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Synthesis of deuterium-labeled cinnamic acids: Understanding the volatile benzenoid pathway in the flowers of the Japanese loquat *Eriobotrya japonica*

Bunta Watanabe[1] | **Shiori Nishitani**[2] | **Takao Koeduka**[2]

[1]Institute for Chemical Research, Kyoto University, Kyoto, Japan

[2]Graduate School of Sciences and Technology for Innovation, Yamaguchi University, Yamaguchi, Japan

Correspondence
Bunta Watanabe, Institute for Chemical Research, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan.
Email: bunta@scl.kyoto-u.ac.jp

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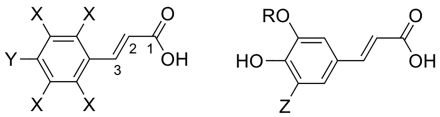
Cinnamic acids are widely distributed in plants, including crops for human use, and exhibit a variety of activities that are beneficial to human health. They also occupy a pivotal position in the biosynthesis of phenylpropanoids such as lignins, anthocyanins, flavonoids, and coumarins. In this context, deuterium-labeled cinnamic acids have been used as tracers and internal standards in food and medicinal chemistry as well as plant biochemistry. Therefore, a concise synthesis of deuterium-labeled cinnamic acids would be highly desirable. In this study, we synthesized deuterium-labeled cinnamic acids using readily available deuterium sources. We also investigated a hydrogen–deuterium exchange reaction in an ethanol-d_1/Et_3N system. This method can introduce deuterium atoms at the *ortho* and *para* positions of the phenolic hydroxy groups as well as at the C-2 position of alkyl cinnamates and is applicable to various phenolic compounds. Using the synthesized labeled compounds, we demonstrated that the benzenoid volatiles, such as 4-methoxybenzaldehyde, in the scent of the flowers of the Japanese loquat *Eriobotrya japonica* are biosynthesized from phenylalanine via cinnamic and 4-coumaric acids. This study provides easy access to a variety of deuterium-labeled (poly)phenols, as well as to useful tools for studies of the metabolism of cinnamic acids in living systems.

KEYWORDS

benzenoid volatiles, biosynthesis, cinnamic acids, *Eriobotrya japonica*, hydrogen–deuterium exchange

1 | INTRODUCTION

Cinnamic acids, such as cinnamic acid (**1a**), ferulic acid (**2a**), sinapic acid (**3a**), and 4-coumaric acid (**4a**), are derivatives of acrylic acid with a substituted or unsubstituted phenyl group at the C-3 position (Figure 1). They are widely distributed in plants that include crops for human use[1–3] and exhibit a variety of beneficial effects on human health such as antioxidant,[1,3,4] anticancer,[1–4] antimicrobial,[1,3,5] antihypercholesterolemia,[1] antiobesity,[4] and antidepressant[6] activity. The simplest member of cinnamic acids (**1a**) is biosynthesized from phenylalanine via the enzymatic reaction of phenylalanine ammonia lyase and subsequently derivatized to cinnamoyl CoA, other cinnamic acids and their CoA esters, cinnamaldehydes, and cinnamyl alcohols. These cinnamic acid derivatives serve as biosynthetic intermediates of diverse end-products of the phenylpropanoid pathway such as lignins, anthocyanins, flavonoids, and coumarins.[7,8]



1a (X = Y = H) **2a** (R = CH_3, Z = H)
1b (X = Y = D) **2b** (R = CD_3, Z = H)
4a (X = H, Y = OH) **3a** (R = CH_3, Z = OCH_3)
4b (X = D, Y = OH) **3b** (R = CD_3, Z = OCD_3)

FIGURE 1 Representative cinnamic acids (**1a**–**4a**) and their deuterium-labeled counterparts (**1b**–**4b**)

To investigate the complicated biosynthesis of phenylpropanoids, stable-isotope-labeled cinnamic acids and their derivatives have frequently been used. For example, using deuterium-labeled cinnamic acids, cinnamaldehydes, and cinnamyl alcohols, Sakakibara and coworkers have quantitated their endogenous counterparts in the seeds of *Carthamus tinctorius* and demonstrated that ferulic acid serves as a biosynthetic precursor of lignans in the plant.[9] Deuterium-labeled cinnamic acids have also been used as tracers in the biosynthetic investigation of coumarins in the root of the cassava plant (*Manihot esculenta* Crantz).[10] Moreover, using deuterium-labeled coniferyl alcohol, Lairez and coworkers have analyzed the polymerization of coniferyl alcohol in a pectin solution as a model system for the first step of lignification.[11]

In addition to plant biochemistry, stable-isotope-labeled cinnamic acids have also been used in the areas of food and medicinal chemistry. For example, they have been used as internal standards for the determination of the concentration of cinnamic acids in milk to establish a method that can determine the type of cattle forage.[12] Piber and coworkers have used deuterium-labeled ferulic acid as a tracer to detect chemical reactions of ferulic acid in flours during bread baking.[13] In addition, using deuterium-labeled cinnamic acids as an authentic sample and an internal standard, Sarkissian and coworkers have quantitated cinnamic acids as one of metabolites of phenylalanine in the plasma and urine of phenylketonuric model mice.[14] The development of efficient synthetic routes to stable-isotope-labeled cinnamic acids and their derivatives is thus strongly desirable.

Herein, we report the synthesis of deuterium-labeled cinnamic acids **1b**–**4b** (Figure 1). We also investigated a hydrogen–deuterium exchange reaction in an ethanol-d_1/Et_3N system that can introduce deuterium atoms at the *ortho* and *para* positions of the phenolic hydroxy group as well as at the C-2 position of alkyl cinnamates. Moreover, using the synthesized labeled compounds, we demonstrated that 4-methoxybenzaldehyde and methyl 4-methoxybenzoate, the major benzenoid volatiles in the flower scent of the Japanese loquat *Eriobotrya japonica*,[15] are biosynthesized from phenylalanine via **1a** and **4a**.

2 | EXPERIMENTAL

2.1 | General information

Melting points (mp) were measured using an AS ONE ATM-01 mp apparatus and are uncorrected. Nuclear magnetic resonance (NMR) spectra were obtained using a JEOL JNM-ECA600 or a Bruker AVANCEIII 600 spectrometer (600 MHz for ^1H; 92 MHz for ^2H; 151 MHz for ^{13}C). Chemical shifts are reported in parts per million relative to the internal standards (tetramethylsilane (0.00 ppm) for ^1H; $CHCl_3$ (7.26 ppm) and CH_3SOCH_3 (2.55 ppm) for ^2H; $CDCl_3$ (77.00 ppm) and CD_3SOCD_3 (39.70 ppm) for ^{13}C). High-resolution mass spectra (HRMS) were recorded using a JEOL JMS-700 or a Bruker timsTOF spectrometer. Electron ionization (EI), fast atom bombardment (FAB) using 3-nitrobenzyl alcohol (NBA) as a matrix, or electrospray ionization (ESI) were used as ionization methods. Flash column chromatography on silica gel was carried out using a Biotage Isolera One chromatograph with SNAP Ultra cartridges (silica gel, 25 μm).

Reagents were used as received from common commercial suppliers unless otherwise stated. **5** (98+% D) was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). CD_3I (99.9% D) and **17** (98.9% D) were purchased from C/D/N Isotopes Inc. (Quebec, Canada). CD_3OD (min 99.8% D) was purchased from Merck KGaA (Darmstadt, Germany). Ethanol-d_1 (EtOD, 99% D) was purchased from Cambridge Isotope Laboratories, Inc. (MA, USA). In the hydrogen–deuterium exchange reaction of phenols in the EtOD/Et_3N system, Et_3N was dried over 3A molecular sieves prior to use, whereas in the Mizoroki–Heck reaction, Et_3N was used as received. **22a**,[16] **24a**,[17] **25a**,[18,19] **31a**,[20] and **33a**[21,22] were prepared according to literature procedures. A pressure tube (Ace pressure tube equipped with front seal plug, Z181099) was purchased from Sigma-Aldrich, Co. (MO, USA). The flowers of *E. japonica* were collected from the farm at the Yoshida campus of Yamaguchi University, Japan in the winter 2019–2020.

