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# Investigation of Biosynthetic Pathways to Hydroxycoumarins During Post-Harvest Physiological Deterioration in Cassava Roots by Using Stable Isotope Labelling

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*Cassava (*Manihot esculenta *Crantz) is an important starch-rich crop, but the storage roots only have a short shelf-life due to post-harvest physiological deterioration (PPD), which includes the over-production and polymerisation of hydroxycoumarins. Key aspects of coumarin secondary-metabolite biosynthesis remain unresolved. Here we exploit the accumulation of hydroxycoumarins to test alternative pathways for their biosynthesis. Using isotopically labelled intermediates (*p*-coumarate-2-$^{13}$C, caffeate-2-$^{13}$C, ferulate-2-$^{13}$C, umbelliferone-2-$^{18}$O and esculetin-2-$^{18}$O), we show that the major biosynthetic pathway to scopoletin and its glucoside, scopolin, in cassava roots during PPD is through* p*-coumaric, caffeic and then ferulic acids. An alternate pathway through 2′,4′-dihydroxycinnamate and umbelliferone leads to esculetin and esculin. We have used C$^{18}O_2$-carboxylate-labelled cinnamic and ferulic acids, and feeding experiments under an atmosphere of $^{18}O_2$, to investigate the* o*-hydroxylation and cyclisation steps. We demonstrate that the major pathway is through* o*-hydroxylation and not via a proposed spirolactone-dienone intermediate.*

## Introduction

Coumarins are pharmacologically active and have important roles in plant development and defence. Despite their importance, key aspects of the biosynthesis of these secondary metabolites remain unresolved.[1,2] Here we exploit the observation that the accumulation of scopoletin and its glucoside, scopolin, increases in cassava roots (*Manihot esculenta* Crantz family Euphorbiaceae) during post-harvest physiological deterioration (PPD)[3,4] to test alternative pathways for the biosynthesis of these hydroxycoumarins.

Cassava is an important crop due to its high root starch content and because of its ability to grow well in poor soil and even under drought conditions. However, harvested cassava roots have a short shelf-life of only 1–3 days due to PPD, thus causing significant wastage and economic loss. This deterioration is not due to microorganisms, but is physiological; possibly due to the over-production and then polymerisation of hydroxycoumarins. Deteriorating cassava roots show blue to black vascular streaking and are unpalatable and unmarketable. The first detectable aspect of PPD is an oxidative burst occurring within 15 minutes of the root's being injured, followed by altered expression of genes, including those involved in antioxidant defence together with the accumulation of secondary metabolites, such as the antioxidant hydroxycoumarins scopoletin and esculetin and their respective glucosides scopolin and esculin.[3–5]

Three hypothetical pathways for the biosynthesis of scopoletin,[6] via 2′,4′-dihydroxycinnamate, 2′,4′,5′-trihydroxycinnamate (6′-hydroxycaffeate), or 4′-hydroxy-3′-methoxycinnamate (ferulate) have been proposed from studies in various plant species (Scheme 1). The observation that esculetin and its glucoside esculin accumulate in deteriorated cassava in lesser amounts than scopoletin and scopolin is consistent with a route via caffeate to esculetin and then scopoletin. In *Agathosma puberula*, the biosynthesis of puberulin (6,8-dimethoxy-7-prenyloxocoumarin) is from umbelliferone via esculetin and then scopoletin;[7,8] this implies hydroxylation and methylation after lactonisation. In addition, in *Cichorium intybus* (chicory), it was confirmed that umbelliferone was converted into esculetin.[9] However, feeding *Nicotiana tabacum* (tobacco) with $^{14}$C-labelled ferulate led to this being actively metabolised by the leaf tissues[10] or tobacco tissue cultures[11] into scopoletin and its glucoside scopolin; this suggests methylation prior to lactonisation. From studies in *Arabidopsis thaliana* it has been proposed that the biosynthesis of scopoletin and scopolin is strongly dependent on the 3′-hydroxylation of *p*-coumarate units catalyzed by CYP98A3;[6] this indicates that scopoletin is biosynthesised in *A. thaliana* from either ferulic or caffeic acid—recently feruloyl-CoA has been proposed as a key precursor in scopoletin biosynthesis in this plant.[12]

We have previously shown that when chopped cassava roots were fed with deuterated (*E*)-cinnamic acid, and PPD was allowed to occur, a certain percentage of labelled scopoletin

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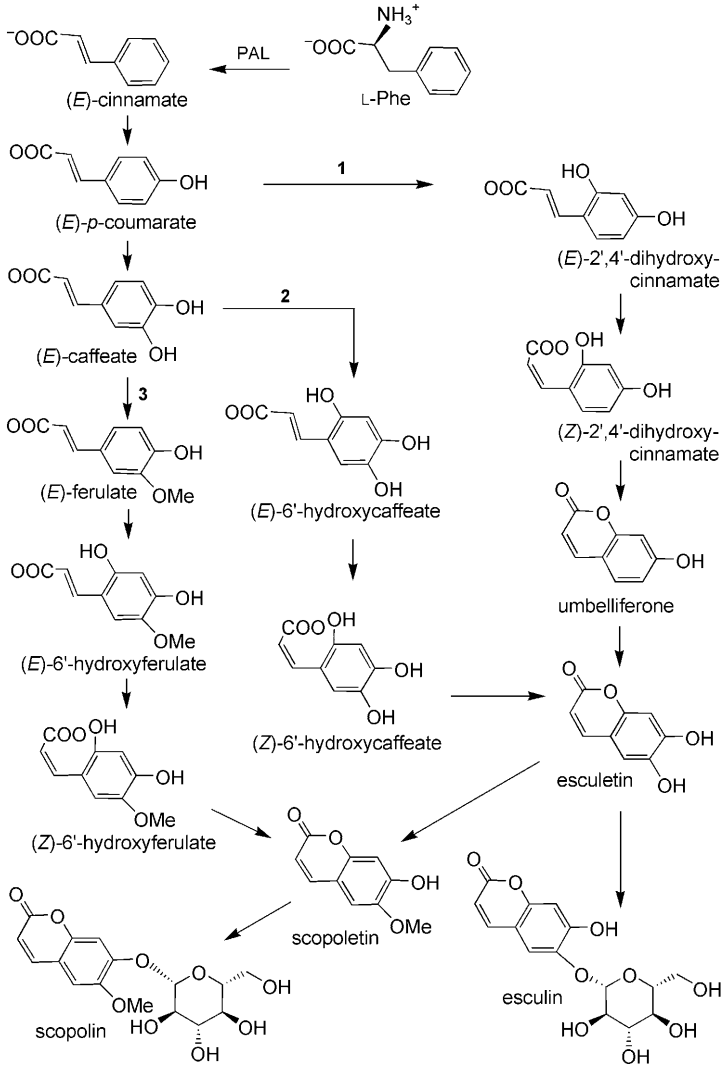
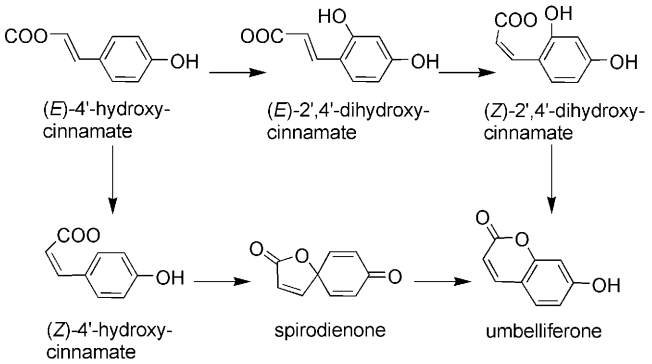



**Scheme 1.** Three proposed metabolic pathways in the biosynthesis of scopoletin. For clarity the carboxylate anion has been omitted after (*E*)-cinnamate; PAL = phenylalanine ammonia lyase.

was produced.[13] In order to investigate the pathway of scopoletin biosynthesis in cassava roots during PPD, direct and competition feeding experiments with potential intermediates were carried out. Here we report feeding experiments in cassava roots under PPD with stable isotopically labelled *p*-coumarate (4′-hydroxycinnamate), caffeate, ferulate, umbelliferone and esculetin. In addition, competition feeding experiments were carried out by feeding the root samples with deuterated (*E*)-cinnamic acid alone as a control and with deuterated ([$D_5$]- and [$D_7$]-) (*E*)-cinnamic acids mixed with putative unlabelled intermediates along the scopoletin biosynthetic pathway that could compete with deuterated (*E*)-cinnamic acid.

