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Synthesis of [^{13}C]- and [^{14}C]-labeled phenolic humus and lignin monomers

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Abstract

Natural phenolic monomers are ubiquitous in the environment and are involved in the stabilization of atmospheric carbon and the transformation of xenobiotics. Investigations on the stabilization of phenolic carbons and their environmental fate are hampered by the unavailability of commercial [^{13}C]- and [^{14}C]-labeled phenols. Here we report the complete chemical synthesis of the lignin and humus structural monomers *p*-coumaric, ferulic, and caffeic acids, *p*-hydroxybenzaldehyde, protocatechualdehyde, vanillin, catechol, and guaiacol, uniformly [^{13}C]- or [^{14}C]-labeled in the aromatic ring, starting from commercially available [U-ring-^{13}C]- or [U-ring-^{14}C]-labeled phenol. The synthesis of these compounds involved selective *ortho*-hydroxylation of the aromatic ring, *Friedel–Crafts* alkylation, and *Knoevenagel* condensation. [U-ring-^{13}C]- or [U-ring-^{14}C]-*p*-coumaric acid was synthesized via *p*-hydroxybenzaldehyde with a 75% yield with respect to phenol. Synthesis of [U-ring-^{13}C]- or [U-ring-^{14}C]-ferulic acid, consisting of six single steps via guaiacol and vanillin, had an overall yield of up to 45%. Uniformly ring-labeled caffeic acid was synthesized either via catechol and protocatechualdehyde in five single steps, yielding [U-ring-^{14}C]-caffeic acid with a 37% yield, or via guaiacol, vanillin, and ferulic acid in seven steps, yielding [U-ring-^{13}C]-caffeic acid with an 18% yield. Ferulic acid, [^{14}C]-labeled at β-C of the propenoic side chain, was synthesized from [2-^{14}C]-malonic acid under *Knoevenagel* conditions with a 67% yield with respect to malonic acid. Demethylation of the [β-^{14}C]-ferulic acid with BBr_3 in CH_3CN resulted in [β-^{14}C]-caffeic acid with a 62% yield. All [U-ring-^{13}C]-labeled phenolic products were analyzed by ^{13}C nuclear magnetic resonance (^{13}C-NMR) spectroscopy and gas chromatography–mass spectrometry (GC–MS).

Keywords: Phenolic acids; Phenolic aldehydes; Catechol; *Friedel–Crafts* alkylation; *Knoevenagel* reaction; ^{13}C-NMR

1. Introduction

Naturally occurring phenolic monomeric compounds, stemming from decomposition of the biopolymer lignin,

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microbial synthesis, and rood exudates, are ubiquitous in the environment (Siqueira et al., 1991). These lignin or humus monomers play important roles in the formation of humic substances (Haider et al., 1975; Dec et al., 2001) and thus in the stabilization of carbon in terrestrial and aquatic ecosystems. Phenols are adsorbed onto or associated with inorganic and organic soil components (Cecchi et al., 2004; Vinken et al., 2005) and are sensitive to oxidation by soil enzymes and metal oxides (Huang, 2000; Dec et al., 2001). Phenols are regarded as allelopathic chemicals (Siqueira et al., 1991; Blum, 1996) and are involved in the transformation of pollutants either as intermediates in the degradation (Schweigert et al., 2001) or by reacting with the pollutants (Adrian et al., 1989; Kim et al., 1997; Park et al., 1999; Wang et al., 2002). The transformation and mineralization of naturally occurring phenols in soil has been widely investigated by using the corresponding ^{14}C-labeled compounds (Martin and Haider, 1980; Haider and Martin, 1981; Cheng et al., 1983; Lehmann et al., 1987; Lehmann and Cheng, 1988; Cecchi et al., 2004); however, the chemical structures of the transformation products present in soil are still largely obscure.

[^{14}C]-labeled compounds allow the determination of the rate of transformation and the distribution of residues, whereas [^{13}C]-labeled compounds are necessary for elucidation of chemical structures. The lack of commercially available [^{13}C]- and [^{14}C]-labeled phenols and their derivatives has hampered investigation on the mechanisms of humus and lignin monomer stabilization in soil.

The synthesis of labeled lignin alcohols and their derivatives has been summarized previously (Haider et al., 1988). Although [^{14}C]-labeled monomeric phenols have been synthesized in various forms, e.g., labeled at the ring, at α-, β-, and γ-C of the propenoic chain of cinnamic acids, or at methoxyl C (Haider and Lim, 1965; Haider, 1966), [U-ring-^{13}C]-labeled naturally occurring phenolic compounds from [U-ring-^{13}C]-phenol have not yet been synthesized. Recently, modified pathways used for the synthesis of catechol, *p*-hydroxybenzaldehyde, protocatechualdehyde, and vanillin with higher yields have been described (Ji and Schäffer, 2002; Ji and Schäffer, 2004). Since many phenolic acids can be synthesized via these compounds (Haider et al., 1988), chemical synthesis with high yields of [^{13}C]- or [^{14}C]-labeled humus and lignin phenolic monomers from commercially available labeled phenol should now be possible.

The present study reports the complete synthesis with high yields of [U-ring-^{14}C]- or [U-ring-^{13}C]-labeled *p*-coumaric, ferulic, and caffeic acids, via *p*-hydroxybenzaldehyde, vanillin, protocatechualdehyde, guaiacol, and catechol, from commercially available [^{14}C]- and [^{13}C]-phenol. Phenolic acids [^{14}C]-labeled in the β-C of the acidic side chain were also synthesized. The [^{13}C]-labeled compounds were analyzed by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy; [^{14}C]-labeled compounds were analyzed by thin layer chromatography (TLC), followed by autoradiography.

2. Materials and methods

2.1. Chemicals

[U-ring-^{14}C]-phenol (**1b**; for the numeration of compounds, see Figs. 1–3) in petrol ether (2.70×10^9 Bq mmol^{-1}, 97% radiochemical purity) and solid [2-^{14}C]-malonic acid (**13**) (2.2×10^9 Bq mmol^{-1}) were supplied by Hartmann Analytic (Braunschweig, Germany). Solid [U-ring-^{13}C]-phenol (**1a**) (99% of ^{13}C atom, 98% chemical purity) was supplied by Euriso-Top (Saarbrücken, Germany).

Piperidine and pyridine were freshly distilled, and dichloromethane (CH_2Cl_2) and acetonitrile (CH_3CN) were dried over molecular sieves (4 Å) before use. The remaining chemicals were purchased from commercial sources.

2.2. Analyses

Reaction products were analyzed by TLC, GC coupled to a mass spectrometer (GC–MS), and NMR spectroscopy. Reaction products were separated using preparative TLC or column chromatography.

For TLC, silica gel 60 plates with a fluorescence indicator (Sil G-25 UV254, 0.25 mm; Macherey-Nagel, Düren, Germany) were used, and separated compounds were viewed under UV light (254 nm). Larger amounts of separated compounds were isolated from preparative silica gel 60 plates (2.0 mm, 20 cm × 20 cm), which were pre-treated by elution with ethyl acetate. TLC plates were autoradiographed using a bioimaging analyzer (Fujifilm BAS-1000; Tokyo, Japan).

