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Synthesis of condensed tannin model compounds regioselectively labeled with a ^{13}C-stable isotope

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Abstract

Condensed tannins exhibit diverse bioactivities that render them promising for applications in the food and medical fields. For the analysis and monitoring of condensed tannins, ^{13}C-labeled derivatives would provide a useful tool. In this study, condensed tannin polymers regioselectively labeled with a stable isotope were synthesized using ^{13}C-labeled acetic acid or ^{13}C-labeled dimethylformamide as the starting material. The resulting synthetic polymers were labeled with ^{13}C at the C-4 or C-2 positions. A GPC analysis revealed that all model polymers comprised mainly tetramer to hexamer structures. According to the ^{13}C-NMR data of the synthetic tannin models and natural condensed tannin obtained from sugi bark, the chemical structure of both compounds was very similar. Furthermore, compared with the natural condensed tannin and unlabeled synthetic polymer, the labeled compound showed more intense C-4 and C-2 ^{13}C signals, indicating that the ^{13}C labeling proceeded regioselectively. These compounds are useful for analyzing the chemical reactions of condensed tannins and monitoring structural transformation processes in vivo.

Keywords Condensed tannin, Synthesis, ^{13}C-NMR, Tannins, Proanthocyanidins, Dimethylformamide

Introduction

Condensed tannins (CTs) are polyflavonoid compounds that are extensively distributed in plants, including algae, ferns, gymnosperms, and angiosperms, especially in the stems, leaves, fruits, roots, bark, and seeds. In particular, a large amount of tannins accumulate in the bark [1]. CTs consist of subunits of flavan-3-ols such as (+)-catechin and (−)-epicatechin and have carbon–carbon bonds at the C-4 and C-8 (or C-6) positions (Fig. 1). CTs produced as secondary metabolites, exhibit protein adsorption capacity [2], metal adsorption capacity [3], antioxidant activity [4], and ultraviolet absorption activity [5]. The physicochemical properties of CTs prevent herbivores and insects from feeding on plants, inhibit pathogen invasion, reduce oxidative stress caused by UV light or reactive oxygen, and absorb toxic heavy metals [6]. Therefore, CTs have a crucial biodefense function in the plant kingdom.

Accordingly, CTs are anticipated to offer practical benefits for humans, including anti-inflammatory effects, improved blood circulation, prevention of arteriosclerosis and diabetes, antiaging properties, and relief from pollen allergy symptoms, which are expected to be applicable in the food and medical fields [1–8]. Although CTs have varying levels of bioavailability, the distinction between their oligomers and polymers (oligomeric procyanidins; OPCs) is not clearly defined [9]. To address this issue, Kuhnert et al. developed a high-performance liquid chromatography method using a diol stationary phase column, for the quantification of individual OPCs in grape seed extract [9]. Nevertheless, as CTs extracted

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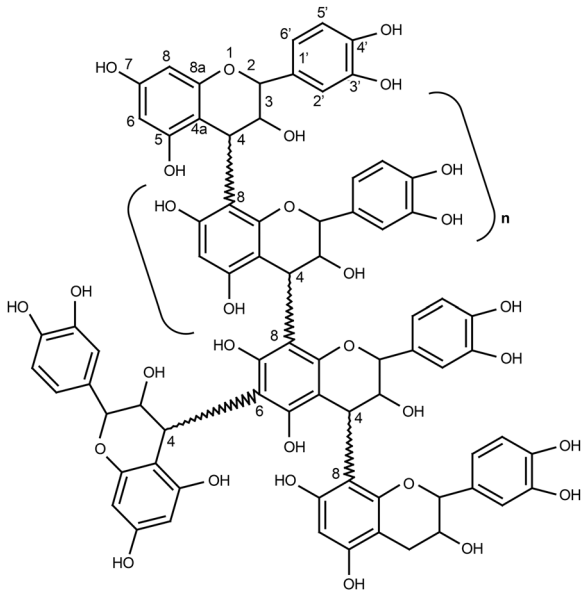

Fig. 1 Model structure of natural condensed tannins

from natural plants exhibit diverse molecular weight distributions ranging from monomers to polymers, the precise quantification of CTs is difficult. Therefore, intense research efforts are being devoted to developing a CT analysis method that is not affected by the degree of polymerization [10–15].

CTs have been suggested to play an essential role in the in vitro study of the reduction of methane gas production after livestock ingestion [16]. In particular, among various plant feeds, acacia showed high probability for reducing methane production, and its activity was partly attributed to the chemical structure and properties of CT in the lumen. Acacia CTs regulate lumen microbes and subsequently control methanogenesis, indicating a potential effect of CTs on the methanogenesis redox ability. For a more precise monitoring of the CT decomposition by microorganisms, ^{13}C-labeled CTs could be a useful tool.

Nay et al. proposed a new method for the precise analysis of CTs using various synthetic techniques [17, 18]. Instead of the traditional C6 unit coupling with the C3–C6 cinnamic acid moiety for the formation of a C6–C3–C6 skeleton, they used racemic 4-[^{13}C] catechin during the synthesis. By introducing ^{13}C-labeled acetic acid at the C-4 position, they synthesized ^{13}C-labeled (−)-procyanidin B3, a catechin dimer. Meanwhile, Yoneda et al. described the polymerization of flavan 3,4-carbonates to generate CTs with high molecular weight ($\overline{Mn}$ > 10,000) [19]. They examined several reactions of cyclic carbonates with CO_2 release, which did not decrease the catalytic activity. They suggested that an appropriate temperature and catalyst selection could provide high molecular weight CTs using a monomer with a cyclic carbonate structure.

Herein, we present a synthetic approach for CTs with a distribution of pentamers to heptamers containing ^{13}C-labeled C-4 and C-2 subunits, the structural analysis of both unlabeled and ^{13}C-labeled CT model compounds, and a comparative analysis with CTs isolated from natural sugi bark extracts.

