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-$^{15}$C]-caffeic acid with an 18% yield. Ferulic acid, $[1^{4}$C]-labeled at $\beta$-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 $[\beta$-$^{14}$C]-ferulic acid with $\text{BBr}_3$ in $\text{CH}_3\text{CN}$ resulted in $[\beta$-$^{14}$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).

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1. Introduction

Naturally occurring phenolic monomeric compounds, stemming from decomposition of the biopolymer lignin,
microbial synthesis, and root 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 13C-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.

[14C]-labeled compounds allow the determination of the rate of transformation and the distribution of residues, whereas [13C]-labeled compounds are necessary for elucidation of chemical structures. The lack of commercially available [13C]- and [14C]-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 [14C]-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 cyclic acids, or at methoxyl C (Haider and Lim, 1965; Haider, 1966), [U-ring-13C]-labeled naturally occurring phenolic compounds from [U-ring-13C]-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 Scha¨ffer, 2002; Ji and Schn′affer, 2004). Since many phenolic acids can be synthesized via these compounds (Haider et al., 1988), chemical synthesis with high yields of [13C]- or [14C]-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-14C]- or [U-ring-13C]-labeled p-coumaric, ferulic, and caffeic acids, via p-hydroxybenzaldehyde, vanillin, protocatechualdehyde, guaiacol, and catechol, from commercially available [14C]- and [13C]-phenol. Phenolic acids [14C]-labeled in the β-C of the acidic side chain were also synthesized. The [13C]-labeled compounds were analyzed by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy; [14C]-labeled compounds were analyzed by thin layer chromatography (TLC), followed by autoradiography.

2. Materials and methods

2.1. Chemicals

[U-ring-14C]-phenol (1b; for the numeration of compounds, see Figs. 1–3) in petrol ether (2.70 × 109 Bq mmol−1, 97% radiochemical purity) and solid [2,14C]-malonic acid (13) (2.2 × 109 Bq mmol−1) were supplied by Hartmann Analytic (Braunschweig, Germany). Solid [U-ring-13C]-phenol (1a) (99% of 13C atom, 98% chemical purity) was supplied by Euriso-Top (Saarbrücken, Germany).

Piperidine and pyridine were freshly distilled, and dichloromethane (CH2Cl2) and acetonitrile (CH3CN) 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-54-0.5μm column (25 m × 0.25 mm, 0.46 μm ﬁlm thickness; CS Chromatographic Service; Langerwehe, Germany) and a Hewlett-Packard 5971A mass selective detector operating in the scan mode (mass range m/z 50–600, 1.5 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)-triﬂuoroacetamide prior to GC–MS analysis (Ji and Schäffer, 2002).

NMR was performed at room temperature on a DPX300 or an AC300 spectrometer (Bruker, Rheinstet-
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-13C]-p-hydroxybenzaldehyde (2a). Compound 1a (500 mg) was mixed with methyl dichloromethyl sulfide (CH₂SCH₂Cl; 1.08 ml) in dry dichloromethane (CH₂Cl₂; 100 ml) and placed in a dry ice/acetone bath (−78 °C); 30 ml of 50% (vol/vol) SnCl₄ in CH₂Cl₂ 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 CH₂Cl₂ phase of the mixture separated from the aqueous phase, and the latter was extracted three times with ethyl acetate (40 ml each). The CH₂Cl₂ phase and the ethyl acetate extract were combined, dried over anhydrous Na₂SO₄, and rotary evaporated 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 Na₂SO₄ 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-13C]-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 CaCl₂. 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 Na₂SO₄, the ethyl acetate extract was extracted five times with 0.1 M NaHCO₃ (20 ml each). The NaHCO₃ solution was acidified with 2 M HCl (6 ml) and extracted with ethyl acetate. The ethyl acetate extract was dried over anhydrous Na₂SO₄ 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 (Rf 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-14C]-p-coumaric acid (3b). A mixture of compound 2b (1.70 × 10⁷ Bq, 4.07 × 10⁶ 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 Na₂SO₄ 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 NaHCO₃ (5 ml each). The NaHCO₃ 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 Na₂SO₄ and rotary evaporated to dryness, resulting in 1.61 × 10⁷ Bq mmol⁻¹ product (3b).

