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Research Article

Synthesis and purification of [1,2-$^{13}C_2$]coniferin

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Summary

The double labelled lignan precursors [1,2-$^{13}C_2$]coniferin and the glucoside of [1,2-$^{13}C_2$]ferulic acid were prepared by classical synthetic methods. Pure double labelled lignan precursors could only be obtained after separation from their contaminating *Z*-isomers and dihydro by-products by high-performance liquid chromatography. Copyright © 2006 John Wiley & Sons, Ltd.

Key Words: monolignols; synthesis; isotopic labelling; carbon 13

Introduction

Lignans are widely distributed plant metabolites associated with a range of biological activities. Podophylotoxin is used as an antiviral agent in the treatment of genital warts,[1] and secoisolariciresinol diglucoside is a natural cancer chemopreventive agent effective against the onset of breast, prostate, and colon cancers.[2] The biosynthetic pathway of lignans is not completely elucidated, as it is specific to each species of plants.[3] Most of the present knowledge about the conversion of monolignols to their dimers lignans and the formation of cell wall lignins in plants comes from incorporation experiments using specifically labelled precursors or substrates.[4,5]

Monolignol glucosides (*p*-glucocoumaryl alcohol, coniferin, syringin) are considered to be the storage or excretion form of monolignols.[6] The synthesis of monolignol glucosides enriched with ^{13}C at specific positions has been used to prove their effective incorporation in lignin.[7] However,

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to obtain detailed information about the biosynthetic route of lignans *in vivo*, the synthesis of multiple labelled precursors becomes necessary.[4] The presence of two consecutive ^{13}C atoms in the monolignol molecules allows their conversion to dimers to be followed, due to the characteristic signals observed in the NMR spectra of intermediates involved. We chose the two atom carbons mostly involved in the dimerization process leading to lignans.

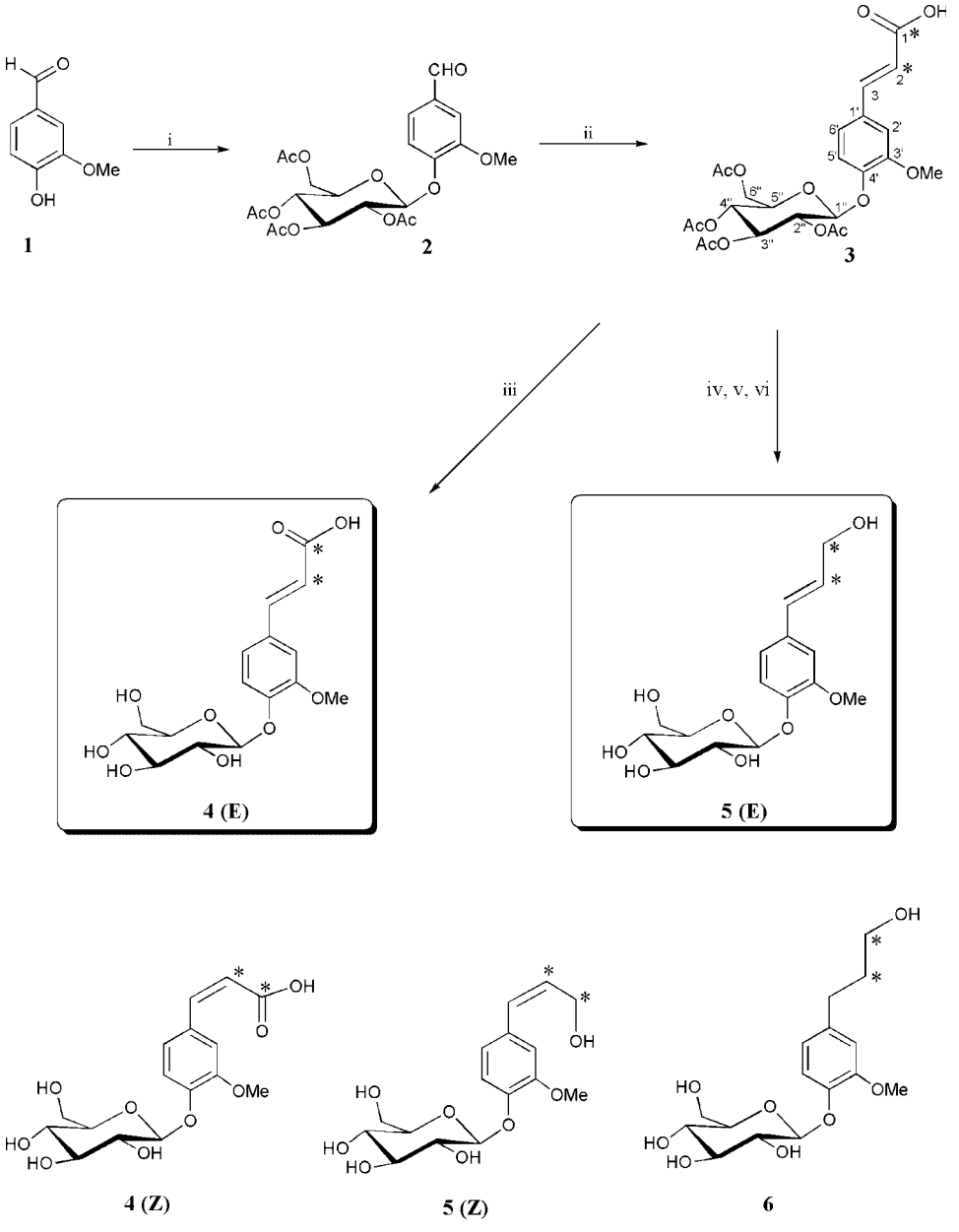
In this paper, we report on the synthesis, chromatographic purification and complete characterization of the labelled monolignol glucoside [1,2-$^{13}C_2$]coniferin, and its precursor, the glucoside of [1,2-$^{13}C_2$]ferulic acid.