GC–MS was conducted on a Hewlett-Packard 5890 Series II gas chromatograph (Agilent Technologies; Waldbronn, Germany) equipped with an FS-SE-54-NB-0.5 column (25 m × 0.25 mm, 0.46 μm film thickness; CS Chromatographie Service; Langerwehe, Germany) and a Hewlett-Packard 5971A mass selective detector operating in the scan mode (mass range *m/z* 50–600, 1.6 scans s^{-1}) at 70 eV. The injection volume was 1 μl (splitless injection). The injector temperature and the transfer line temperature were 250 and 280 °C, respectively. A temperature program (50 °C for 5 min, 10 °C min^{-1} to 280 °C, then 280 °C for 5 min) was used. Samples were silylated with *N*,*O*-bis-(trimethylsilyl)-trifluoracetamide prior to GC–MS analysis (Ji and Schäffer, 2002).

NMR was performed at room temperature on a DPX300 or an AC300 spectrometer (Bruker, Rheinstet-

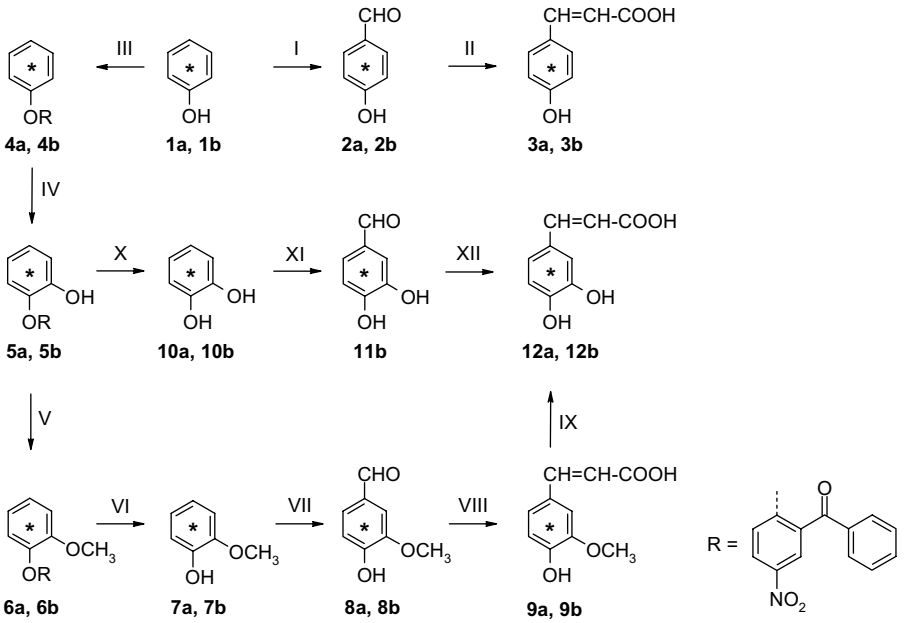


Fig. 1. Synthesis of uniformly [U-ring-^{13}C]-labeled (**a**) or [U-ring-^{14}C]-labeled (**b**) phenolic compounds. (I) $CH_3SCHCl_2 + SnCl_4$ in CH_2Cl_2, −78 °C, 5 min, HCl; (II) $CH_2(COOH)_2$ + piperidine in pyridine, 60–70 °C, 8 h, HCl; (III) 2-chloro-5-nitrobenzophenone + NaH in tetrahydrofuran, 70 °C, 8.5 h, NaOH; (IV) 35% H_2O_2 in H_2SO_4/acetic acid, 70 min; (V) CH_2N_2 in diethyl ether/methanol, 4 h, 5 °C; (VI) piperidine, 110 °C, 90 min, HCl; (VII) $CH_3SCHCl_2 + SnCl_4$ in CH_2Cl_2, −78 °C, HCl; (VIII) the same as (II); (IX) BBr_3 in CH_2Cl_2, 2 h, HCl; (X) the same as (VI); (XI) $CH_3SCHCl_2 + SnCl_4$, −20 °C, 1 min, HCl; (XII) the same as (II).

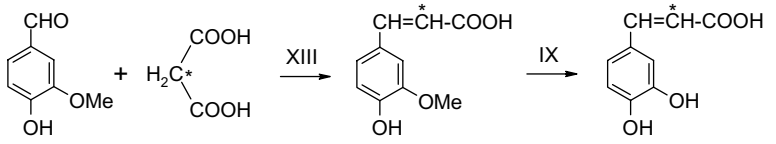


Fig. 2. Synthesis of [β-^{14}C]-labeled ferulic (**9c**) and caffeic (**12c**) acids from [2-^{14}C]-labeled malonic acid (**13**). (XIII) piperidine/pyridine, 65 °C, 8.5 h.

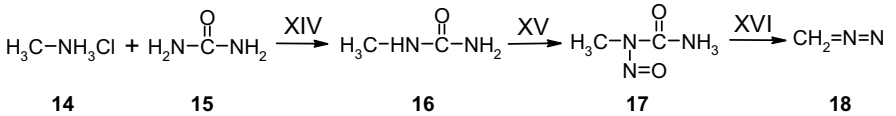


Fig. 3. Synthesis of diazomethane (**18**). (XIV) 140 °C, 3 h; (XV) $NaNO_2$, H_2SO_4, 0 °C; (XVI) 40% KOH in diethyl ether, 0 °C.

ten, Germany) equipped with a 5-mm QNP probe head and a 7-T magnetic field. Samples in 5-mm NMR tubes were dissolved in DMSO-d_6 (for catechol) or acetone-d_6 (for all other compounds) containing 1% tetramethylsilane as a standard, at a concentration of 40–140 mg ml^{-1} for non-labeled compounds and at approximately 20 mg ml^{-1} for labeled compounds under an Ar atmosphere. For ^{1}H-NMR, 16 scans (for non-labeled compounds) or 256 scans (for labeled compounds) with a 2.65-s acquisition time were recorded and analyzed with 0.3-Hz line broadening. For ^{13}C-NMR, 128 scans with a 0.72-s acquisition time and ^{1}H decoupling were recorded and analyzed with 1.0-Hz line broadening.

Column chromatography was conducted with silica gel 60 (particle diameter 0.04–0.063 mm, Merck, Germany; equilibrated with *n*-hexane or *n*-pentane) in a glass column with a diameter of 4 cm. The column was

eluted with a mixture of ethyl acetate and *n*-pentane or *n*-hexane, containing 0.5% formic acid in some cases (see the individual methods). Fractions of 20 ml were collected. Products in the fractions were identified by TLC with UV detection and/or with a bioimaging analyzer.

Radioactivity in liquid samples was determined quantitatively with a liquid scintillation counter (LS6500, Beckman Coulter; California, USA) using the scintillation cocktail Lumasafe Plus (Lumac LSC; Groningen, The Netherlands).