2.2 | Ethyl (*E*)-3-[$^2H_5$]phenylacrylate (6b), ethyl (*Z*)-3-[$^2H_5$]phenylacrylate (7), and 1-ethoxy-3-[$^2H_5$]phenyl-1-trimethylsiloxyallene (8)

2.2.1 | Preparation of the Grignard reagent

A suspension of magnesium turnings (1.62 g, 66.6 mmol) in anhydrous THF (10 ml) in the presence of a catalytic amount of iodine was treated with approximately 1/20th

of a solution of **5** (10.4 g, 64.2 mmol) in anhydrous THF (30 ml) at room temperature under a nitrogen atmosphere. The mixture was heated until the solvent began to boil gently to initiate the formation of the Grignard reagent. Then, the mixture was treated dropwise with the rest of the solution of **5** under cooling by an ice bath. After completion of the addition, the mixture was stirred for 30 min at room temperature and then left to stand for 29 h at room temperature under a nitrogen atmosphere. The black supernatant (35 ml, ca. 56 mmol) was used in the next step.

2.2.2 | Reaction of the Grignard reagent with ethyl propiolate

A mixture of CuI (2.90 g, 15.2 mmol) and LiCl (1.30 g, 30.7 mmol) in anhydrous THF (200 ml) was stirred for 15 min at room temperature under a nitrogen atmosphere, and then ethyl propiolate (3.0 ml, 30 mmol) and Me_3SiCl (5.0 ml, 39 mmol) were successively added to the mixture at −78°C. After 10 min of stirring at −78°C, the Grignard reagent solution prepared as described in the previous paragraph was added dropwise to the mixture over 1 h, and the reaction mixture was stirred for an additional hour at the same temperature. Then, the reaction was quenched by the dropwise addition of 2 M HCl (30 ml), and the mixture was allowed to warm to room temperature. The mixture was diluted with water (150 ml), and the separated aqueous layer was extracted with EtOAc (3 × 50 ml). The combined organic layers were washed successively with water (2 × 100 ml) and brine (50 ml), dried over anhydrous $MgSO_4$ and filtered, before the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc = 98/2 → 95/5, v/v). **8** (538 mg, 7.2%) eluted first followed by **7** (1.25 g, 23%), while **6b** (3.18 g, 59%) eluted last. **6b**–**8** were obtained as colorless oils. **6b**: ^{1}H NMR (600 MHz, $CDCl_3$, δ): 1.34 (3H, t, J = 7.1 Hz), 4.27 (2H, q, J = 7.1 Hz), 6.44 (1H, d, J = 16.2 Hz), 7.69 (1H, d, J = 16.2 Hz). ^{13}C NMR (151 MHz, $CDCl_3$, δ): 14.30, 60.48, 118.25, 127.60 (2C, d, J = 23.8 Hz), 128.34 (2C, d, J = 24.6 Hz), 129.68 (d, J = 23.8 Hz), 134.29, 144.52, 167.00. HRMS-EI (m/z): $[M]^+$ calcd for $C_{11}H_7D_5O_2$, 181.1151; found, 181.1149. **7**: ^{1}H NMR (600 MHz, $CDCl_3$, δ): 1.24 (3H, t, J = 7.1 Hz), 4.17 (2H, q, J = 7.1 Hz), 5.95 (1H, d, J = 12.5 Hz), 6.95 (1H, d, J = 12.5 Hz). ^{13}C NMR (151 MHz, $CDCl_3$, δ): 14.06, 60.26, 119.84, 127.44 (2C, t, J = 24.6 Hz), 128.44 (t, J = 24.6 Hz), 129.25 (2C, t, J = 24.6 Hz), 134.66, 142.89, 166.21. HRMS-EI (m/z): $[M]^+$ calcd for $C_{11}H_7D_5O_2$, 181.1151; found, 181.1151. **8**: ^{1}H NMR (600 MHz, $CDCl_3$, δ): 0.24 (9H, s), 1.22 (3H, t, J = 7.1 Hz), 4.20 (2H, q, J = 7.1 Hz), 6.82 (1H, s). ^{13}C NMR (151 MHz, $CDCl_3$, δ): −1.68 (3C), 14.13, 60.42, 127.73 (2C, t, J = 23.8 Hz), 127.78 (2C, t, J = 24.6 Hz), 127.89 (t, J = 25.3 Hz), 136.34, 138.61, 141.24, 171.99. HRMS-EI (m/z): $[M]^+$ calcd for $C_{14}H_{15}D_5O_2Si$, 253.1546; found, 253.1541.

2.3 | (*E*)-3-[$^2H_5$]Phenylacrylic acid (1b)

A mixture of **6** (500 mg, 2.76 mmol), EtOH (2.0 ml), and an aqueous NaOH solution (10% w/v, 2.0 ml) was stirred for 1 h at room temperature, and 2 M HCl (5.0 ml) and water (5.0 ml) were then added to the mixture. The precipitate was collected using suction filtration, washed successively with water and hexane, and dried in vacuo to give **1b** (330 mg, 78%) as a colorless solid. mp 134–135°C (lit.[23] mp 133°C). ^{1}H NMR (600 MHz, $CDCl_3$, δ): 6.46 (1H, d, J = 16.0 Hz), 7.81 (1H, d, J = 16.0 Hz). The carboxylic acid proton was not observed. ^{2}H NMR (92 MHz, $CHCl_3$, δ): 7.46 (br s), 7.62 (br s). ^{13}C NMR (151 MHz, $CDCl_3$, δ): 117.32, 127.94 (2C, t, J = 23.8 Hz), 128.44 (2C, t, J = 23.8 Hz), 130.22 (t, J = 24.6 Hz), 133.86, 147.04, 172.59. HRMS-EI (m/z): $[M]^+$ calcd for $C_9H_3D_5O_2$, 153.0838; found, 153.0837.

2.4 | 4-Hydroxy-3-[$^2H_3$] methoxybenzaldehyde (10)

NaH (3.20 g, 60% dispersion in mineral oil, 80 mmol) was added portionwise to an ice-cold solution of **9** (5.00 g, 36.2 mmol) in anhydrous DMSO (90 ml). After 30 min of stirring at room temperature, a solution of CD_3I (5.40 g, 37.3 mmol) dissolved in anhydrous DMSO (10 ml) was added dropwise to the mixture. The reaction mixture was stirred for 11 h at room temperature and quenched by the dropwise addition of 2 M HCl (30 ml) at 0°C. The mixture was diluted with water (200 ml) and extracted with toluene (3 × 75 ml). The combined organic layers were washed successively with water (2 × 50 ml) and brine (50 ml), dried over anhydrous $MgSO_4$ and filtered, before the filtrate was concentrated in vacuo. The combined aqueous layers were extracted again with $CHCl_3$ (3 × 75 ml) because the toluene extraction was not sufficient. The $CHCl_3$ layers were combined and washed with brine (50 ml), dried over anhydrous $MgSO_4$ and filtered, before the filtrate was concentrated in vacuo. The crude products thus obtained were combined and purified by flash column chromatography on silica gel (hexane/EtOAc = 80/20 → 60/40, v/v) to give **10** (4.33 g, 77%) as a pale-yellow solid. mp 82°C (lit.[11,24] mp 81–83°C). ^{1}H NMR (600 MHz, $CDCl_3$, δ): 6.25 (1H, s), 7.05 (1H, d, J = 7.9 Hz), 7.42–7.44 (2H, m), 9.83 (1H, s). ^{13}C NMR

