

Stable Isotope Tracer Methods in Lignin Research (Laboratory Manual for a Beginner)

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Abstract

Synthetic methods for ¹³C-labeled monolignol glycosides (coniferin, syringin and *p*-glucocoumaryl alcohol), with the label on the α -, β -, or γ -carbon of the side chain or 4-, 5-, 3-, or 1-carbon in the aromatic ring, are described. Synthetic procedures are given for dehydrogenation polymers (DHPs) from the coniferin and ¹³C-enriched cell wall-DHPs using isolated tree soft parts. Experimental procedures are also described for the ¹³C-enrichment of specific carbon atoms of cell wall lignin by feeding the ¹³C-labeled coniferin to ginkgo tree shoot. A beginner's guide to these chemical experiments is included.

Keywords: *lignin; monolignol glucosides; ¹³C-labelling; ²H-labelling; DHP (dehydrogenation polymer)*

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1. INTRODUCTION

The tracer method employing the technique of ¹³C-enrichment at specific carbon of lignin combined with various types of mass spectrometry and NMR analyses can provide highly reliable information on lignin structure and reactions that is not obtained by any other analytical methods. Monolignol glucosides are the best precursors for specific ¹³C-enrichment of lignin in plant cell walls.

In order to obtain successful results, the following two requirements must be satisfied at least.

- (1) Experimental plan must be logically designed to hit research target exactly. The idea of research plan (setting of **valuable target**, and **detail of the experimental procedure** to reach the target) is most important.
- (2) Every step in the procedure must be carried out in a correct way, confirming the correctness at every step. Even a small mistake in the long course of synthesis involving many steps makes all efforts nothing. To prevent experimental failure, make every effort to follow a **fail-safe procedure** in the laboratory. One of the best fail-safe procedures is to do **practice experiments using unenriched compounds**. The practice experiment must be carried out using the same tools and equipment before doing the synthesis of labeled compounds. It is strongly recommended to **divide the precious labeled compound into several parts, and to do experiments several times using a part of them**.

2. GENERAL REQUIREMENTS

The syntheses of the isotope-labeled compounds must be carried out in a correct laboratory procedure. The correct procedure means a procedure by which a worker can get a pure target compound in a good yield, always without fail. A beginner is strongly recommended to satisfy following requirements for doing laboratory work in a correct way.

2.1. Keep everywhere (desk, floor, draft, and shelf) clean in the laboratory.

A clean desktop enables us to recover a spilled sample. If the dirty material is organic, wipe out with a sheet of kitchen paper wetted with acetone. If it is inorganic, apply a small amount of concentrated hydrochloric acid, and then wipe out with a wet sheet of kitchen paper.

2.2. Keep glassware, tool and equipment clean and in good condition.

Glassware: Clean them by the use of the most appropriate cleaning agents (such as soap, ethanol, hydrochloric acid, nitric acid, etc.) depending on the nature of the material to be washed out. Frequent use of abrasive cleanser is not recommended. It is recommended to wrap the metal-wire part of cleaning brush with a plastic tape or cloth so that the naked wire part do not hurt the inside surface of the flask. Do not put many flasks in a washing basin, because even a slight touch of a water-filled flask with other flasks may make a stir-like crack. Check star-like cracks carefully before using for experiments. Even if the crack is a tiny one, discard the flask, because the use of a flask with a star-like crack may cause a dangerous accident.

Water bath: A clean water bath enables us to recover a spilled sample.

Oil bath: Use a stainless steel pan or heat-resistant glass pan with a temperature controller, stirrer and colorless oil. Clean oil enables us to watch inside of the flask clearly.

Water jet pump (water-circulating type): Keep it clean and in good condition by checking with a vacuum gauge (< 30 mmHg).

Vacuum oil pump: Keep it in good condition by checking with a vacuum gauge (< 1 mmHg) and change the oil with fresh one if necessary. Contamination of the oil with water and acidic materials should be prevented by the use of appropriate traps.

Stirring motor: Explosion-free motor of about 1000 rpm (max) should be employed.

Glass capillary: A glass capillary is prepared by yourself using a gas burner. If a glass-work burner on the glass-work desk is not available, you can use a small portable gas burner (commercially available) in a draft chamber where all flammable materials must be completely removed. Heat a disposable glass pipet rotating on the blue flame, then extend the softened red part outside the flame. The best way to cut the capillary is to make a small crack by gently touching it with a folded edge of an abrasive paper, then fold the capillary.

Small stirring blade: If you have no small stirring blade suitable for a small three (or two) necked (heart shape or round) flask, you can make it by yourself. At first, heat one end (about 2 cm) of a glass rod (ϕ : 6–8 mm \times 20–30 cm, glass-work quality) to a dull red, and press the red part with crucible tongs to form a paddle shape in the out of the gas flame. Then, heat the flat paddle part to a dull red, and twist it slightly into a screw shape. The twisting direction should be chosen so that the stirred solution in the reaction flask is ejected downward to the bottom center of the flask. Minor correction for the symmetry of the stirring blade can be made by the use of an abrasive paper if necessary.

Setting of stirring units: The stirring blade must not be connected directly to the stirring motor. At first, set a short glass rod (ϕ : 6–8 mm \times 10 cm) to the stirring motor, and connect the lower end of this rod to the upper end of the stirring blade using a short rubber tube, creating a few millimeters of gap between them. And the middle part of the glass stirring blade is held vertically through a hole (ϕ : 7–8 mm) made on a cork stopper (ϕ : 3–4 cm) held by a clamp. High speed stirring without vibration is achieved by making the connection gap and using a cork holder.

2.3. Use tools and materials in a correct way.

Clamp: Apply a drop of oil on the screw part so that most appropriate power can be applied to grip glassware firmly. Apply a drop of oil to the cork holder of stirring glass rod.

Stopper: Fresh cork stopper should be softened by the use of cork-softener and wrap it with weighing paper. It is recommended to use a cork stopper during recrystallization or storage of an organic solution in an Erlenmeyer flask (conical flask). The use of a ground glass stopper is not recommended for above purposes.

Ground glass joint and stopper: Do not apply vacuum grease except only when its application is necessary to make a high vacuum. When a ground glass joint cannot be disconnected, try the following ways,

- (1) Apply a suitable solvent to dissolve the material bonding both parts of the joint.
- (2) Put the whole glassware in a pan containing water, heat the pan to boiling for a while, and allow it to cool slowly overnight.
- (3) Wrap the joint part with a cloth, and slowly pour plenty of boiling water (2-3 liters) from a kettle. After a few minutes, try to separate the joint. It is recommended to use rubber gloves or a rubber band to grip the glassware firmly.

Large glass pipet: Use a large pipet and a small cotton ball for the filtration of a small amount of liquid without any mechanical loss.

Dental tweezers: For handling small items, such as a small cut filter paper, it is recommended to use dental tweezers.

Label: Using a black pencil, write the structure and name of the compound, date and the name of worker (Note 2.3-1). Then, cover the pasted label with Scotch tape. It is recommended to record the exact weight of the labeled empty bottle (tare) on the Scotch tape with a black pencil before putting the compound into the bottle. This enables us to know the exact amount of the compound remained in the bottle after partial use of the compound.

Note 2.3-1. For the naming of the labeled compounds in this laboratory manual, the same nomenclature used in the reference publications is employed. In your future publications, naming by the IUPAC nomenclature is recommended.

2.4. Precautions against possible laboratory hazards or unexpected experimental failures

- (1) Do not use a flask with a flat surface (Erlenmeyer [conical] flask, or flat-bottomed round flask) for evaporation or distillation under reduced pressure. The flat part may be broken by atmospheric pressure ($\sim 1 \text{ Kg / cm}^2$) explosively.
- (2) Wear safety goggles when vacuum evaporation or reaction is carrying out.
- (3) Do not disconnect electric line by pulling out a plug from the socket while an apparatus power is ON. Electric discharge may cause a fire if a flammable solvent is used nearby in the laboratory.
- (4) When a separatory funnel is put on a ring holder, place an empty flask under the funnel. This is an important precaution against unexpected leak of the sample solution from the funnel.

2.5. Before starting synthesis

Write down the reaction using chemical structure, stoichiometry and detailed procedure in your notebook. If you have not enough fundamental knowledge on the following three points, it is strongly recommended to make a thorough search through appropriate books. Theoretical and practical knowledge, combined with working experiences in the laboratory, will improve your creative ability in future research.

- (1) Chemistry of the reaction involved in the present synthesis and related reactions.

- (2) The reason why each step of procedure is essential for the present work, and general laboratory procedures (mixing, heating, cooling, extraction, distillation, recrystallization, drying, etc.) not only involved in the present synthesis but also in other syntheses.
- (3) Chemical and physical properties of reagents and compounds involved in the present synthesis.

2.6. Use of following dictionaries and books is recommended.

- 1) *Beilstein's Handbuch der Organischen Chemie*
- 2) *The Merck Index of Chemicals and Drugs* (Merck & Co.)
- 3) *Organic Syntheses* (Wiley)
- 4) *Organic Reactions* (Wiley)
- 5) Fieser and Fieser, *Reagents for Organic Synthesis* (Wiley)
- 6) R. L. Shriner et al., *The Systematic Identification of Organic Compounds* (Wiley)

NOTE 2-6. Two travelers A and B were making a trip from start town S to target town T. From the travel agent, A obtained a detailed information about the course, "First take a bus from S to S1, then take a subway to S2, finally take a train to T". The traveler B obtained the same information and a guide map from his travel agent. B enjoyed his travel looking at the guide map during the trip. After completion of their travel, A obtained only a linear knowledge and experience along his travel route. While, B obtained a wider two-dimensional knowledge and experiences about other towns, K and L, and other possible routs. For next travel from K to T, A had to ask his travel agent again about the route, while B could find many possible routs from any town to T by himself. In addition, B will find a greater joy in creating his own travel from any town to any attractive town in the future.

3. SYNTHESIS OF ¹³C-ENRICHED MONOLIGNOL GLUCOSIDES^{1,2)}

The chemicals, tools and other materials described in the procedure can be replaced with those of the same quality and efficiency. See Recommended books 1, 2 and 5 in the previous section 2.6. and commercial catalogs for guidance.

3.1. Coniferin-[α-¹³C] (1)

3.1.1. Acetovanillone-[CO-¹³C] (2)

To a two-necked heart-shaped flask (50 ml) with a motor-driven stirrer, conc. phosphoric acid (85%, 17 g) is placed. Phosphorus pentoxide (18 g) is added carefully in portions with stirring (Note 3.1.1-1.). After the initial generation of heat subsided, the mixture is heated in an oil bath at 100°C with stirring under the exclusion of moisture to dissolve the white powder of phosphorus pentoxide completely. After about 0.5 h, colorless viscous polyphosphoric acid is formed.

To a mortar (i.d. = approximately 6 cm), sodium acetate-[1-¹³C] (1.0 g, 12.04 mmol) is placed. Guaiacol (2.5 g, 20 mmol) is added, and mixed thoroughly. The whole mixture becomes hot and solidifies. The solid mixture is crashed to a fine powder.