2.3. Syntheses

2.3.1. p-Coumaric acid (3)

*2.3.1.1. [U-ring-^{13}C]-p-hydroxybenzaldehyde (**2a**).* Compound **1a** (500 mg) was mixed with methyl dichloromethyl sulfide (CH_3SCHCl_2; 1.08 ml) in dry dichloromethane (CH_2Cl_2; 100 ml) and placed in a dry ice/acetone bath (−78 °C); 30 ml of 50% (vol/vol) $SnCl_4$ in CH_2Cl_2 at −78 °C was then added rapidly with magnetic stirring. A yellow precipitate formed immediately. After another 5 min of stirring, 20 ml of 2 M HCl was added to terminate the reaction. The mixture was allowed to warm to room temperature and then stirred for 30 min to dissolve the precipitate. The CH_2Cl_2 phase of the mixture separated from the aqueous phase, and the latter was extracted three times with ethyl acetate (40 ml each). The CH_2Cl_2 phase and the ethyl acetate extract were combined, dried over anhydrous Na_2SO_4, and rotary evaporated at 40 °C to dryness, resulting in a red-brown sticky residue. Deionized water (50 ml) was added to the residue, and the mixture was heated under reflux for 1 h after the entire residue dissolved. The mixture was extracted three times with ethyl acetate at room temperature. The ethyl acetate extract was dried over anhydrous Na_2SO_4 and rotary evaporated to dryness. The raw product was sublimated for 3 h under 4 Pa at 105 °C, cooled to 0 °C, resulting in 532 mg of a white product (**2a**).

*2.3.1.2. [U-ring-^{13}C]-p-coumaric acid (**3a**).* Compound **2a** (294 mg, 2.30 mmol) and malonic acid (256 mg, 2.46 mmol) were dissolved in pyridine (4 ml) containing piperidine (40 μl) in a 50-ml round-bottom flask connected to a drying tube filled with $CaCl_2$. The mixture was heated at 70 °C for 8 h with magnetic stirring and then cooled to room temperature; 6 M HCl (10 ml) was added drop-wise to terminate the reaction. The color of the mixture changed from yellow to orange. The mixture was extracted four times with ethyl acetate (50 ml each). After drying over anhydrous Na_2SO_4, the ethyl acetate extract was extracted five times with 0.1 M $NaHCO_3$ (20 ml each). The $NaHCO_3$ solution was acidified with 2 M HCl (6 ml) and extracted with ethyl acetate. The ethyl acetate extract was dried over anhydrous Na_2SO_4 and rotary evaporated to approximately 5 ml. Ethanol (1 ml) was added to dissolve the solids formed in the flask. The solution was loaded onto a silica gel column (300 ml), which was then sequentially eluted with mixtures of *n*-hexane (A) and ethyl acetate (B) at the following A:B (vol:vol) ratios: 100% A (200 ml), 10:1 (110 ml), 9:1 (100 ml), 8:1 (100 ml), 6:1 (100 ml), 4:1 (100 ml), 3:1 (400 ml), 2:1 (400 ml), and 1:1 (500 ml). The last three solutions also contained 0.5% formic acid. Fractions containing compounds **2a** and **3a** were identified by TLC using *n*-hexane:ethyl acetate:acetic acid (1:1:0.01, by vol.) as the eluent (R_f value of **2a** and **3a**: 0.40 and 0.29, respectively) and rotary evaporated to dryness, resulting in 25 mg of compound **2a** and 356 mg of compound **3a**.

*2.3.1.3. [U-ring-^{14}C]-p-coumaric acid (**3b**).* A mixture of compound **2b** (1.70×10^7 Bq, 4.07×10^8 Bq mmol^{-1}, synthesized according to Ji and Schäffer (2004)) and malonic acid (11.5 mg) in pyridine (1.5 ml) containing piperidine (15 μl) was heated at 60 °C for 8 h with stirring, and the reaction was terminated by addition of 2 M HCl (13 ml) (as for **3a**). The mixture was extracted three times with ethyl acetate (10 ml each). The ethyl acetate extract was dried over anhydrous Na_2SO_4 and analyzed by TLC (as for **3a**). Autoradiography of the TLC plate showed that compound **3b** had a radiochemical purity of 94%. The ethyl acetate extract was extracted twice with 0.1 M $NaHCO_3$ (5 ml each). The $NaHCO_3$ extract was acidified with 2 M HCl (4 ml) and then extracted three times with ethyl acetate (10 ml each). The ethyl acetate was dried over Na_2SO_4 and rotary evaporated to dryness, resulting in 1.61×10^7 Bq product (**3b**).

2.3.2. Ferulic acid (9)

*2.3.2.1. 2-([U-ring-^{13}C]-2-methoxyphenoxy)-5-nitrobenzophenone (**6a**).* 2-([U-ring-^{13}C]-2-hydroxyphenoxy)-5-nitrobenzophenone (**5a**, 4.668 g, containing 183 mg 2-chloro-5-nitrobenzophenone), which was synthesized from compound **1a** (1.345 g, 13.45 mmol) via 2-([U-ring-^{13}C]-phenoxy)-5-nitrobenzophenone (**4a**) according to Ji and Schäffer (2002), was dissolved in a mixture of diethyl ether (40 ml) and methanol (20 ml) in a 100-ml flask at 5 °C. Freshly prepared diazomethane (**18**)/diethyl ether solution (40 ml, 1 M, 0 °C; for the preparation, see Section 2.3.4) was added drop-wise with stirring at 5 °C. During the addition, hydrogen gas was released. After 2 h of incubation, another 40 ml of the diazomethane/diethyl ether solution was added. The reaction mixture was stirred for another 2 h, and acetic acid (3 ml) at 5 °C was added to remove the residual diazomethane. Rotary evaporation of the mixture at 40 °C to dryness resulted in a yellow solid, which was then dissolved in CH_2Cl_2 and loaded onto a silica gel column (300 ml). The column was eluted with mixtures of *n*-pentane (A) and ethyl acetate (B) at the following A:B ratios (vol:vol): 4:1 (800 ml), 3:1 (400 ml), and 2:1 (300 ml). The product

(**6a**) was identified by TLC using toluene:chloroform (1:3, vol:vol) as the eluent (R_f value of **5a** and **6a**: 0.27 and 0.71, respectively) and resulted in 4.455 g of a white solid, which was colorless in its crystalline form.

*2.3.2.2. 2-([U-ring-^{14}C]-2-methoxyphenoxy)-5-nitrobenzophenone (**6b**).* 2-([U-ring-^{14}C]-2-hydroxyphenoxy)-5-nitrobenzophenone (**5b**, 4.12×10^7 Bq, 7.28×10^7 Bq mmol^{-1}, synthesized from compound **1b** with a 91% yield as described previously (Ji and Schäffer, 2002)), was dissolved in mixture of diethyl ether (4 ml) and methanol (2 ml), and methylated with diazomethane (4 ml, 1 M in ether) at 5 °C using procedures similar to those for the synthesis of compound **6a**. Subsequent separation and purification resulted in 4.08×10^7 Bq of compound **6b** with a radiochemical purity of 98.3%.