(151 MHz, $CDCl_3$, δ): 55.22 (septet, $J = 22.2$ Hz), 108.77, 114.39, 127.49, 129.72, 147.15, 151.75, 190.98. The spectra were in good accordance with those reported.[24]

2.5 | Ethyl (*E*)-3-(4-hydroxy-3-[$^2H_3$] methoxyphenyl)acrylate (11b) and its (*Z*)-isomer (12)

A mixture of **10** (3.75 g, 24.2 mmol) and (carbethoxymethylene)triphenylphosphorane (9.00 g, 25.8 mmol) in anhydrous toluene (100 ml) was stirred for 3 h at 90°C. The reaction mixture was cooled to room temperature before the addition of silica gel (100 ml). The solvent was evaporated, and the residue was subjected to flash column chromatography on silica gel (hexane/EtOAc = 85/15 → 50/50, v/v). **12** (838 mg, 15%) eluted first, followed by **11b** (4.60 g, 85%). Both **11b** and **12** were obtained as oils at first and solidified during storage in a refrigerator. **11b**: Colorless solid. mp 55–58°C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 1.34 (3H, t, $J = 7.2$ Hz), 4.26 (2H, q, $J = 7.2$ Hz), 5.88 (1H, s), 6.29 (1H, d, $J = 16.2$ Hz), 6.92 (1H, d, $J = 8.2$ Hz), 7.03 (1H, d, $J = 1.7$ Hz), 7.07 (1H, dd, $J = 8.2$, 1.7 Hz), 7.61 (1H, d, $J = 16.2$ Hz). ^{13}C NMR (151 MHz, $CDCl_3$, δ): 14.27, 55.03 (septet, $J = 22.2$ Hz), 60.31, 109.30, 114.71, 115.48, 122.91, 126.91, 144.66, 146.75, 147.91, 167.30. HRMS-EI (*m*/*z*): $[M]^+$ calcd for $C_{12}H_{11}D_3O_4$, 225.1080; found, 225.1080. **12**: Colorless solid. mp 37–39°C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 1.29 (3H, t, $J = 7.1$ Hz), 4.19 (2H, q, $J = 7.1$ Hz), 5.81 (1H, d, $J = 13.1$ Hz), 5.85 (1H, s), 6.79 (1H, d, $J = 13.1$ Hz), 6.88 (1H, d, $J = 8.1$ Hz), 7.11 (1H, dd, $J = 8.1$, 2.1 Hz), 7.77 (1H, d, $J = 2.1$ Hz). ^{13}C NMR (151 MHz, $CDCl_3$, δ): 14.17, 55.08 (septet, $J = 21.9$ Hz), 60.12, 112.72, 113.79, 116.73, 125.55, 127.13, 143.65, 145.86, 147.00, 166.50. HRMS-EI (*m*/*z*): $[M]^+$ calcd for $C_{12}H_{11}D_3O_4$, 225.1080; found, 225.1081.

2.6 | (*E*)-3-(4-Hydroxy-3-[$^2H_3$] methoxyphenyl)acrylic acid (2b)

A mixture of **11** (660 mg, 2.93 mmol), EtOH (4.0 ml), and an aqueous NaOH solution (10% w/v, 4.0 ml) was stirred for 2 h at room temperature. Additional aqueous NaOH solution (10% w/v, 8.0 ml) was added to the mixture, and the reaction mixture was stirred for a further 5 h at room temperature. The mixture was acidified (pH = 1) with 2 M HCl before being poured onto water (50 ml). The precipitate was collected using suction filtration, washed successively with water and hexane, and dried in vacuo to give **2b** (393 mg, 68%) as a pale-yellow solid. mp 172–173°C. ^{1}H NMR (600 MHz, CD_3SOCD_3, δ): 6.37 (1H, d, $J = 15.8$ Hz), 6.80 (1H, d, $J = 8.1$ Hz), 7.09 (1H, dd, $J = 8.1$, 1.8 Hz), 7.28 (1H, d, $J = 1.8$ Hz), 7.50 (1H, d, $J = 15.8$ Hz), 9.55 (1H, s), 12.14 (1H, br s). ^{2}H NMR (92 MHz, CH_3SOCH_3, δ): 3.77 (br s). ^{13}C NMR (151 MHz, CD_3SOCD_3, δ): 55.07 (septet, $J = 21.7$ Hz), 111.33, 115.72, 115.83, 123.01, 125.99, 144.74, 148.12, 149.28, 168.21. HRMS-EI (*m*/*z*): $[M]^+$ calcd for $C_{10}H_7D_3O_4$, 197.0767; found, 197.0766.

2.7 | (*E*)-3-(4-Hydroxy-3-[$^2H_3$] methoxyphenyl)prop-2-enol (13)

A solution of **11** (2.60 g, 11.5 mmol) in anhydrous CH_2Cl_2 (150 ml) was treated dropwise with a solution of diisobutylaluminum hydride (1.0 M in toluene, 48 ml, 48 mmol) at −78°C under an argon atmosphere, and the mixture was stirred for 2 h at room temperature. The reaction was quenched by the dropwise addition of an aqueous solution of potassium sodium tartrate tetrahydrate (50% w/v, 100 ml) at −78°C. The mixture was stirred for a further 21 h at room temperature and then slightly acidified (pH = 6) with 2 M HCl. The separated aqueous layer was extracted with $CHCl_3$ (2 × 50 ml), and the combined organic layers were washed with brine (100 ml), dried over anhydrous $MgSO_4$ and filtered, before the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc = 50/50 → 20/80, v/v) to give **13** (1.95 g, 92%) as a colorless solid. mp 74–75°C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 1.84 (1H, br s), 4.29 (2H, br d, $J = 5.5$ Hz), 5.83 (1H, s), 6.20 (1H, dt, $J = 15.8$, 5.5 Hz), 6.51 (1H, br d, $J = 15.8$ Hz), 6.85 (1H, d, $J = 8.2$ Hz), 6.87 (1H, dd, $J = 8.2$, 1.5 Hz), 6.89 (1H, d, $J = 1.5$ Hz). ^{13}C NMR (151 MHz, $CDCl_3$, δ): 54.99 (septet, $J = 22.2$ Hz), 63.75, 108.30, 114.44, 120.20, 126.05, 129.17, 131.30, 145.51, 146.60. HRMS-FAB-NBA (*m*/*z*): $[M]^+$ calcd for $C_{10}H_9D_3O_3$, 183.0975; found, 183.0972.

2.8 | (*E*)-3-(4-Hydroxy-3-[$^2H_3$] methoxyphenyl)prop-2-enal (14)

DDQ (1.00 g, 4.41 mmol) was added portionwise to an ice-cold solution of **13** (734 mg, 4.01 mmol) in anhydrous 1,4-dioxane (40 ml), and the mixture was stirred for 30 min at 0°C. After a further 2.5 h of stirring at room temperature, the insoluble materials were filtered off, and the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc = 65/35 → 50/50, v/v) to give a yellow oil, which was dissolved in EtOAc (100 ml). The organic layer was washed successively with a saturated aqueous

solution of $NaHCO_3$ and brine (40 ml each), dried over anhydrous $MgSO_4$ and filtered, before the filtrate was concentrated in vacuo to give **14** (397 mg, 55%) as a pale-yellow solid. mp 80–82°C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 6.28 (1H, br s), 6.60 (1H, dd, J = 15.8, 7.7 Hz), 6.96 (1H, d, J = 8.2 Hz), 7.06 (1H, d, J = 2.1 Hz), 7.12 (1H, dd, J = 8.2, 2.1 Hz), 7.41 (1H, d, J = 15.8 Hz), 9.64 (1H, d, J = 7.7 Hz). ^{13}C NMR (151 MHz, $CDCl_3$, δ): 55.12 (septet, J = 22.2 Hz), 109.47, 114.93, 123.99, 126.25, 126.54, 146.95, 148.97, 153.23, 193.70. HRMS-FAB-NBA (m/z): $[M + H]^+$ calcd for $C_{10}H_8D_3O_3$, 182.0896; found, 182.0891.