Methods

Reagents

Sugi bark was kindly provided by Niwa Mokuzai Co., Ltd. The reagents, 60% sodium hydride (NaH, oily), trifluoroacetic anhydride (TFAA), 47% boron trifluoride diethyl ether complex (BF_3Et_2O), catechol and osmium (VIII) oxide microcapsules were purchased from FUJIFILM Wako Pure Chemical Co., Ltd. Phloroglucinol anhydrous (99.0%) and *N*-methyl morpholine-*N*-oxide were purchased from Tokyo chemical industry Co., Ltd. Toyopearl HW-40EC was obtained from TOSOH Co., Ltd. ^{13}C-labeled acetic acid and *N*,*N*-dimethylformamide (DMF; 1 g) were purchased from Nippon Sanso Co., Ltd. Stabilizer free tetrahydrofuran (THF) for GPC analysis was purchased from FUJIFILM Wako Pure Chemical Co., Ltd. Standard polystyrenes (oligomer kit, type A-5000 and A-2500) were purchased from TOSOH Co., Ltd. As ^{13}C NMR solvents, chloroform-*d* ($CDCl_3$) and acetone-d_6 (FUJIFILM Wako Pure Chemical Co., Ltd.) were used.

Extraction and purification of condensed tannin from sugi bark

100 g of sugi bark was successively extracted with *n*-hexane and 70% acetone water, respectively. The extract with 70% acetone water was concentrated in a rotary evaporator and lyophilized. The dried residues were fractionated in 35% methanol with Toyopearl HW-40EC to remove low molecular weight tannins. The sample absorbed in Toyopearl HW-40EC was eluted with 70% acetone water, concentrated, and residual aqueous suspension was washed with ethyl acetate. The aqueous layer was concentrated and lyophilized, and finally the condensed tannin polymer powder 1 g of yield 1% was recovered.

Synthesis of condensed tannin model compounds

For the synthesis of condensed tannin model compounds, unlabeled, C-2-labeled, and C-4-labeled precursors and polymers were synthesized separately.

Compounds 2 and 3

After completely dissolving 24 g of phloroglucinol (compound 1) in 158 mL of pyridine, an acetic anhydride solution (158 mL, 161 g) was added and reacted at

room temperature (r.t.) for 20 h under dark conditions. The reaction solution was then precipitated by dripping into ice water. The precipitated sample was dissolved in heated ethanol and recrystallized again. After filtering and drying the sample, 42.14 g of compound 2 with an acetyl protecting group was obtained.

Compound 2 (15.0 g) was dissolved in 300 mL of DMF at a temperature of 0 °C. Then, 26.93 g of benzyl chloride and 17.02 g of 60% NaH were slowly added while stirring under a N_2 purge. Next, 3.22 g of distilled water was carefully and slowly dripped into the stirring solution using a syringe, to avoid rapid expansion of the reaction. After 24 h at r.t., the solution was crystallized in ice water. The resulting white crystals were subsequently dissolved in heated ethanol and recrystallized at −10 °C to obtain 14.98 g of compound 3 with a benzyl protecting group.

Compound 4

Compound 3 (10.2 g) was dissolved in 50 mL of anhydrous dichloromethane (CH_2Cl_2) under stirring in a N_2 atmosphere at 0 °C. Subsequently, a mixture of 13.1 mL of TFAA and 5.0 mL of acetic acid (including 0.5 mL of ^{13}C-labeled acetic acid) was slowly introduced with a syringe for 10 min. This mixture was subjected to a Friedel–Crafts acylation reaction for 1.5 h [17, 18]. The reaction solution was diluted using CH_2Cl_2 and then neutralized with a saturated sodium hydrogen carbonate solution. The mixture was repeatedly washed with brine solution and dried over anhydrous sodium sulfate (Na_2SO_4). The resulting filtered solution was then concentrated using a rotary evaporator. The residual material was purified using a Wakogel C-300HG column with CH_2Cl_2: *n*-hexane (2:1, v/v) as an eluent to obtain 10.73 g of ^{13}C-labeled compound 4 as a yellow paste. ^{13}C NMR: δ_C (100 MHz, $CDCl_3$) 32.76 (–$COCH_3$), 70.35, 70.66 (C-3–OCH_2Ph, C-1, C-5–OCH_2Ph), 93.51 (C-2, C-4), 115.12 (C-6), 127.20–128.80 (–CH_2Ph), 157.28 (C-1, C-5), 161.24 (C-3), 201.81 (labeled, –CO). ^{1}H NMR: δ_H (400 MHz, $CDCl_3$) 2.47 (3H, s, –$COCH_3$), 4.99 (2H, s, C-5–OCH_2Ph), 5.04 (4H, s, C-1-, C-3–OCH_2Ph), 6.23 (2H, s, C-2-, C-4-H), 7.25–7.40 (15H, m, –CH_2Ph).

Compound 5

A solution (0.7 mL) containing anhydrous CH_2Cl_2 and $TiCl_4$, was stirred, and 5.04 g of compound 4 was dissolved in 100 mL of anhydrous CH_2Cl_2 and stirred in a N_2 environment at −10 °C. The $TiCl_4$ solution was then added dropwise using a glass pipette. After stirring for 4 h at −10 °C, 100 mL of water was added and the CH_2Cl_2 layer was washed using a separatory funnel. The CH_2Cl_2 layer was filtered through anhydrous Na_2SO_4 and a Wakogel C-300HG column using CH_2Cl_2 as the eluent. The resulting filtered solution was then concentrated using a rotary evaporator, and the resulting yellow paste residue was dissolved in methanol at 50 °C and sonicated. The resulting solution was then crystallized at −50 °C and stored. Finally, the crystals were filtered to obtain 3.02 g of compound 5. ^{13}C NMR: δ_C (100 MHz, $CDCl_3$) 33.46 (–$COCH_3$), 70.36, 71.19 (C-3–OCH_2Ph, C-5–OCH_2Ph), 92.45 (C-2, C-4), 94.78 (C-6), 127.76–128.85 (–CH_2Ph), 162.07 (C-1), 165.18 (C-5), 167.66 (C-3), 203.30 (labeled, –CO). ^{1}H NMR: δ_H (400 MHz, $CDCl_3$) 2.54 (3H, s, –$COCH_3$), 5.05 (1H, s, C-1–OH), 6.09 (1H, d, J = 2.3 Hz, C-2-H or C-4-H), 6.16 (1H, d, J = 2.3 Hz, C-2-H or C-4-H), 7.25–7.43 (10H, m, –CH_2Ph).