*2.3.2.3. [U-ring-^{13}C]-guaiacol (**7a**).* Pure compound **6a** (4.055 g, 11.41 mmol) was dissolved in piperidine (6 ml) in a 50-ml flask connected to a reflux condenser. The solution was heated under an Ar atmosphere at 110 °C under reflux with stirring for 90 min; the solution turned brown. The reaction mixture was cooled to room temperature and diluted with 30 ml of CH_2Cl_2. Then 50 ml of 2 M HCl was added slowly with stirring. The mixture was extracted three times with CH_2Cl_2 (10 ml each). The extract was rotary evaporated to approximately 5 ml, diluted with ethyl acetate (15 ml), and extracted four times with 1 M O_2-free NaOH (20 ml each). The NaOH extract was immediately added to 2 M HCl to avoid oxidation of compound **7a** under alkaline conditions. The acidic mixture was extracted with CH_2Cl_2. After drying over anhydrous Na_2SO_4, the solvent CH_2Cl_2 was slowly removed by rotary evaporation under 3×10^4 Pa at 40 °C, resulting in a brown liquid. Pure compound **7a** (liquid; 1.142 g) was obtained by distilling the raw product under 200 Pa at 65 °C and then cooling to 0 °C in a sublimation tube for 150 min.

*2.3.2.4. [U-ring-^{14}C]-guaiacol (**7b**).* Compound **6b** (4.08×10^7 Bq, 7.28×10^7 Bq mmol^{-1}) was dissolved in 3 ml piperidine and heated under reflux under an Ar atmosphere for 90 min (as for **7a**). The subsequent separation and purification procedures were similar to those for compound **7a**, except that the sublimation was carried out under 10 Pa at 55 °C; after cooling to 0 °C, 1.69×10^7 Bq compound **7b** was obtained. TLC using *n*-pentane:ethyl acetate (10:1, vol:vol) as eluent (R_f value of **6b** and **7b**: 0.17 and 0.36, respectively) and autoradiography showed a radiochemical purity of **7b** of 99.9%.

*2.3.2.5. [U-ring-^{13}C]-vanillin (**8a**).* Compound **7a** (1.02 g, 7.84 mmol) in 50 ml of dry CH_2Cl_2 (at −78 °C) was added drop-wise at −78 °C with stirring within 16 min to a mixture of CH_3SCHCl_2 (3.16 ml, 31.4 mmol) and $SnCl_4$ (3.72 ml, 31.4 mmol) in 20 ml of dry CH_2Cl_2. The reaction mixture was stirred for another 5 min at −78 °C and then poured into 50 ml of 2 M HCl (0 °C) with stirring. After warming to room temperature, the mixture was stirred for 30 min and extracted four times with CH_2Cl_2 (50 ml each). The extract was dried over anhydrous Na_2SO_4 and rotary evaporated to dryness, resulting in a brown sticky residue. Compound **8a** (964 mg) and [U-ring-^{13}C]-*iso*-vanillin (204 mg) were obtained by boiling the raw product in deionized water for 4 h, as for the synthesis of compound **2a**, and purifying by column chromatography with *n*-hexane:ethyl acetate (3:1; vol:vol) as eluent.

*2.3.2.6. [U-ring-^{13}C]-ferulic acid (**9a**).* Compound **9a** was synthesized following procedures similar to those for compound **3a**. A mixture of compound **8a** (514 mg, 3.25 mmol) and malonic acid (0.355 g, 3.42 mmol) was heated in pyridine (6 ml) containing piperidine (60 μl) in a 50-ml pear-shaped flask at 65 °C for 8 h, and the reaction was terminated with 2 M HCl (6 ml) and 6 M HCl (5 ml). The subsequent extraction with $NaHCO_3$ and column chromatography resulted in 530 mg compound **9a** and 50 mg non-reacted compound **8a**.

*2.3.2.7. [U-ring-^{14}C]-ferulic acid (**9b**).* A mixture of compound **8b** (1.15×10^7 Bq, 3.96×10^8 Bq mmol^{-1}, radiochemical purity 89.8%, synthesized according to Ji and Schäffer (2004)) and malonic acid (6.6 mg) was heated in pyridine (1.5 ml) containing piperidine (15 μl) at 70 °C with stirring for 8 h. The reaction was terminated with 2 M HCl (4 ml) and 6 M HCl (2 ml) (as for **3a**). The mixture was extracted three times with ethyl acetate (20 ml each). After drying over anhydrous Na_2SO_4 and rotary evaporation to 0.5 ml, the mixture was loaded onto a preparative TLC plate. The plate was developed twice in *n*-hexane:ethyl acetate:formic acid (100:100:1, by vol.). The bands of the product ferulic acid and the non-reacted vanillin were scraped from the plate and extracted seven times with ethyl acetate (15 ml each), resulting in 8.43×10^6 Bq **9b** and 8.14×10^5 Bq **8b**. TLC using *n*-hexane:ethyl acetate:formic acid (1:1:0.01, by vol.) as solvent (R_f value of compounds **8b** and **9b**: 0.55 and 0.43, respectively) and autoradiography showed a radiochemical purity of 99.8% for both **9b** and **8b**.

*2.3.2.8. [β-^{14}C]-ferulic acid (**9c**).* Vanillin in pyridine (0.36 ml of 0.1 mmol ml^{-1}), 1 ml pyridine, and 15 μl piperidine were added to [2-^{14}C]-malonic acid (**13**) (2.05×10^7 Bq, 6.82×10^8 Bq mmol^{-1}). The mixture was heated at 65 °C for 8.5 h. The subsequent acidification with HCl, extraction with ethyl acetate, and purification by preparative TLC were the same as for compound

9b. Compound **9c** (1.38×10^7 Bq) with a radiochemical purity of 98.7% was obtained.

*2.3.3. Caffeic acid (**12**)*

*2.3.3.1. [U-ring-^{13}C]-caffeic acid (**12a**).* BBr_3 (1 M in CH_2Cl_2, 1.66 ml) was added drop-wise at room temperature with stirring to compound **9a** (316 mg, 1.58 mmol) in dry CH_3CN (40 ml) in a 100-ml flask. A yellow precipitate formed. The mixture was stirred at room temperature for 2 h, and 50 μl of deionized water was added to terminate the reaction. The precipitate disappeared immediately, and the mixture became clear and yellow/orange. The mixture was rotary evaporated to dryness, and the solids were dissolved in ethanol and loaded onto a silica gel column (300 ml). The column was sequentially eluted with mixtures of *n*-hexane (A) and ethyl acetate (B) at the following A:B ratios (vol:vol) containing 0.5% formic acid: 100% A (200 ml), 4:1 (100 ml), 3:1 (400 ml), 2:1 (450 ml), and 1:1 (1000 ml). Fractions containing the product compound **12a** and non-reacted compound **9a** were identified by TLC using *n*-hexane:ethyl acetate:acetic acid (1:1:0.01, by vol.) as an eluent (R_f value of **9a** and **12a**: 0.28 and 0.17, respectively) and UV detection. Compound **12a** (120.5 mg) and non-reacted compound **9a** (141.4 mg) were obtained.