Two routes have been proposed for the key benzo-2-pyrone cyclisation reaction of 4′-hydroxycinnamic acid (Scheme 2).[1] Oxidative cyclisation via a spirodienone intermediate would yield umbelliferone in which the lactone ether oxygen is derived from the carboxyl group. The spirolactone-dienone intermediate pathway has been previously established in *Streptomyces niveus* for novobiocin biosynthesis through elegant work by Kenner and co-workers,[14] and also proposed from UV studies in cultures of the plant *Ammi majus* L. (Apiaceae, Bishop's flower, large bullwort)[15] following work by Grisebach and Ollis.[16] Alternatively the 2′-hydroxylation of 4′-hydroxycinnamic acid might be followed by *E*-to-*Z* isomerisation and lactonisation. In this case the lactone ether oxygen would be derived from the 2′-hydroxy group and not from the carboxylic acid. Although *o*-hydroxylation of cinnamic (*p*-coumaric, caffeic or ferulic) acids is of pivotal importance for the biosynthesis of coumarins, it remains a missing link in phenylpropanoid biosynthesis.[2]

NIH shift (the migration of the H-atom to a neighbouring position and its partial retention) is an excellent tool in the prediction of isotopic patterns in *o*-hydroxylation in phenylpropanoids (deuterium abundance was in agreement with the theoretical results in methyl salicylate and in coumarins).[17] Labelled cinnamic acid (*o*-$^{3}$H, ring-1-$^{14}$C) was fed to *Melilotus alba* shoots, and the retention of label was 78% upon conversion into coumarin and 92% upon conversion into *o*-coumaric acid in *Gaultheria procumbens* leaves, so *o*-coumaric acid biosynthesis involves an efficient migration and retention of the *o*-proton, evidence that in coumarin the lactone ether oxygen is introduced by *o*-hydroxylation.[18] Recently, in *A. thaliana*, it was shown that an $Fe^{II}$- and 2-oxoglutarate-dependent dioxygenase (rather than a P450 enzyme) catalyses the *o*-hydroxylation of feruloyl-CoA in scopoletin biosynthesis.[12] It is notable that the biosynthesis of salicylic acid and the origin of its *o*-hydroxyl group is also still a matter of debate and shows differences between species.[19] In *Nicotiana benthamiana*, it was determined that salicylic acid is biosynthesised from isochorismate,[20] while in phenylalanine ammonia lyase (PAL)-suppressed *N. tabacum* the level of salicylic acid was decreased,[21] thus suggesting that salicylic acid was synthesised from phenylalanine[22] through benzoic acid,[23] which was then *o*-hydroxylated to salicylic acid. Following the $^{18}$O-labelling of salicylic acid, it was proposed that benzoic acid *o*-hydroxylase




**Scheme 2.** Proposed routes for the conversion of 4′-hydroxycinnamate into umbelliferone.

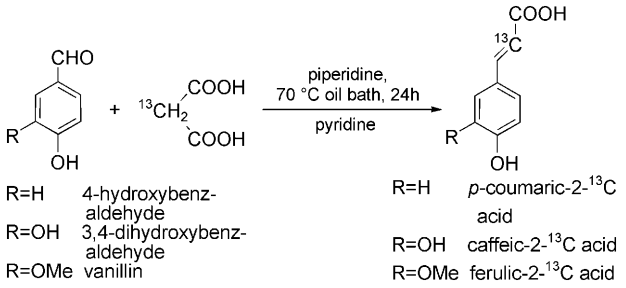
  

is an oxygenase that specifically hydroxylates the *o*-position of benzoic acid. This hydroxylase enzyme was purified and found to belong to a novel class of soluble, high-molecular-weight cytochrome P450 enzymes.[24] Therefore, in order to investigate the origin of the lactone ether oxygen in coumarins biosynthesised in cassava roots during PPD, we designed and carried out feeding experiments with $C^{18}O_2$-enriched (*E*)-cinnamic and ferulic acids by spraying an aqueous solution of each labelled cinnamic acid on cubes of freshly harvested cassava roots. Separately, in an atmosphere rich in $^{18}O_2$, the scopoletin and scopolin labelling patterns resulting from deuterated [$D_7$]cinnamic acid feeding were obtained and compared with those from untreated roots kept in the same vacuum desiccator.

## Results and Discussion

### Synthesis of isotopically labelled intermediates

*Synthesis of* p-*coumaric-2-$^{13}$C acid, caffeic-2-$^{13}$C acid and ferulic-2-$^{13}$C acid*: *p*-Coumaric-2-$^{13}$C acid, caffeic-2-$^{13}$C acid and ferulic-2-$^{13}$C acid were synthesised by a Knoevenagel reaction[25,26] through the nucleophilic addition of 2-$^{13}$C-malonic acid to the corresponding aldehyde (Scheme 3), and the three desired (*E*)-cinnamic acid derivatives were recrystallized from water.




**Scheme 3.** Synthesis of labelled substituted cinnamic-2-$^{13}$C acids.

The chemical structures, and therefore the position of the $^{13}$C-labels, were confirmed by $^1$H and $^{13}$C NMR spectroscopy (Tables 1 and 2). The $^1$H NMR spectra (Table 1) showed H-2 doublet of doublets due to H-2-$^{13}$C-2 coupling ($^1J_{CH} = \sim 161$ Hz) and *trans*-H-2-H-3 coupling ($^3J_{HH} = 16$), H-3 is also a doublet of doublets including $^2J_{CH} = 3$, and such *J* values are consistent with the reported literature values.[27] In all the $^{13}$C NMR spectra, the signal for C-2 at $\delta = 116$ was approximately 90-fold more intense (99% enriched) than the natural abundance peaks for the other positions. This intense signal is superimposed on the doublet ($^1J_{CC} = 71$) resulting from natural abundance $^{13}$C at C-3 (or at C-1), while the peaks at $\delta = 171$ and 147, corresponding to C-1 and C-3, respectively, are doublets ($^1J_{CC} = 74$ or 71), due to this $^1J$ coupling. Peaks corresponding to C-2′ and C-6′ are doublets ($^3J_{CC} = 5$) due to long-range coupling to the enriched C-2. Interestingly, quaternary C-1′ was an apparent singlet ($^2J_{CC} < 1.5$), due to a combination of being a quaternary carbon and being β to the enriched $^{13}$C-atom. Why the $^3J_{CC} = 5$ is greater than $^2J_{CC} < 1.5$ is complex, but has literature precedent, for example, in geraniol where $^3J_{CC} = 5$ and $^2J_{CC} = 1.3$;[28] however, this is not always the case as $^2J_{CC}$ can be equal to or slightly greater than $^3J_{CC}$, but always in the range 2–7 Hz,[29] and such long-range $^{13}$C–$^{13}$C coupling constants can now be detected at natural abundance with the appropriate pulse-sequence.[30] The $^{13}$C NMR spectra (Table 2) clearly show that introducing an *o*-phenolic group, from *p*-coumarate to caffeate, causes a shift of −12–16 ppm whilst a gain of 30 ppm is seen at the carbon that now carries the oxygen (relative to proton), and a shift of −8 ppm at the *para*-carbon. Therefore we assign 149.5 (from 161.2) to 4′ and 146.8 (from 116.8) to 3′. However, on converting the hydroxy into a methoxy, that is, from caffeate to ferulate, the gain would be 30.2 ppm at the attached carbon, with a shift of −14.7 ppm at the *ortho*-carbons, according to tables.[27] We cannot unambiguously assign 150.5 and 149.3 ppm to 3′ and 4′, respectively, but this order is consistent with tables, that is, shifting further from TMS with a methoxy (relative to a hydroxy) and shifting slightly nearer (2 ppm) to TMS with an *o*-methoxy than with an *o*-hydroxy.[27] These $^1$H and $^{13}$C NMR assignments are also consistent with those few literature data that have been reported for cinnamic-2-$^{13}$C acid[31] and ferulic-2-$^{13}$C acid.[32]