Compound 7

DMF (4.5 g), ^{13}C-labeled DMF (0.5 g), and 25.9 g of phosphoryl chloride were added in a three-necked flask under a N_2 at 0 °C for 30 min. Then, 8.9 g of catechol (compound 6) was added, and the mixture was heated at 120 °C in an oil bath for 2 h. After stopping the reaction by adding 200 mL of water, the sample was diluted with *n*-hexane: ethyl acetate (1:1, v/v) and washed repeatedly with brine. The solution was dried over anhydrous Na_2SO_4. The resulting concentrated residue was fractionated using normal-phase medium-pressure chromatography (Sepacore Silica, FlashPure EcoFlex C18 40 g, BUCHI, Switzerland) to remove any unreacted catechol. Finally, 6.28 g of ^{13}C-labeled compound 7 was obtained.

Compound 8

Benzyl chloride (11.0 g), potassium carbonate (16.0 g), and potassium iodide (4.0 g) were added to DMF (100 mL) and then stirred for 10 min at r.t. under N_2. Compound 7 (4.54 g) was added in three portions at 30 min intervals and stirred for 4.5 h. After the reaction was stopped by adding 100 mL of water, the suspension was diluted with diethyl ether. Then, the mixture was repeatedly washed with 1 N NaOH (aq.) and brine, and the diethyl ether layer was concentrated using a rotary evaporator to obtain a pale yellow residue. The residue was dissolved in a small quantity of ethyl acetate and then concentrated with a rotary evaporator until crystallization occurred. ^{13}C-Labeled compound 8 (6.68 g) was obtained after recrystallization in a freezer at −50 °C by adding a small amount of methanol. ^{13}C NMR: δ_C (100 MHz, $CDCl_3$) 70.88, 71.02 (C-3′, C-4′–OCH_2Ph), 112.31, 113.10 (C-2′, C-5′), 126.86–128.78 (–CH_2Ph), 130.35 (C-1′), 136.63 (C-6′), 149.24 (C-4′), 154.32 (C-3′), 190.97 (labeled, –CO). ^{1}H NMR: δ_H (400 MHz, $CDCl_3$) 5.21 (2H, s, –CH_2Ph), 5.25 (2H, s, –CH_2Ph), 7.03 (1H, d, J = 8.2 Hz, C-5′-H), 6.16 (1H, d, J = 2.3 Hz, C-2′-H), 7.25–7.43 (11H, m, C-6′-H, –CH_2Ph), 9.80 (1H, s, –CHO).

Compound 9

Compound 5 (3.37 g) was dissolved in 35 mL of DMF, and 696 mg of 60% NaH was added and stirred under N_2 at −10 °C. Next, compound 8 (3.08 g), also dissolved in DMF, was gradually added to the compound 5 solution using a syringe. After 1 h at room temperature, the reaction was stopped by adding 1 N HCl (aq.) and neutralized with saturated sodium carbonate (aq.). CH_2Cl_2 was added to the reaction mixture, and the solution was washed with brine and dried over anhydrous Na_2SO_4. The concentrated residue was dissolved in a small quantity of CH_2Cl_2 while simultaneously adding *n*-hexane and stored in a −50 °C for crystallization. After filtration of the resulting crystals, ^{13}C-labeled compound 9 (5.48 g) was obtained. ^{13}C NMR (finally labeled C-4): δ_C (100 MHz, $CDCl_3$) 70.41 (C-3–OCH_2Ph), 71.18 (C-5–OCH_2Ph), 92.78 (C-2, C-4), 95.11 (C-6), 106.61 (C-1′), 114.52 (C-2′), 115.20 (C-5′), 122.34 (C-6′), 125.86 (C-α), 127.20–128.97 (–CH_2Ph), 142.82 (C-β), 148.75 (C-3′), 150.73 (C-4′), 161.65 (C-1), 165.18 (C-5), 168.61 (C-3), 192.66 (labeled, –CO). ^{13}C NMR (finally labeled C-2): δ_C (100 MHz, $CDCl_3$) 70.41 (C-3–OCH_2Ph), 71.18 (C-5–OCH_2Ph), 92.78 (C-2, C-4), 95.11 (C-6), 106.61 (C-1′), 114.51 (C-2′), 115.20 (C-5′), 122.35 (C-6′), 125.86 (C-α), 127.21–128.97 (–CH_2Ph), 142.83 (labeled, C-β), 148.75 (C-3′), 150.73 (C-4′), 161.66 (C-1), 165.18 (C-5), 168.63 (C-3), 192.65 (–CO). ^{1}H NMR: δ_H (400 MHz, $CDCl_3$) 4.93 (2H, s, –CH_2Ph), 5.05 (2H, s, –CH_2Ph), 5.08 (2H, s, –CH_2Ph), 5.20 (2H, s, –CH_2Ph), 6.15 and 6.22 (2H, d, *J* = 2.2, 2.3 Hz, C-2-H and C-4-H), 6.60–7.46 (23H, m, C-2′-, C-5′-, and C-6′-H, –CH_2Ph), 7.66 (1H, d, *J* = 15.1 Hz, C-β-H), 7.76 (1H, d, *J* = 15.5 Hz, C-α-H).