*2.3.3.2. [U-ring-^{14}C]-caffeic acid (**12b**).* A mixture of compound **11b** (3.67×10^6 Bq, 7.33×10^7 Bq mmol^{-1}, synthesized according to Ji and Schäffer (2004)), and malonic acid (10.0 mg) was heated in pyridine (1 ml) containing piperidine (10 μl) at 60 °C for 8 h; the color of the mixture changed from orange to brown-black. The subsequent separation and purification procedures were similar to those for compound **9b**, except that the TLC plate was developed with *n*-pentane:ethyl acetate:formic acid at two different ratios, first at 100:100:4 (by vol.) and then with 150:50:2 (by vol.). Compound **12b** (2.05×10^6 Bq) was obtained with a radiochemical purity of 98%, as shown by TLC using *n*-hexane:ethyl acetate:formic acid (1:1:0.01, by vol.) as eluent (R_f value of **11b** and **12b**: 0.43 and 0.33, respectively) and autoradiography.

*2.3.3.3. [β-^{14}C]-caffeic acid (**12c**).* Compound **9c** (6.51×10^6 Bq, 6.6×10^8 Bq mmol^{-1}) was diluted with compound **9** (12.6 mg) to a final ^{14}C-specific radioactivity of 7.33×10^7 Bq mmol^{-1} and dissolved in 3 ml dry CH_3CN. BBr_3 (0.5 ml of 1 M) in CH_2Cl_2 was added drop-wise to the solution with stirring at room temperature. The reaction was terminated after 2 h by adding 2 M HCl (30 μl). The mixture was rotary evaporated to approximately 0.5 ml and loaded onto a preparative TLC plate, which was developed three times in *n*-hexane:ethyl acetate:formic acid (100:100:2, by vol.). Bands of compounds **9c** and **12c** were scraped from the plate and extracted seven times with ethyl acetate (15 ml each), resulting in 4.01×10^6 Bq **12c** and 2.05×10^6 Bq non-reacted **9c** at a radiochemical purity of 99% and 99.5%, respectively.

*2.3.4. Diazomethane (**18**)*

*2.3.4.1. N-nitroso-N-methylurea (**17**).* The synthesis of compound **17** was according to Arndt (1943) with modifications. An aqueous solution (40 ml) of methylamine hydrochloride (**14**, 33.7 g) and urea (**15**, 30 g) was heated under reflux in a 140 °C oil bath for 3 h, forming methyl urea (**16**). The mixture was cooled to room temperature. $NaNO_2$ (41.4 g) was added, and the mixture turned yellow. The mixture was then slowly added drop-wise to 1.6 M H_2SO_4 (200 ml) in an ice-NaCl bath with continuous stirring. A slightly yellow foamy product (**17**) was formed. The reaction temperature was kept <0 °C. The product was separated from the solvent by filtration through filter paper and washed four times with deionized water at 0 °C (20 ml each) and twice with deionized water at room temperature (5 ml each) to remove the salts. The pellet (**17**) was dried over P_2O_5 under vacuum at 4 °C, resulting in 17.2 g compound **17**. Compound **17** was stored at −15 °C in a flask containing filter paper impregnated with 2 ml glacial acetic acid.

*2.3.4.2. Diazomethane (**18**) solution (1 M).* Compound **17** (0.96 g) was slowly added in several small portions to a mixture of KOH (40%, 4 ml, aqueous) and diethyl ether (10 ml) in a 50-ml Erlenmeyer flask with continuous stirring at 0 °C. The mixture was stirred for another 10 min, and the yellow organic phase containing compound **18** was separated and dried over Na_2SO_4. The solution of compound **18** was stored in an ice bath until used (Becker et al., 1999).

Warning: Compound **17** (*N*-nitroso-*N*-methylurea) is a carcinogen that causes inheritable injuries; contact with skin should be avoided. Compound **18** (diazomethane) is carcinogenic, and explosive as a gas.

3. Results and discussion

The synthesis of ring-labeled compounds starting from [U-ring-^{13}C]- or [U-ring-^{14}C]-labeled phenol (**1a** or **1b**) and of β-C-labeled compounds starting from [2-^{14}C]-malonic acid (**13**) is outlined in Figs. 1 and 2, respectively. The yields and chemical or radiochemical purities of the products are summarized in Table 1.

3.1. Synthesis of compounds *3*, *9*, and *12*

Compounds **3** and **9** were synthesized in a *Knoevenagel* reaction by heating malonic acid with the corresponding labeled aldehydes (*p*-hydroxybenzaldehyde (**2**) and vanillin (**8**)) in freshly distilled dry pyridine,

Table 1
Yields in the synthesis of [^{14}C]- and [^{13}C]-labeled phenolic compounds. Pathways and compounds are shown in Figs. 1 and 2

Compound [labeling]	Synthetic step	Yield (%) with respect to		Purity[a] (%)
		Converted reactant	Applied reactant	
2a [ring-^{13}C]	**I**	–[b]	82.8	99.3
2b [ring-^{14}C]	**I**	96[c]	88[c]	97[c]
3a [ring-^{13}C]	**II**	99.7	91.0	99.0
3b [ring-^{14}C]	**II**	–	94.7	97.2
6a [ring-^{13}C]	**III + IV + V**	–	93.2	99.0
6b [ring-^{14}C]	**V**	–	99.0	98.3
7a [ring-^{13}C]	**VI**	–	76.7	99.9
7b [ring-^{14}C]	**VI**	–	41.4	99.9
8a [ring-^{13}C]	**VII**	–	77.8	99.5
8b [ring-^{14}C]	**VII**	81[c]	75[c]	99[c]
9a [ring-^{13}C]	**VIII**	90.2	81.4	98.0
9b [ring-^{14}C]	**VIII**	88.8	81.8	99.8
9c [β-^{14}C]	**XIII**	–	67.3	98.7
10a [ring-^{13}C]	**X**	–	82.3[d]	99.7[d]
10b [ring-^{14}C]	**X**	–	88.2[d]	99.7[d]
11b [ring-^{14}C]	**XI**	88[c]	83[e]	99[c]
12a [ring-^{13}C]	**IX**	74.0	41.0	97.0
12b [ring-^{14}C]	**XII**	–	55.9	98.0
12c [β-^{14}C]	**IX**	89.8	61.6	99.0

[a] Radiochemical purity of ^{14}C-labeled compounds was determined by autoradiography; chemical purity of ^{13}C-labeled compounds was determined by ^{1}H-NMR.
[b] Not determined.
[c] From Ji and Schäffer (2004).
[d] From Ji and Schäffer (2002).

catalyzed by piperidine (1% pyridine, vol/vol) at 60–70 °C for approximately 8 h, followed by hydrolysis with HCl (2–6 M). Malonic acid was added in slight excess when the ring was labeled (reactions II and VIII), and compounds **3** and **9** were purified with yields (with respect to the aldehydes) of >91% and >81%, respectively (Table 1). However, the reactions were not complete after 8 h, and approximately 9% of the reactant aldehyde remained in the mixture. Therefore, extraction with $NaHCO_3$ only did not result in pure products. For example, [U-ring-^{14}C]-*p*-coumaric acid (**3b**) was only 97% pure (Table 1). Purification by preparative TLC or column chromatography was necessary. In the synthesis of [β-^{14}C]-ferulic acid (**9c**), vanillin was added in excess (Fig. 3), and the yield was only 67%, which was much lower than the yield of [U-ring-^{14}C]-ferulic acid (**9b**) (82%) synthesized under similar reaction conditions.