2.9 | 4-Hydroxy-3,5-di[$^2H_3$] methoxybenzaldehyde (16)

CD_3OD (8.5 ml) was removed from a solution of CD_3ONa (prepared from sodium (1.15 g, 50.0 mmol) and CD_3OD (25 ml)) under reflux with stirring, and the solution was cooled to room temperature. A mixture of **15** (1.77 g, 6.32 mmol) and $CuCl_2$ (680 mg, 5.06 mmol) in anhydrous DMF (12.5 ml) was added to the solution. The reaction mixture was stirred for 1 h at 110°C with the removal of CD_3OD (13 ml) and cooled to room temperature. The mixture was diluted with water (50 ml), acidified (pH = 1) at 0°C with concentrated HCl, and extracted with EtOAc (3 × 50 ml). The combined organic layers were washed with brine (40 ml), dried over anhydrous $MgSO_4$ and filtered, before the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc = 60/40 → 30/70, v/v) to give **16** (1.03 g, 87%) as a colorless solid. mp 112–113°C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 6.05 (1H, s), 7.15 (2H, s), 9.82 (1H, s). ^{13}C NMR (151 MHz, $CDCl_3$, δ): 55.56 (2C, septet, J = 22.2 Hz), 106.59 (2C), 128.25, 140.79, 147.28 (2C), 190.72. HRMS-FAB-NBA (m/z): $[M + H]^+$ calcd for $C_9H_5D_6O_4$, 189.1034; found, 189.1034.

2.10 | (*E*)-3-(4-Hydroxy-3,5-di[$^2H_3$] methoxyphenyl)acrylic acid (3b)

A mixture of **16** (988 mg, 5.25 mmol), malonic acid (1.20 g, 11.5 mmol), and aniline (64.7 mg, 0.695 mmol) in anhydrous pyridine (2.7 ml) was stirred for 20 h at 60°C and then cooled to room temperature. Crushed ice (5.6 g) and concentrated HCl (3.3 ml) were added successively to the reaction mixture. After 30 min of stirring at room temperature, the precipitate was collected using suction filtration and dried in vacuo to give **3b** (994 mg, 82%) as a pale-yellow solid. mp 194–195°C. ^{1}H NMR (600 MHz, CD_3SOCD_3, δ): 6.43 (1H, d, J = 15.8 Hz), 6.99 (2H, s), 7.51 (1H, d, J = 15.8 Hz), 8.92 (1H, br s), 12.16 (1H, br s). ^{2}H NMR (92 MHz, CH_3SOCH_3, δ): 3.76 (br s). ^{13}C NMR (151 MHz, CD_3SOCD_3, δ): 55.50 (2C, septet, J = 21.9 Hz), 106.27 (2C), 116.30, 124.86, 138.29, 145.10, 148.28 (2C), 168.24. HRMS-EI (m/z): $[M]^+$ calcd for $C_{11}H_6D_6O_5$, 230.1061; found, 230.1061.

2.11 | 4-Bromo[2,3,5,6-$^2H_4$]phenol (18)

Tetrabutylammonium tribromide (25.0 g, 51.8 mmol) was added portionwise to a solution of **17** (5.10 g, 50.9 mmol) in MeOH (200 ml) and CH_2Cl_2 (300 ml) at room temperature. The mixture was stirred for a further 30 min at room temperature and then concentrated in vacuo. Water (150 ml) was added to the residue, and the aqueous layer was extracted with toluene (1 × 100 ml, 2 × 50 ml). The combined organic layers were washed successively with water (100 ml) and brine (50 ml), dried over anhydrous $MgSO_4$ and filtered, before the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc = 90/10 → 60/40, v/v) to give **18** (6.29 g, 70%) as a pale-yellow oil, which solidified during storage in a refrigerator. mp 61–65°C. ^{13}C NMR (151 MHz, $CDCl_3$, δ): 112.64, 116.81 (2C, t, J = 24.6 Hz), 132.06 (2C, t, J = 25.3 Hz), 154.46.

2.12 | Ethyl (*E*)-3-(4-hydroxy[2,3,5,6-$^2H_4$] phenyl)[2-^{2}H]acrylate (19′)

2.12.1 | Mizoroki–Heck reaction

Under a nitrogen atmosphere, a mixture of **18** (5.92 g, 33.4 mmol), tri(*o*-tolyl)phosphine (2.00 g, 6.57 mmol), ethyl acrylate (7.00 g, 69.9 mmol), Pd $(OAc)_2$ (250 mg, 1.11 mmol), and Et_3N (35 ml) was stirred for 23 h under reflux. The reaction mixture was cooled to room temperature and concentrated in vacuo. The residue was suspended in CH_2Cl_2, and AcOH (10 ml) was added to the suspension. This mixture was subjected to flash column chromatography on silica gel (hexane/EtOAc = 80/20 → 15/85, v/v) to give **19** as a pale-yellow solid (6.53 g, quant). From its ^{1}H NMR spectrum, the deuterium content at the *ortho* position of the phenolic hydroxy group and the C-2 position of **19** was estimated to be 55% and 18%, respectively.

2.12.2 | Re-incorporation of deuterium

19 (400 mg, 2.05 mmol) was transferred to a pressure tube, the interior of which was flushed with argon.

EtOD (12 ml, 0.21 mol) and Et_3N (0.60 ml, 4.3 mmol) were added to the tube, and the upper space of the tube was flushed with argon again before the tube was plugged. The plugged tube was heated at 100°C for 72 h with stirring. The mixture was cooled to room temperature, concentrated in vacuo, and purified by flash column chromatography on silica gel (hexane/EtOAc = 80/20 → 20/80, v/v) to give a colorless solid (390 mg, 97%). Part of the product from the first cycle (384 mg, 1.95 mmol) was treated and purified as described above to give a colorless solid (382 mg, 99%). Part of the product from the second cycle (373 mg, 1.89 mmol) was treated with EtOD (11 ml, 0.19 mol) and Et_3N (0.55 ml, 3.9 mmol) at 100°C for 72 h and purified chromatographically to give **19′** as a colorless solid (368 mg, 99%). mp 75–77°C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 1.34 (3H, t, $J = 7.0$ Hz), 4.26 (2H, q, $J = 7.0$ Hz), 5.66 (1H, br s), 7.63 (1H, s). ^{13}C NMR (151 MHz, $CDCl_3$, δ):14.20, 60.80, 114.52 (t, $J = 23.8$ Hz), 115.57 (2C, t, $J = 23.8$ Hz), 126.34, 129.62 (2C, t, $J = 23.8$ Hz), 145.06, 158.39, 168.45. HRMS-ESI (m/z): $[M - H]^-$ calcd for $C_{11}H_6D_5O_3$, 196.1028; found, 196.1029. ^{1}H NMR analysis of the products of each operation revealed that the deuterium content at the *ortho* position of the phenolic hydroxy group was 84% (first cycle), 94% (second cycle), and 98% (third cycle) and that at the C-2 position was 91% (first cycle), 98% (second cycle), and 98% (third cycle).

2.13 | (*E*)-3-(4-Hydroxy[2,3,5,6-$^2H_4$]phenyl)[2-^{2}H]acrylic acid (4b′)

A solution of **19′** (192 mg, 0.973 mmol) in EtOH (1.5 ml) was treated dropwise with an aqueous NaOH solution (10% w/v, 4.5 ml) at room temperature. After 3 h of stirring at room temperature, the mixture was acidified (pH = 1) with 2 M HCl at 0°C. The precipitate was collected using suction filtration, washed with ice-cold water, and dried in vacuo to give **4b′** (150 mg, 91%) as a colorless solid. The mp of **4b′** was difficult to measure, as it sublimed gradually from approximately 180–219°C. ^{1}H NMR (600 MHz, CD_3SOCD_3, δ): 7.49 (1H, br s). Active protons (CO_2H and phenolic OH) were not clearly observed. ^{2}H NMR (92 MHz, CH_3SOCH_3, δ): 6.31 (br s), 6.82 (br s), 7.54 (br s). ^{13}C NMR (151 MHz, CD_3SOCD_3, δ): 115.28 (t, $J = 23.8$ Hz), 115.60 (2C, t, $J = 23.8$ Hz), 125.31, 129.93 (2C, t, $J = 23.1$ Hz), 144.28, 159.74, 168.20. HRMS-ESI (m/z): $[M - H]^-$ calcd for $C_9H_2D_5O_3$, 168.0715; found, 168.0714.