Results and discussion

The synthetic strategy[8] is shown in Scheme 1. Conversion of vanillin (**1**) to its corresponding β-glucoside was performed with tetra-*O*-acetyl-α-D-glucopyranosyl bromide and silver oxide in quinoline to give **2** in 73% yield. The introduction of the double ^{13}C labelling was accomplished by condensation of **2** with readily available [1,2,3-$^{13}C_3$]malonic acid leading, after decarboxylation, to compound **3** in 70% yield. The typical pattern of consecutive labelling was observed in the ^{13}C NMR spectrum of compound **3** in $CDCl_3$, the two doublets appear at δ 171.5 (C-1) and δ 116.6 (C-2) with a $J_{1,2} = 74$ Hz.

Since the glucoside of ferulic acid is also an interesting substrate for biosynthetic studies, we decided to perform the deprotection of the sugar moiety in NaOMe–methanol. Deacetylation and purification by flash chromatography on silica gel lead to the acid **4**. Disappointingly, the ^{13}C NMR spectrum of deacetylated glucoside of labelled acid **4** in CD_3OD showed the presence of a second product, its *Z*-isomer, which had not been detected on the NMR spectrum of the protected acid **3**. Previous synthesis of unlabelled coniferin using the same synthetic route[9] did not report about the formation of the *Z*-isomer, however in our products the presence of the double labelling allows its easy detection by ^{13}C NMR. On the other hand, as the *Z*-isomer has been previously obtained by other methods,[10,11] the additional NMR doublets could be undoubtedly assigned. For the major *E*-acid two doublets appear at δ 170.7 (C-1) and δ 117.9 (C-2) with a $J_{1,2} = 73.2$ Hz whereas the corresponding resonances of the minor *Z*-acid are δ 170.8 (C-1) and δ 120.3 (C-2) with a $J_{1,2} = 71.2$ Hz.

It is important at this stage to separate the two isomers to avoid contamination of our biosynthetic studies by the less common *Z*-isomer. All attempts to perform separation of *E*- and *Z*-isomers before deacetylation (compound **3**) were unsuccessful. We decided to purify the deacetylated acid **4**. Using a Prevail C-18 column and a gradient of water (containing 0.2% of acetic acid)–acetonitrile (Figure 1(a)), first was eluted the natural glucoside of



Scheme 1. Synthesis of labelled ferulic acid and coniferin. Reagents: (i) Tetra-*O*-acetyl-α-D-glucopyranosyl bromide, Ag_2O, quinoline, r.t., 2 h; (ii) $^{13}CH_2(^{13}COOH)_2$, pyridine, piperidine, 1 h at 55°C and 2 h at 85°C; (iii) MeONa, MeOH, r.t., overnight; (iv) $(COCl)_2$, CH_2Cl_2, r.t., overnight; (v) $NaBH_4$, THF, r.t., 4 h; (vi) NH_3, MeOH, r.t., overnight