Compound **12** could be synthesized via two pathways: via compound **11** in a *Knoevenagel* reaction (reaction XII) or via compound **9** by demethylation with BBr_3 in dry CH_3CN at room temperature for 2 h (reaction IX). The yield via the former pathway was higher than the yield via the latter pathway (Table 1), but the complete synthesis of compound **12** from compound **1** via compound **9** also provided three phenolic compounds (**7**, **8**, **9**) of interest. Therefore, the choice of the pathway for the synthesis of compound **12** depends on the availability of the precursors and whether phenols in addition to compound **12** are needed.

In this study, [U-ring-^{13}C]-protocatechualdehyde (**11a**) was not synthesized; therefore, [U-ring-^{13}C]-caffeic acid (**12a**) was synthesized via [U-ring-^{13}C]-ferulic acid (**9a**), and [U-ring-^{14}C]-caffeic acid (**12b**) was synthesized via [U-ring-^{14}C]-protocatechualdehyde (**11b**). [β-^{14}C]-caffeic acid (**12c**) was also synthesized in reaction IX via compound **9c**. Demethylation of compound **9** to form compound **12** was not complete within 2 h; non-reacted compound **9** was recovered (45% and 32% in the synthesis of compounds **12a** and **12c**, respectively). Demethylation for longer than 2 h did not increase the yield of compound **12**, although the amount of compound **9** recovered decreased (data not shown). The yield of compound **12b** was much lower than that of compounds **3a**, **3b**, **9a**, and **9b**, which were synthesized under the same reaction conditions (Table 1), and no reactant was recovered, as in the synthesis of compound **9**. Tazaki et al. (2001) have reported a yield of compound **12** of up to 93% with respect to compound **11** when the synthesis reaction was incubated at room temperature for 1 week. The lower yield (56%) obtained in our study was probably caused by a relatively small amount of the reactant **11b** (7 mg) and the loss of a

portion of the product **12b**, which is sensitive to oxidation by O_2, during the separation and purification procedures, particularly in preparative TLC.

Compounds **3**, **9**, and **12** have been usually synthesized from aromatic aldehydes and malonic acid in a *Knoevenagel* reaction, traditionally in pyridine catalyzed by piperidine or aniline, and recently with some modifications, e.g., irradiated with microwave or ultrasound, using solid catalysts, or in solvent-free systems (Mitra et al., 1999; Kumar et al., 2000; Li et al., 2001; Karchgaudhuri et al., 2002; Perez et al., 2003). Caffeic acid has also been obtained by enzymatic (peroxidase) oxidation of ferulic acid (Bossoli et al., 1988) or by fungal transformation from *p*-coumaric acid (Alvarado et al., 2003). However, our results showed that the traditional *Knoevenagel* reaction is still the easiest method for synthesizing labeled compounds in small amounts with high yields.

3.2. Synthesis of compounds *2*, *8*, and *11*

[U-ring-^{14}C]-labeled aldehydes (**2b**, **11b**, and **8b**) were synthesized in a *Friedel–Crafts* alkylation reaction, described originally by Gross and Matthey (1964) and later modified by Ji and Schäffer (2004), by rapid addition of $SnCl_4$ to a mixture of the respective precursor (phenol (**1b**), catechol (**10b**), and guaiacol (**7b**)) and CH_3SCHCl_2 in CH_2Cl_2 or CH_3CN at −78 or −20 °C, followed by hydrolysis with HCl (2–6 M). Yields of 88%, 75%, and 83%, respectively, have been obtained (Ji and Schäffer, 2004). Under similar reaction conditions, [U-ring-^{13}C]-*p*-hydroxybenzaldehyde (**2a**) was synthesized using a **1a**:CH_3SCHCl_2:$SnCl_4$ molar ratio of 1:2:2.

[U-ring-^{13}C]-vanillin (**8a**) was synthesized by dropwise addition of [U-ring-^{13}C]-guaiacol (**7a**) to a mixture of CH_3SCHCl_2 and $SnCl_4$ at a **7a**:CH_3SCHCl_2:$SnCl_4$ molar ratio of 1:4:4. By adding compound **7a** slowly, CH_3SCHCl_2 and $SnCl_4$ were always in excess of compound **7a**. The yields of compounds **2a** and **8a** were similar to those of compounds **2b** and **8b** (Table 1). Since compound **10a** was only available in small amounts, [U-ring-^{13}C]-protocatechualdehyde was not synthesized. The byproduct *iso*-vanillin (approximately 17%) in the synthesis of compound **8** can be recycled for the synthesis of compound **11** by demethylation with BBr_3 in CH_2Cl_2 under reflux for 2 h (Haider, 1966). Demethylation of [U-ring-^{14}C]-*iso*-vanillin and compound **8b** resulted in compound **11b** with a yield of 24% and 64%, respectively (experimental procedures not shown).

3.3. Synthesis of compounds *7* and *10*

Compound **7** was synthesized from compound **6** (reaction VI), similar to the synthesis of compound **10** from compound **5** (reaction X) (Ji and Schäffer, 2002), by cleavage of compound **6** in piperidine under reflux for 90 min, followed by hydrolysis with HCl (2–6 M). Kratzl and Vierhapper (1971) have used the same reaction for the synthesis of [1-^{14}C]-guaiacol, but the purification procedures used in our study are simpler. Rotary evaporation under vacuum in the separation and purification procedures had to be carried out very carefully to avoid producing a vacuum higher than 3×10^4 Pa. The lower yield of compound **7b** compared to compound **7a** was caused by too high of a vacuum (10 Pa) in the sublimation of compound **7b**, which led to a portion of compound **7b** being drawn out of the sublimation tube. Compound **7** evaporated and condensed on the wall of the drip-catcher connecting the sample flask and the rotary system when the vacuum in the system was $<3 \times 10^4$ Pa (at 40 °C). CH_2Cl_2 was used instead of ethyl acetate to extract compound **7** from the alkaline aqueous extract since CH_2Cl_2 could be completely removed from compound **7** under a vacuum higher than 3×10^4 Pa. Compound **7** is O_2 sensitive in an aqueous alkaline solution. To avoid autooxidation of compound **7**, O_2 was removed from the NaOH solution by boiling. The NaOH phase in the mixture had to lie under the organic phase during the extraction procedure, and the NaOH extract was acidified immediately after separation (for details, see Section 2).