Compounds 10 and 11

Compound 9 (4.49 g) was dissolved in 90 mL of 2-methylethanol in an oil bath at 90 °C. Then, 0.296 g of sodium borohydride ($NaBH_4$) was added, and the mixture was stirred for 1 h. Then, the solution was diluted with ethyl acetate at 0 °C. After repeatedly washing with brine and drying over anhydrous Na_2SO_4, 120 µL of 47% BF_3Et_2O in anhydrous 2.3 mL of CH_2Cl_2 was added. The reaction was performed for 20 min at r.t. under N_2. The reaction mixture was diluted with ethyl acetate, washed with water and brine, and dried over anhydrous Na_2SO_4. The concentrated residue was dissolved in a small quantity of diethyl ether. Compound 11 (3.36 g) was obtained after crystallization in a freezer at −50 °C.

Compound 11 ^{13}C NMR (finally labeled C-4): δ_C (100 MHz, $CDCl_3$) 70.18–71.30 (C-2,–CH_2Ph), 93.92 (C-6), 95.13 (C-8), 105.05 (C-4a), 114.22 (C-2′), 114.72 (C-5′), 119.08 (labeled, C-4), 120.63 (C-3), 127.31–128.71 (–CH_2Ph), 134.08 (C-6′), 137.24 (C-3′), 149.05 (C-4′), 154.92 (C-5), 155.41 (C-7), 160.37 (C-8a). ^{1}H NMR: δ_H (400 MHz, $CDCl_3$) 4.92–5.19 (8H, s, –CH_2Ph), 5.56 (1H, dd, *J* = 3.7 Hz, 3.6 Hz, C-3-H or C-4-H), 5.73 (1H, dd, *J* = 1.9 Hz, 1.9 Hz, C-2-H), 6.11 (1H, d, *J* = 2.3 Hz, C-6-H), 6.19 (1H, d, *J* = 2.3 Hz, C-8-H), 6.91 (1H, dd, *J* = 8.2 Hz, 2.2 Hz, C-4-H or C-3-H), 7.06–7.44 (23H, m, C-2′-, C-5′-, and C-6′-H, –CH_2Ph). ^{13}C NMR (finally labeled C-2): δ_C (100 MHz, $CDCl_3$) 70.18–71.30 (labeled, C-2,–CH_2Ph), 93.93 (C-6), 95.14 (C-8), 105.06 (C-4a), 114.23 (C-2′), 114.73 (C-5′), 119.08 (C-4), 120.64 (C-3), 127.31–128.71 (–CH_2Ph), 134.09 (C-6′), 136.75 (C-3′), 149.06 (C-4′), 154.93 (C-5), 155.42 (C-7), 160.38 (C-8a). ^{1}H NMR: δ_H (400 MHz, $CDCl_3$) 4.93–5.19 (8H, s, –CH_2Ph), 5.56 (1H, dd, *J* = 3.2 Hz, 3.7 Hz, C-3-H or C-4-H), 5.73 (1H, dd, *J* = 1.9 Hz, 1.9 Hz, C-2-H), 6.12 (1H, d, *J* = 2.3 Hz, C-6-H), 6.20 (1H, d, *J* = 1.9 Hz, C-8-H), 6.91 (1H, dd, *J* = 8.7 Hz, 1.8 Hz, C-4-H or C-3-H), 7.20–7.48 (23H, m, C-2′-, C-5′-, and C-6′-H, –CH_2Ph).

Compound 12

Compound 11 (2.49 g) was dissolved in 261 mL of acetone and 0.276 g of osmium (VIII) oxide microcapsules was added. After stirring at r.t. under N_2 for 30 min, 1.38 mL of *N*-methyl-morpholine-*N*-oxide (50% in water 4.8 mol/L) was injected with a syringe, and mixture was allowed to react at r.t. under N_2 for 24 h. The solution was diluted with acetone, and osmium (VIII) oxide microcapsules were recovered from the solution. The acetone solution was concentrated in a rotary evaporator, and the residue was dissolved in CH_2Cl_2 and washed with 1 M sodium thiosulfate (aq.). The solution was repeatedly washed with brine and dried over anhydrous Na_2SO_4. The concentrated white residue was dissolved with a small quantity of diethyl ether and stored in a freezer at −50 °C for crystallization to obtain compound 12 (1.79 g).

Compound 13

Compound 12 (1.5 g) was dissolved completely in 750 mL of toluene, and then 0.73 g of *N*, *N*′-carbonyldiimidazole (CDI) was added. After refluxing for 72 h, the solution was repeatedly washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated using a rotary evaporator. The residue was dissolved in a small quantity of CH_2Cl_2, and *n*-hexane was added for crystallization in a freezer at −50 °C to obtain compound 13 (1.39 g) after filtration.

^{13}C NMR data of ^{13}C-labeled C-4 compound 13: δ_C (100 MHz, $CDCl_3$) 70.30, 70.47, 71.25, 71.57, 75.52, and 75.93 (C-2, C-3, C-4, and –CH_2Ph), 94.98, 95.23, and 98.87 (C-4a, C-6, and C-8), 113.99, 114.86, 120.76 (C-2′, C-5′, and C-6′), 127.22, 127.31, 127.59, 127.99, 128.02, 128.24, 128.36, 128.62, 128.79, 136.19, 137.00, and 137.08 (benzylated group, $C_6H_5CH_2$–), 149.20, 149.85, 154.38,

157.19, 159.80, and 162.28 (C-3′, C-4′, C-5, C-7, C-8a, and C=O).

^{1}H NMR: δ_H (400 MHz, $CDCl_3$) 4.76 (2H, m, C-2-H and C-3-H), 5.00–5.18 (8H, m, $-CH_2Ph$), 5.80 (1H, m, C-4-H), 6.21 and 6.31 (2H, d, J = 2.2 and 2.3 Hz, C-6-H and C-8-H), 6.96 and 7.03 (3H, s, C-2′-, C-5′-, and C-6′-H), 7.25–7.45 (20H, m, $-CH_2Ph$).