To the flask containing polyphosphoric acid, the mixture of sodium acetate and guaiacol is added with vigorous stirring at 100°C. After 10 min, the whole mixture becomes dark red. The mixture is cooled and poured into cold water (~ 60 ml). On standing, crystals of acetovanillone separate. The crystals are collected by filtration, and the filtrate is extracted with diethyl ether (30 ml × 5). The crystal is dissolved in the ether solution, and the combined ether solution is washed with brine (15 ml

$\times 2$), and dried on anhydrous sodium sulfate. After evaporation of ether, the residue is recrystallized from ethanol to give 1.28 g (7.66 mmol, 63.6% based on sodium acetate) + α g (from the mother liquor) of acetovanillone-[CO- ^{13}C] **2**.

Note 3.1.1-1. Phosphorus pentoxide is a highly hygroscopic powder. Handle it quickly and carefully. Do not use the solidified part that may be found in an old bottle. On contact of phosphorus pentoxide with phosphoric acid, an intense heat is generated. Local heating may break the flask. Do not cool it with cold water, because cooling may break the flask causing a serious hazard.

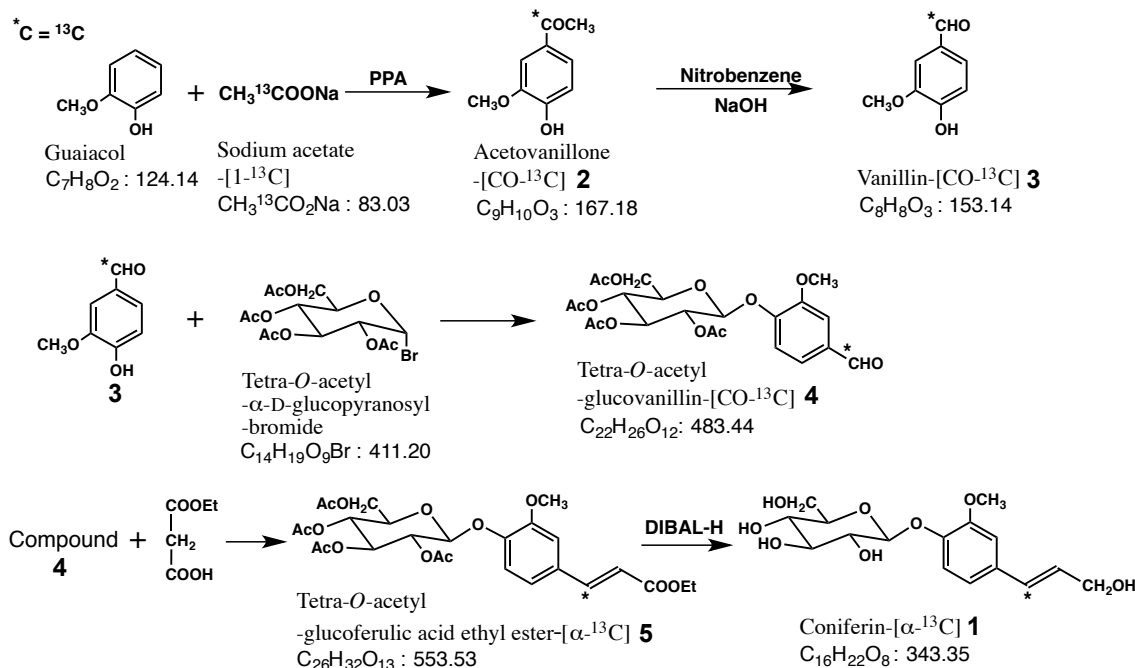


Fig.1. Synthesis of coniferin-[α - ^{13}C] (**1**).

3.1.2. Vanillin-[CHO- ^{13}C] (**3**)

To five stainless steel bombs (volume: 110 ml each $\times 5 = 550$ ml), acetovanillone-[CO- ^{13}C] (**2**), (1.08 g $\times 5 = 5.4$ g, 32.3 mmol), nitrobenzene (8.5 ml $\times 5 = 42$ ml) and 2N sodium hydroxide solution (62 ml $\times 5 = 310$ ml) are placed. For effective agitation, stainless-steel nuts are added to the bombs. The bombs are closed tightly using Teflon packing, and heated in an oil bath with rotating device at 180°C for 2 h (Note 3.1.2-1). After cooling, the reaction mixture is extracted with ether (200 ml $\times 3$). The residual aq. solution is then acidified (pH <1) with dil. hydrochloric acid (1:3), and extracted again with diethyl ether (100 ml $\times 3$). The ether solution is extracted with 20% aq. sodium hydrogen sulfite (NaHSO₃) (50 ml $\times 4$) (Note 3.1.2-2). The aqueous solution is then acidified (pH <1) by addition of dil. sulfuric acid (H₂SO₄: water, 1: 2), and blow air into warmed solution at about 60°C to remove SO₂ gas. The solution is then extracted with ether (150 ml $\times 3$), and the ether solution is washed with brine (80 ml $\times 2$), and dried over anhydrous sodium sulfate. The ether solution is evaporated to give crystals of vanillin-[CHO- ^{13}C] (**3**), (3.0 + α g [crude]). Recrystallization from ether, and from a mixture of CH₂Cl₂-ether-petroleum ether gives pale yellow crystals (2.9 g, 19 mmol, 58.7% + α g from the mother liquor) (Note 3.1.2-3). ¹H-NMR of compound **3** shows no CH₃ signal of the starting material **2**.

Note 3.1.2-1. If a rotating device is not available, a stainless-steel bomb containing the reaction mixture is held vertically in the oil bath (180°C) that is placed on a heating-magnetic stirrer, and nitrobenzene oxidation is carried out by heating and magnetic stirring. Effective stirring yields vanillin.

Note 3.1.2-2. This extraction is to isolate vanillin from the reaction mixture that may contain acetovanillone or vanillic acid. Only vanillin forms a water-soluble addition compound with NaHSO_3 . $\text{RCHO} + \text{NaHSO}_3 \rightarrow \text{R-C(OH)(H)-SO}_3\text{Na}$ (Cf. Reference book 6 in the previous section 2.6, and Reference 2). Because the formation of the addition compound takes place slowly, extraction by vigorous shaking in a separatory funnel should be carried out for more than a few minutes for one extraction.

Note 3.1.2-3. If the vanillin is not pure enough as indicated by brown colored sticky material, purification by column chromatography is strongly recommended as follows: The impure material is dissolved in a small amount of diethyl ether in a small flask and a small amount of dry silica gel (Wakosil C-200) is added. Removal of ether using a rotatory evaporator gives silica gel powder coated with impure vanillin. A glass column (3 cm \times 30 cm) with a thin conical cotton and circular filter paper at its lower end is held perpendicularly and filled slowly with the Wakosil C-200 while rotating the column to give about 10 cm gel layer. The crude vanillin-coated silica gel is added on the top of the column, and covered with a circular filter paper. A small amount of developer (n-hexane: ethyl acetate = 1: 1) is added repeatedly so that the developer permeates downwards without forming air bubbles inside of the column. After the colored elution band reaches to one third of the column height, a larger amount of developer is added. Every 30 ml of the eluted developer is collected in different flasks, and the fraction containing purified vanillin (detection by thin-layer chromatography, TLC) is combined and the removal of developer solvent gives purified vanillin as almost colorless crystal.

3.1.3. Tetra-O-acetyl-glucovanillin-[CHO-¹³C] (4)

Vanillin-[CHO-¹³C] (**3**) (2.81 g, 18.3 mmol) and silver (I) oxide (Ag_2O , >99%, Aldrich Chemical Co., 2.13 g, 9.2 mmol) is mixed thoroughly in a dry mortar. In a tall beaker (50 ml), acetobromo-D-glucose (2,3,4,6-tetra-O-acetyl- α -D-bromoglucose, 7.55 g, 18.36 mmol) is dissolved in quinoline (20 ml), and the mixture of vanillin and silver oxide is added portionwise with vigorous stirring using a glass blade (Note 3.1.3-1) at 0–5°C. The whole mixture becomes a viscous slurry, and stirring is continued for a further 60 min at room temperature. Acetic acid (20 ml) is added with stirring. The whole mixture is then poured into cold water (approximately 700 ml) with stirring and allowed to stand for 30 min to precipitate products. The fine precipitate is collected by filtration. If filtration takes too long time, a layer of Celite is laid on the filter paper as a filtration aid. The residue on the filter is extracted three times with hot ethanol (150 ml \times 3). Concentration of the combined ethanol solution gives crystals. Recrystallization from ethanol affords pure compound **4** (7.03 g, 14.5 mmol, 79.2% + α g from the mother liquor).

Note 3.1.3-1. Effective stirring of this viscous mixture is important to obtain the product in a good yield. The effective stirring is achieved by the use of a glass rod with a blade part slightly twisted to a screw shape at its end. If you have no suitable stirring blade, this tool can be prepared by yourself as explained in the former section 2.2.

3.1.4. Tetra-O-acetyl-glucoferulic acid ethyl ester-[α -¹³C] (5)

A mixture of compound **4** (6.52 g, 13.5 mmol), monoethyl malonate (2.14 g, 16.18 mmol) (Note 3.1.4-1.), pyridine (5.5 ml) and piperidine (0.21 ml) are placed in a round bottomed flask equipped with a reflux condenser and a calcium chloride tube and heated at 100°C for 2 h. The mixture is poured into water (300 ml) with stirring to precipitate compound **5**. The precipitation is effected by addition of dil. hydrochloric acid to neutralize the solution, and the aggregate of compound **5** is crashed to powder in the water. The precipitate is collected by filtration and washed with water. Recrystallization from ethanol gives compound **5** (6.76 g, 12.2 mmol, 90.5% + α g from the mother liquor).

Note 3.1.4-1. Monoethyl malonate is prepared according to Niwayama and Cho³⁾. In a 200 ml-size one-necked flask, equipped with a magnetic stirrer, is placed 25.73 g (0.16 mol) of diethyl malonate, and 6 ml of acetonitrile is added to dissolve the diethyl malonate. After the solution is stirred for one min, the reaction mixture is cooled to 0°C with an ice-water bath maintained at 0–4°C. To this mixture, 60 ml of water is added and the mixture is stirred for 30 min. To this reaction mixture, 32 ml of 5 M aqueous KOH (0.16 mol) solution is added dropwise

with continuous stirring for a period of 15 min using an addition funnel. When this addition is completed, the reaction mixture is stirred for one additional hour while covered with a stopper and immersed in the ice-water bath maintained at 0–4°C. The reaction is monitored by TLC using a staining solution prepared with bromocresol green (pH indicator; pH 3.8: yellow, pH 5.4: blue) (40 mg) dissolved in absolute ethanol (100 ml).

The reaction mixture is acidified with 24 ml of 12 M aqueous HCl solution in an ice-water bath, then saturated with NaCl, and extracted with five 100 ml portions of ethyl acetate using a one-liter-separatory funnel. The extract is washed with 100 ml of a saturated aqueous NaCl solution. The ethyl acetate extract is dried over approximately 10 g of anhydrous sodium sulfate. After the drying agent is removed by filtration, the ethyl acetate solution is concentrated by a rotary evaporator, and the residue is transferred to a vacuum distillation apparatus (Note 3.1.4-2.). Under slightly reduced pressure, the ethyl acetate remaining in the monoester is removed completely, and then distilled under high vacuum at 120 °C (oil bath temperature). The temperature should not exceed higher than 125°C, because monoester decomposes at higher temperature. The yield of monomethyl malonate is 16.37g (77%).

Note 3.1.4-2. The use of distillation apparatus with 100-ml heart-shape flask, Claisen-type head, Liebig-type condenser is recommended.