**Table 1.** $^1$H NMR (400 MHz) spectral data.

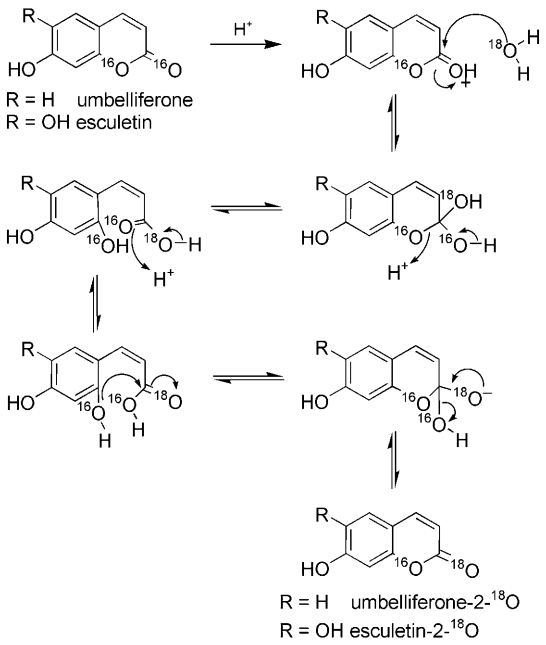
| Compound position | $^1$H multiplicity (*J*) *p*-coumaric-2-$^{13}$C acid | caffeic-2-$^{13}$C acid | ferulic-2-$^{13}$C acid |
|---|---|---|---|
| 2 | 6.28 dd (161, 16) | 6.22 dd (160, 16) | 6.31 dd (161, 16) |
| 3 | 7.60 dd (16, 3) | 7.53 dd (16, 3) | 7.59 dd (16, 3) |
| 2′ | 7.45 d (9) | 7.04 d (2) | 7.17 d (2) |
| 3′ | 6.81 d (9) | – | – |
| 5′ | 6.81 d (9) | 6.78 d (8) | 6.81 d (8) |
| 6′ | 7.45 d (9) | 6.94 dd (8, 2) | 7.06 dd (8, 2) |
| $OCH_3$ | – | – | 3.89 s |

**Table 2.** $^{13}$C NMR (100 MHz) spectral data ($^1$H broadband decoupled).

| Compound position | $^{13}$C multiplicity (*J*) *p*-coumaric-2-$^{13}$C acid | caffeic-2-$^{13}$C acid | ferulic-2-$^{13}$C acid |
|---|---|---|---|
| 1 | 171.0 d (74) | 171.0 d (74) | 171.0 d (74) |
| 2 | 115.6 (99% enriched) | 115.5 (99% enriched) | 115.9 (99% enriched) |
| 3 | 146.7 d (71) | 147.0 d (71) | 146.9 d (71) |
| 1′ | 127.2 | 127.8 | 127.7 |
| 2′ | 131.1 d (5) | 115.0 d (5) | 111.6 d (5) |
| 3′ | 116.8 | 146.8 | 150.5 (149.3) |
| 4′ | 161.2 | 149.5 | 149.3 (150.5) |
| 5′ | 116.8 | 116.5 | 116.2 |
| 6′ | 131.1 d (5) | 122.8 d (5) | 124.0 d (5) |
| $OCH_3$ | – | – | 56.4 |

*$^{18}$O enrichment of umbelliferone and esculetin*: $^{18}$O enrichment of umbelliferone and esculetin with $H_2{}^{18}O$ is a practical method of introducing selectively only one label into these compounds (Scheme 4). This mechanism yields coumarins labelled only in the lactone carbonyl, the lactone ether and phenolic oxygen atoms are not exchanged. The percentage of $^{18}$O-enrichment was monitored for umbelliferone after 3 h (8%), 4 days (61%) and 7 days (82%). Esculetin was $^{18}$O-enriched for 7 days (82%),




**Scheme 4.** Isotopic enrichment of umbelliferone or esculetin with $^{18}O$.

as this gave the highest percentage enrichment for umbelliferone.

*$C^{18}O_2$ enrichment of* (E)*-cinnamic acid and ferulic acid*: $C^{18}O_2$-enriched (*E*)-cinnamic acid was synthesised by double exchange with $H_2{}^{18}O$ catalysed by concentrated HCl. The percentage $^{18}O$ enrichment was monitored over 3 h, 1 day, 2 days and 4 days in order to optimise the yield of the $^{18}O_2$-enriched species (Table 3). $C^{18}O_2$-enriched ferulic acid was then synthesised by the same method as (*E*)-cinnamic acid for 4 days.

**Table 3.** HR ESI MS data of cinnamate labelling showing the percentage of $^{18}O$-enriched (*E*)-cinnamic acid.

| Duration of the reaction | % unlabelled cinnamic acid (*m/z* 149) | % $^{18}O$ enrichment (*m/z* 151) | % $^{18}O_2$ enrichment (*m/z* 153) |
|---|---|---|---|
| 3 h | 86.2 | 13.8 | 0 |
| 1 d | 9.3 | 43.5 | 47.2 |
| 2 d | 1.9 | 23.7 | 74.4 |
| 4 d | 0 | 14.0 | 86.0 |

### Biosynthetic feeding experiments

*Feeding with* p*-coumaric-2-$^{13}$C acid, caffeic-2-$^{13}$C acid and ferulic-2-$^{13}$C acid*: The relative importance of the pathways labelled 1, 2 and 3 in Scheme 1 was explored by direct feeding of cassava roots with the above three $^{13}C$-labelled acids, which are later intermediates than cinnamic acid in the biosynthesis of scopoletin. *p*-Coumaric acid is an intermediate in all three proposed pathways, caffeic acid in pathways 2 and 3, while ferulic acid is in pathway 3 only. The percentages of labelled scopolin and scopoletin were calculated and are summarized in Tables 4 and 5. These results showed an increase in the percentage of labelled scopoletin and scopolin above the natural abundance

**Table 4.** HR ESI MS data of the HPLC peak at 7.1 min showing the percentage of labelled scopolin.

| Cassava cv MCOL 22 fed with | Scopolin[a] | Scopolin-3-$^{13}$C | % of scopolin-3-$^{13}$C | % $^{13}$C scopolin[b] |
|---|---|---|---|---|
| untreated (control) | 355.1033 | 356.1073 | 13.9 | – |
| *p*-coumaric-2-$^{13}$C acid | 355.1050 | 356.1087 | 31.2 | 17.3 |
| caffeic-2-$^{13}$C acid | 355.1033 | 356.1079 | 28.2 | 14.3 |
| ferulic-2-$^{13}$C acid | 355.1039 | 356.1068 | 27.5 | 13.6 |

[a] Scopolin signifies *m/z* found of scopolin, $C_{16}H_{19}O_9$ $[M+H]^+$ (calcd 355.1024). Scopolin-3-$^{13}$C signifies *m/z* found of scopolin-3-$^{13}$C, $C_{15}{}^{13}CH_{19}O_9[^{13}C,M+H]^+$ (calcd 356.1057). [b] Adjusted for natural abundance.

**Table 5.** HR ESI MS data of the HPLC peak at 24.7 min and the percentage of labelled scopoletin.

| Cassava cv MCOL 22 fed with | Scopoletin[a] | Scopoletin-3-$^{13}$C | % of scopoletin-3-$^{13}$C | % $^{13}$C scopoletin[b] |
|---|---|---|---|---|
| untreated (control) | 193.0487 | 194.0537 | 7.5 | – |
| *p*-coumaric-2-$^{13}$C acid | 193.0488 | 194.0533 | 22.6 | 15.1 |
| caffeic-2-$^{13}$C acid | 193.0489 | 194.0529 | 19.1 | 11.6 |
| ferulic-2-$^{13}$C acid | 193.0487 | 194.0529 | 23.0 | 15.5 |

[a] Scopoletin signifies *m/z* found of scopoletin, $C_{10}H_9O_4$ $[M+H]^+$ (calcd 193.0495). Scopoletin-3-$^{13}$C signifies *m/z* found of scopoletin-3-$^{13}$C, $C_9{}^{13}CH_9O_4$ $[^{13}C,M+H]^+$ (calcd 194.0529). [b] Adjusted for natural abundance.

for all three precursors. Ferulic-2-$^{13}$C acid incorporation confirms that pathway 3 is involved in the biosynthesis of scopoletin in cassava roots during PPD. However, as all three labelled precursors gave very similar enrichment of the product hydroxycoumarins, biosynthetic pathways 1 and 2 cannot be completely excluded.