2.14 | General procedure for the hydrogen–deuterium exchange reaction in the EtOD/Et_3N system

In the cases of **11a**, **12**, **20a**, and **22a**–**31a**, which contain one phenolic hydroxy group, the substrate (1.35–1.44 mmol) was transferred to a pressure tube, the interior of which was flushed with argon. EtOD (8.0 ml, 0.14 mol, ca. 100 equiv.) and Et_3N (0.40–0.42 ml, 2.9–3.0 mmol, ca. 2.1 equiv.) was added to the tube, and the upper space of the tube was flushed with argon again before the tube was plugged. The plugged tube was heated at 100°C for 72 h with stirring. The mixture was cooled to room temperature, concentrated in vacuo, and purified by flash column chromatography on silica gel. The deuterium content in the product was determined using ^{1}H NMR. The same reaction conditions were applied to **6a** and **37**. In the cases of **21a**, **33a**, **34a**, and **36a**, which contain two phenolic hydroxy groups, 0.670–0.700 mmol of the substrate was treated with approximately 3 equiv. of Et_3N (0.28–0.30 ml) and 200 equiv. of EtOD (8.0 ml). The same conditions were applied to **4a**. In the cases of **32a** and **35a**, which contain three phenolic hydroxy groups, 0.469–0.470 mmol of the substrate was treated with approximately 4 equiv. of Et_3N (0.26 ml) and 300 equiv. of EtOD (8.0 ml). For detailed conditions, see the Supporting Information.

2.15 | Method for feeding the flower petals with 1b and 4b′ and GC-MS analysis of the benzenoid volatiles

Petals of opened flowers (100 mg fresh weight) were harvested and immersed in a 12-well cell culture plate (well size: 23 mm i.d. and 18 mm in height), in which **1b** or **4b′** dissolved in an aqueous $NaHCO_3$ solution (5% w/v) had been incorporated. The feed concentration was 50 mM and feeding was performed via vacuum infiltration with a vacuum desiccator and pump. After 5 h of incubation, the flower petals were homogenized in liquid nitrogen with a mortar and pestle, and the volatile compounds were extracted using 3 ml of EtOAc containing isobutyl benzene (5 μg) as an internal standard. The resulting volatiles were analyzed using a gas chromatography–mass spectrometry (GC-MS) instrument (QP-2010 Plus, Shimadzu Corporation, Kyoto, Japan) equipped with a DB-5ms column (30 m × 0.25 mm i.d. × 0.4 μm film thickness, Agilent Technologies, Inc., CA, USA). The column temperature was programmed as follows: 40°C for 2 min, 10°C min^{-1} ramp to 170°C, 20°C min^{-1} ramp to 240°C, 10 min hold. Helium was

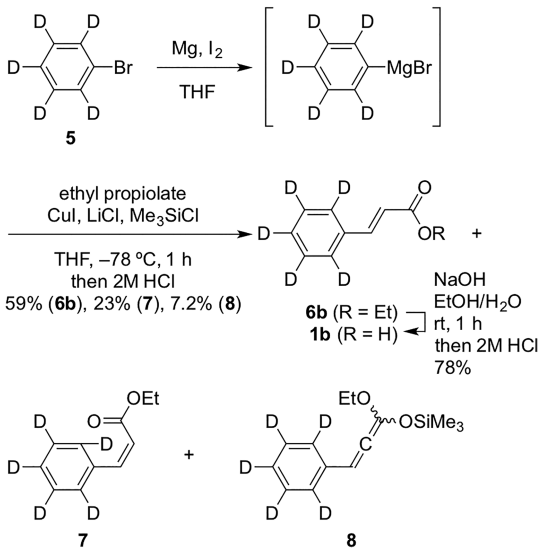
used as the carrier gas. The MS was operated in the electron ionization mode at 70 eV. The ion source and interface temperature were set to 240°C.

3 | RESULTS AND DISCUSSION

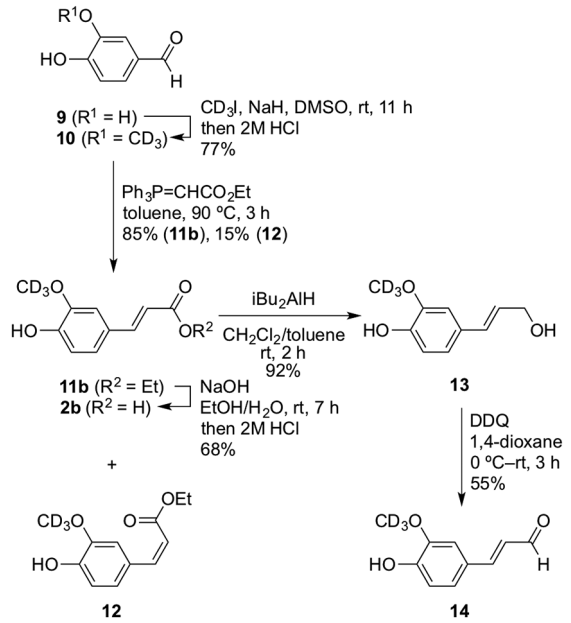
3.1 | Synthesis of deuterium-labeled cinnamic acids

For the synthesis of cinnamic acids, Knoevenagel condensations between benzaldehydes and malonic acid have frequently been used.[9–13,25–27] Knoevenagel condensations involving monoalkyl malonates have also been employed for the synthesis of alkyl cinnamates,[9,28] in addition to Wittig reactions between benzaldehydes and stable ylides,[29,30] and Mizoroki–Heck reactions between aryl halides and alkyl acrylates.[31] In this study, we selected previously established reaction conditions considering both the availability of deuterium sources and that of non-deuterated starting materials.

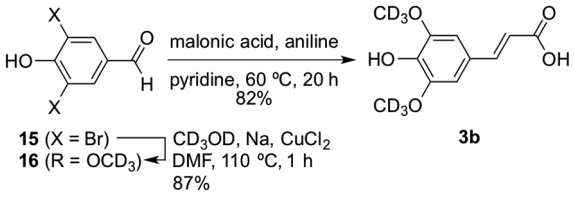
The synthesis of **1b** is illustrated in Scheme 1, whereby bromobenzene-d_5 (**5**) was used as the deuterium source. In Schemes 1–4, "D" represents a deuterium content greater than 98%, as the deuterium content of the starting materials is ≥98%. We used a Me_3SiCl-mediated catalytic carbocupration, considering that the reaction between PhMgBr and ethyl propiolate under the reported conditions furnishes ethyl cinnamate in high yield with *E*-selectivity (91%, $E/Z = 95/5$).[32] In our experiment, the desired product **6b** and its *Z*-isomer **7** were obtained in 59% and 23% yield, respectively. Allene **8** (7.2%), the intermediate in the carbocupration, was also obtained after chromatographic purification on silica gel. Such intermediates were not isolated in the original report.[32] Basic hydrolysis of **6b** furnished **1b** in 78% yield.