3.1.5. Coniferin-[α -¹³C] (1)

Compound **5** (6.66 g, 12 mmol) is dissolved in dry toluene (360 ml) by slight warming, in a four-necked flask (1000 ml) (Note 3.1.5-1) equipped with a motor-driven efficient stirrer (with an efficient bearing), a dropping funnel, and a nitrogen gas inlet and outlet connected to two gas washing bottles (the first one is empty for safety, the next one contains a small amount of sulfuric acid to prevent moisture). The flask is cooled in an ice water bath (0–5°C), and the air is removed by a gentle flow of nitrogen gas through the flask.

Diisobutylaluminum hydride (DIBAL-H, 1.5 M in toluene, 102 ml, 153 mmol) (Note 3.1.5-2) is slowly added from the dropping funnel under vigorous stirring at 0–5°C. After 30 min, addition is completed, and stirring is continued for another one hour. The color of the solution becomes yellow at first and becomes pale yellow at the end of the reaction. The reaction mixture is carefully quenched by slow addition of absolute ethanol (50 ml) (Note 3.1.5-3) with vigorous stirring at 0–5°C. The whole mixture is transferred into a 2000 ml round-bottomed flask, and the solvent is removed thoroughly under reduced pressure at 50°C. Water (200 ml) is added to the residue, and shaken to form a suspension of yellowish white jelly material. A small amount of toluene separated on the water layer is removed again under reduced pressure at 50°C. The residual aqueous suspension is warmed in a boiling water bath and filtered. The extraction with hot water is repeated twice (200 ml × 2). The combined water solution is concentrated under reduced pressure at 60°C to give compound **1**. Recrystallization from hot water gives fine white needles of compound **1** (2.8 g, 8.15 mmol, 68% + α g from the mother liquor). From the mother liquor, an additional amount of compound **1** can be obtained by column chromatography employing silica gel (Wako C-200) and acetone: ethyl acetate: water = 10:10:1 as a developer ²⁾.

Note 3.1.5-1. Three-necked flask with a Y-shape adapter can be used in place of a four-necked flask. A flask with a vertical side neck and a Y-shape adapter with vertical arm is preferred because the dropping funnel must be held vertically on one of the neck or arms. An inclined neck is also not suitable for vigorous stirring of toluene solution. All apparatus must be dried in an oven or by blowing hot air using a hairdryer just before use.

Note 3.1.5-2. DIBAL-H is highly hygroscopic and flammable. For transfer of the necessary amount from a commercially available glass bottle or a metal cylinder, it is strongly recommended to use a combination of a glass syringe (50 or 100 ml, with Luer lock) and a double-needle for liquid-transfer/gas-purge (Luer lock, See Catalog of Aldrich Chemical Co.).

Note 3.1.5-3. If 95% ethanol is used, jelly material is partly formed and it prevents efficient removal of solvent and filtration of hot water solution in the next step.

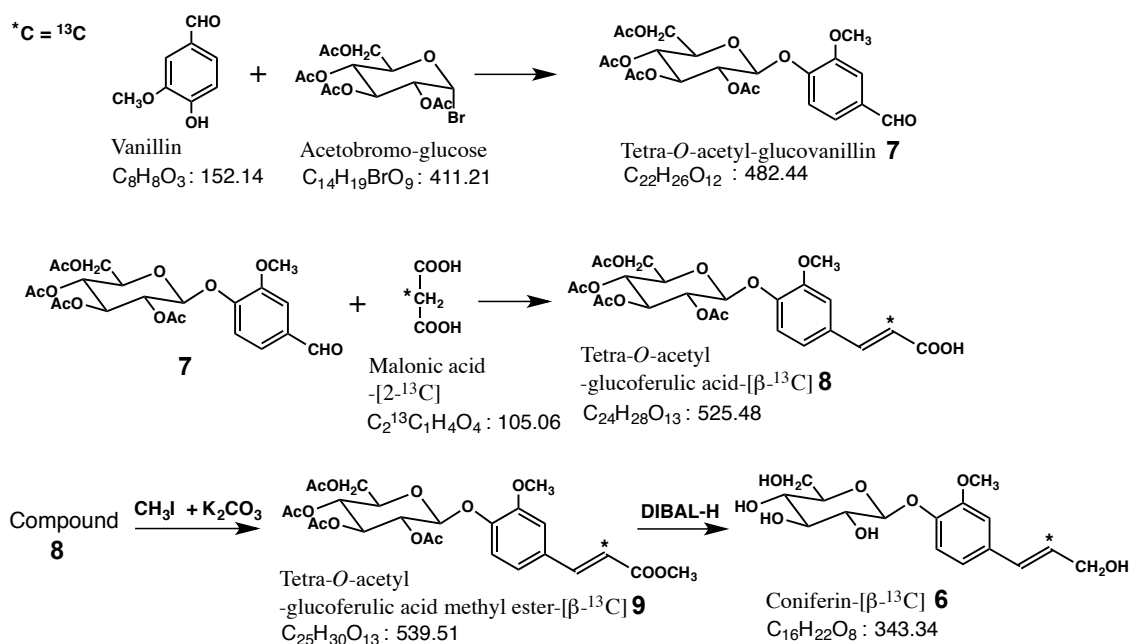


Fig. 2. Synthesis of coniferin-[- ^{13}C] (**6**).

3.2. Coniferin-[- ^{13}C] (**6**)

3.2.1. Tetra-*O*-acetyl-glucovanillin (**7**)

Compound **7** is prepared by the same procedure described for the preparation of compound **4** employing unenriched vanillin instead of vanillin-[CHO- ^{13}C].

3.2.2. Tetra-*O*-acetyl-glucoferulic acid-[- ^{13}C] (**8**)

A mixture of compound **7** (8.38 g, 17.4 mmol), malonic acid-[2- ^{13}C] (1.66 g, 15.8 mmol), pyridine (7.0 ml) and piperidine (0.22 ml) is placed in a flask (50 ml) fitted with a reflux condenser and a calcium chloride tube for the exclusion of moisture, and heated at 100°C for 1.5 h. Malonic acid (0.40 g, 3.84 mmol) is added to the reaction flask, and heating is continued further for 1.5 h. Evolution of CO_2 gas ceases at the end of the reaction. After cooling, the reaction mixture is poured slowly into a mixture of dil. HCl (about 10 ml) and water (300 ml) with stirring. Dil. HCl (1:3) is added to make the solution acidic (pH < 2) to precipitate the product **8** completely. The precipitate is collected by filtration and recrystallized from ethanol to give compound **8** (8.29 g, 15.8 mmol, 80.4% based on total malonic acid + α g from the mother liquor).

3.2.3. Tetra-*O*-acetyl-glucoferulic acid methyl ester-[- ^{13}C] (**9**)

A mixture of compound **8** (3.97 g, 7.55 mmol), fine powder of dry potassium carbonate (1.56 g, 11.2 mmol) (Note 3.2.3-1) and methyl iodide (2.68 g, 18.8 mmol) in dry acetone (18 ml) (Note 3.2.3-2) is heated to reflux under the exclusion of moisture for 1 h. Methyl iodide (1.1 g, 7.5 mmol) is added and refluxed for further 3 hrs. Then, the solvent and excess methyl iodide are removed by evaporation. To the residue, chloroform (70 ml) is added and shaken, and then water is added and shaken. The chloroform layer (the lower part in the separatory funnel) is separated and washed with fresh water. The chloroform solution is dried with anhydrous sodium sulfate. The residue obtained after the

evaporation of chloroform under reduced pressure is recrystallized from methanol to give compound **9** (3.60 g, 6.67 mmol, 88.4%, + α g from the mother liquor).

Note 3.2.3-1. Use of a mortar dried thoroughly by blowing hot air with a hairdryer is recommended to prepare the dry fine powder of K_2CO_3 .

Note 3.2.3-2. Acetone dried over anhydrous K_2CO_3 is used.

3.2.4. Coniferin- $[\beta\text{-}^{13}\text{C}]$ (**6**)

Reduction of compound **9** to compound **6** is carried out by the same manner described for the preparation of coniferin- $[\alpha\text{-}^{13}\text{C}]$.

3.3. Coniferin- $[\gamma\text{-}^{13}\text{C}]$ (**10**)

Compound **10** is prepared by the same manner described for the preparation of coniferin- $[\beta\text{-}^{13}\text{C}]$ using malonic acid- $[1,3\text{-}^{13}\text{C}]$ instead of malonic acid- $[2\text{-}^{13}\text{C}]$ (See Figure 3).

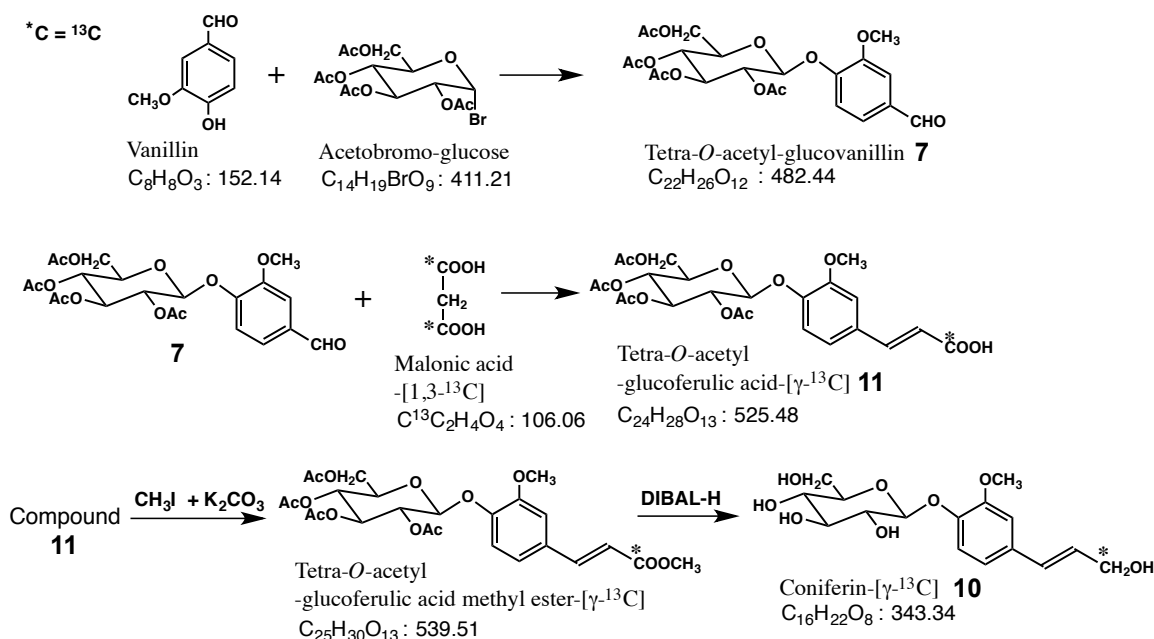


Fig. 3. Synthesis of coniferin- $[\gamma\text{-}^{13}\text{C}]$ (**10**).