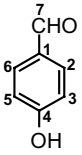
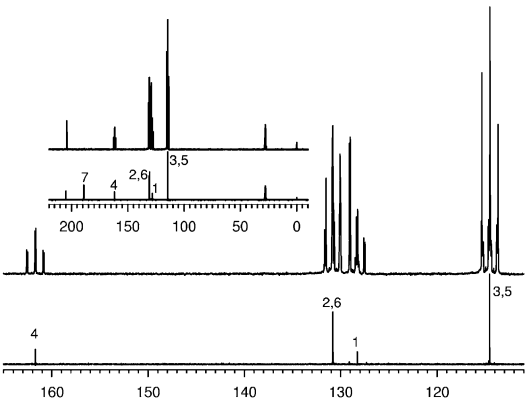
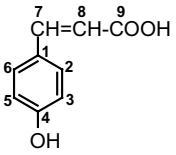
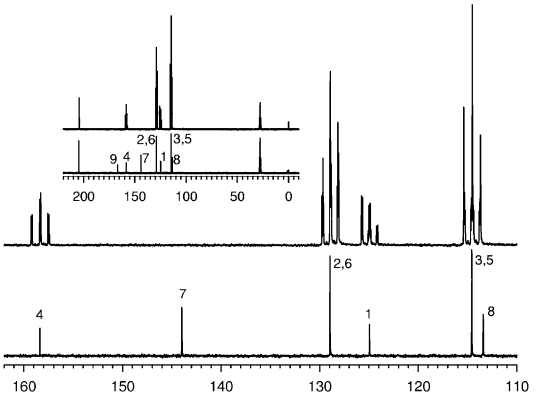
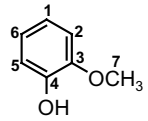
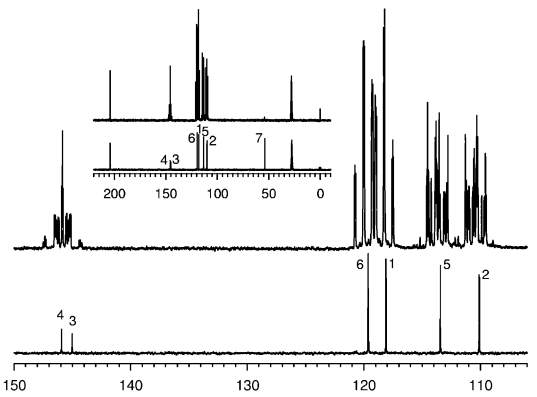
Compound **6** was synthesized from compound **5** according to Kratzl and Vierhapper (1971), except that compound **5** was methylated with diazomethane (**18**, Fig. 3) in diethyl ether at 5 °C for 4 h. Autoradiography showed that 95% of compound **5** was already methylated, forming compound **6**, within 2 h. Methylation of compound **5** by CH_3I and NaH in tetrahydrofuran at room temperature overnight was also successful, and a yield of 76% was obtained (experimental procedures not shown).

3.4. NMR and MS of [^{13}C]-labeled compounds

The NMR spectra of the [^{13}C]-labeled compounds and the mass spectra of their silylated derivatives are summarized in Table 2. Owing to C–C coupling, the ^{13}C-signals of the aromatic carbons occur as a triplet or a multiplet with spans of 1.4–2.0 ppm. Inside the triplet, each peak occurred again in a duplet with spans of approximately 0.1 ppm. Even though splitting of the signals into triplets or multiplets decreases the signal intensity in the NMR spectra, the triplet signals can be used to identify residues of the phenols bound to or sequestrated by humic substances, as shown with the fungicide cyprodinil (Dec et al., 1997a,b).

[^{13}C]-labeling makes the signals of the aromatic ring carbons distinct in NMR spectra, and thus comparison of the NMR spectra of labeled compounds and their corresponding non-labeled compounds allows the correct assignment of chemical shifts to the carbon atoms. For instance, the chemical shifts of the C-5 and C-8

Table 2
^{13}C-NMR spectra and MS data of [U-ring-^{13}C]-labeled phenolic compounds

Compound	Comparison of ^{13}C-NMR spectra of [U-ring-^{13}C]-labeled compounds (upper lines) and the corresponding compounds with natural ^{13}C abundance (lower lines)[a]	Chemical shifts (ppm)[b]	MS fragments of [U-ring-^{13}C]-labeled silylated compounds (m/z)[c]
2a 		C-1: 128.28 C-2, C-6: 130.83 C-3, C-5: 114.55 C-4: 161.70 C-7: 189.20	200 (M^+, 68.8), 185 (100), 167 (9.0), 157 (70.8), 141 (12.1), 127 (3.0), 111 (6.6), 97 (22.2), 92 (21.5), 82 (12.6), 73 (34.8), 59 (6.5), 55 (17.0)
3a 		C-1: 124.95 C-2, C-6: 128.95 C-3, C-5: 114.58 C-4: 158.38 C-7: 143.99 C-8: 113.43 C-9: 166.90	314 (M^+, 25.9), 299 (33.7), 255 (21.2), 225 (45.2), 209 (4.3), 197 (4.0), 185 (11.3), 167 (2.3), 147 (4.5), 142 (8.9), 121 (7.5), 108 (4.0), 94 (3.3), 73 (100), 59 (4.2)
7a 		C-1: 118.39 C-2: 110.39 C-3: 145.30 C-4: 146.22 C-5: 113.73 C-6: 119.91 C-7: 54.09	202 (M^+, 19.7), 187 (25.1), 172 (100), 157 (23.6), 142 (15.5), 127 (1.5), 113 (1.5), 97 (3.1), 89 (2.5), 83 (2.9), 73 (12.5), 59 (7.54)

(*continued on next page*)

Table 2 (*continued*)