^{13}C NMR data of ^{13}C-labeled C-2 compound 13: δ_C (100 MHz, $CDCl_3$) 70.31, 70.34, 70.45, 71.22, 71.53, 75.52, and 75.93 (C-2, C-3, C-4, and $-CH_2Ph$), 94.95, 95.22, and 98.94 (C-4a, C-6, and C-8), 113.92, 114.81, 120.75 (C-2′, C-5′, and C-6′), 127.22, 127.30, 127.59, 127.99, 128.02, 128.24, 128.36, 128.62, 128.80, 136.18, 136.99, and 137.07 (benzylated group, $C_6H_5CH_2-$), 149.17, 149.82, 154.39, 157.19, 159.78, and 162.27 (C-3′, C-4′, C-5, C-7, C-8a, and C=O).

^{1}H NMR: δ_H (400 MHz, $CDCl_3$) 4.75 (2H, m, C-2-H and C-3-H), 5.00–5.17 (8H, m, $-CH_2Ph$), 5.80 (1H, m, C-4-H), 6.21 and 6.31 (2H, d, J = 2.3 and 1.9 Hz, C-6-H and C-8-H), 6.96 and 7.03 (3H, s, C-2′-, C-5′-, and C-6′-H), 7.25–7.45 (20H, m, $-CH_2Ph$).

Synthesis of polymeric tannin model compounds

As a precursor, compound 13 (0.33 mmol, 228.9 mg) was dissolved in 10 mL of anhydrous CH_2Cl_2 and stirred in a dry ice–acetone bath at −20 °C in a N_2 atmosphere. A solution of 47% BF_3Et_2O (0.26 mmol, 0.04 mL) in 1.96 mL of anhydrous CH_2Cl_2 was added to the reaction solution with a syringe and allowed to react for 24 h in a freezer at −20 °C. The solution was diluted with ethyl acetate, washed repeatedly with water and brine, dried over anhydrous Na_2SO_4, and concentrated using a rotary evaporator. The concentrated residue was vacuum dried, affording compound 14 (226.6 mg).

Then, compound 14 (226.6 mg) was dissolved in 100 mL of THF/ethanol (4:1; v/v), and 1.84 g of palladium on activated carbon (10% Pd-/C) was added and stirred. The reaction was performed under a H_2 atmosphere at 37 °C, for 48 h. The solution was diluted with methanol, and centrifuged at 3300 rpm at 4 °C for 10 min to remove Pd-/C. The methanol-diluted solution was then filtered with a column packed with 10 g of Wakogel C-300HG (lower layer) and 10 g of cellulose powder (upper layer). The recovered methanol solution was filtered through Toyopearl HW-40EC to recover low-molecular-weight oligomers. The polymer was eluted with 70% acetone (aq.), and the acetone was then removed using a rotary evaporator. The polymer solution was lyophilized, and the dried sample was resuspended in water. The residual low-molecular-weight compounds were completely removed by repeatedly washing with ethyl acetate. Finally, the polymer solution was lyophilized to obtain 83.1–85.4 mg of debenzylated unlabeled and C-4 and C-2 ^{13}C-labeled CT model compounds, respectively.

GPC analysis

For the GPC analysis, LC solution (Shimadzu, Ltd.) and Chromato PRO-GPC software (Runtime Instruments Co., Ltd) were used. The column was Shodex KF-602 (size 6.0 i.d. 150 mm, Japan), and the analysis was conducted at a flow rate of 0.5 mL/min using THF as a solvent, a detection wavelength of 280 nm (UV-975 JAS, Co.), the analysis of standard substances conducted at a wavelength of 254 nm, and an oven temperature 30 °C (CTO-10AC, SHIMADZU). The samples were acetylated prior to analysis. The acetyl protected samples were dissolved in THF to achieve a concentration of 5.0 mg/mL, and the injection volume was 20 μL.

NMR analyses

^{13}C and ^{1}H NMR analyses were conducted using a NMR spectrometer Z JNM-ECZS series, JEOL Ltd. (400 MHz for ^{1}H and ^{13}C, Delta V5 model software).

The assignment of signals derived from small amounts of residual solvent impurities, such as ethyl acetate, remaining during each step of the synthesis process was adapted from that reported by Gottlieb et al. [20]. ^{13}C NMR data of each synthesized compound was confirmed based on the chemical shifts (1024 scans, default) with $CDCl_3$ solvent. Finally, the polymerized CT model compounds and natural sugi tannin samples were acetylated prior to analysis and dissolved in acetone-d_6 to perform the ^{13}C NMR analysis.

Results and discussion

The synthesis steps are shown in Fig. 2. For the synthesis of condensed tannin model compounds, unlabeled, C-2-labeled, and C-4-labeled precursors and polymers were synthesized separately.

Synthesis of precursors

We used ^{13}C-labeled acetic acid to introduce ^{13}C into the C-ring according to previous reports [18, 21]. Using TFAA as a strong Lewis acid catalyst, we synthesized ^{13}C-labeled phloroacetophenone (compound 4) with high yields of over 90%. In natural CTs, the C-4 position generally serves as an interunit binding site. Therefore, a regioselective labeling at the C-4 position is imperative as a preliminary step in the preparation of CTs. The ^{13}C spectrum showed a strong peak at 201.81 ppm, which can be ascribed to a carbonyl carbon atom, and other signals that were consistent with the expected molecular structure of the compound. These results demonstrate the successful synthesis of C-4-labeled compound 4. The