3.4. Coniferin- $[\text{ring-4-}^{13}\text{C}]$ (**12**)⁴⁾

3.4.1. Methoxyacetonitrile- $[\text{CN-}^{13}\text{C}]$ (**13**)

Sodium cyanide- $[\text{CN-}^{13}\text{C}]$ (1000 mg, 20 mmol, Cambridge Isotope Laboratories, Inc. Andover, MA, USA) is dissolved in water (2 ml) in a glass vial (diameter: 18 mm, 10 ml) which is kept in a water bath at 20–25°C. Paraformaldehyde (600 mg, 20 mmol) is added slowly (Note 3.4.1-1) to the cyanide solution under vigorous stirring over a period of 30 min (Note 3.4.1-2). The reaction mixture is stirred for an additional 30 min at 20–25°C after addition is completed. The mixture is then cooled to 13°C, and dimethylsulfate (2 ml, 2700 mg, 21 mmol, Merck co.) is added slowly through a pipet (Note

3.4.1-3) under vigorous stirring over a period of 20 min. After completion of addition, the mixture is stirred for additional 30 min. The mixture is cooled to 5°C, and transferred to a small vial (d = 10 mm). The oily layer appeared at the upper part is separated using a pipet to a vial containing a small amount (50 mg) of anhydrous sodium sulfate to give crude methoxyacetonitrile-[CN-¹³C] (**13**). The lower layer is transferred again to the reaction vial, and second methylation is carried out using

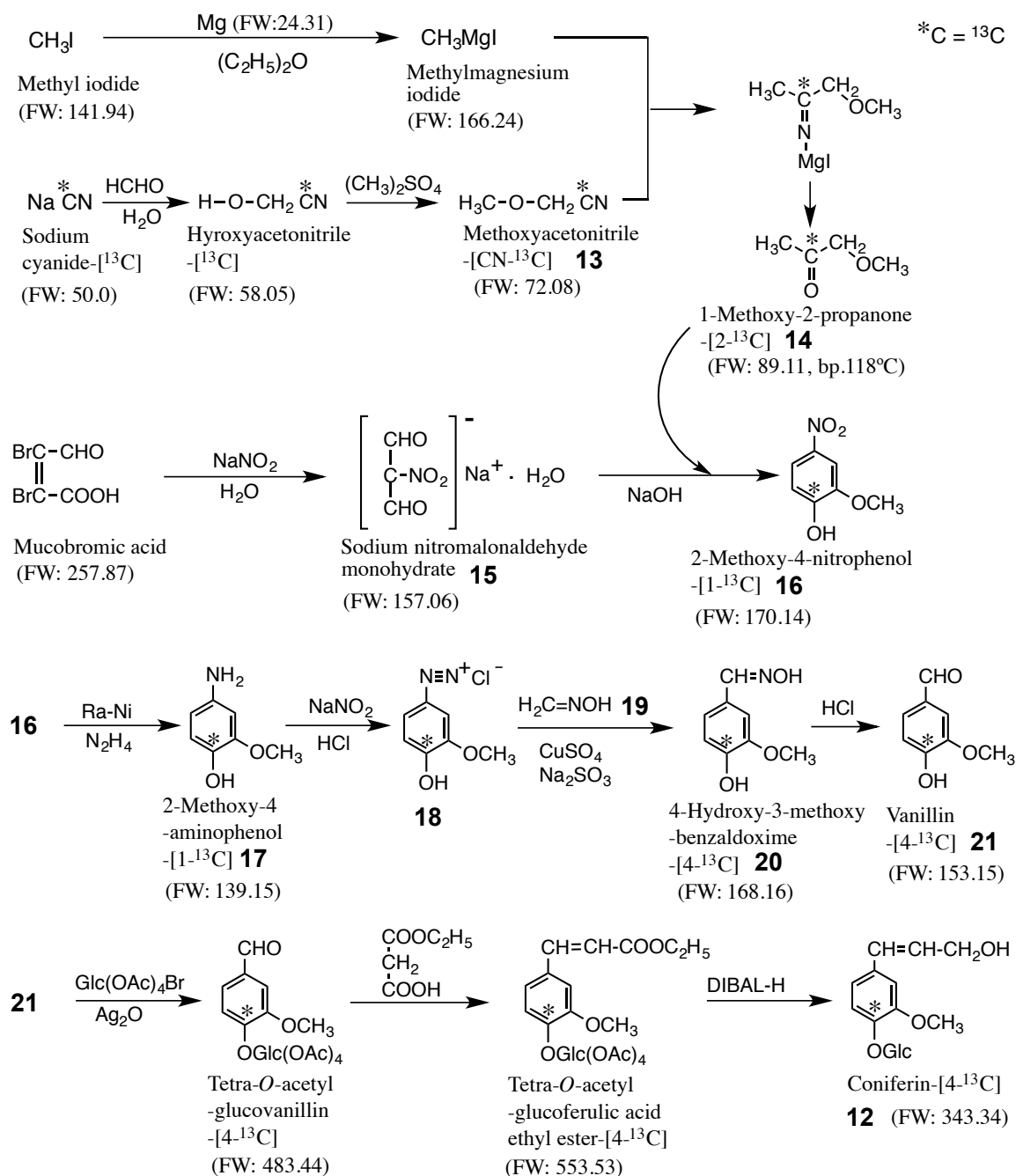


Fig. 4. Synthesis of coniferin-[ring-4-¹³C] (**12**).

dimethylsulfate (2 ml, 2700 mg, 21 mmol) in the same manner as the first methylation to give additional amount of crude oily methoxyacetone nitrile-[CN-¹³C]. The first and second oily fractions are distilled under reduced pressure separately (Note 3.4.1-4). The first and second crude nitrile fractions are distilled again separately under atmospheric pressure maintaining the oil-bath temperature at 160°C. After distillation, a small amount (100 mg) of unenriched methoxyacetone nitrile is added to the residue and distilled to recover the enriched material. Total yield is 946 mg (13.12 mmol), (846 mg [11.74 mmol, 60.0%] of enriched + 100 mg of unenriched).

Note 3.4.1-1. A combination of a short glass pipet (d = 7 mm, l = 50 mm) and a short stainless steel wire is used as a powder funnel to add paraformaldehyde in small portions.

Note 3.4.1-2. Effective stirring is achieved by the use of a glass stirring blade (See section 2.2).

Note 3.4.1-3. A Pasteur pipet with a fine capillary end is used. The end of the capillary is immersed under the surface of the vigorously stirred reaction mixture.

Note 3.4.1-4. Mechanical loss is minimized by the use of a specially designed one-piece distilling apparatus composed of a small pear-shape flask, a Claisen distilling head, and a cooling receiver. The distilling flask is connected directly to a water aspirator pump, and bath temperature is kept at 85°C during distillation.

3.4.2. Methoxyacetone-[CO-¹³C] (1-methoxy-2-propanone-[2-¹³C]) (**14**)

Magnesium turnings (352 mg, 14.5 mmol, Aldrich Chemical Co.), dry ether (5 ml) (Note 3.4.2-1), a small piece of iodine and a stirring magnet bar are placed in a two-necked heart-shape flask (50 ml) fitted with a reflux condenser with calcium chloride tube at its top and a sealed septum at a side neck (Note 3.4.2-2). Under gentle stirring, a solution of iodomethane (2090 mg, 14.7 mmol) in dry ether (3 ml) is added slowly through a septum using a glass syringe. The rate of addition is adjusted to keep gentle refluxing of ether (in about 20 min). After the addition is finished, the flask is put in an oil bath (45°C) to keep gentle refluxing with stirring. After about 1 h, most magnesium is dissolved, (Note 3.4.2-3).

The magnetic stirrer bar is removed, and a glass stirring-blade is attached with a Teflon seal for powerful stirring by an electric motor. The oil bath is changed to an ice-salt bath to cool the flask at < -5°C. A disposable syringe containing anhydrous calcium chloride pellet in place of a piston is attached by inserting needle to the seal septum at the side neck. Under vigorous stirring, methoxyacetone nitrile-[CN-¹³C] (**13**) (946 mg, 13.14 mmol) in dry ether (3 ml) is added slowly through the septum using a glass syringe. A grayish-white addition product appears immediately (Note 3.4.2-4). After the addition is finished, stirring is continued for 1 h at room temperature. Then the flask is cooled again with ice, and under stirring, ice-water (7 ml) is added slowly, then cold dilute sulfuric acid (5 ml, H₂SO₄: water = 1: 2) is added dropwise carefully (Note 3.4.2-5).

The stirring unit is removed and a Claisen-K-type distilling head, a Liebig-type condenser and a 20 ml test tube as a receiver are attached (Note 3.4.2-6). A steam inlet glass tube is inserted from the side arm to near the bottom of the flask. A steam generator flask is connected with a rubber tubing to the distillation flask via a T-shape connector, as a safety trap (Note 3.4.2-7). The steam generator flask is put in the oil bath and the temperature is raised to 140°C to generate steam constantly and steam appears from the opening at the foot of the T connector (Note 3.4.2-8). The opening at the foot of the T connector is carefully closed with a rubber tube and a pinchcock. Steam is introduced carefully into the distilling flask. When most of ether is removed by introducing steam, the distillation flask is put in an oil bath at 120°C. When the distillate becomes 10 ml, the receiver test tube is changed to a new one, and 10 ml of second distillate is collected. After third 10 ml distillate is collected, distillation is stopped by releasing the pinchcock of the T connector. The distillate in the three test tubes is saturated with potassium carbonate by shaking the screw-capped tube under cooling in an ice bath (Note 3.4.2-9). On standing for 30 min, methoxyacetone-[CO-¹³C] (**14**) is separated on the surface as an oily layer. The oily product is collected using a Pasteur pipet. The residual saturated solution is extracted with ether (3 ml each) and the ether is evaporated to leave a small amount of methoxyacetone. The total yield of compound **14** is 1321 mg (14.8 mmol containing a small amount of ether).

Note 3.4.2-1. Ether should be dried over metallic sodium for more than one day. The dry ether available from Aldrich Chem. Co. can be used without further drying.

Note 3.4.2-2. All glass apparatus should be dried by blowing hot air using a hairdryer.

Note 3.4.2-3. After about 1 hour, a very small amount of magnesium still remains undissolved.

Note 3.4.2-4. If a big aggregate is formed, this should be crushed using a spatula quickly preventing exposure to moisture. Vigorous stirring and slow addition under the exclusion of moisture usually do not form big aggregates.

Note 3.4.2-5. Evolution of heat and gas may cause bumping of the mixture. Water and acid must be added carefully.

Note 3.4.2-6. A screw-capped test tube (20 ml) is suitable to collect a small amount of methoxyacetone by salting out from the distillate.

Note 3.4.2-7. Generation of steam is conveniently achieved as follows: a four-necked round-bottomed flask (300 ml) is equipped with glass tube (about 80 cm) and rubber stopper at its center reaching the end of the tube almost bottom of the flask. Water (150 ml), a magnetic stirring bar and boiling stones are introduced. A steam outlet is connected with rubber (or silicone) tubing via a T-shape connector to the steam inlet tube of the side arm of the distillation flask. The vertical end of the T-shape connector, equipped with a short rubber (approximately 5 cm) and a pinchcock, can be used as a steam trap and an emergency opening.

Note 3.4.2-8. The safety opening of the T connector should be open until enough steam is generated. Steam must be generated constantly. This is achieved by effective magnetic stirring of the hot water and effective boiling stones. If steam generation stops during distillation, the reaction mixture will flow in the reverse way to the steam generator. This can be prevented by opening the T-shape safety trap.

Note 3.4.2-9. Carbon dioxide gas evolved during shaking must be allowed to escape by releasing the cap carefully.

