PBS and 4.0 mmol H_2O_2 in 25 mL PBS were added dropwise to the monolignol solutions over 3 h, and the reaction was continued for another 13 h. The resulting suspensions were centrifuged, and the precipitates were washed with Milli-Q water three times. The residues were freeze-dried to give DHPs (56.8 mg from coniferyl alcohol; 63.1 mg from coniferyl alcohol-*d*₇).

NMR analyses

The obtained DHP products were treated by the addition/evaporation of CD_3OD three times. Fifty milligrams of samples were dissolved in 0.45 mL of DMSO-*d*₆, and the complete dissolution of the samples was visually confirmed. The edited HSQC spectra were obtained

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using 256 scans using the standard Bruker pulse program (hsqcedetgpsp.3) with the following parameters for acquisition: TD = 2048 (F2), 192 (F1); SW = 11.1082 ppm (F2), 209.9969 ppm (F1); SWH = 5555.556 Hz (F2); 26,411.512 Hz (F1); IN_F = 37.8623 ms; AQ 0.1843200 s (F2), 0.0036348 s (F1); FIDRES = 5.425347 Hz (F2), 275.119934 Hz (F1); FW = 240,000,000.000 Hz. The resulting data were processed with the TopSpin 4.1.3. The thresholds in Fig. 3a–d were set as follows: Base level 1.4E10 (Positive) and −1.4E10 (Negative); Level increment 1.200 (Positive and Negative); Number of levels 16. Threefold magnification in Fig. 3b was obtained at the Base level 1.4/3E10 (Positive) and −1.4/3E10 (Negative). The thresholds in Fig. 3e, f were set as follows: Base level 1.4E9 (Positive) and −1.4E9 (Negative); Level increment 1.200 (Positive and Negative); Number of levels 16.

Abbreviations
AcOH: Acetic acid; Bn: Benzyl; CA: Cinnamyl alcohol; DHP: Dehydrogenative polymer; dipp: 2,5-Diisopropylphenyl; DMF: *N*, *N*-Dimethylformamide; DMSO: Dimethyl sulfoxide; EtOAc: Ethyl acetate; GC: Gas chromatography; HSQC: Heteronuclear single quantum coherence; Im: Imidazolium; LCC: Lignin–carbohydrate complex; MS: Mass spectrometry; NMP: *N*-Methylpyrrolidone; NMR: Nuclear magnetic resonance; PBS: Phosphate-buffered saline; Tf: Trifluoromethanesulfonyl; THF: Tetrahydrofuran; TIC: Total ion chromatogram; Ts: *p*-Toluenesulfonyl.

Author contributions
KS conceived and planned the experiments. MT and KS carried out the experiment. KS wrote the manuscript. KS and YU supervised the project. All authors read and approved the final manuscript.

Availability of data and materials
All data generated or analyzed during this study are included in this published article. The spectral data of NMR are available from the corresponding author on reasonable request.

Declarations

Competing interests
The authors declare that they have no competing interests.