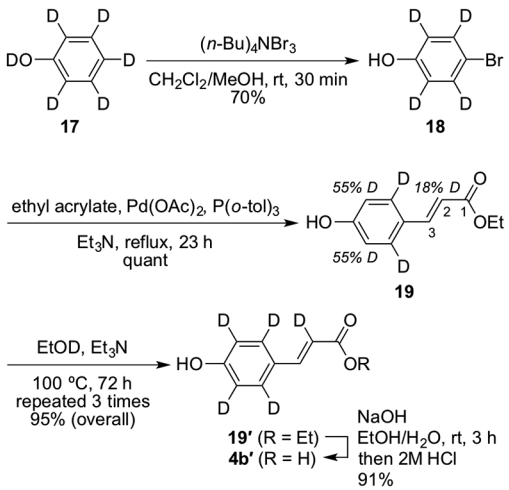
SCHEME 1 Synthesis of **1b**



SCHEME 2 Synthesis of **2b**, **13**, and **14**



SCHEME 3 Synthesis of **3b**



SCHEME 4 Synthesis of deuterium-labeled 4-coumaric acid **4b′**

The synthesis of **2b** is illustrated in Scheme 2, whereby CD_3I was used as the deuterium source. As described in the literature,[11,33] the 3-hydroxy group of 3,4-dihydroxybenzaldehyde (**9**) was regioselectively alkylated to produce **10** in 77% yield. We used a Wittig reaction for the construction of the cinnamate skeleton, because the reaction between 4-hydroxy-3-methoxybenzaldehyde and (carbethoxymethylene) triphenylphosphorane has been reported to provide ethyl ferulate in 95% yield.[29] In our reaction, the Wittig reaction between **10** and the stable ylide provided **11b** and its *Z*-isomer **12** in 85% and 15% yield, respectively. Basic hydrolysis of **11b** furnished **2b** in 68% yield. Employing the reported procedure,[30] **11b** was also reduced with diisobutylaluminum hydride to give **13** in 92% yield, which was oxidized with DDQ to afford **14** in 55% yield (Scheme 2).

The synthesis of **3b** is illustrated in Scheme 3, whereby CD_3OD was used as the deuterium source. The procedure of Rao and Stuber[34] was applied to 3,5-dibromo-4-hydroxybenzaldehyde (**15**) to produce **16** in 87% yield. Lan and coworkers have synthesized **16** from 4-hydroxy-3,5-diiodobenzaldehyde in 76% yield under similar conditions.[35] We used a Knoevenagel condensation in the next step, because the reaction between 4-hydroxy-3,5-dimethoxybenzaldehyde and malonic acid in the presence of aniline has been reported to afford sinapic acid in 86% yield.[27] Using these reaction conditions, **16** was converted to **3b** in 82% yield.

Our attempt to synthesize **4b** is illustrated in Scheme 4, whereby phenol-d_6 (**17**) was used as the deuterium source. We used a Mizoroki–Heck reaction for the construction of the cinnamate skeleton, because the reaction between 4-bromophenol and methyl acrylate in the presence of $Pd(OAc)_2$ and $P(o\text{-tol})_3$ in refluxing Et_3N has been reported to produce methyl 4-coumarate in 90% yield.[31] Therefore, **17** was first brominated with tetrabutylammonium tribromide[36] to provide **18** in 70% yield and then subjected to a Mizoroki–Heck reaction with ethyl acrylate using the reported conditions.[31] This reaction quantitatively provided deuterium-labeled ethyl 4-coumarate **19**; however, approximately half the deuterium atoms at the *ortho* position of the phenolic hydroxy group proved to be substituted with hydrogen atoms based on the ^1H NMR analysis. Interestingly, ca. 20% of the hydrogen atoms at the C-2 position of **19** were exchanged for deuterium atoms. Because the Mizoroki–Heck reaction was not carried out under strict anhydrous conditions, hydrogen atoms from water in the reaction system may have been exchanged with the deuterium atoms of **18** and **19**. Another possibility is the migration of the hydrogen atoms on the phenolic hydroxy groups of **18** and **19**. In both cases, Et_3N is considered to promote the exchange reaction.

Therefore, the re-incorporation of the deuterium atoms on **19** was carried out using ethanol-d_1 (EtOD, ca. 100 equiv.) as the deuterium source. EtOD has been used for the deuteration of the methyl ketone moiety of a steroidal compound in the presence of catalytic amount of PhLi.[37] The reaction was carried out in the presence of Et_3N (ca. 2.1 equiv.) at 100°C using a pressure tube. The progress of the reaction was monitored after 24, 48, and 72 h using ^1H NMR. The deuterium content at the *ortho* position of the phenolic hydroxy group reached its maximum value after 72 h, whereas that at the C-2 position reached its maximum after 24 h. The reaction did not proceed at room temperature, and only the hydrogen atoms at the C-2 position were slowly exchanged by deuterium atoms at 70°C. The use of CD_3CO_2D instead of Et_3N did not affect the deuterium content of **19**.

After 72 h of reaction, the ratios of deuterium atoms at the *ortho* position of the phenolic hydroxy group and the C-2 position were 84% and 91%, respectively. When the reaction product was subjected to the second operation, the deuterium content at the former and latter positions increased to 94% and 98%, and that at the former also reached 98% after the third operation. The yield of each reaction was nearly quantitative (97%, 99%, 99%). The thus-obtained **19′** was hydrolyzed under basic conditions to give **4b′** in 91% yield. No loss of deuterium atoms was observed in the basic hydrolysis. Recently, Chen and coworkers prepared **4b** using a Mizoroki–Heck reaction between **18** and acrylic acid in an aqueous K_2CO_3 solution.[38] They reported neither the loss of deuterium atoms at the *ortho* position of the phenolic hydroxy group nor the incorporation of deuterium at the C-2 position of **4b**.

3.2 | Hydrogen–deuterium exchange reaction of phenolic compounds in the EtOD/Et_3N system

To assess the scope and limitations of the hydrogen–deuterium exchange reaction, we investigated a series of alkyl cinnamates and other phenolic compounds as substrates. In this study, approximately 100 equiv. of EtOD and 2.1 equiv. of Et_3N per equiv. of substrates bearing one phenolic hydroxy group were employed; the ratio of reagents per substrate was increased in the cases of polyphenolic compounds (for detailed conditions, see Section 2 and the Supporting Information). The results after a single operation at 100°C for 72 h are summarized in Figure 2.

Except in the case of free carboxylic acid **4a**, which decomposed under the reaction conditions, hydrogen–