3.4.3. Sodium nitromalonalddehyde monohydrate (**15**)⁵⁾

In a three-necked round-bottomed flask (250 ml) equipped with a mechanical stirrer, a thermometer, a dropping funnel and gas vent (Note 3.4.3-1), sodium nitrite (25.8 g, 374 mmol) is dissolved in water (25 ml) by heating with stirring. The temperature of the mixture is kept in a water bath at $54 \pm 1^\circ\text{C}$. A solution of mucobromic acid (25.8 g, 100 mmol) in 95% ethanol (25 ml) is added dropwise under stirring over the period of 70 min. A mildly exothermic reaction occurs, and the solution becomes deep red, and gas is evolved. The temperature is kept at $54 \pm 1^\circ\text{C}$ during the reaction, and the gas is vented to a water jet pump (Note 3.4.3-1). After the mixture is stirred for an additional 10 min at $54 \pm 1^\circ\text{C}$, the mixture is cooled to $0\text{--}5^\circ\text{C}$ by the application of an ice bath. The fine yellow precipitate is collected on a chilled Büchner funnel. The slightly moist cake of crude product is transferred to a 100 ml flask and heated to boiling with a mixture of 40 ml of 95% ethanol and 10 ml of water. The hot solution is filtered to remove a fine yellow solid, and the clear red filtrate is cooled to $0\text{--}5^\circ\text{C}$. The recrystallized product is collected on a Büchner funnel and dried in air at room temperature. The yield of compound **15** is 5.82 g (37.1%) as pink needles (Note 3.4.3-2).

Note 3.4.3-1. A Claisen Y-shape adapter is attached to one of the necks. A dropping funnel and a hose connector are attached to the top parts of the Claisen adapter. The hose adapter is connected to a T connector and a water jet pump with rubber tubing. The foot part of the T connector is open when the water jet pump is working to vent the highly dangerous hydrogen cyanide gas evolved during the reaction.

Note 3.4.3-2. The sodium salt of nitromalonalddehyde is impact-sensitive and thermally unstable. It should be handled as a potentially explosive material and should be kept in a refrigerator.

3.4.4. 2-Methoxy-4-nitrophenol-[1-¹³C] (5-nitroguaiacol-[3-¹³C]) (**16**)

Methoxyacetone-[CO-¹³C] (**14**) (1321 mg, 14.8 mmol) is added to a solution of sodium nitromalonalddehyde monohydrate (**15**) (3015 mg, 19.2 mmol) in 10% sodium hydroxide (40 ml) under stirring at room temperature to form an orange-red precipitate. After stirring for 5 h, the mixture is acidified with diluted hydrochloric acid (concentrated HCl:water = 1:3), and extracted with ether

(50 ml \times 4). The ether extract is washed with brine (20 ml \times 2) and dried over anhydrous sodium sulfate. Evaporation of ether gives crude 2-methoxy-4-nitrophenol-[1- ^{13}C] (**16**) (1287 mg, 7.56 mmol, 51.1% from methoxyacetone).

3.4.5. 2-Methoxy-4-aminophenol-[1- ^{13}C] hydrochloride (5-aminoguaiacol-[3- ^{13}C]) (**17**)

A solution of 2-methoxy-4-nitrophenol-[1- ^{13}C] (**16**) (1058 mg, 6.22 mmol) in absolute ethanol (30 ml) is placed in a two-necked heart-shaped flask (50 ml). Nitrogen is slowly introduced through the side neck using a syringe needle and a seal septum to protect aminoguaiacol from oxidation in the air. Under vigorous stirring and cooling with water, hydrazine hydrate (total 1.6 ml) and Raney nickel are added in small portions. In about 1-2 h, red color of nitroguaiacol becomes yellow and finally almost colorless. Reduction of the nitro-group to amino group is confirmed by TLC (Rf: 0.57 for 2-methoxy-4-nitrophenol- [1- ^{13}C], 0.17 for 2-methoxy-4-aminophenol-[1- ^{13}C], using n-hexane:ethyl acetate = 1:1). After reduction is completed, ethanol is added (total 50 ml) and warmed under gentle stirring to dissolve most of aminoguaiacol. Stirring is stopped, and the warm supernatant is separated from the Raney-nickel by pipetting. The aminoguaiacol and Raney-nickel remaining in the flask are warmed again in ethanol (50 ml), and quickly separated by filtration. The whole filtrate is transferred to a round-bottomed flask (300 ml) and ethanol is removed quickly under reduced pressure to dryness (Note 3.4.5-1). The flask is connected directly to a water jet pump and then to an oil vacuum pump with slight warming to completely dry the aminoguaiacol (**17**). A mixture of conc. hydrochloric acid (6.22mmol \times 2.5 = 15.55 mmol hydrogen chloride: 568 mg \approx 1.3 ml conc. HCl) and water (1.3 ml) is added to dissolve the aminoguaiacol. The solution is transferred to a two-necked heart-shaped flask (50 ml) and additional amount of water (20 ml) is employed for the complete transfer of aminoguaiacol hydrochloride. (Note 3.4.5-2).

Note 3.4.5-1. 2-Methoxy-4-aminophenol is unstable material, because it is oxidized quickly in the air. Exposure to air for long time is not recommended.

Note 3.4.5-2. Aminoguaiacol hydrochloride is stable to oxidation in the air.

3.4.6. Diazonium salt of 2-methoxy-4-aminophenol-[1- ^{13}C] (**18**)

To the two-necked heart-shaped flask (50 ml) containing compound **17**, a glass stirrer blade is inserted near its bottom. Under vigorous stirring, and cooling at 0–5°C in ice-water, a solution of sodium nitrite (472 mg, 6.84 mmol) in water (0.7 ml) is introduced slowly below the surface of the amine hydrochloride solution (Note 3.4.6-1). After the addition is finished, stirring is continued for a further 15 min at 0–5°C to form a brown-colored diazonium salt solution. Sodium acetate (363 mg, 4.43 mmol) in water (1 ml) is added to make the pH around 4 (by pH test paper). If necessary, an additional amount of sodium acetate is added to make the pH around 4. The diazonium salt solution is kept at 0–5°C.

Note 3.4.6-1. A special pipet with fine capillary tip is used. This pipet is made by heating a Pasteur pipet in a gas flame and elongating the tip to a capillary. Then the tip is cut using a folded abrasive paper.

3.4.7. Formaldoxime (**19**)

A mixture of paraformaldehyde (372 mg, 12.4 mmol) and hydroxylamine hydrochloride (862 mg, 12.4 mmol) in water (6.0 ml) is placed in a 20 ml sealed test tube, and heated in a boiling water bath with occasional shaking and releasing pressure inside of the tube until a clear solution is obtained. Then, sodium acetate (1017 mg, 12.4 mmol) is added and heated again in a boiling water bath for 30 min to obtain a 10% solution of formaldoxime.

3.4.8. 4-Hydroxy-3-methoxy-benzaldoxime-[4-¹³C] (20)

In a two necked flask (50 ml) equipped with a mechanical stirrer, the solution of formaldoxime **19** prepared above (section 3.4.7) is placed. A mixture of copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 322 mg, 1.29 mmol), sodium sulfite (Na_2SO_3 , 50 mg, 0.4 mmol) and sodium acetate (CH_3COONa , 4762 mg, 58 mmol) in water (13 ml) is added. Under vigorous stirring at 10–15°C, the diazonium salt solution **18** prepared above (Section 3.4.6) is introduced slowly using a pipet with fine capillary tip. The tip is introduced below surface of the formaldoxime solution. The dark-colored reaction mixture of 4-hydroxy-3-methoxy-benzaldoxime-[4-¹³C] (**20**) and pasty material is stirred for additional 40 min.

3.4.9. Vanillin-[4-¹³C] (21)

The mechanical stirrer at the center of the flask containing 4-hydroxy-3-methoxy-benzaldoxime-[4-¹³C] (**20**) is replaced with a reflux condenser. A magnetic stirrer bar and conc. hydrochloric acid (11 ml) is placed and heated on an oil bath at 100°C with stirring for 1 h (Note 3.4.9-1). After cooling, the whole mixture is extracted with ether (40 ml \times 5). The ether solution is extracted with 20% sodium hydrogen sulfite (20 ml \times 5). The vanillin adduct is decomposed by addition of sulfuric acid (1:3, 20 ml) to the solution, and air is bubbled into the warmed solution (approximately at 60°C) to remove sulfur dioxide (Note 3.4.9-2). After cooling, the solution is extracted with ether (30 ml \times 4), washed with brine (20 ml \times 2) and dried over anhydrous sodium sulfate. Ether is evaporated, and the residue is put in a vacuum desiccator (containing NaOH pellets) for complete removal of volatile material to give crude vanillin-[4-¹³C (43 atom%-¹³C)] (**21**), (183 mg, 1.19 mmol, 19.2% from 2-methoxy-4-nitrophenol-[1-¹³C] (**16**)).

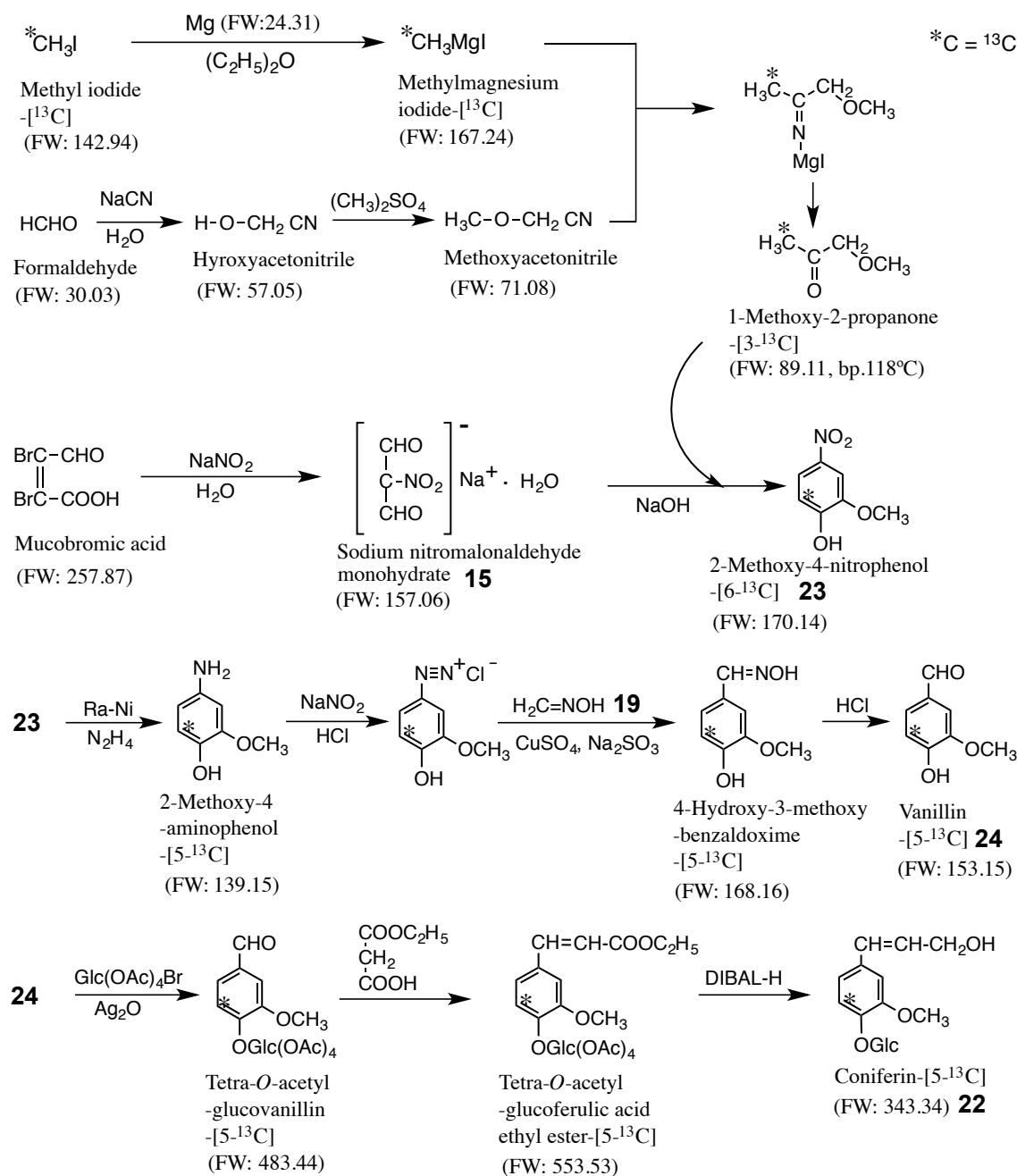
Note 3.4.9-1. Formation of vanillin in the reaction mixture can be confirmed by TLC as follows. A few drops of the reaction mixture are extracted with a small amount of ether in a small vial. The ether extract and ether solution of authentic vanillin are spotted on a silica gel TLC plate (1.5 \times 6 cm) and developed with a mixture of n-hexane and ethyl acetate (1:1, v/v) in a wide mouth vial. For the detection of the vanillin spot after development, the TLC plate is put in a vial containing a small amount of iodine covered with glass wool. The spot of the labeled vanillin is easily detected by comparison of authentic vanillin at an R_f value around 0.4. In a small scale TLC experiment using easily volatile hexane, the developer is prepared conveniently by weighing 330 mg of hexane (approx. 0.5 ml) and 451 mg of ethyl acetate (0.5 ml) directly in a vial. The fine capillary for spotting on TLC can be prepared by heating a Pasteur pipet on a gas burner to a bright red and elongate to a fine capillary. Cutting is achieved best using a folded abrasive paper as a file.