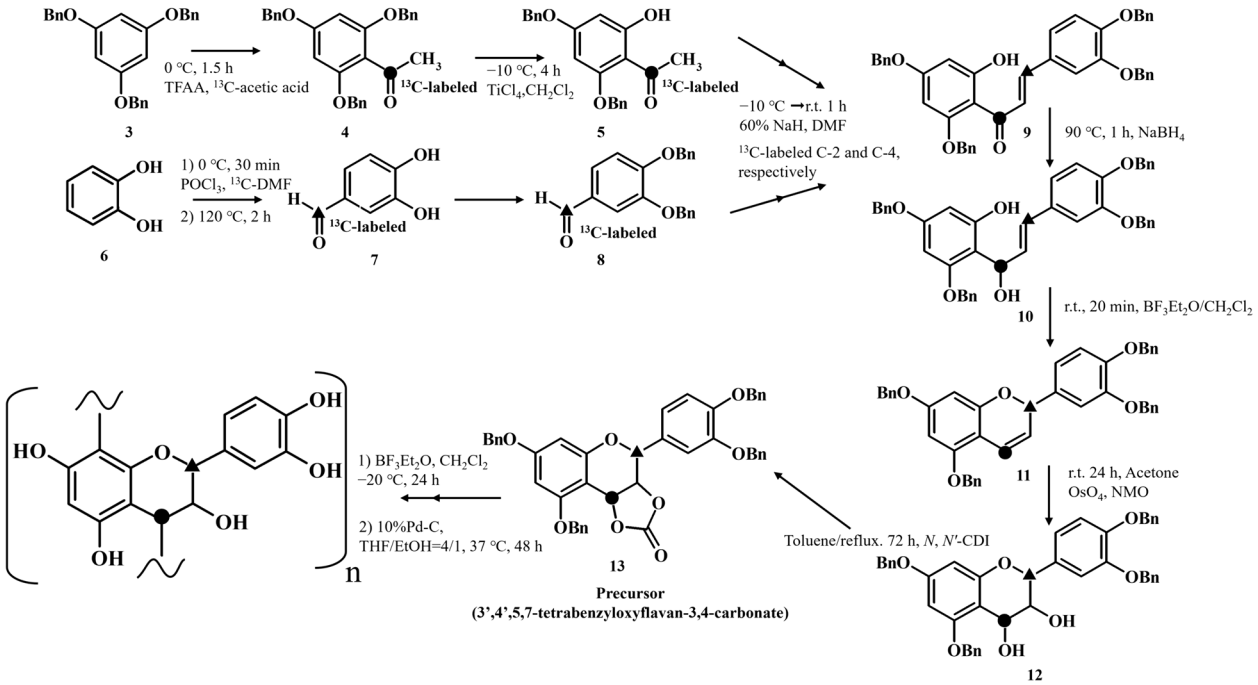

Fig. 2 Synthetic routes for C-2 and C-4 ^{13}C-labeled precursor. Synthetic routes for compounds 2 and 3 are not shown

synthesis of compounds 5 was carried out by the same method as reported by Nay et al. with similar results [17].

Compound 7 was synthesized via the Vilsmeier–Haack reaction using a catechol monomer and labeled with ^{13}C-labeled DMF. The labeled C-2 position of the aldehyde group is the bonding site between the B and C rings. Crystallization was attempted to separate the unreacted catechol from compound 7 produced during the synthesis. Attempts to crystallize pure compound 7 using diverse organic solvents, temperature, and pH conditions failed. The catechol and compound 7 dissolved in ethyl acetate were filtered on a Wakogel C-300HG (40–60 μm) packed column using ethyl acetate: *n*-hexane (2:1, v/v) as the eluent to remove side reaction products. Although the yield was as low as 20%–26%, purification was essential to obtain high-purity compound 7 crystals. The synthesized compound 7 was benzylated to facilitate aldol condensation. Subsequently, the structure of benzylated ^{13}C-labeled compound 8 was determined via ^{13}C NMR spectroscopy. The synthesis of compounds 9–12 was carried out by the same method as reported by Nay et al. with similar results [17].

The reaction between CDI and compound 12 yielded approximately 90% of C-2 and C-4 ^{13}C-labeled precursors after 72 h of reflux. The steric hindrance of the cyclic carbonate substructure in compound 13 afforded a 3,4-cis-structure, leading to the precursor exclusively possessing the 3,4-*cis*-ester structure. Hence, on the basis of previous studies on the synthesis of polymeric CTs [21–24], we selected the relevant C-2/C-4 ^{13}C-labeled precursors as the initial monomers for the polymerization reaction. A comparison of the ^{13}C NMR spectral features of the unlabeled and C-2 and C-4 ^{13}C-labeled precursors revealed a considerable strength of the C-2 and C-4 ^{13}C labeled signals in the C-ring, confirming the successful labeling.

Precursor polymerization reactions and GPC analysis

The synthesized precursors were polymerized under BF_3Et_2O catalysis for 24 h at −20 °C to obtain a pure, organochemically synthesized polymeric tannin compound model. Kawamoto et al. reported that changing the polymerization time could afford polymers with diverse masses [22]. Debenzylation of the polymer was performed using 10% Pd/C in THF/ethanol (4:1, v/v) under a H_2 atmosphere. To remove trace amounts of residual low-molecular-weight oligomeric synthetic CTs, the polymeric tannin powder was suspended in distilled water, washed with ethyl acetate, and lyophilized to obtain a high-purity polymeric CT powder. The synthesized CT polymers were acetyl derivatized for GPC analysis. After acetylation, the remaining pyridine and low-molecular-weight byproducts were removed by washing with 0.2 N HCl (aq.).