2.3.2. Ferulic acid (9)

2.3.2.1. 2-((U-ring-13C)-2-methoxyphenoxy)-5-nitrobenzophenone (6a). 2-((U-ring-13C)-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-13C)-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 CH₂Cl₂ 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 (Rf 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-14C)-2-methoxyphenoxy]-5-nitrobenzophenone (6b). 2-[(U-ring-14C)-2-hydroxyphenoxy]-5-nitrobenzophenone (5b) \( \cdot \) \( 10^{-7} \) Bq, 7.28 \( \times \) \( 10^{-7} \) 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 \( \times \) \( 10^{-7} \) Bq of compound 6b with a radiochemical purity of 98.3%.

2.3.2.3. [U-ring-13C]-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\(_2\)Cl\(_2\). Then the brown liquid was filtered and extracted four times with CH\(_2\)Cl\(_2\) (50 ml each). The extract was rotary evaporated to approximately 5 ml, diluted with ethyl acetate (15 ml), and extracted four times with 1 M \( \text{O}_2\)-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\(_2\)Cl\(_2\). The solvent CH\(_2\)Cl\(_2\) was slowly removed by rotary evaporation under 3 \( \times \) \( 10^{-6} \) 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-14C]-guaiacol (7b). Compound 6b (4.08 \( \times \) \( 10^{-7} \) Bq, 7.28 \( \times \) \( 10^{-7} \) 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 \( \times \) \( 10^{-7} \) Bq compound 7b was obtained. TLC using n-pentane:ethyl acetate (10:1, vol:vol) as eluent (Rf 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-13C]-vanillin (8a). Compound 7a (1.02 g, 7.84 mmol) in 50 ml of dry CH\(_2\)Cl\(_2\) (at \(-78^\circ\)C) was added drop-wise at \(-78^\circ\)C with stirring within 16 min to a mixture of CH\(_2\)SCH\(_2\)Cl \((3.16 \text{ ml}, 31.4 \text{ mmol})\) and \( \text{SnCl}_4 \) \((3.72 \text{ ml}, 31.4 \text{ mmol})\) in 20 ml of dry CH\(_2\)Cl\(_2\). The reaction mixture was stirred for another 5 min at \(-78^\circ\)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\(_2\)Cl\(_2\) (50 ml each). The extract was dried over anhydrous Na\(_2\)SO\(_4\) and rotary evaporated to dryness, resulting in a brown sticky residue.

Compound 8a (964 mg) and [U-ring-13C]-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-13C]-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 \( \mu \)l) 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 extract 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-14C]-ferulic acid (9b). A mixture of compound 8b (1.15 \( \times \) \( 10^{-7} \) Bq, 3.96 \( \times \) \( 10^{-8} \) 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 \( \mu \)l) 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\(_2\)SO\(_4\) 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 \( \times \) \( 10^{-6} \) Bq 9b and 8.14 \( \times \) \( 10^{-6} \) Bq 8b. TLC using n-hexane:ethyl acetate:formic acid (1:1:0.01, by vol.) as solvent (Rf 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. [\( ^{3} \text{H} \)]-ferulic acid (9c). Vanillin in pyridine (0.36 ml of 0.1 mmol ml\(^{-1}\)), 1 ml pyridine, and 15 ml piperidine were added to [2-\(^{14}\text{C}\)]-malonic acid (13) \((2.05 \times \) \( 10^{-7} \) Bq, 6.82 \( \times \) \( 10^{-8} \) 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-13C]-caffeic acid (12a). BBr₃ (1 M in CH₂Cl₂, 1.66 ml) was added drop-wise at room temperature with stirring to compound 9a (316 mg, 1.58 mmol) in dry CH₂CN (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 (Rf 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-14C]-caffeic acid (12b). A mixture of compound 11b (3.67 × 10^6 Bq, 7.33 × 10^4 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 (Rf value of 11b and 12b: 0.43 and 0.33, respectively) and autoradiography.