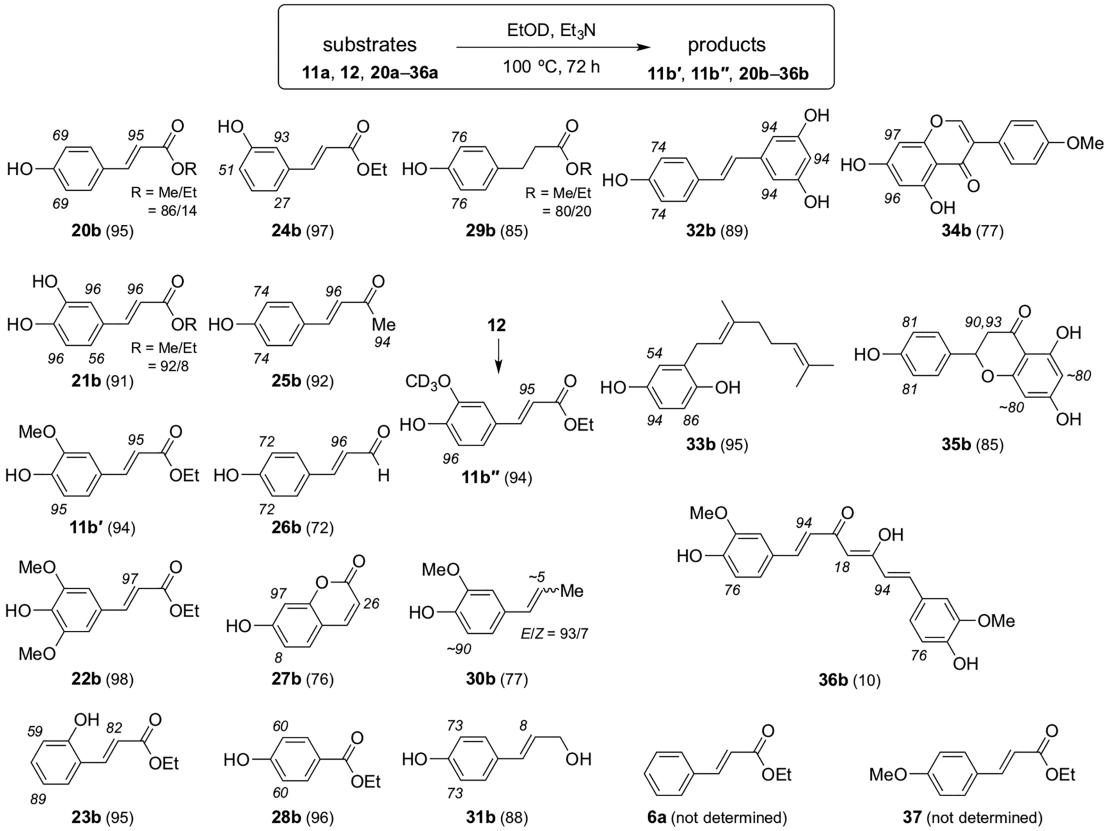


FIGURE 2 Deuterium incorporation ratios of phenolic compounds in the EtOD/Et_3N system. The numbers in italics in each structure represent the percentage of deuterium atom incorporation at the designated positions. The number in parentheses represents the yield of the reaction after chromatographic purification

deuterium exchange occurred at the *ortho* positions of the phenolic hydroxy groups of all the compounds tested, although the *meta* positions were totally inactive. Hydrogen–deuterium exchange also occurred at the C-2 position of alkyl cinnamates **20a**, **21a**, **11a**, **22a**, and **23a**, as well as the *para* positions of the phenolic hydroxy groups of **21a**, **23a**, and **24a**. Although the C-2 position of **24a** was inactive, the corresponding position was deuterated in **25a**–**27a**. Considering these results, it is obvious that the deuterated positions can resonate with the anion of the phenolic hydroxy groups. As seen in the cases of **28a** and **29a**, the hydrogen–deuterium exchange reaction proceeds regardless of the presence or absence of an electron-withdrawing group on a benzene ring. Roughley and Whiting have reported the deuteration of **11a** and (*E*)-4-(4-hydroxy-3-methoxyphenyl)but-3-en-2-one, in which a methoxy group is introduced to the benzene ring of **25a**, in D_2O (ca. 50 equiv.)/1,4-dioxane in the presence of Et_3N (1 equiv.) at 100°C for 3 days.[25] They observed the incorporation of the deuterium atoms at the *ortho* positions of their phenolic hydroxy groups as well as the C-2 position of **11a** and the corresponding position of the latter compound. However, neither chemical yield nor deuterium contents were reported, and a systematic investigation into this type of hydrogen–deuterium exchange reaction was not carried out.

In the reaction, the *Z*-double bond of **12** was completely isomerized to the *E* configuration to give **11b″**, whereas the *E*/*Z* ratio of isoeugenol (**30a**) did not change during the reaction. A small amount (ca. 5%–8%) of the olefinic hydrogen atoms were exchanged for deuterium atoms in the cases of **30a** and **31a**. In contrast, no deuteration was observed at the olefinic position of resveratrol (**32a**). In addition, as seen in the cases of geranylhydroquinone (**33a**) and biochanin A (**34a**), the olefinic positions without conjugation with the anions of the phenolic hydroxy groups are also inactive. Kirby and Ogunkoya have reported the deuteration of the *ortho* position of the hydroxy group of **30a** under basic conditions (0.5 equiv. of Et_3N or NaOD in D_2O at 100°C),[39] but detailed results were not described.

As seen in the cases of **25a** and naringenin (**35a**), the hydrogen atoms of methyl or methylene groups adjacent to a ketone carbonyl group were exchanged with deuterium atoms, but the less-acidic hydrogen atoms at the α-position of the ester carbonyl group of **29a** were

inactive. Curcumin (**36a**), which has active hydrogen atoms between the two carbonyl groups, was unstable, probably due to aldol and/or Michael addition reactions, and the yield of the deuterium-labeled product **36b** was 10%, even though the hydrogen–deuterium exchange proceeded efficiently. In the cases of **6a** and **37**, in which a free phenolic hydroxy group is not present, the hydrogen–deuterium exchange did not proceed. Despite the presence of a large excess of EtOD, the extent of the ester exchange reaction (methyl to ethyl) was relatively small (8%–20%), as seen in the cases of **20b**, **21b**, and **29b**.

D_2O has been frequently used in combination with acid catalysts for the deuteration of benzene rings.[12,26,35,40] Recently, Sajiki and coworkers have developed a hydrogen–deuterium exchange reaction using D_2O as a deuterium source in the presence of platinum-group catalysts.[41–43] This method can deuterate not only arenes but also saturated aliphatic compounds. In contrast to these methods, to our best of knowledge, only a few examples of base-catalyzed hydrogen–deuterium exchange on the benzene ring of phenolic compounds have been reported.[25,39] However, Zhan and co-workers have recently developed a method for the deuteration of phenols in D_2O in the presence of NaOH (0.5 equiv.) under microwave irradiation.[44] Under these basic conditions, as seen in this study, the *ortho* and/or *para* positions of phenolic hydroxy groups are regioselectively deuterated. As summarized in Figure 2, the yield of the hydrogen–deuterium exchange reaction in the EtOD/Et_3N system is satisfactory (72%–98%), and the deuterium content can be increased by repeating the reaction, as seen in the case of **19**. In addition, not only alkyl cinnamates, but also a variety of phenolic compounds, are suitable substrates of the reaction. Furthermore, EtOD is a readily available, inexpensive, and easy-to-handle reagent, and can dissolve a wide range of substrates compared with D_2O. Therefore, the hydrogen–deuterium exchange reaction of phenolic compounds in the EtOD/Et_3N system should provide a useful alternative to previously reported methods.

3.3 | Tracking the biosynthesis of benzenoid volatiles in the flowers of *E. japonica*

With the desired deuterium-labeled cinnamic acids in hand, we investigated the biosynthetic pathway of benzenoid volatiles in the flowers of the Japanese loquat *E. japonica*. Kuwahara and coworkers have reported that (2-nitroethyl)benzene, 4-methoxybenzaldehyde (**38a**), and methyl 4-methoxybenzoate (**39a**) are the major components of the flower scent of *E. japonica*.[15] In their study, 4-methoxybenzyl alcohol (**40a**) was detected as a minor component, and the benzenoids **38a**–**40a** were speculated to be biosynthesized from tyrosine.[45] In their proposed pathway, the involvement of cinnamic acids **1a** and **4a** was not considered. To assess this point, we administered deuterium-labeled **1b** and **4b′** to the flowers of *E. japonica* and analyzed the profile of the benzenoid volatiles after 5 h of incubation. The results using **1b** are shown in Figure 3.