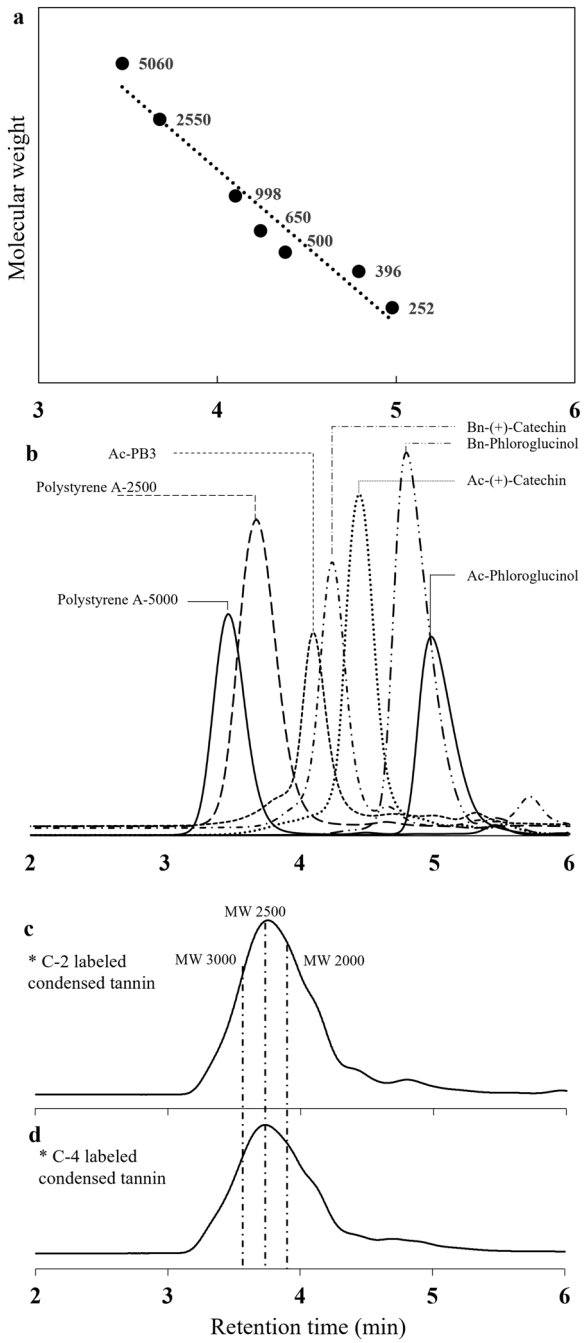

Fig. 3 GPC analysis of standard substances and ^{13}C-labeled model compounds. Polystyrene A-5000 (MW = 5.06×10^3), polystyrene A-2500 (MW = 2.55×10^3), Ac-PB3 (acetylated procyanidin B3; MW = 998), benzylated catechin (Bn-(+) Catechin; MW = 650), acetylated catechin (Ac-(+) Catechin; MW = 500), benzylated phloroglucinol (Bn-Phloroglucinol; MW = 396), acetylated phloroglucinol (Ac-Phloroglucinol; MW = 252) **a** The calibration curve of standard substances, **b** GPC analysis results of standard substances, **c**, **d** GPC analysis results of C-2 and C-4 labeled condensed tannin

The molecular weight distribution of the CT model compounds was determined with GPC system using a standard calibration curve, including polystyrene A-5000, polystyrene A-2500, acetylated procyanidin B3, benzylated catechin, acetylated catechin, benzylated phloroglucinol and acetylated phloroglucinol, as a set of standard substances (Fig. 3a, b). The peak top molecular weight of the synthesized CTs was around 2500 (Fig. 3c, d). Furthermore, the molecular weight of the synthesized CTs was mainly in the range of 2000 to 3000. Since the molecular weight of acetylated catechin (monomer) is 500, the main component is estimated to be tetramer to hexamer.

^{13}C NMR analysis of the polymer

The ^{13}C NMR spectrum of the acetylated unlabeled CT model compound agrees with the analysis of high-molecular-fraction polymeric CTs extracted from natural sugi bark (Fig. 4a, b). It has been reported the polymerization reaction using flavan 3,4-carbonates proceeds with stereoselectivity in favor of the 3,4-trans-configuration [19]. The C-ring of natural CTs exhibits a 3,4-trans-stereochemistry [10, 12]. Therefore, it was demonstrated that CT model compounds synthesized from unlabeled precursors were structurally similar to natural CTs. We conducted a ^{13}C NMR analysis of the C-2 and C-4 ^{13}C-labeled CT model compounds and compared the results with those of the unlabeled CT compound (Fig. 5). The ^{13}C-labeled C-2 carbon of the C-ring derived from ^{13}C-DMF gave rise to a signal at δ_C 77–80 ppm, which was strong compared with those of the unlabeled compound and natural sugi tannin (Fig. 5a, b). In addition, the C-4 ^{13}C-labeled carbon of the C-ring derived from ^{13}C-labeled acetic acid gave a relatively strong signal at δ_C 36–37 ppm (Fig. 5a, c).

Conclusions

For the first time, ^{13}C-labeling was performed at the C-2 and C-4 positions of CTs to synthesize regioselective stable isotope labeled CTs of high molecular weight. The ^{13}C-labeled CT model compounds, which resemble natural compounds, are potentially valuable for studies on pharmacology, microbial enzyme degradation mechanisms in nature, and the digestion process upon herbivore consumption of tannins by tracking the properties and degradation processes of CTs.