2.3.3.3. [β-14C]-caffeic acid (12c). Compound 9c (6.51 × 10^6 Bq, 6.6 × 10^4 Bq mmol⁻¹) was diluted with compound 9 (12.6 mg) to a final 14C-specific radioactivity of 7.33 × 10⁴ Bq mmol⁻¹ and dissolved in 3 ml dry CH₂CN. BBr₃ (0.5 ml of 1 M) in CH₂Cl₂ 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 × 10⁶ Bq 12c and 2.05 × 10⁶ 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. NaNO₂ (41.4 g) was added, and the mixture turned yellow. The mixture was then slowly added drop-wise to 1.6 M H₂SO₄ (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 P₂O₅ 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. 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 P₂O₅ 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.

3. Results and discussion

The synthesis of ring-labeled compounds starting from [U-ring-13C]- or [U-ring-14C]-labeled phenol (1a or 1b) and of β-C-labeled compounds starting from [2,14C]-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,
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 NaHCO₃ only did not result in pure products. For example, [U-ring-14C]-p-coumaric acid (3b) was only 97% pure (Table 1). Purification by preparative TLC or column chromatography was necessary. In the synthesis of [β-14C]-ferulic acid (9b), vanillin was added in excess (Fig. 3), and the yield was only 67%, which was much lower than the yield of [U-ring-14C]-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 BBr₃ in dry CH₃CN 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-13C]-protocatechualdehyde (11a) was not synthesized; therefore, [U-ring-13C]-caffeic acid (12a) was synthesized via [U-ring-13C]-ferulic acid (9a), and [U-ring-14C]-caffeic acid (12b) was synthesized via [U-ring-14C]-protocatechualdehyde (11b). [β-14C]-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

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

a Radiochemical purity of 14C-labeled compounds was determined by autoradiography; chemical purity of 13C-labeled compounds was determined by 1H-NMR.
b Not determined.
c From Ji and Scha¨ffer (2004).
d From Ji and Scha¨ffer (2002).
portion of the product 12b, which is sensitive to oxidation by O₂, 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- 14C]-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 SnCl₄ to a mixture of the respective precursor (phenol (1b), catechol (10b), and guaiacol (7b)) and CH₃SCHCl₂ in CH₂Cl₂ or CH₃CN at −78 or −20 °C, followed by hydrolysis with HCl (2–6 M). Yields of 88%, 75%, and 83%, respectively, have 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.

[U-ring- 13C]-vanillin (8a) was synthesized by drop-wise addition of [U-ring- 13C]-guaiacol (7a) to a mixture of CH₃SCHCl₂ and SnCl₄ at a 7a:CH₃SCHCl₂:SnCl₄ molar ratio of 1:4:1.4. By adding compound 7a slowly, CH₃SCHCl₂ and SnCl₄ 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- 13C]-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 Br₂ in CH₂Cl₂ under reflux for 2 h ( Haida er, 1966). Demethylation of [U-ring- 13C]-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 [1-14C]-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 × 10⁻⁴ Pa (at 40 °C). CH₂Cl₂ was used instead of ethyl acetate to extract compound 7 from the alkaline aqueous extract since CH₂Cl₂ could be completely removed from compound 7 under a vacuum higher than 3 × 10⁻⁴ Pa. Compound 7 is O₂ sensitive in an aqueous alkaline solution. To avoid autooxidation of compound 7, O₂ 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 CH₃I 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 [ 13C]-labeled compounds

The NMR spectra of the [ 13C]-labeled compounds and the mass spectra of their silylated derivatives are summarized in Table 2. Owing to C–C coupling, the 13C-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 sequestered by humic substances, as shown with the fungicide cyprodinil ( Dec et al., 1997a,b).