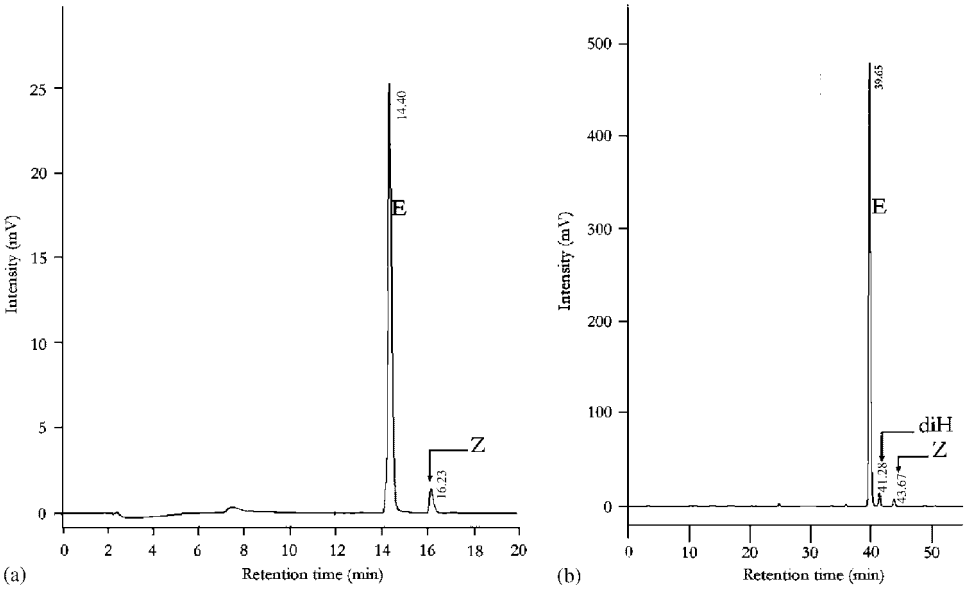


Figure 1. (a) HPLC elution profile of deacetylated glucoside of labelled ferulic acid 4. Prevail C-18 column, gradient water (containing 0.2% of acetic acid)–acetonitrile. t_R 14.40 min = *E*-ferulic acid glucoside, t_R 16.23 min = *Z*-isomer, λ = 280 nm. (b) HPLC elution profile of labelled coniferin 5. Kromasyl C-18 column, gradient water (containing 0.2% of acetic acid)-acetonitrile. t_R 39.65 min = *E*-coniferin, t_R 41.28 min = dihydroconiferin, t_R 43.67 min = *Z*-isomer, λ = 280 nm

E-ferulic acid, then the contaminant *Z*-isomer (t_R 14.40 and 16.23 min, respectively). After purification, the ^{13}C NMR spectra of **4** showed only the doublets corresponding to the desired *E*-isomer.

The synthesis of the labelled monolignol from the protected acid **3** was performed through the acyl chloride derivative. As we have previously reported,[8] after deacetylation, the desired alcohol **5** was obtained, together with the reduced double bond by-product **6**.

As the pure *E*-acid could only be purified by HPLC in their deacetylated form, we needed to perform a reacetylation step before its conversion into the acyl chloride and reduction to alcohol. Unfortunately, some degradation was observed during the reacetylation step, and in addition, traces of pyridine or acetic anhydride resulted in low yields of the following borohydride reduction step. Therefore, the acetylated acid **3** (*E*/*Z* mixture) obtained before was directly used for reduction. In spite of different chromatographic conditions, only two peaks were observed for the acetylated alcohol prepared. MS and NMR analysis showed that they corresponded to the *Z*-alcohol and an inseparable mixture of the *E*-alcohol and the dihydro derivative, respectively.

As the complete separation was not possible at this stage, the crude alcohol was deacetylated. Separation of *E*- and *Z*-isomers of some monolignol glucosides has been previously reported using two columns in series,[12] but these conditions were unsuccessful for obtaining pure *E*-isomers without the dihydrogenated by-products. Reversed-phase HPLC on a Kromasyl C-18 column allowed us to obtain the three components on different peaks of the elution profile, as it is shown for the labelled coniferin **5** (Figure 1(b)): first was eluted the *E*-isomer, then the dihydrogenated by-product, and finally the *Z*-isomer (t_R 39.65, 41.28 and 43.67 min, respectively).