Note 3.4.9-2. For extraction of vanillin as NaHSO_3 adduct, refer to Note 3.1.2-2 for the preparation of vanillin-[α -¹³C] (**3**).

According to the same procedure described for synthesis of coniferin-[side chain carbon-¹³C], coniferin-[ring-4-¹³C] (**12**) is synthesized from vanillin-[4-¹³C] (**21**).

3.5. Coniferin-[ring-5-¹³C] (22)

Coniferin-[ring-5-¹³C] (**22**) is prepared by the same procedure described for the synthesis of coniferin-[ring-4-¹³C] (**12**) using iodomethane-[¹³C] and unenriched potassium cyanide as shown in the scheme of synthesis (Fig. 5). Commercially available methoxyacetonitrile (Aldrich Chemical Co.) can be also used. To increase the ¹³C-yield in coniferin-[ring-5-¹³C] from iodomethane-[¹³C], a 5–10% excess amount of magnesium and methoxyacetonitrile should be employed. The best yield of vanillin is 362 mg from 1.48 g nitroguaiacol (27%).

Fig. 5. Synthesis of coniferin-[ring-5- ^{13}C] (**22**).

3.6. Coniferin-[ring-3- ^{13}C] (**25**)

Coniferin-[ring-3- ^{13}C] (**25**) is prepared by the same procedure described for the synthesis of coniferin-[ring-4- ^{13}C] (**12**) using paraformaldehyde- ^{13}C and unenriched sodium cyanide as shown in the scheme of synthesis (Fig. 6). Commercially available methylmagnesium iodide can be also used. To increase the ^{13}C -yield in coniferin-[ring-3- ^{13}C] from paraformaldehyde- ^{13}C , a 10% excess amount of sodium cyanide and methoxymagnesium iodide should be employed.

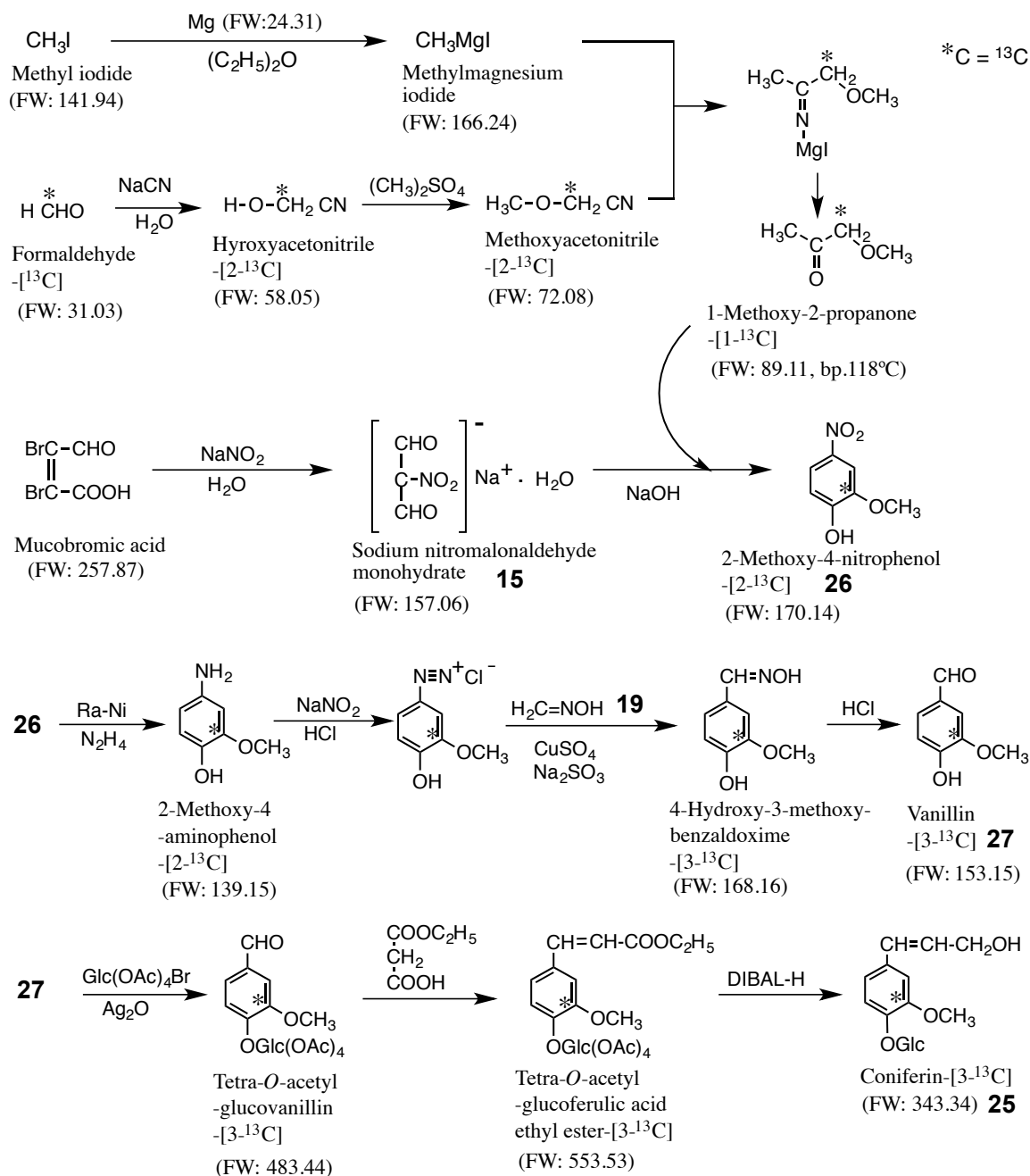


Fig. 6. Synthesis of coniferin-[ring-3- ^{13}C] (**25**).

3.7. Coniferin-[ring-1- ^{13}C]

Coniferin-[ring-1- ^{13}C] can be synthesized from $\text{Br}^{13}\text{CH}_2\text{COOH}$ ⁶⁾. However, further improvement is necessary, because the final yield is low. Another method for the synthesis of vanillin-[ring-1- ^{13}C] giving a better yield has been reported ⁷⁾.

3.8. Coniferin-unenriched (26)²⁾

Unenriched coniferin is prepared by the same manner described for preparation of coniferin- $[\alpha\text{-}^{13}\text{C}]$ (1) using vanillin instead of vanillin- $[\text{CHO-}^{13}\text{C}]$.

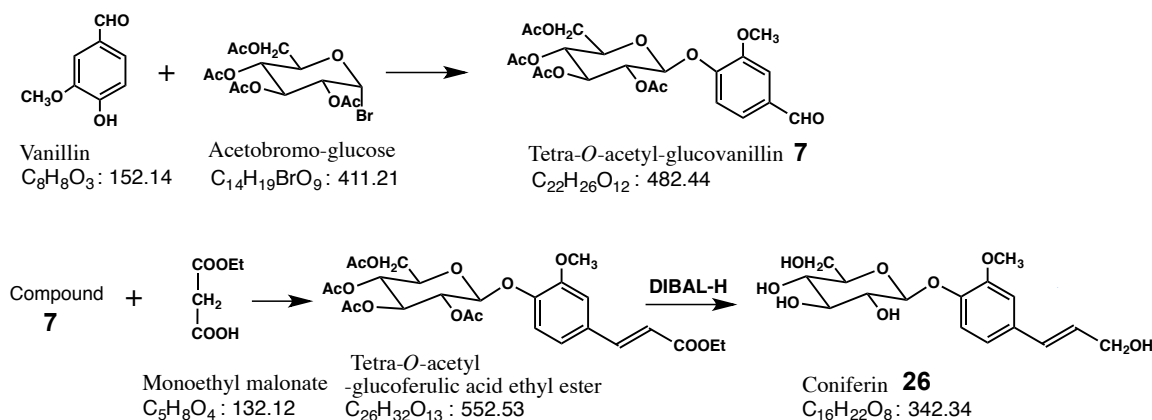


Fig. 7. Synthesis of coniferin-unenriched (26).

3.9. *p*-Glucocoumaryl alcohol-[side chain and ring- ^{13}C]²⁾

p-Glucocoumaryl alcohol-[side chain- ^{13}C] is prepared in the same manner as described for preparation of coniferin-[side chain- ^{13}C] using *p*-hydroxybenzaldehyde instead of vanillin. *p*-Glucocoumaryl alcohol-[ring- ^{13}C] is prepared in the same manner as described for preparation of coniferin-[ring- ^{13}C] using acetone- $[\text{ring-}^{13}\text{C}]$ instead of methoxyacetone- $[\text{ring-}^{13}\text{C}]$.