[13C]-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
13C-NMR spectra and MS data of [U-ring-13C]-labeled phenolic compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Comparison of 13C-NMR spectra of [U-ring-13C]-labeled compounds (upper lines) and the corresponding compounds with natural 13C abundance (lower lines)^a</th>
<th>Chemical shifts (ppm)^b</th>
<th>MS fragments of [U-ring-13C]-labeled silylated compounds (m/z)^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td><img src="image1" alt="2a spectrum" /></td>
<td>C-1: 128.28</td>
<td>200 (M^+, 68.8), 185 (100), 167 (9.0), 157 (70.8), 141</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-2, C-6: 130.83</td>
<td>(12.1), 127 (3.0), 111 (6.6), 97 (22.2), 92 (21.5),</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-3, C-5: 114.55</td>
<td>82 (12.6), 73 (34.8), 59 (6.5), 55 (17.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-4: 161.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-7: 189.20</td>
<td></td>
</tr>
<tr>
<td>3a</td>
<td><img src="image2" alt="3a spectrum" /></td>
<td>C-1: 124.95</td>
<td>314 (M^+, 25.9), 299 (33.7), 255 (21.2), 225 (45.2), 209</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-2, C-6: 128.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-3, C-5: 114.58</td>
<td>197 (4.0), 185 (11.3), 167 (2.3), 147 (4.5), 142</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-4: 158.38</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-7: 143.99</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>C-8: 113.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-9: 166.90</td>
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<tr>
<td>7a</td>
<td><img src="image3" alt="7a spectrum" /></td>
<td>C-1: 118.39</td>
<td>202 (M^+, 19.7), 187 (25.1), 172 (100), 157 (23.6), 142</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-2: 110.39</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-3: 145.30</td>
<td>(15.5), 127 (1.5), 113 (1.5), 97 (3.1), 89 (2.5), 83 (2.9),</td>
</tr>
<tr>
<td></td>
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<td>C-4: 146.22</td>
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<tr>
<td></td>
<td></td>
<td>C-5: 113.73</td>
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<td>C-6: 119.91</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>C-7: 54.09</td>
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</table>

(continued on next page)
Table 2 (continued)

<table>
<thead>
<tr>
<th>Compound</th>
<th>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)$^a$</th>
<th>Chemical shifts (ppm)$^b$</th>
<th>MS fragments of [U-ring-$^{13}$C]-labeled silylated compounds (m/z)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>8a</td>
<td><img src="image" alt="8a NMR spectra" /></td>
<td>C-1: 128.67 230 (M+, 27.1), C-2: 108.84 215 (41.0), C-3: 146.83 200 (100), 185 C-4: 151.35 169 C-5: 113.82 157 (3.8), C-6: 125.01 142 (8.6), C-7: 189.13 127 (1.5), 107 C-8: 54.25 6 (6.5), 85 (4.5), 73 (33.7), 59 (16.5)</td>
<td></td>
</tr>
<tr>
<td>10a</td>
<td><img src="image" alt="10a NMR spectra" /></td>
<td>C-1, C-6: 119.17 260 (M+, 7.23), 245 (1.97), C-2, C-5: 115.57 172 (1.52), 157 (2.63), 142 C-3, C-4: 145.16 (2.25), 180 (1.05), 73 (100), 59 (1.34)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Data from Table 2 in the original article.

$^b$ Chemical shifts in ppm relative to TMS as internal standard.

$^c$ MS fragments in m/z units with relative intensity (in parentheses).
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 13C-NMR spectrum of [13C]-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-13C coupling, the 1H-NMR signals of the H-atoms at the [13C]-labeled aromatic rings were also split into two signals and overlapped (spectra not shown). However, 1H-NMR 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-13C]- or [U-14C]-labeled in the aromatic ring or [13C]-labeled at the β-C of the propenoic side chain, starting from commercially available [U-ring-13C]- and [U-ring-14C]-labeled phenols or [2,14C]-malonic acid. The availability of these 13C-labeled phenols will facilitate studies on their fate in the environment and on the structural elucidation of their transformation products in the environment.

### Acknowledgement

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### References


Tazaki, H., Taguchi, D., Hayashida, T., Nabeto, K., 2001. Stable isotope-labeling studies on the oxidative coupling of