In the ^{13}C NMR spectra of the mixture obtained, the major product (*E*) presents two doublets at δ 128.9 (C-2) and 63.7 (C-1) with a $J_{1,2} = 47.4$ Hz. The *Z*-isomer, at δ 131.9 (C-2) and 59.9 (C-1) with a $J_{1,2} = 46.3$ Hz, and the dihydrogenated by-product showed two resonances at δ 62.2 (C-1) and 35.5 (C-2) with a $J_{1,2} = 37.6$ Hz. The ^{13}C NMR spectra of the pure [1,2-$^{13}C_2$] coniferin **5** showed only the expected two typical doublets.

Experimental

Melting points were determined on a Büchi 535. Optical rotations were measured with a Perkin Elmer Model 343 polarimeter using a sodium lamp at 20°C. High-resolution electrospray mass spectra in the positive ion mode were obtained on a Q-TOF Ultima Global hybrid quadrupole/time-of-flight instrument (Waters-Micromass, Manchester, UK), equipped with a pneumatically assisted electrospray (*Z*-spray) ion source and an additional sprayer (Lock Spray) for the reference compound. ^1H NMR spectra were recorded with a Bruker AC 500 spectrometer, ^{13}C NMR spectra were recorded with a Bruker AC 300 spectrometer.

HPLC

The instrumentation in these purifications employed a Waters Model Prep LC 4000 solvent delivery system and a Waters Model 2487 dual λ absorbance. Preparative chromatographic separation of deacetylated acids used a Prevail C18 (250 mm × 22 mm, 5 μm, stainless steel) column. Elution was performed with water (containing 0.2% of acetic acid)–acetonitrile, linear gradient 100:0 – 82:18 (10 min) and kept for additional 20 min. The flow rate was 18 ml min^{-1}, and detection at 254 and 280 nm. Preparative chromatographic separation of deacetylated alcohols used a Kromasyl C18 (250 mm × 10 mm, 5 μm, stainless steel) column. Elution was performed with water (containing 0.2% of acetic acid)–acetonitrile, linear gradient 100:0–86:14 (54 min). The flow rate was 4.7 ml min^{-1}, and detection at 254 and 280 nm.

4′-O-(2″,3″,4″,6″-Tetra-O-acetyl-β-D-glucopyranosyl)vanillin (**2**)

Vanillin (0.222 g, 1.46 mmol) and acetobromoglucose (1.026 g, 2.5 mmol) were dissolved in distilled quinoleine (10 ml) and silver oxide (0.336 g, 1.7 mmol) was added in portions at 0°C. The reaction mixture was stirred for 2 h in the dark at room temperature. After the addition of acetic acid (20 ml), the whole mixture was poured into water (200 ml) to form a precipitate, which was collected by filtration. Purification by silica gel column chromatography (3:7 cyclohexane–ethyl acetate) gave **2** (0.513 g, 73% yield) as a white solid: m.p. 143.2–144.9°C (lit.[13] 143–144°C).

[1,2-$^{13}C_2$]-4′-O-β-D-glucopyranosylferulic acid (**4**)