*Feeding with $^{18}$O-umbelliferone and $^{18}$O-esculetin compared with ferulic-2-$^{13}$C acid*: The relative importance of these three possible pathways (Scheme 1) was further explored by feeding cassava roots with the above three compounds, later intermediates in these three pathways where umbelliferone is in pathway 1, esculetin is in both pathways 1 and 2, but ferulic acid is only in pathway 3. The percentages of labelled scopolin, scopoletin and esculin are summarized in Tables 6, 7, and 8. These results showed an increase in the percentage of labelled scopoletin and scopolin above the natural abundance for ferulic-2-$^{13}$C acid, together with a relatively small increase in the percentage of labelled scopoletin and scopolin above the natural abundance for umbelliferone and esculetin; we interpret this as confirmation that pathway 3 is the main pathway for the biosynthesis of scopoletin and scopolin in cassava roots during PPD (Scheme 5). It is noteworthy that the accurate mass of the $[M+2]$ isotope peak for scopolin (Table 6) obtained from the experiment with $^{18}O$-labelled umbelliferone and esculetin did not agree with calculated value for $C_{16}H_{19}O_8{}^{18}O$. Thus, the peak observed at this mass does not arise entirely from $^{18}O$-labelled scopolin, which would reduce the apparent enrichment observed. A similar discrepancy was observed in the

**Table 6.** HR MS data of the HPLC peak at 7.1 min showing the percentage of labelled scopolin.

| Feeding cassava cv MNGA 19 with | Scopolin[a] | Isotope peak monitored | *m/z* found of labelled scopolin (calcd) | % of total labelled scopolin | % of enriched scopolin |
|---|---|---|---|---|---|
| ferulic-2-$^{13}$C acid | 355.1012 | $C_{15}{}^{13}CH_{19}O_9$ | 356.1053 (356.1057) | 33.3 | 19.4 |
| umbelliferone-2-$^{18}$O | 355.1036 | $C_{16}H_{19}O_8{}^{18}O$ | 357.1396 (357.1066) | 9.4 | 4.8 |
| esculetin-2-$^{18}$O | 355.1024 | $C_{16}H_{19}O_8{}^{18}O$ | 357.1102 (357.1066) | 8.3 | 3.7 |

[a] Scopolin signifies *m/z* found of scopolin, $C_{16}H_{19}O_9$ $[M+H]^+$ (calcd 355.1024).

**Table 7.** HR MS data of the HPLC peak at 24.7 min showing the percentage of labelled scopoletin.

| Feeding cassava cv MNGA 19 with | Scopoletin[a] | Isotope peak monitored | *m/z* found of labelled scopoletin (calcd) | % of total labelled scopoletin | % of enriched scopoletin |
|---|---|---|---|---|---|
| ferulic-2-$^{13}$C acid | 193.0499 | $C_9{}^{13}CH_9O_4$ | 194.0532 (194.0529) | 15.7 | 8.2 |
| umbelliferone-2-$^{18}$O | 193.0491 | $C_{10}H_9O_3{}^{18}O$ | 195.0524 (195.0538) | 1.7 | 0.4 |
| esculetin-2-$^{18}$O | 193.0493 | $C_{10}H_9O_3{}^{18}O$ | 195.0493 (195.0538) | 1.6 | 0.3 |

[a] Scopoletin signifies *m/z* found of scopoletin, $C_{10}H_9O_4$ $[M+H]^+$ (calcd 193.0495).

**Table 8.** HR MS data of the HPLC peak at 6.0 min.

| Feeding cassava cv MNGA 19 with | Esculin[a] | *m/z* found of esculin-2-$^{18}$O | % of total labelled esculin |
|---|---|---|---|
| umbelliferone-2-$^{18}$O | 341.0864 | 343.0921 | 19.8 |
| esculetin-2-$^{18}$O | 341.0871 | 343.0907 | 67.7 |

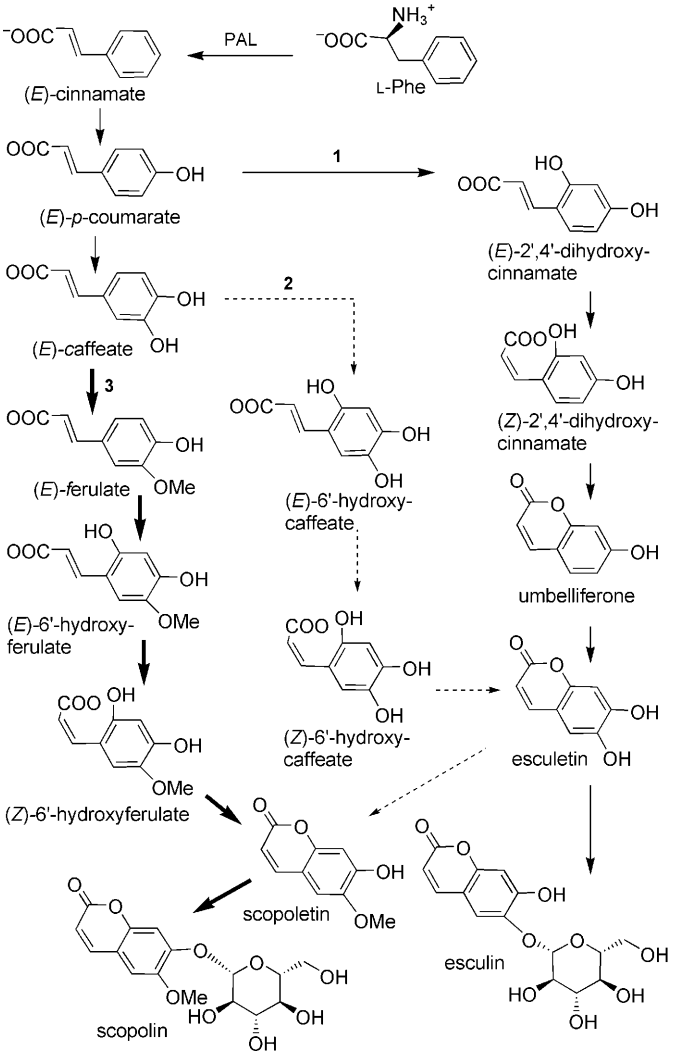
[a] Esculin signifies *m/z* found of esculin, $C_{15}H_{17}O_9$ $[M+H]^+$ (calcd 341.0867). Esculin-2-$^{18}$O signifies *m/z* found of esculin-2-$^{18}$O, $C_{15}H_{17}O_8{}^{18}O$-$[^{18}O,M+H]^+$ (calcd 343.0910).

accurate mass of scopoletin derived from $^{18}$O-labelled esculetin (Table 7). Experimental error was evaluated by calculating the $^{13}$C-natural abundance of ten (unlabelled) scopoletin samples. The percentage of scopoletin containing $1\times{}^{13}$C was $7.7\pm0.6$% (s.d.) and that of $2\times{}^{13}$C-labelled scopoletin was $1.27\pm0.05$% (s.d.), for example, found 195.0557 and 195.0562, calculated as 195.0562 for $C_8{}^{13}C_2H_9{}^{16}O_4$. Furthermore, this small $[M+2]$ isotope peak for scopoletin is not due to the presence of (for example) $C_{10}H_9{}^{16}O_3{}^{18}O$ calculated as 195.0538, an argument which we develop further below with respect to results obtained from feeding experiments with $C^{18}O_2$-labelled (*E*)-cinnamic and ferulic acids. The low level of error in these reproducible feeding experiments, for example, from umbelliferone-2-$^{18}$O, allows us to assign (Table 7) 195.0524 as $C_{10}H_9O_3{}^{18}O$ which requires 195.0538 (and therefore it is not 195.0562 for $C_8{}^{13}C_2H_9{}^{16}O_4$), so calculating only 0.4% of $^{18}$O-enriched scopoletin.

The incorporation of umbelliferone into esculin (Table 8) suggests that pathway 1 (Scheme 5) operates for the biosynthesis of esculetin with only a small level of *O*-methylation to scopoletin. Pathway 2 cannot be excluded. The key first intermediate in pathway 1, 2′,4′-dihydroxycinnamic acid, was investigated in a competition experiment with (*E*)-[$D_7$]cinnamic acid in a further attempt to elucidate the pathway.