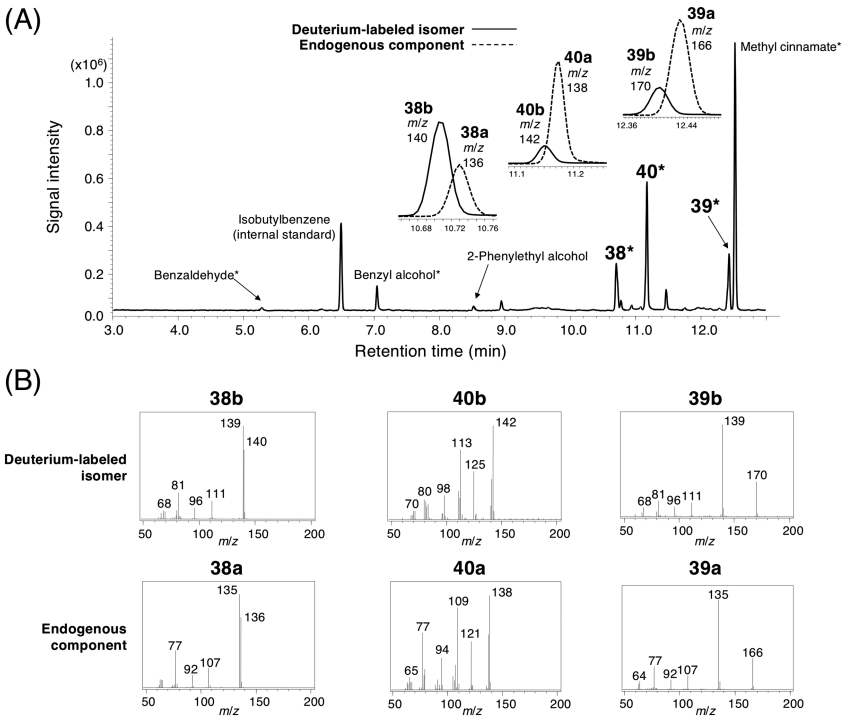
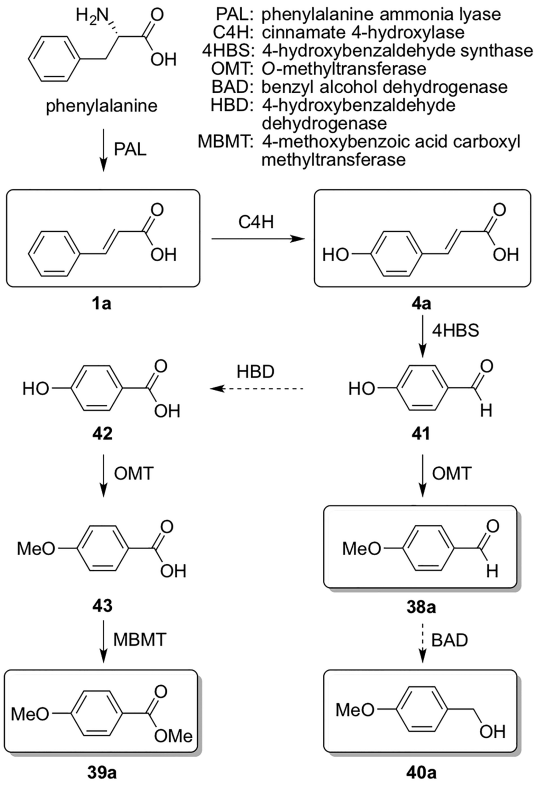


FIGURE 3 GC-MS analysis of the benzenoid volatiles in the flowers of *Eriobotrya japonica* after 5 h incubation with **1b**. (A) Total ion chromatogram of EtOAc extracts of the loquat flowers. Deuterium-labeled volatiles are indicated by asterisks. (B) MS profiles obtained from the peaks corresponding to **38** (left), **40** (middle), and **39** (right)

As shown in Figure 3, the conversion of the administered **1b** to deuterium-labeled **38b–40b** was observed. In our experiment, not only **38a** and **39a** but also **40a**, were detected as the major endogenous components in the scent of *E. japonica* flowers. Similarly, the formation of **38b–40b** was observed after the administration of **4b′** (Supporting Information). Because **1a** is biosynthesized from phenylalanine, and the activity of tyrosine ammonia lyase, which converts tyrosine to **4a**, is generally limited in Poaceae (monocot grass family),[46] our results indicate that the biosynthetic precursor of benzenoids **38a–40a** in the flowers of *E. japonica* is phenylalanine but not tyrosine. Accordingly, we propose a putative biosynthetic route for benzenoids **38a–40a** in the flowers of *E. japonica* as illustrated in Scheme 5.

As shown in Scheme 5, the first two steps of the biosynthesis of benzenoids **38a–40a** in the flowers of *E. japonica* are believed to be mediated by the well-known enzymes phenylalanine ammonia lyase and cinnamate 4-hydroxylase. The former converts phenylalanine to **1a**, whereas the latter subsequently oxidizes **1a** to



SCHEME 5 Putative biosynthetic route of benzenoids **38a–40a** in the flowers of *Eriobotrya japonica*. The deuterium-free structures of the administered cinnamic acids and their metabolites are enclosed in rounded rectangles and shadowed rounded rectangles, respectively. The solid arrows represent reactions catalyzed by identified enzymes in the plants. The dashed arrows represent the reactions mediated by unidentified enzymes

4a. Next, the acrylic side chain of **4a** would be shortened by 4-hydroxybenzaldehyde synthase[47] to produce 4-hydroxybenzaldehyde (**41**). The catalytic activity of the enzyme has been observed in tissue cultures of the vanilla orchid *Vanilla planifolia*.[47] The subsequent methylation of the phenolic hydroxy group of **41** would be catalyzed by an *O*-methyltransferase that we have previously characterized from the flowers of *E. japonica*.[48] The successive reduction of the formyl group of **38a** provides **40a** (Scheme 5). Because both benzaldehyde **38b** and benzyl alcohol **40b**, which bear a methoxy group on the benzene ring, were detected as the metabolites of administered **1b** and **4b′**, the phenolic hydroxy group of **41** is believed to be methylated prior to the reduction of the formyl group.

In the route leading to **39a**, the formyl group of **41** would be oxidized to yield 4-hydroxybenzoic acid (**42**) (Scheme 5). Although 4-hydroxybenzaldehyde dehydrogenase, which catalyzes this step, has not been identified in plants, enzymatic activities that transform **4a** to **42** via **41** have been reported in cell-free extracts from the tuber of the potato *Solanum tuberosum*[49] as well as cell cultures of *Lithospermum erythrorhizon*[50] and the carrot *Daucus carota*.[51,52] The authors did not note whether the transformation was catalyzed by a single enzyme or not. However, the above-mentioned 4-hydroxybenzaldehyde synthase does not produce **42**,[47] and cell-free extracts generally contain a number of enzymes. Therefore, the transformation of **4a** to **42** via **41** in the flowers of *E. japonica* would be mediated by two distinct enzymes, that is, 4-hydroxybenzaldehyde synthase, which converts **4a** to **41**, and 4-hydroxybenzaldehyde dehydrogenase, which oxidizes **41** to **42**. It is reported that cell-free extracts from *L. erythrorhizon* and *D. carota* recognize **41** as the best substrate in the benzaldehyde oxidation step.[50,53]

The subsequent methylation of the phenolic hydroxy group of **42** by the *O*-methyltransferase[48] provides 4-methoxybenzoic acid (**43**), followed by the esterification of the carboxy group of **43**, which affords **39a** (Scheme 5). Recently, we characterized 4-methoxybenzoic acid carboxyl methyltransferase from *E. japonica*, which strongly prefers **43** as a substrate and gives **39a** as a product.[54] Therefore, the methylation of the phenolic hydroxy group of **42** would occur in advance of the esterification of the carboxylic group, as depicted in Scheme 5.

4 | CONCLUSIONS

In this study, deuterium-labeled cinnamic acids **1b–3b** and **4b′** were synthesized using readily available deuterium sources. We also developed a hydrogen–deuterium

exchange reaction in an EtOD/Et_3N system, which can introduce deuterium atoms at the *ortho* and the *para* positions of the phenolic hydroxy groups of (poly)phenols as well as at the C-2 position of alkyl cinnamates. Using **1b** and **4b′**, we demonstrated that the benzenoid volatiles **38a**–**40a** in the scent of *E. japonica* flowers would be biosynthesized from phenylalanine via **1a** and **4a**.

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CONFLICT OF INTEREST

The authors declare the absence of any potential conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article

ORCID

Bunta Watanabe https://orcid.org/0000-0003-3645-5712

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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