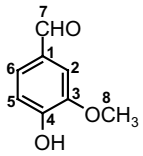
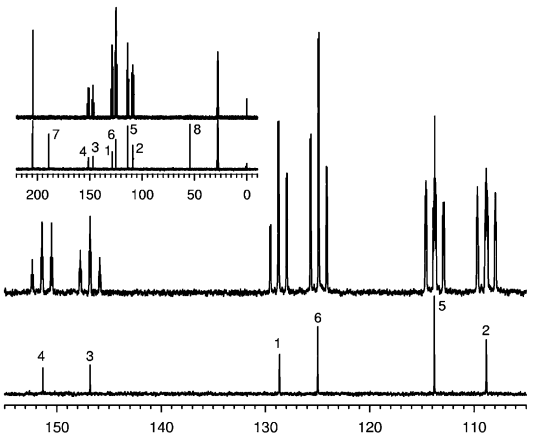
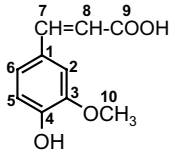
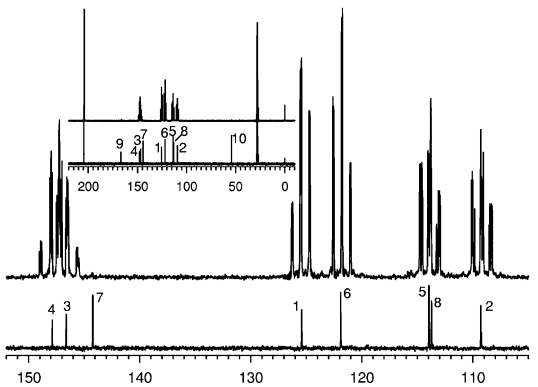
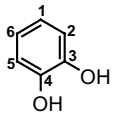
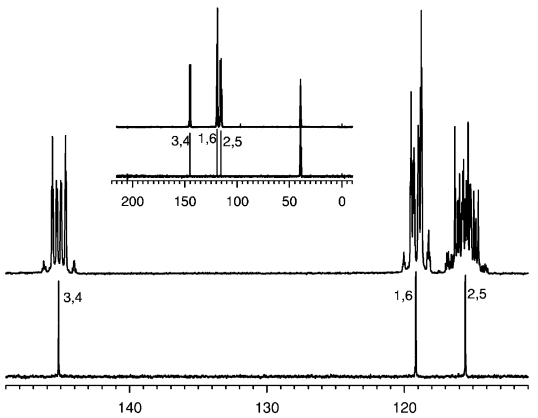
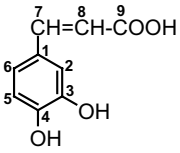
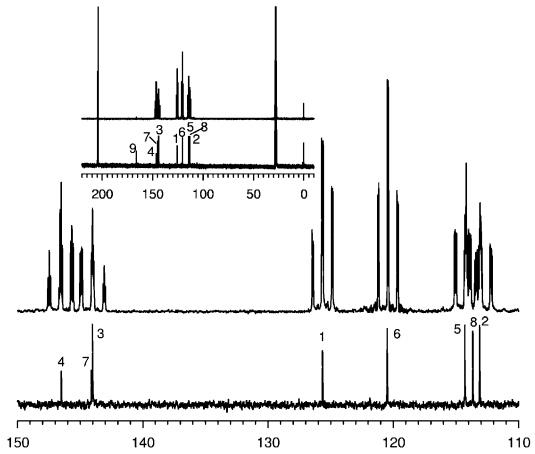
Compound	Comparison of ^{13}C-NMR spectra of [U-ring-^{13}C]-labeled compounds (upper lines) and the corresponding compounds with natural ^{13}C abundance (lower lines)[a]	Chemical shifts (ppm)[b]	MS fragments of [U-ring-^{13}C]-labeled silylated compounds (m/z)[c]
8a 		C-1: 128.67 C-2: 108.84 C-3: 146.83 C-4: 151.35 C-5: 113.82 C-6: 125.01 C-7: 189.13 C-8: 54.25	230 (M^+, 27.1), 215 (41.0), 200 (100), 185 (5.5), 169 (8.0), 157 (3.8), 142 (8.6), 127 (1.5), 107 (6.5), 97 (4.5), 85 (4.5), 73 (33.7), 59 (16.5)
9a 		C-1: 125.42 C-2: 109.29 C-3: 146.42 C-4: 147.87 C-5: 113.96 C-6: 121.91 C-7: 144.22 C-8: 113.73 C-9: 166.72 C-10: 54.29	344 (M^+, 32.2), 329 (18.7), 314 (19.1), 299 (11.9), 285 (3.4), 255 (20.1), 239 (2.1), 225 (12.1), 209 (4.6), 197 (7.5), 181 (7.3), 166 (5.5), 149 (11.1), 142 (5.8), 121 (4.9), 94 (6.6), 73 (100), 59 (14.7)
10a 		C-1, C-6: 119.17 C-2, C-5: 115.57 C-3, C-4: 145.16	260 (M^+, 7.23), 245 (1.97), 172 (1.52), 157 (2.63), 142 (2.25), 180 (1.05), 73 (100), 59 (1.34)

Table 2 (*continued*)

Compound	Comparison of ^{13}C-NMR spectra of [U-ring-^{13}C]-labeled compounds (upper lines) and the corresponding compounds with natural ^{13}C abundance (lower lines)[a]	Chemical shifts (ppm)[b]	MS fragments of [U-ring-^{13}C]-labeled silylated compounds (*m*/*z*)[c]
12a 		C-1: 125.67 C-2: 113.13 C-3: 144.03 C-4: 146.52 C-5: 114.31 C-6: 120.51 C-7: 144.13 C-8: 113.67 C-9: 166.26	402 (M^+, 16.8), 387 (4.1), 313 (3.0), 299 (2.5), 273 (0.8), 255 (3.0), 239 (1.6), 225 (48.2), 197 (10.1), 181 (2.0), 166 (1.8), 147 (3.5), 133 (2.5), 94 (2.0), 73 (100), 59 (2.9)

[a] Full spectra (inset) and expanded spectra covering the aromatic carbons are shown. Positions of the numbered C atoms are given in the corresponding structures in the first column. The horizontal axes represent the chemical shift in ppm relative to the internal standard tetramethylsilane. NMR spectra were obtained on a 300-MHz spectrometer, using ^{1}H-decoupling and 1-Hz line broadening (for details, see Materials and methods). Signals at 204.2 and 27.8 ppm and at 39.4 ppm were attributed to the solvents acetone-d_6 and DMSO-d_6, respectively. The signals of non-labeled C atoms of the labeled compounds are too low to be seen in the figures.

[b] Data listed are peaks determined with natural ^{13}C abundance. The corresponding peaks of aromatic carbons in the [U-ring-^{13}C]-labeled compounds are split into triplets or multiplets, with spans of approximately 1.4–2.0 ppm.

[c] Numbers in parentheses are relative abundance.

atoms of ferulic acid (**9**) are very close; comparison of the spectra of labeled and non-labeled ferulic acid (Table 2) allows the assignment of the 113.73 ppm signal to C-8. The ^{13}C-NMR spectrum of [^{13}C]-labeled ferulic acid (Table 2) in this study indicated that the chemical shifts of C-6 and C-8 of *cis*-ferulic acid in an NMR database (Ralph et al., 2004) are probably inversely assigned.

Owing to strong H-^{13}C coupling, the ^{1}H-NMR signals of the H-atoms at the [U-^{13}C]-labeled aromatic rings were also split into two signals and overlapped (spectra not shown). However, ^{1}H-spectra analysis can still be used to quantitatively determine the product purity.

The MS data (Table 2) of the silylated labeled compounds were consistent with MS data of the corresponding non-labeled compounds (data not shown) with respect to the fragment patterns. The GC–MS and NMR data confirmed the chemical structures of the synthesized compounds.

4. Conclusion

The present study provides improved procedures for the synthesis in high yields of phenolic humus and lignin monomers [U-^{13}C]- or [U-^{14}C]-labeled in the aromatic ring or [^{14}C]-labeled at the β-C of the propenoic side chain, starting from commercially available [U-ring-^{13}C]- and [U-ring-^{14}C]-labeled phenols or [2-^{14}C]-malonic acid. The availability of these ^{13}C-labeled phenols will facilitate studies on their fate in the environment and on the structural elucidation of their transformation products in the environment.

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