*Competition feeding experiments with deuterium-labelled cinnamic acid and unlabelled intermediates*: Competition experiments were carried out by feeding the root samples with (*E*)-[$D_7$]cinnamic acid alone as a control, and with (*E*)-[$D_7$]-cinnamic together with unlabelled 2′,4′-dihydroxycinnamic, ferulic or caffeic acids. It was postulated[6–8,12] that if any of these unlabelled compounds were intermediates in the biosynthesis of scopoletin, they would compete with (*E*)-[$D_7$]cinnamic acid and decrease the amount of deuterium labelled scopoletin produced. The results of these experiments are summarized in Tables 9 and 10.

A further competition experiment was carried out between labelled (*E*)-cinnamic-2′,3′,4′,5′,6′-[$D_5$]acid and umbelliferone or esculetin, which are in




**Scheme 5.** Results of feeding experiments supporting pathway 3 as the major pathway for the biosynthesis of scopoletin in cassava roots during PPD.

**Table 9.** HR MS data of the HPLC peak at 7.1 min showing the percentage of labelled scopolin.

| Feeding cassava cv MCOL 22 with | Scopolin[a] | [$D_3$]Scopolin | % of [$D_7$]scopolin |
|---|---|---|---|
| [$D_7$]cinnamic acid | 355.1027 | 358.1222 | 7.8 |
| [$D_7$]cinnamic + ferulic acids | 355.1037 | 358.1224 | 8.1 |
| [$D_7$]cinnamic + caffeic acids | 355.1038 | 358.1217 | 14.1 |
| [$D_7$]cinnamic + 2′,4′-dihydroxycinnamic acids | 355.1037 | 358.1224 | 7.5 |

[a] Scopolin signifies *m/z* found of scopolin, $C_{16}H_{19}O_9$ $[M+H]^+$ (calcd 355.1024). [$D_3$]Scopolin signifies *m/z* found of [$D_3$]scopoletin, $C_{16}H_{16}D_3O_9$ [$D_3$,$M$+H]$^+$ (calcd 358.1212).

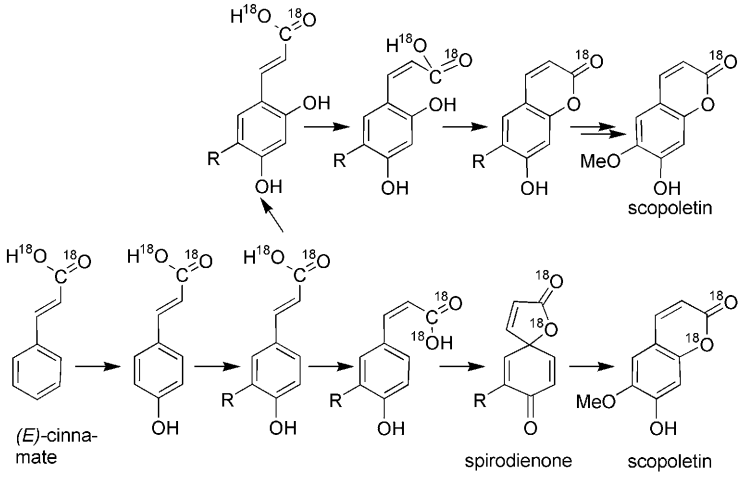
**Table 10.** HR MS data of the HPLC peak at 27.4 min showing the percentage of labelled scopoletin.

| Feeding cassava cv MCOL 22 with | Scopoletin[a] | [$D_3$]Scopoletin | % of [$D_3$]scopoletin |
|---|---|---|---|
| [$D_7$]cinnamic acid | 193.0502 | 196.0694 | 5.8 |
| [$D_7$]cinnamic + ferulic acids | 193.0489 | 196.0687 | 6.0 |
| [$D_7$]cinnamic + caffeic acids | 193.0496 | 196.0692 | 8.3 |
| [$D_7$]cinnamic + 2′,4′-dihydroxycinnamic acids | 193.0500 | 196.0688 | 4.3 |

[a] Scopoletin signifies *m/z* found of scopoletin, $C_{10}H_9O_4$ $[M+H]^+$ (calcd 193.0495). [$D_3$]Scopoletin signifies *m/z* found of [$D_3$]scopoletin, $C_{10}H_6D_3O_4$ [$D_3$,$M$+H]$^+$ (calcd 196.0684).

the same pathway (1) as 2′,4′-dihydroxycinnamate (Scheme 5). The results of these experiments are summarized in Tables 11 and 12. Only minor reductions in the enrichment of labelled scopolin and scopoletin were observed with 2′,4′-dihydroxycinnamate and umbelliferone; this confirmed the minor role of this pathway in the biosynthesis of scopoletin. The presence of unlabelled caffeic acid (Tables 9 and 10) or esculetin (Tables 11 and 12) resulted in an unexpected increase in the percentage of labelling in scopoletin and scopolin. It is possible that this effect arises from the inhibition of PAL,[33] thus reducing the flux of unlabelled substrate through the pathway.

*Feeding experiments with C$^{18}O_2$-labelled* (E)*-cinnamic and ferulic acids*: If the biosynthesis of scopoletin occurs via a spirodienone intermediate, then feeding experiments with C$^{18}O_2$-enriched (*E*)-cinnamic acid would yield $^{18}O_2$-scopoletin enriched in both the lactone ether and carbonyl oxygen atoms, whereas biosynthesis through 2′-hydroxylation of 4′-hydroxycinnamic acid would yield $^{18}O_1$-scopoletin enriched only in the carbonyl oxygen atom (Scheme 6). Both doubly $^{18}$O-labelled (*E*)-cinnamic and ferulic acids were incorporated only into scopoletin-2-$^{18}$O and scopolin-2-$^{18}$O, that is, scopoletin and scopolin enriched only in the carbonyl oxygen. No HR MS peaks corresponding to $C_{10}H_9{}^{16}O_2{}^{18}O_2$ or $C_{16}H_{19}{}^{16}O_7{}^{18}O_2$ were found; this is evidence that their biosynthesis in cassava roots




**Scheme 6.** Pathways from feeding C$^{18}O_2$-enriched (*E*)-cinnamic acid depending on the route of the biosynthesis of scopoletin in cassava roots.

**Table 11.** HR ESI MS data of peak 1 at 7.1 min showing the percentage of labelled scopolin.

| Feeding cassava cv MCOL 22 with | Scopolin[a] | [$D_2$]Scopolin | % of [$D_2$]scopolin |
|---|---|---|---|
| [$D_5$]cinnamic acid | 355.1018 | 357.1150 | 16.8 |
| [$D_5$]cinnamic acid + umbelliferone | 355.1036 | 357.1174 | 14.0 |
| [$D_5$]cinnamic acid + esculetin | 355.1028 | 357.1150 | 25.8 |

[a] Scopolin signifies *m/z* found of scopolin, $C_{16}H_{19}O_9$ $[M+H]^+$ (calcd 355.1024). [$D_2$]scopoletin signifies *m/z* found of [$D_2$]scopoletin, $C_{16}H_{17}D_2O_9$ [$D_2$,$M$+H]$^+$ (calcd 357.1149).

**Table 12.** HR ESI MS data of peak 2 at 24.7 min showing the percentage of labelled scopoletin.

| Feeding cassava cv MCOL 22 with | Scopoletin[a] | [$D_2$]Scopoletin | % of [$D_2$]scopoletin |
|---|---|---|---|
| [$D_5$]cinnamic acid | 193.0501 | 195.0633 | 8.1 |
| [$D_5$]cinnamic acid + umbelliferone | 193.0502 | 195.0623 | 4.8 |
| [$D_5$]cinnamic acid + esculetin | 193.0503 | 195.0631 | 14.9 |

[a] Scopoletin signifies *m/z* found of scopoletin, $C_{10}H_9O_4$ $[M+H]^+$ (calcd 193.0495). [$D_2$]Scopoletin signifies *m/z* found of [$D_2$]scopoletin, $C_{10}H_7D_2O_4$ [$D_2$,$M$+H]$^+$ (calcd 195.0621).

during PPD occurs through *o*-hydroxylation and not via a spirodienone intermediate.