3.10. Syringin-[side-chain and ring- ^{13}C] (27)^{1, 2)}

For preparation of syringin- $[\alpha\text{-}^{13}\text{C}]$ (27), syringaldehyde- $[\alpha\text{-}^{13}\text{C}]$ (29) is prepared from vanillin- $[\alpha\text{-}^{13}\text{C}]$ (3) by introducing OCH_3 at the ring position 5 (Fig.8). Syringin- $[\beta\text{-}^{13}\text{C}]$ and syringin- $[\gamma\text{-}^{13}\text{C}]$ are prepared by the same way as described for the syntheses of coniferin- $[\beta\text{-}^{13}\text{C}]$ and coniferin- $[\gamma\text{-}^{13}\text{C}]$ using malonic acid- $[\text{2-}^{13}\text{C}]$ and malonic acid- $[\text{1,3-}^{13}\text{C}]$ as starting materials, respectively. Syringin-[ring- ^{13}C] is also prepared from syringaldehyde-[ring- ^{13}C] derived from vanillin-[ring- ^{13}C] by introducing $-\text{OCH}_3$ at the ring position 5.

3.10.1. 5-Bromovanillin- $[\alpha\text{-}^{13}\text{C}]$ (28)⁸⁾

Vanillin- $[\text{CHO-}^{13}\text{C}]$ (3) (2.40 g, 15.77 mmol) is dissolved in glacial acetic acid (16 ml). In a draft chamber, bromine (2.8 g, 35 mmol) is added dropwise under stirring with a magnetic stirrer. After addition of bromine, stirring is continued for a further 30 min. The reaction mixture is poured into ice water (30 ml) under stirring. The precipitate is collected by filtration, washed with water, and recrystallized with ethanol to give 5-bromovanillin- $[\alpha\text{-}^{13}\text{C}]$ (3.25 g, 14.0 mmol, 88.8%).



3.10.2. Syringaldehyde- $[\alpha\text{-}^{13}\text{C}]$ (**29**)⁸⁾

A 25-ml round-bottomed flask is assembled with a Claisen distillation head, a thermometer, a Liebig cooler, a receiving adapter connected to a flask and a calcium chloride tube. A piece of metallic sodium (1.38 g, 60 mmol), a small magnetic stirrer bar and methanol (28 ml) are placed in the flask. Sodium dissolves with the evolution of heat, and methanol is distilled. Then the flask is heated in an oil bath at 110°C to distill methanol. After about 5 ml of methanol is distilled, a solution of 5-bromovanillin- $[\alpha\text{-}^{13}\text{C}]$ (**28**) (3.25 g, 14.0 mmol) and anhydrous copper (II) chloride (CuCl_2 , 0.76 g, 5.65 mmol) (Note 3.10.2-1) dissolved in DMF (14 ml) is added, and distillation is continued for one hour with stirring. The color of the reaction mixture becomes from dark bluish-black at the beginning to dark brown after about one hour. After another 20 min, the flask is cooled and the whole mixture is diluted with water (30 ml), acidified by addition of 6 M hydrochloric acid (15 ml). The mixture is extracted with ethyl acetate (25 ml \times 3), and the ethyl acetate fraction is washed with brine (15 ml \times 2). The ethyl acetate solution is dried with anhydrous sodium sulfate and the solvent is removed by evaporation under reduced pressure. The residue is recrystallized from ethanol to give syringaldehyde- $[\alpha\text{-}^{13}\text{C}]$ (**29**) (1.93 g, 59.3%).

Note 3.10.2-1. Hydrated copper chloride (blue-colored) is dehydrated by heating in an oven at 110 °C overnight to give brown-colored anhydrous copper chloride.

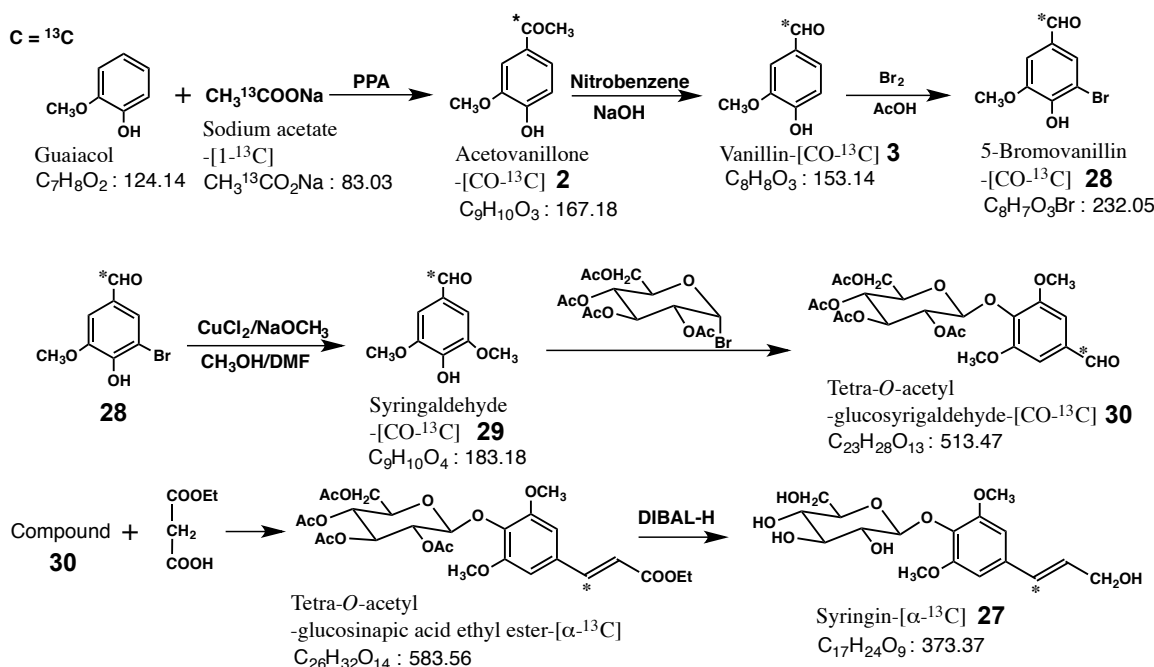


Fig. 8. Synthesis of syringin- $[\alpha\text{-}^{13}\text{C}]$ (**27**).

4. SYNTHESIS OF DHP- $[\text{C}^{13}]$ FROM MONOLIGNOLGLUCOSIDE- $[\text{C}^{13}]$

In the apoplast of lignifying plant cell walls, the polymerization of monolignols occur in a hydrophilic environment where monolignols are supplied as water-soluble monolignol glucosides^{9,10)}. The lignin polymer models (dehydrogenation polymer: DHP) can be prepared from monolignol glucosides as shown in Fig. 9. The linkage type analysis by thioacidolysis indicates that the structural

resemblance of those DHPs to that of native lignin is closer than that of DHPs prepared from water-insoluble free monolignols by the conventional method¹¹⁾.

p-Hydroxyphenyl-guaiacyl DHP-[¹³C] and guaiacyl-syringyl DHP-[¹³C] can be prepared by the same way as the preparation of guaiacyl-DHP from a mixture of corresponding monolignol glucosides-[¹³C].

4.1. Synthesis of guaiacyl-DHP-[¹³C] from coniferin-[¹³C]

In a 100 ml Erlenmeyer flask with a “loose cap”, coniferin (150 mg, Note 4.1-1) is dissolved in phosphate buffer (15 ml, pH 6.0, Note 4.1-2). β -Glucosidase (Sigma Co., 28.5 units, 10 mg dissolved in 0.5 ml water), glucose oxidase (Sigma Co., 210 units, 1 mg dissolved in 0.25 ml water) and peroxidase (Sigma Co., 116 units, 1 mg dissolved in 0.25 ml water) are added. The flask is kept at 35°C in a water bath with gentle magnetic stirring for 24 h (Note 4.1-3, and 4.1-4). The pH goes down to 4.5 during 24 h, and the pH is adjusted to 6.0 by the addition of dil. aq. sodium hydroxide. The three enzymes are added again in the same amount, and gentle stirring is continued at 35°C. The adjustment of pH is done every 24 h, and after 4 days, the DHP is collected by centrifugation and dried in a vacuum desiccator (Note 4.1-5). The crude DHP is dissolved in a mixture of 1,2-dichloroethane and ethanol (2:1, v/v, 0.5 ml), and centrifuged. Dissolution of the precipitate in a mixture of 1,2-dichloroethane-ethanol and centrifugation is repeated again. The combined supernatant is added dropwise to stirred dry ether (20 ml). The precipitated DHP is collected by centrifugation, and the DHP is washed with a few ml of petroleum ether by mixing and centrifugation. The DHP is finally dried in a vacuum desiccator (yield, 40–50 mg, 51–63%).

Note 4.1-1. For the study of structure and reaction of DHP by the ¹³C-NMR difference spectroscopy, suitable ¹³C-enrichment in the DHP is 5–10 times of natural abundance (¹³C content is about 5–10%, minimum 2%). The highly ¹³C-enriched coniferin is mixed with unenriched coniferin to give suitable enrichment in DHP.

Note 4.1-2. The pH 6.0 buffer is prepared by taking 12.3 ml of 0.2 M disodium monohydrogen phosphate (1.433 g Na₂HPO₄•12H₂O in 20 ml water) and 87.7 ml of 0.2 M monosodium dihydrogen phosphate (2.76 g NaH₂PO₄•H₂O in 100 ml water) in a 200 ml volumetric flask, and filling up with water. The pH should be confirmed by using a calibrated pH meter.

Note 4.1-3. In the preparation of a series of ¹³C-enriched DHPs as reported by Parkâs et al.^{14,15)}, a mixture of coniferin (300 mg in 30 ml buffer), β -glucosidase (60 units in 1 ml H₂O), glucose oxidase (70 units in 1 ml H₂O) and peroxidase (70 units in 1 ml H₂O) was placed in a 300 ml Erlenmeyer flask with a “loose cap” and kept at 32 °C with occasional stirring by hand or gentle stirring by a magnetic stirrer. If the size of the Erlenmeyer flask is large enough to provide enough surface area for the dissolution of oxygen from the air by occasional stirring by hand, DHP was obtained after 4 days in 52–62% of the theoretical yield. Repeated DHP preparations gave identical ¹³C-NMR spectra.

Note 4.1-4. Alternatively, mixing solution A (coniferin) and solution B (β -glucosidase) using a pump into a flask containing peroxidase and glucose-oxidase under gentle stirring for supplying O₂ from the air also gives DHP in good yield.

Note 4.1-5. To prevent splashing and contamination during vacuum drying, the wet DHP is spread on the inside surface of centrifugal tube by tapping it on the hand or desktop. Each tube must be covered with a weighing paper with many small holes and put in a beaker separately to prevent unexpected contaminations.

4.2. Synthesis of HG type or GS type DHPs

Any type of mixture DHPs can be prepared in good yield by mixing the solutions of H, G and/or S type monolignol glucosides, β -glucosidase, glucose oxidase and peroxidase using two or more pumps under gentle stirring supplying O₂ from the air.