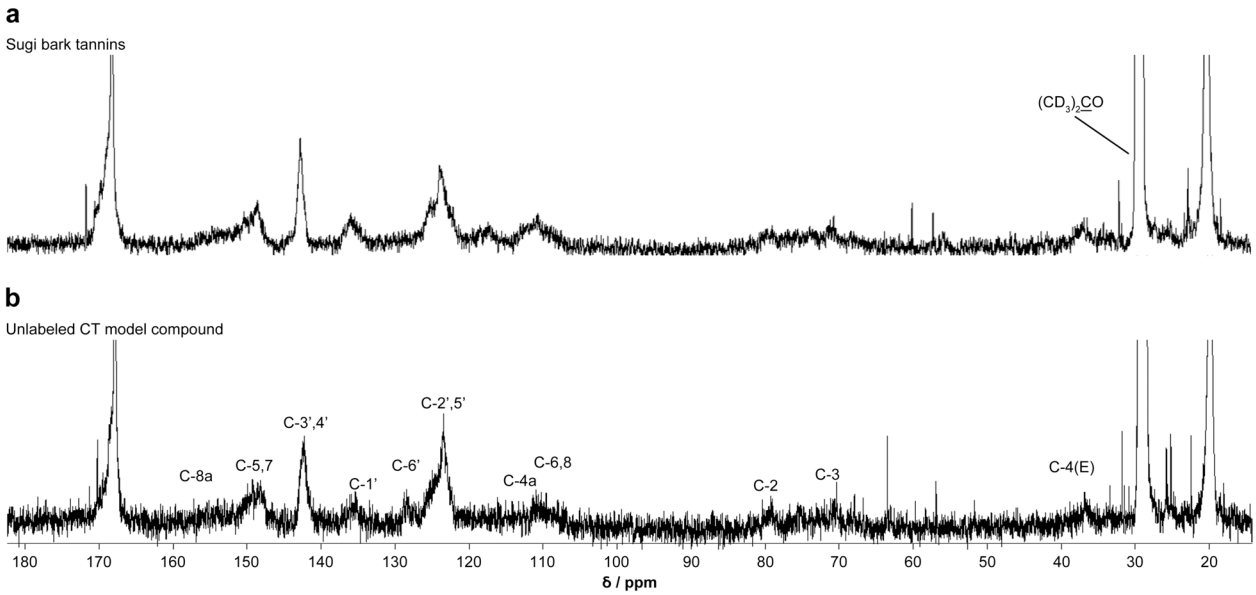

Fig. 4 ^{13}C NMR spectra of the bark tannin and the tannin model compound. The bark tannin and the tannin model compound were acetylated before ^{13}C NMR analysis. **a** ^{13}C NMR spectrum of the acetylated sugi bark tannins, **b** ^{13}C NMR spectrum of the acetylated unlabeled condensed tannin model compound

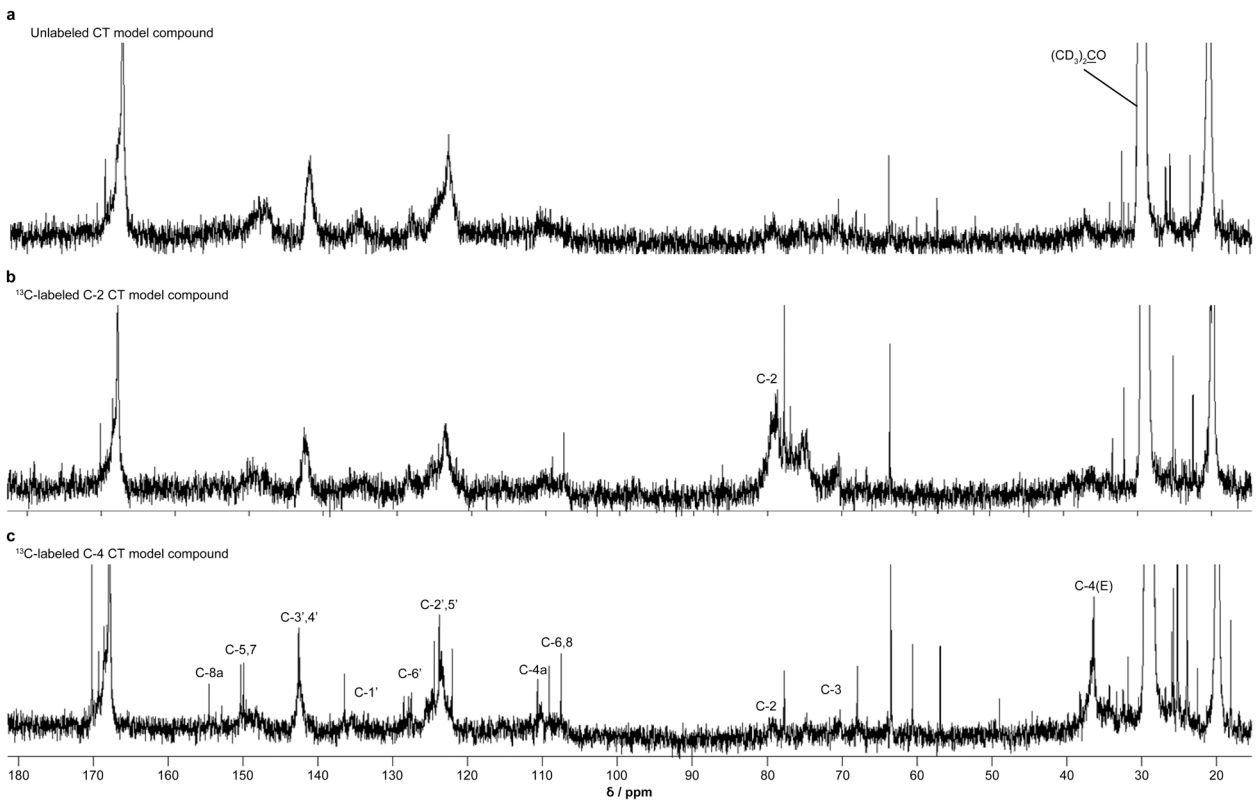

Fig. 5 ^{13}C NMR spectra of acetylated tannin model compounds. **a** ^{13}C NMR spectrum of the acetylated unlabeled condensed tannin model compound, **b** ^{13}C NMR spectrum of the acetylated ^{13}C-labeled C-2 condensed tannin model compound, **c** ^{13}C NMR spectrum of the acetylated ^{13}C-labeled C-4 condensed tannin model compound

Abbreviations

CDI	*N,N′*-Carbonyldiimidazole
CTs	Condensed tannins
DMF	*N,N*-Dimethylformamide
OPC	Oligomer procyanidin
r.t.	Room temperature
TFAA	Trifluoroacetic anhydride
THF	Tetrahydrofuran

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Author contributions
SJM performed ^{13}C-labeled CT synthesis and prepared the manuscript. YK performed unlabeled CT synthesis and the chromatography assay. HK designed and supervised this study. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used in the current study 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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