A mixture of **2** (0.5 g, 1 mmol), [1,2,3-$^{13}C_3$]malonic acid (0.155 g, 1.3 mmol), pyridine (3.5 ml) and piperidine (four drops) was heated at 55°C for 1 h then at 85°C for 2 h. After cooling, water (5 ml) was added and crystals of [1,2-$^{13}C_2$]-4′-*O*-(2″,3″,4″,6″-tetra-*O*-acetyl-β-D-glucopyranosyl)-ferulic acid (**3**) were obtained by acidification with HCl. Pyridine and piperidin were co-evaporated with toluene. Purification by silica gel column chromatography (6:4–3:7 cyclohexane–ethyl acetate) gave **3** (0.398 g, 73% yield) as a white solid. Compound **3** was dissolved in a NH_3 solution (7 N in methanol) and was stirred for 12 h. The solvent was removed under reduced pressure and the residue was dissolved in water and then lyophilized. A mixture of two isomers of **4**, *E*/*Z* was obtained (0.252 g, 93% yield), which were separated by reverse phase HPLC (Prevail C18, 5 μ, 250 × 22 mm, water 0.2% acetic acide:acetonitrile), to give pure **4**, as a white solid: m.p. 141.5–143.5°C, $[\alpha]_D$-7.8 (*c* 0.62, CH_3OH). ^{1}H NMR (500 MHz, CD_3OD): δ (ppm) 3.40 (dd, 1 H, $H_{4''}$, $J_{4'',3''}$ = 8.3 Hz, $J_{4'',5''}$ = 9.6 Hz); 3.45 (ddd, 1 H, $H_{5''}$, $J_{4'',5''}$ = 9.6 Hz, $J_{5'',6''a}$ = 5.3 Hz, $J_{5'',6''b}$ = 2.2 Hz); 3.48 (dd, 1 H, $H_{3''}$, $J_{3'',2''}$ = 9.3 Hz, $J_{3'',4''}$ = 8.3 Hz); 3.52 (dd, 1 H, $H_{2''}$, $J_{2'',1''}$ = 7.4 Hz, $J_{2'',3''}$ = 9.3 Hz); 3.70 (dd, 1 H, $H_{6''a}$, $J_{6''a,6''b}$ = 12.1 Hz, $J_{6''a,5''}$ = 5.3 Hz); 3.88 (dd, 1 H, $H_{6''b}$, $J_{6''b,5''}$ = 2.2 Hz, $J_{6''b,6''a}$ = 12.1 Hz); 3.90 (s, 3 H, OCH_3); 4.97 (d, 1 H, $H_{1''}$, $J_{1'',2''}$ = 7.4 Hz); 6.39 (ddd, 1 H, H_2, $J_{2,3}$ = 15.9 Hz, $J_{2,C2}$ = 161.0 Hz, $J_{2,C1}$ = 2.6 Hz); 7.15 (dd, 1 H, $H_{6'}$, $J_{6',5'}$ = 8.4 Hz, $J_{6',2'}$ = 1.7 Hz); 7.18 (d, 1 H, $H_{5'}$, $J_{5',6'}$ = 8.4 Hz); 7.24 (d, 1 H, $H_{2'}$, $J_{2'6'}$ = 1.7 Hz); 7.61 (ddd, 1 H, H_3, $J_{3,2}$ = 15.9 Hz, $J_{3,C2}$ = 2.8 Hz, $J_{3,C1}$ = 6.7 Hz). ^{13}C NMR (75.5 MHz, CD_3OD): δ (ppm) 56.8 (CH_3O); 62.5 ($C_{6''}$); 71.3 ($C_{4''}$); 74.8 ($C_{2''}$); 77.9 ($C_{3''}$); 78.3 ($C_{5''}$); 102.2 ($C_{1''}$); 112.5 (d, $C_{2'}$, $J_{C2',C2}$ = 4.5 Hz); 117.9 (d, C_2, $C_{5'}$, $J_{C2,C1}$ = 73.2 Hz); 123.4 (d, $C_{6'}$, $J_{C6',C2}$ = 5.3 Hz); 130.6 (d, $C_{1'}$, $J_{C1',C1}$ = 7.0 Hz); 146.0 (d, C_3, $J_{C3,C2}$ = 70.6 Hz); 151.0 (d, $C_{4'}$, $J_{C4',C2}$ = 1.2 Hz); 151.0 ($C_{3'}$); 170.7 (d, C_1, $J_{C1,C2}$ = 73.2 Hz). ESI-HRMS [M + Na]: Calculated for $^{12}C_{14}$ $^{13}C_2H_{20}O_9Na$: *m*/*z* 381.1072. Found: 381.1075.