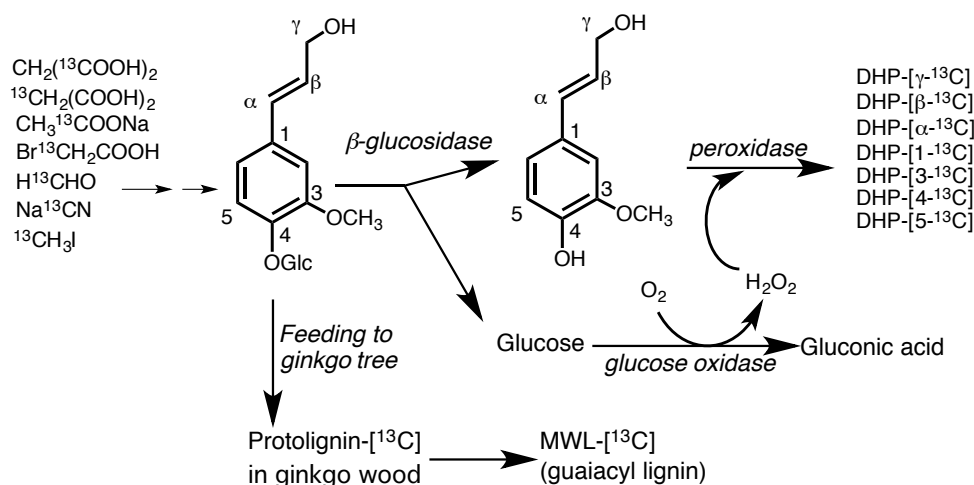


Fig. 9. Synthesis of DHP-[^{13}C] and ginkgo milled-wood lignin (MWL)-[^{13}C] from coniferin-[^{13}C]. For the preparation of DHPs from monolignol glucosides, a hypothetical scenario for cell wall lignification^{9,12)} has been tentatively employed. Glucose (the glycone part) liberated from monolignol glucosides is oxidized by glucose oxidase to gluconic acid (eventually converted into gluconolactone) producing H_2O_2 . However, glucose oxidase is not known to be present in the lignifying plant cell walls. Another hypothetical scenario for the generation of H_2O_2 from the liberated glycone part glucose in the lignification site has been proposed¹³⁾.

4.3. Syntheses of cell-wall-DHPs on isolated tree soft xylem

4.3.1. Example 1¹⁶⁾

Two spruce (*Picea abies*) trees (approximately 20–30 cm in diameter at breast height) were cut outside the city of Stockholm, Sweden, in July. The bark was carefully peeled off and the lignifying xylem (soft xylem) was collected from the surface of the logs by scraping off the soft tissue with a blunt tool. The material was immediately frozen in batches of 200 ml wet tissue. A total of about 4 l of wet cell-wall material was collected.

The frozen soft xylem samples were thawed and, thereafter, immediately dispersed for 3×15 min in 0.2 M CaCl_2 to remove soluble enzymes. After thoroughly rinsing in distilled water, the samples were dried by pressing between filter papers. About 500 mg moist tissue (about 30% dry weight) was put in a 25 ml glass-vial and soaked in 1.5 ml phosphate buffer (0.2M, pH 6.0) containing 5 μl antibiotics (penicillin-streptomycin-neomycin, 2 mg/ml, Sigma-Aldrich AB, Sweden) and 20 mg unenriched or β - ^{13}C -enriched coniferin. After 7 days, another 20 mg coniferin in 1.5 ml buffer was added to the sample. All reactions were performed at room temperature. During the experiment, the vials were regularly ventilated (twice a day) to expose the tissue to air (oxygen), and every third day, 5 μl antibiotics (2 mg/ml) were added to the samples. After 18 days, the samples were washed with water (20 ml, 3×15 min) and stored in a refrigerator.

4.3.2. Example 2¹⁷⁾

Depending on the purpose of the experiment, a 3 days treatment without the addition of antibiotics is enough to prepare the ^{13}C -enriched cell wall (CW)-DHPs. The differentiating xylem prepared in the same way as **Example 1** was washed with water to ensure that no bark remained in the sample. In a typical experiment, 200 mg (o.d.) xylem was transferred to a glass test tube (35 ml) and 86 units of β -glucosidase (from almonds, Sigma-Aldrich Sweden AB) and 86 units of glucose oxidase (from *Aspergillus niger*, Sigma-Aldrich Sweden AB) were added. The sample was then diluted to a total volume of 27 ml with a phosphate buffer (pH 6.0, 0.1M). A total of 100 mg unenriched or

enriched coniferin was added in small portions during a period of 48 h. All experiments were performed at room temperature. After the completion of the CW-DHPs formation, the samples were collected on filter paper and washed thoroughly with water.

5. SPECIFIC ^{13}C -ENRICHMENT OF CELL WALL LIGNIN BY FEEDING MONOLIGNOL GLUCOSIDES- ^{13}C TO GROWING PLANTS

Monolignol glucosides are the essential intermediates in the biosynthesis of cell wall lignin¹⁰⁾. For the effective ^{13}C -enrichment of specific carbon in cell wall lignin by feeding monolignol glucosides- ^{13}C to plants, various factors must be taken into consideration, because incorporation of the precursors can be achieved only by feeding them to actively lignifying cell walls. Important factors are plant species, feeding season, feeding procedure, harvesting, and sample preparations for ^{13}C analysis etc. Ginkgo (*Ginkgo biloba*) may be one of the suitable plant species among various trees. Feeding during June and July is the best season in Japan for the effective incorporation of ^{13}C -monolignol glucosides into Ginkgo xylem cell walls^{18,19)}. Among gramineous plants, wheat (*Triticum aestivum*) was one of the suitable plants for the ^{13}C -enrichment of culm lignin. The precursor is selectively incorporated only into lignifying cell walls in the lower part of the culm near the node²¹⁾.

5.1. Example 1: Feeding to ginkgo shoot^{18,19)}

Ginkgo trees (5-years-old) were grown in pots (Note 5.1-1). The shoot was cut into 15 cm to 20 cm long, having 20 to 30 leaves (Note 5.1-2). The lower end of the shoot was cut again with a sharp knife in the water (Note 5.1-3), and immediately put in a small vial containing an aqueous solution of coniferin- ^{13}C , or unenriched coniferin as an unfed control. Feeding of the total precursor solution (300 mg coniferin in 300 ml water) was carried out in portions (50 ml \times 6 times) in a small vial. A little before the small vial became empty, 50 ml of the precursor solution was added. This portionwise feeding is to prevent slowing down of absorption due to growing of bacteria or the deposition of resinous material at the end of the shoot (Note 5.1-4). When the absorption of the precursor solution became too slow, the end of the shoot was cut off again in the water. After feeding the precursor solution for about one week under natural conditions (sunlight in daytime and dark at night) or in a growth chamber, the shoots were allowed to grow further for three weeks in flask containing plenty of fresh water (Note 5.1-5). Then, the bark was removed, and newly formed xylem was collected and extracted with acetone and hot water, and dried in a vacuum desiccator (Note 5.1-6).

Note 5.1-1. Younger ginkgo shoot, 2–4 years-old, grown in the field, can be used in the season when new soft xylem formation is most active, June to July in Japan. Other trees belonging to gymnosperms and angiosperms can be used in the same way.

Note 5.1-2. The number of leaves is adjusted for the balance between the absorption speed of the precursor solution and normal photosynthetic activity to form new xylem cell walls. A shoot with many large leaves will waste the labeled precursor.

Note 5.1-3. In order to prevent mechanical damages to the lignifying soft xylem under the bark, handling of the shoot must be gentle by wearing soft gloves on the gripping hands.

Note 5.1-4. In the case of pine shoots, the deposition of resinous material sometimes prevents the smooth absorption of the precursor solution. Frequent cleaning is necessary by cutting off a few millimeters of the lower end of the shoot in water.

Note 5.1-5. Monolignol glucosides are quickly incorporated into lignifying cell walls as shown by the microautoradiographic study²⁰⁾. Depending on the purpose of the research, feeding period can be shortened.

Note 5.1-6. After three weeks from the end of feeding, the lignin in the newly formed xylem close to the cambium is not ^{13}C -enriched. The mature cell walls containing ^{13}C -enriched lignin can be obtained by collecting the xylem of about 800 μm thick from the cambium¹⁹⁾ using a microtome³¹⁾ or a peeler.

5.2. Example 2: Feeding to wheat straw ²¹⁾

Dwarf wheat (*Triticum aestivum* L. cv. Marshall, MN.) was grown in a pot and, just after the head appeared, an aliquot (ca. 0.1–0.2 ml) of the aq. solution of coniferin (10 mg /ml) was injected using a fine glass capillary into a culm to fill up the inner cavity of the second internode from the top of the plant stem. After growing for one further month, the mature wheat plants were harvested and dried. The straw internodes fed with coniferin were carefully cut out from the plants, and the top and bottom directions of the internode were noted (the bottom part is ¹³C-enriched more than the top part). The straws were extracted thoroughly with ether and hot water (70 °C) successively and dried in a vacuum desiccator. Incorporation of ¹³C occurs only into the lignifying cell walls of the culm. No incorporation of ¹³C into the leaf lignin was observed.

6. INFORMATIONS OBTAINED BY THE STABLE ISOTOPE TRACER METHODS

6.1 Specific ¹³C-enrichment

6.1.1. Determine the accurate difference between enriched and unenriched specimens.

In the case of feeding monolignol glucosides-[¹³C] (¹³C/¹²C > 99%) to ginkgo shoot, the ¹³C-enrichment of lignin is not so high¹⁹⁾ due to dilution of the fed monolignol glucosides-[¹³C] with a large amount of native monolignol glucosides present at the lignin-depositing site. However, determination of the difference NMR spectrum between enriched and unenriched samples can provide qualitative and quantitative information on the structure of cell wall lignin²²⁾. For accurate determination of the difference NMR, mass spectrum, etc. between enriched and unenriched specimens, they must be prepared in the same manner under the same conditions.

6.1.2. Consideration to the heterogeneous nature of cell wall lignin is necessary.

In the tracheids of most representative gymnosperms, the deposition process and final concentration of lignin in the compound middle lamella (CML) is quite different from those in the secondary cell walls^{23, 24)}. The absolute amount of lignin in the CML is small, but a large part of the CML lignin cannot be completely solubilized in most solvents even after fine grinding²⁵⁾. This is because the macromolecular structure of the CML lignin is a three-dimensional condensed structure that is quite different from the structure of the secondary wall lignin²⁶⁾. Examination by the non-destructive methods such as high-resolution solid state NMR^{27,28)} or other non-destructive spectroscopic methods²⁹⁾ is recommended for obtaining information on the whole lignin including CML lignin. The mechanical isolation of CML fraction introduced by Whiting²⁵⁾ or by Westermarck³⁰⁾ combined with the ¹³C tracer methods will be useful for better understanding of the heterogeneous nature of lignin in the tracheid walls.

Every plant forms a variety of lignin macromolecule that is specific to the lignified tissue to fulfill the role of the tissue in the growing plant. Therefore, proper and careful consideration of the heterogeneous nature of cell wall lignin must be given not only in devising the strategy of the ¹³C-tracer experiments but also in explanation of the experimental results.

6.2. Specific labeling with ²H (deuterium)

Monolignol glucosides in which the ring H is specifically replaced with ²H can be prepared by the same methods as those for the preparation of the ³H-labeled lignin precursors^{31,32)}. Examination of the specifically ²H-labeled lignin or DHP can provide useful information on the structure and reaction of lignin.

6.3. Specific labeling with ^{13}C at glycone glucose of monolignol glucosides

The physical, chemical and biological properties of wood depend on the supramolecular assembly of cellulose microfibrils, hemicelluloses and lignin in the growing cell walls. Based on the ^{13}C -tracer studies of ginkgo xylem formation by feeding coniferin labeled with ^{13}C at glycone part glucose, a hypothetical scenario for the role of monolignol glucosides in the assembly was proposed³³.

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