The accurate mass of the [$M$+2] isotope peak for scopoletin obtained from feeding experiments with C$^{18}O_2$-labelled (*E*)-cinnamic and ferulic acids was found to 195.0541 or 195.0543, agreeing with the value calculated for $C_{10}H_9{}^{16}O_3{}^{18}O$ (195.0538). The [$M$+2] peak is not due to the presence of two $^{13}$C-isotopes at natural abundance, which is calculated as 195.0562 for $C_8{}^{13}C_2H_9{}^{16}O_4$. From Einstein's Theory of Relativity, $E=mc^2$, some of the energy used for holding the extra neutrons in the nucleus has come from the mass. Thus, although at a superficial

level adding two neutrons to make $^{18}O$ from $^{16}O$ looks like adding any other two neutrons, for example, incorporating 2× $^{13}C$ or 2×D, it is not the same mass gain, and this small, but measurable difference is called the mass defect.

*Feeding experiments under an atmosphere of 20% $^{18}O_2$*: The doubly $^{18}O$-labelled cinnamic acid could lose one $^{18}O$ atom during the pathway to scopoletin by conversion into its CoA ester or into the shikimate or quinate ester;[6] such esters have recently been shown to be involved during the insertion of the 3′-hydroxy group in several species.[6] Although ferulate is a later component of the pathway, the involvement of such an ester of ferulate in the *E–Z* isomerisation step cannot be ruled out. In order to confirm our interpretation of the above results, feeding experiments under an atmosphere rich in $^{18}O_2$ were designed and carried out by using a vacuum desiccator evacuated to 10–20 mbar (1 mbar is 100 Pa, 100 $N m^{-2}$) and then filled with anhydrous nitrogen to a pressure of only 800 mbar, then taken to 1000 mbar with $^{18}O_2$ to afford an atmosphere of approximately $N_2/^{18}O_2$ 4:1 (*v*/*v*).

It has been established that both 4′-hydroxylation and 3′-hydroxylation of cinnamate involve cytochrome P450 enzymes[2] and that the inserted oxygen atom is derived from molecular oxygen. 2′-Hydroxylation is thought to involve a $Fe^{II}$- and 2-oxoglutarate-dependent dioxygenase[12] which also utilises molecular oxygen. Thus, if the biosynthesis of scopoletin occurs via a spirodienone intermediate, feeding experiments under $^{18}O_2$ would yield $^{18}O_2$-enriched scopoletin, doubly labelled in the methoxy and hydroxy oxygen groups, whereas biosynthesis through *o*-hydroxylation would yield $^{18}O_3$-enriched scopoletin, labelled in the methoxy, hydroxy and lactone ether oxygen atoms (Scheme 7). The results of scopoletin biosynthesis under $^{18}O_2$ and of feeding cassava roots with [$D_7$]cinnamic acid under $^{18}O_2$ are summarized in Tables 13 and 14, and two representative HR mass spectra are shown in Figure 1.

These results (Figure 1) unequivocally show that the major isotopic peak was $^{18}O_3$-enriched scopoletin, and thus the major pathway in the biosynthesis of scopoletin in cassava roots during PPD is through *o*-hydroxylation not via a spirolactone-

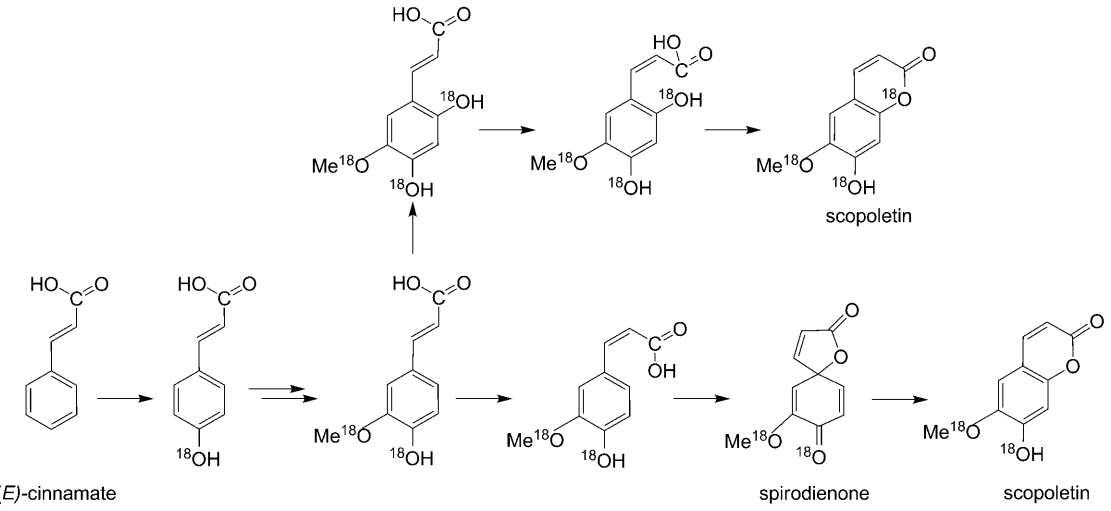
**Table 13.** HR ESI MS data of scopoletin showing the percentage of labelled scopoletin when cassava cv MNGA 2 was stored under 20% $^{18}O_2$.

| Scopoletin and its isotope peak monitored $[M+H]^+$ | *m/z* found of labelled scopoletin | Calcd | % of scopoletin isotopomers |
|---|---|---|---|
| $C_{10}H_9{}^{16}O_4$ | 193.0496 | 193.0495 | 8.8 |
| $C_{10}H_9O^{16}O_3{}^{18}O$ | 195.0544 | 195.0538 | 10.7 |
| $C_{10}H_9{}^{16}O_2{}^{18}O_2$ | 197.0581 | 197.0580 | 33.3 |
| $C_{10}H_9{}^{16}O^{18}O_3$ | 199.0624 | 199.0623 | 47.2 |

**Table 14.** HR ESI MS data of scopoletin showing the percentage of labelled scopoletin when cassava cv MNGA 2 was fed with [$D_7$]cinnamic acid under $^{18}O_2$.

| Scopoletin and its isotope peak monitored $[M+H]^+$ | *m/z* found of labelled scopoletin | Calcd | % of scopoletin isotopomers |
|---|---|---|---|
| $C_{10}H_9{}^{16}O_4$ | 193.0502 | 193.0495 | 2.3 |
| $C_{10}H_9{}^{16}O_3{}^{18}O$ | 195.0571 | 195.0538 | 1.8 |
| $C_{10}H_9{}^{16}O_2{}^{18}O_2$ | 197.0606 | 197.0580 | 4.5 |
| $C_{10}H_9{}^{16}O^{18}O_3$ | 199.0656 | 199.0623 | 25.2 |
| $C_{10}H_6D_3{}^{16}O_4$ | 196.0684 | 196.0684 | 3.2 |
| $C_{10}H_6\ D_3{}^{16}O_3{}^{18}O$ | 198.0719 | 198.0726 | 9.9 |
| $C_{10}H_6\ D_3{}^{16}O_2{}^{18}O_2$ | 200.0768 | 200.0769 | 24.1 |
| $C_{10}H_6\ D_3{}^{16}O^{18}O_3$ | 202.0804 | 202.0811 | 29.0 |

dienone intermediate. Although there is $^{18}O_2$-enriched scopoletin, this is mainly due to the presence of a low percentage of residual air (that is, of $^{16}O_2$) trapped in the plant material. This was confirmed by the presence of small amounts of both unlabelled scopoletin (2.3%) and $^{18}O_1$-enriched scopoletin (1.8%, Table 14). Feeding cassava roots with [$D_7$]cinnamic acid under $^{18}O_2$ resulted in the formation of a small amount of $^{16}O_4$-[$D_3$]scopoletin (9.9%), and there was a similar pattern of $^{18}O$-labelling superimposed on [$D_3$]scopoletin. Thus, as well as the definitive $[M+6]^+$ peak for $^{18}O_3$-scopoletin $[M+H]^+$ (Figure 1A), the corresponding $[M+6]^+$ peak for $^{18}O_3$-[$D_3$]scopoletin $[M+Na]^+$ (Figure 1B) is also observed with a sufficiently satis-