*[1,2-$^{13}C_2$]Coniferin (**5**)*

Compound **3** (0.300 g, 0.57 mmol) was dissolved in dried dichloromethane (25 ml) under argon and cooled in an ice water bath. Oxalyl chloride (0.15 ml) was added dropwise. After addition, the ice water bath was removed and the reaction was left for 12 h at room temperature. The solvent was removed under reduced pressure to give a paleyellow precipitate. Sodium borohydride (96 mg) was dissolved in dried THF (20 ml) and [1,2-$^{13}C_2$]-4′-*O*-(2″,3″,4″,6″-tetra-*O*-acetyl-*β*-D-glucopyranosyl)feruloyl chloride was slowly added dissolved in dried THF (20 ml) under stirring under argon. The reaction mixture was left at room temperature for 5 h. The completion of the reaction was confirmed by mass spectrometry. Methanol was added to react with the excess of sodium borohydride and then the solvents were removed under reduced pressure. Several evaporations with methanol gave a residue which was purified by column chromatography on silica gel (4:6 cyclohexane–ethylacetate). A mixture of [1,2-$^{13}C_2$]-2″,3″,4″,6″-tetra-*O*-acetylconiferin (0.271 g, 70% yield), the isomer *Z* and [1,2-$^{13}C_2$]-2″,3″,4″,6″-tetra-*O*-acetyldihydroconiferin was obtained. The mixture (0.21 g) was dissolved in a NH_3 solution (7 N in methanol) (10 ml) and was stirred for 12 h. The solvent was removed under reduced pressure without heating in absence of light, then the residue was redissolved in water and lyophilized. A mixture of **5** (0.178 g, 92% yield) and two isomers: the isomer *Z* and dihydroconiferin **6** was obtained. The three compounds could be separated by reverse-phase HPLC: (Prevail C18, 5 μ, 250 × 22 mm, water 0.2% acetic acide:acetonitrile) to give pure **5**, as a white solid: m.p. 189.3–190.5°C, $[\alpha]_D$-1.4 (*c* 0.7, CH_3OH). ^{1}H NMR (500 MHz, CD_3OD): δ (ppm) 3.43–3.37 (m, 2 H, $H_{4''}$, $H_{5''}$); 3.46 (dd, 1 H, $H_{3''}$, $J_{3'',2''} = 9.1$ Hz, $J_{3'',4''} = 10.1$ Hz); 3.50 (dd, 1 H, $H_{2''}$, $J_{2'',1''} = 7.3$ Hz, $J_{2'',3''} = 9.1$ Hz); 3.69 (m, 1 H, $H_{6''a}$, $J_{6''a,6''b} = 12.1$ Hz, $J_{6''a,5''} = 5.1$ Hz); 3.87 (s + dd, 4 H, OMe, $H_{6''b}$, $J_{6''b,5''} = 1.6$ Hz, $J_{6''b,6''a} = 12.1$ Hz); 4.21 (dddd, 2 H, 2 H_1, $J_{1,C1} = 141.5$ Hz, $J_{1,3} = 1.4$ Hz, $J_{1,2} = 5.7$ Hz, $J_{1,C2} = 4.5$ Hz); 4.89 (d, 1 H, $H_{1''}$, $J_{1'',2''} = 7.3$ Hz); 6.28 (dddt, 1 H, H_2, $J_{2,3} = 16.1$ Hz, $J_{2,C2} = 151.5$ Hz, $J_{2,1} = 5.7$ Hz, $J_{2,C1} = 3.9$ Hz); 6.55 (ddl, 1 H, H_3, $J_{3,2} = 16.1$ Hz, $J_{3,C1} = 7.0$ Hz); 6.95 (dd, 1 H, $H_{6'}$, $J_{6',5'} = 8.4$ Hz, $J_{6',2'} = 1.9$ Hz); 7.07 (d, 1 H, $H_{2'}$, $J_{2'6'} = 1.9$ Hz); 7.12 (d, 1 H, $H_{5'}$, $J_{5',6'} = 8.4$ Hz). ^{13}C NMR (75.5 MHz, CD_3OD): δ (ppm) 56.7 (CH_3O); 62.5 ($C_{6''}$); 63.7 (d, C_1, $J_{C1,C2} = 47.4$ Hz); 71.3 ($C_{4''}$); 74.9 ($C_{2''}$); 77.8 ($C_{3''}$); 78.2 ($C_{5''}$); 102.7 ($C_{1''}$); 111.3 (d, $C_{2'}$, $J_{C2',C2} = 4.5$ Hz); 117.8 ($C_{5'}$); 120.7 (d, $C_{6'}$, $J_{C6',C2} = 5.0$ Hz); 128.9 (d, C_2, $J_{C2,C1} = 47.4$ Hz); 131.3 (dd, C_3, $J_{C3,C2} = 72.8$ Hz, $J_{C3,C1} = 3.5$ Hz); 133.6 (d, $C_{1'}$, $J_{C1',C1} = 6.3$ Hz); 147.6 (d, $C_{4'}$, $J_{C4',C2} = 1.1$ Hz); 150.8 ($C_{3'}$). ESI-HRMS [M + Na]: Calculated for $^{12}C_{14}$ $^{13}C_2H_{22}O_8Na$: *m*/*z* 367.1279. Found: 367.1265.

Conclusion

During our synthesis of the double labelled glucoside of [1,2-$^{13}C_2$]ferulic acid and [1,2-$^{13}C_2$]coniferin, we could detect the formation of the *Z*-isomer in the condensation step of vanillin with labelled malonic acid, and the partial hydrogenation of the double bond as a side reaction in the reduction step. For both lignans precursors, a purification by reversed-phase chromatography was necessary to avoid contamination by their double labelled synthetic by-products during plant inoculation.

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