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References
1. Pedreño MA, Barceló AR, Sabater F, Muñoz R (1989) Control by pH of cell wall peroxidase activity involved in lignification. Plant Cell Physiol 30:237–241
2. Aminzadeh S, Zhang L, Henriksson G (2017) A possible explanation for the structural inhomogeneity of lignin in LCC networks. Wood Sci Technol 51:1365–1376
3. Takahama U (1995) Oxidation of hydroxycinnamic acid and hydroxycinnamyl alcohol derivatives by laccase and peroxidase. Interactions among *p*-hydroxyphenyl, guaiacyl and syringyl groups during the oxidation reactions. Physiol Plant 93:61–68
4. Grabber JH, Hatfield RD, Ralph J (2003) Apoplastic pH and monolignol addition rate effects on lignin formation and cell wall degradability in Maize. J Agric Food Chem 51:4984–4989
5. van Parijs FRD, Morreel K, Ralph J, Boerjan W, Merks RMH (2010) Modeling lignin polymerization. I. Simulation model of dehydrogenation polymers. Plant Physiol 153:1332–1344
6. Fournand D, Cathala B, Lapierre C (2003) Initial steps of the peroxidase-catalyzed polymerization of coniferyl alcohol and/or sinapyl aldehyde: capillary zone electrophoresis study of pH effect. Phytochemistry 62:139–146
7. Demont-Caulet N, Lapierre C, Jouanin L, Baumberger S, Me´chin S (2010) Arabidopsis peroxidase-catalyzed copolymerization of coniferyl and sinapyl alcohols: kinetics of an endwise process. Phytochemistry 71:1673–1683
8. Aoyama W, Sasaki S, Matsumura S, Mitsunaga T, Hirai H, Tsutsumi Y, Nishida T (2002) Sinapyl alcohol-specific peroxidase isoenzyme catalyzes the formation of the dehydrogenative polymer from sinapyl alcohol. J Wood Sci 48:497–504
9. Elschner T, Adam J, Lesny H, Joseph Y, Eischer S (2022) Growing of artificial lignin on cellulose ferulate thin films. Biomacromol. https://doi.org/10.1021/acs.biomac.2c00096
10. Lyu Y, Matsumoto T, Taira S, Ijiri K, Yoshinaga A, Shigetomi K, Uraki Y (2021) Influences of polysaccharides in wood cell walls on lignification in vitro. Cellulose 28:9907–9917
11. Matsushita Y, Ko C, Aoki D, Hashigaya S, Yagami S, Fukushima K (2015) Enzymatic dehydrogenative polymerization of monolignol dimers. J Wood Sci 61:608–619
12. Shimizu K, Matsushita Y, Aoki D, Mitsuda H, Fukushima K (2021) Reactivity of a benzylic lignin-carbohydrate model compound during enzymatic dehydrogenative polymerization of coniferyl alcohol. Holzforschung. https://doi.org/10.1515/hf-2020-0216
13. Ralph J, Landucci LL (2011) NMR of lignins. In: Heitner C, Dimmel DR, Schmidt JH (eds) Lignin and lignans: advances in chemistry. CRC Press, Taylor & Francis Group, Boca Raton, pp 137–244
14. Wen JL, Sun SL, Xue BL, Sun RC (2013) Recent advances in characterization of lignin polymer by solution-state nuclear magnetic resonance (NMR) Methodology. Materials 6:359–391
15. Tsuji Y, Chen F, Yasuda S, Fukushima K (2004) The behavior of deuterium-labeled monolignol and monolignol glucosides in lignin biosynthesis in angiosperms. J Agric Food Chem 52:131–134
16. Tsuji Y, Chen F, Yasuda S, Fukushima K (2005) Unexpected behavior of coniferin in lignin biosynthesis of *Ginkgo biloba* L. Planta 222:58–69
17. Freudenberg K, Jovanovic V, Topfmeier F (1961) Versuche mit deuteriertem Coniferylalkohol zur Bestimmung der Substitution am C-Atom 5 der Coniferyleinheit des Lignins. Chem Ber 94:3227–3238
18. Freudenberg K, Jovanovic V (1963) Weitere Versuche mit deuteriertem Coniferylalkohol. Chem Ber 96:2178–2181
19. Kato MJ, Chu A, Davin LB, Lewis NG (1998) Biosynthesis of antioxidant lignans in *Sesamum indicum* seeds. Phytochemistry 47:583–591
20. Umezawa T, Shimada M (1996) Formation of the lignan (+)-secoisolariciresinol by cell-free extracts of *Arctium lappa*. Biosci Biotechnol Biochem 60:736–737
21. Sakakibara N, Nakatsubo T, Suzuki S, Shibata D, Shimada M, Umezawa T (2007) Metabolic analysis of the cinnamate/monolignol pathway in *Carthamus tinctorius* seeds by a stable-isotope-dilution method. Org Biomol Chem 5:802–815
22. Suzuki S, Umezawa T, Shimada M (2002) Stereochemical diversity in lignan biosynthesis of *Arctium lappa* L. Biosci Biotechnol Biochem 66:1262–1269

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23. Miyauchi T, Ozawa S (1998) Formation of (+)-eudesmin in *Magnolia kobus* DC. var borealis Sarg. Phytochemistry 47:665–670

24. Watanabe B, Nishitani S, Koeduka T (2021) Synthesis of deuterium-labeled cinnamic acids: Understanding the volatile benzenoid pathway in the flowers of the Japanese loquat *Eriobotrya japonica*. J Label Compd Radiopharm 64:403–416

25. Ralph J, Zhang Y, Ede RM (1998) Preparation of synthetic lignins with superior NMR characteristics *via* isotopically labeled monolignols. J Chem Soc, Perkin Trans 1(16):2609–2613

26. Geng H, Chen X, Gui J, Zhang Y, Shen Z, Qian P, Chen J, Zhang S, Wang W (2019) Practical synthesis of C1 deuterated aldehydes enabled by NHC catalysis. Nat Catal 2:1071–1077

27. Ye X, Jhonson MD, Diao T, Yates MH, Stahl SS (2010) Development of safe and scalable continuous-flow methods for palladium-catalyzed aerobic oxidation reactions. Green Chem 12:1180–1186

28. Sakakibara Y, Osada K, Uraki Y, Ubukata M, Shigetomi K (2021) Direct deuteration of hinokitiol and its mechanistic study. Biosci Biotechnol Biochem 85:215–222

29. Regner M, Bartuce A, Padmakshan D, Ralph J, Karlen SD (2018) Reductive cleavage method for quantitation of monolignols and low-abundance monolignol conjugates. Chemsuschem 11:1600–1605

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