**Scheme 7.** Possible biosynthetic routes to scopoletin in cassava roots in the presence of $^{18}O_2$.

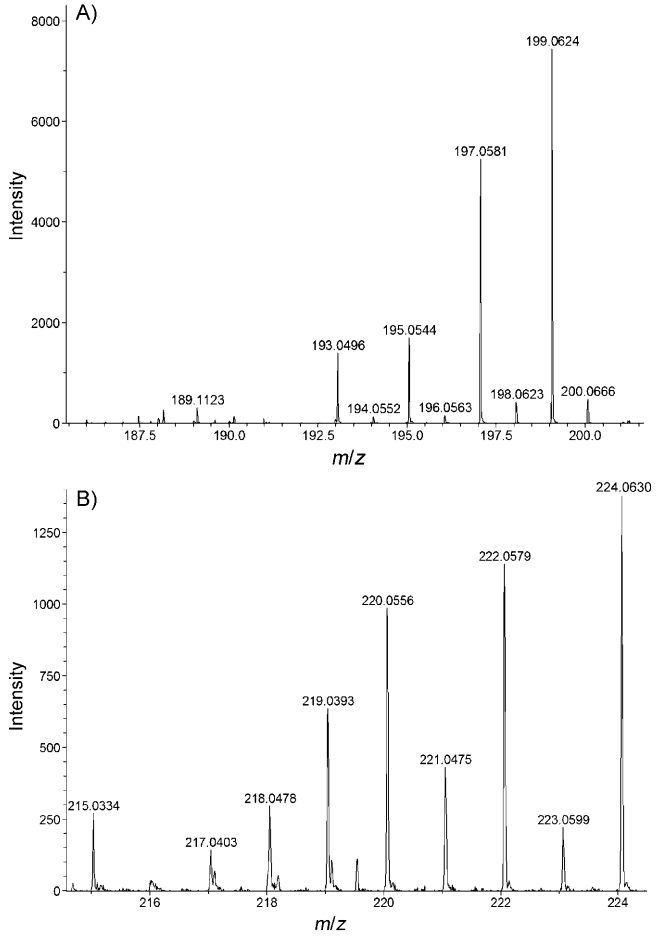



**Figure 1.** A) Mass spectrum of scopoletin $[M+H]^+$ after allowing PPD in 20% $^{18}O_2$. B) Mass spectrum of scopoletin $[M+Na]^+$ after feeding with $[D_7]$cinnamic acid in 20% $^{18}O_2$.

factorily high resolution (within 5 ppm) as to be unambiguous, HR MS found 224.0630, $C_{10}H_5D_3Na^{16}O^{18}O_3$ requires 224.0630.

## Conclusions

The accumulation of hydroxycoumarins in cassava roots during PPD makes cassava a good model for studying the biosynthesis of scopoletin and scopolin by using various stable isotopic intermediates along the biosynthetic pathway. The major pathway for the biosynthesis of scopoletin and scopolin in cassava roots during PPD was shown to be via *p*-coumaric, caffeic and then ferulic acids, which are *o*-hydroxylated, isomerised and lactonised into scopoletin and then glucosylated into scopolin (pathway 3, Scheme 5). Although we have no evidence for the order of the *o*-hydroxylation and *E–Z* isomerisation steps, the well-known facile closure of δ-lactones makes lactonisation a rapid step once the *Z* geometry is established in the presence of a 2′-hydroxyl group. The biosynthesis of esculetin and esculin is via 2′,4′-dihydroxycinnamic acid and then umbelliferone. Methylation of esculetin to scopoletin occurs only to a small extent.

## Experimental Section

**Plant material**: Root tubers of different cultivars (MCOL 22, MNGA 19 and MNGA 2) were harvested from cassava plants growing in the tropical glass house at the University of Bath under the following conditions: 22–28 °C, relative humidity (R.H.; 40–80%) and a light period of 14 h per day. The cultivar MCOL 22 shows high susceptibility to PPD, MNGA 2 shows medium susceptibility, MNGA 19 has not been determined according to the Centro Internacional de Agricultura Tropical (CIAT, Colombia).

**General methods**: Chemicals were obtained routinely from Sigma–Aldrich, except (*E*)-2′,3′,4′,5′,6′-$[D_5]$cinnamic acid, which was obtained from CDN Isotopes (Pointe-Claire, Québec, Canada) and $H_2{}^{18}O$ 95 atom% $^{18}O$ from CK GAS Products Ltd. (Hook, UK). The HPLC instrument was a Jasco PU-980 pump, monitored at 360 nm with a Jasco UV-975 detector, using 16% acetonitrile/84% aq. formic acid (0.1%), flow rate 4 mL min$^{-1}$ at 20 °C. HPLC data were recorded on a Goerz Metrawatt Servogor 120 recorder, HPLC columns were purchased from Phenomenex Inc.: Phenomenex Gemini 10 μ C18 110A 250×10 mm with guard column Phenomenex Gemini 5 μ C18 10×10 mm. Samples were injected by using a 100 μL loop. HR ESI MS was carried out on a Bruker microTOF mass spectrometer in the Department of Pharmacy and Pharmacology, University of Bath or on a Micromass Quattro II in the EPSRC National Mass Spectrometry Service Centre, University of Wales, Swansea. NMR spectra were obtained on a Varian Mercury Spectrometer at 400 MHz ($^1$H) or 100 MHz ($^{13}$C) in $CD_3OD$, all chemical shifts are reported in parts per million (ppm) relative to internal tetramethylsilane, and coupling constants (*J*) are absolute values in Hz.

**General feeding procedure**: Cassava roots (typically 1 kg) were peeled, then cut into approximately 1 cm$^3$ cubes and divided into groups (typically 100 g). One group was immediately crushed and extracted with EtOH (fresh cassava extract) and another (control group) was stored under controlled conditions (20 °C, 80–90% R.H.) until crushing and extraction. Where exogenous substrates were introduced, this was by spraying a group with the substrate (typically 30 mg dissolved in aq. 4% $Na_2CO_3$ (3 mL) then adjusted to pH 7.5 with 1 M HCl) with a simple hand-pumped aerosoliser. As the highest amount of scopoletin and scopolin accumulation occurs between days 3 and 4 (this is cultivar dependent[3]), half the group was crushed and macerated after three days (EtOH, 200 mL, 3 days), and the other half was crushed after 4 days and then macerated (EtOH, 200 mL, 2 days). The combined ethanolic extracts were filtered. A second round of maceration (EtOH, 400 mL, 2 days) was performed. The combined EtOH extracts, were filtered and evaporated under reduced pressure at 35–40 °C. The residue was dissolved in MeOH (0.2 g mL$^{-1}$) and purified by HPLC. From repeat injections ($n=12$, each of 100 μL) two peaks of retention time 7.1 min and 24.7 min (scopolin and scopoletin respectively, the latter by comparison with a commercial authentic sample) were detected, collected, concentrated, dissolved in MeOH and identified by HR ESI MS, in order to separate isotopes and to identify the percentage of labelled scopoletin and scopolin in relation to the unlabelled coumarins.

**Synthesis of *p*-coumaric-2-$^{13}$C acid, caffeic-2-$^{13}$C acid and ferulic-2-$^{13}$C acid**: *p*-Hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde or vanillin (4-hydroxy-3-methoxybenzaldehyde) (1.2 mmol) and malonic-2-$^{13}$C acid (99 atom% $^{13}$C; 276 mg, 2.63 mmol) were dissolved in pyridine (660 μL). Piperidine (13 μL, 130 nmol; 0.00011 equiv) was added, the reaction was heated to 70 °C (oil bath) for 24 h. Water (7.5 mL) and then concentrated HCl (0.4 mL) were added

dropwise until a precipitate appeared, which was collected and recrystallised. The product purity was checked by TLC with *n*-hexane/ethyl acetate/acetic acid (1:1:0.01, *v/v/v*).

*p*-Coumaric-2-$^{13}$C acid: white crystals; m.p. 208–210 °C ($H_2O$; 61% relative to *p*-hydroxybenzaldehyde); TLC: $R_f = 0.36$; $^1$H NMR and $^{13}$C NMR data: see Tables 1 and 2; $C_8{}^{13}CH_7O_3$ requires 164.0434, HR ESI MS gave *m/z* 164.0434 $[M-H]^-$.

Caffeic-2-$^{13}$C acid: yellowish-white crystals; m.p. 217–220 °C ($H_2O$; 64% relative to 3,4-dihydroxybenzaldehyde); TLC: $R_f = 0.22$; $^1$H NMR and $^{13}$C NMR data: see Tables 1 and 2; $C_8{}^{13}CH_7O_4$ requires 180.0383, HR ESI MS gave *m/z* 180.0381 $[M-H]^-$.

Ferulic-2-$^{13}$C acid: white crystals; m.p. 168–171 °C ($H_2O$; 64% relative to vanillin); TLC: $R_f = 0.35$; $^1$H NMR and $^{13}$C NMR data: see Tables 1 and 2; $C_9{}^{13}CH_9O_4$ requires 194.0540, HR ESI MS gave *m/z* 194.0538 $[M-H]^-$.

**$^{18}$O enrichment of umbelliferone and esculetin**: Following the literature procedure,[34,35] umbelliferone (20 mg, 0.124 mmol) or esculetin (20 mg, 0.112 mmol), $H_2{}^{18}O$ (0.5 mL, 95 atom% $^{18}$O) and conc. HCl (10 μL) in acetonitrile (1 mL) were maintained at 70 °C in a sealed tube for 3 h, 4 days, and 7 days. $^{18}$O-enriched umbelliferone: $C_9H_7O_2{}^{18}O$ requires 165.0432, HR ESI MS found 165.0430 $[M+H]^+$, $C_9H_7O_3$ requires 163.0390, found 163.0430 $[M+H]^+$. $^{18}$O-enriched esculetin: $C_9H_7O_3{}^{18}O$ requires 181.0381, HR MS found 181.0373 $[M+H]^+$ and $C_9H_7O_4$ requires 179.0339, found 179.0328 $[M+H]^+$. Percentage of $^{18}$O-enrichment of umbelliferone (% of *m/z* 165/163) was 8% (after 3 h), 61% (after 4 days) and 82% (after 7 days). Percentage of $^{18}$O-enrichment of esculetin was 82% (after 7 days).

**$^{18}$O enrichment of (*E*)-cinnamic acid and ferulic acid**: (*E*)-Cinnamic acid (20 mg, 0.135 mmol) or ferulic acid (20 mg, 0.103 mmol), $H_2{}^{18}O$ (0.5 mL, 95 atom% $^{18}$O) and conc. HCl (10 μL) in acetonitrile (1 mL) were maintained at 70 °C in a sealed tube in for 3 h, 1 day, 2 days and 4 days. $^{18}$O-enriched (*E*)-cinnamic acid after 4 days: $C_9H_9O^{18}O$ requires 151.0640, HR ESI MS found 151.0662 $[M+H]^+$ and $C_9H_9{}^{18}O_2$ requires 153.0682, found 153.0687 $[M+H]^+$. The percentage of $^{18}$O-enrichment of (*E*)-cinnamic acid after each period is listed in Table 3. $^{18}$O-enriched (*E*)-ferulic acid after 4 days: $C_{10}H_{11}O_4$ requires 195.0652, HR MS found 195.0661 $[M+H]^+$ 2%, $C_{10}H_{11}O_3{}^{18}O$ requires 197.0694, found 197.0705 $[M+H]^+$ 20%, and $C_{10}H_{11}O_2{}^{18}O_2$ requires 199.0737, found 199.0743 $[M+H]^+$ 78%.

**Feeding experiments with *p*-coumaric-2-$^{13}$C acid, caffeic-2-$^{13}$C acid and ferulic-2-$^{13}$C acid**: By using the general feeding procedure, cassava roots (0.8 kg, cv MCOL 22) were peeled (0.64 kg) and divided into four groups, one as a control (40 g, the roots left to deteriorate for 3 and 4 days without feeding with any intermediates) and three equal groups (85 g), fed with *p*-coumaric-2-$^{13}$C acid, caffeic-2-$^{13}$C acid or ferulic-2-$^{13}$C acid (20 mg of each acid) dissolved in aq. 4% $Na_2CO_3$ (2 mL). A representative sample of the combined EtOH extract (2 g) was then purified by HPLC. HR MS data of the HPLC peaks at 7.1 and 24.7 min of scopolin and scopoletin are listed in Tables 4 and 5.

**Feeding experiments with $^{18}$O-labelled esculetin and umbelliferone and ferulic-2-$^{13}$C acid**: By using the general feeding procedure, cassava roots (0.69 kg, cv MNGA 19) were peeled (0.49 kg) and divided into three equal groups (85 g). One was fed with $^{18}$O-carbonyl-labelled esculetin (20 mg), one with $^{18}$O-carbonyl-labelled umbelliferone (20 mg), and the other with ferulic-2-$^{13}$C acid (20 mg). Each sample was dissolved in DMSO (1 mL), then diluted with water (1 mL). A representative sample of the combined EtOH extract (2 g, except group 1: 0.7 g) was then purified by HPLC. HR MS data of the HPLC peaks at 7.1, 24.7 and 6.0 min for scopolin, scopoletin and esculin, respectively, are listed in Tables 6, 7 and 8.

**Competition feeding experiments between deuterium labelled (*E*)-[$D_7$]cinnamic acid and unlabelled intermediates**: By using the general feeding procedure, cassava roots (1.23 kg, cv MCOL 22) were peeled (1 kg) and divided into four equal groups, the first group was fed with (*E*)-[$D_7$]cinnamic acid (30 mg) dissolved in aq. 4% $Na_2CO_3$ (3 mL). The second, third and fourth groups were fed with (*E*)-[$D_7$]cinnamic acid (30 mg) dissolved in aq. 4% $Na_2CO_3$ (3 mL), then unlabelled caffeic, ferulic or (*E*)-2′,4′-dihydroxycinnamic acid (30 mg) prepared in the same way as the substrate. A representative sample of the combined EtOH extract (4 g) was then purified by HPLC. HR MS data of the HPLC peaks at 7.1 and 24.7 min of scopolin and scopoletin are listed in Tables 9 and 10.

**Competition feeding experiments between deuterium-labelled (*E*)-2′,3′,4′,5′,6′-[$D_5$]cinnamic acid and unlabelled umbelliferone and esculetin**: By using the general feeding procedure, cassava roots (0.68 kg, cv MCOL 22) were peeled (0.46 kg) and divided into three equal groups (115 g), the first was fed with (*E*)-[$D_5$]cinnamic acid (20 mg) dissolved in DMSO (1 mL), then water (1 mL) was added. The second group was fed with (*E*)-[$D_5$]cinnamic acid (20 mg) and umbelliferone (20 mg) dissolved in DMSO (1 mL), then water (1 mL) was added. The third group was fed with (*E*)-[$D_5$]cinnamic acid (20 mg) and esculetin (20 mg) dissolved in DMSO (1 mL), then water (1 mL) was added. A representative sample of the combined EtOH extract (3.5 g) was then purified by HPLC. HR MS data of the HPLC peaks at 7.1 (scopolin) and 24.7 min (scopoletin) are listed in Tables 11 and 12.

**Feeding experiments with (*E*)-C$^{18}O_2$-cinnamic acid and C$^{18}O_2$-ferulic acid**: By using the general feeding procedure, cassava roots (0.69 kg, cv MNGA 19) were peeled (0.49 kg). One group (85 g) was fed with C$^{18}O_2$-(*E*)-cinnamic acid (20 mg), and the other group (65 g) was fed with C$^{18}O_2$-ferulic acid (10 mg) dissolved in aq. 4% $Na_2CO_3$ (2 mL). A representative sample of the combined EtOH extract (2 g) was then purified by HPLC. From the feeding experiments with C$^{18}O_2$-(*E*)-cinnamic acid, the HPLC peak at 7.1 mins (scopolin), $C_{16}H_{19}O_9$ requires 355.1024, HR MS *m/z* found 355.1039 $[M+H]^+$ and $C_{16}H_{19}O_8{}^{18}O$ requires 357.1066, found 357.1082 $[M+H]^+$. The percentage of labelled scopolin was 12%. The HPLC peak at 24.7 min (scopoletin), naturally occurring scopoletin $C_{10}H_9O_4$ requires 193.0495, HR MS *m/z* found 193.0494 $[M+H]^+$ and $C_{10}H_9O_3{}^{18}O$ requires 195.0538, found 195.0543 $[M+H]^+$. The percentage of labelled scopoletin was 4.8%.

From the feeding experiments with C$^{18}O_2$-ferulic acid, the HPLC peak at 24.7 min (scopoletin), naturally occurring scopoletin $C_{10}H_9O_4$ requires 193.0495, HR MS *m/z* found 193.0489 $[M+H]^+$ and $C_{10}H_9O_3{}^{18}O$ requires 195.0538, found 195.0541 $[M+H]^+$. The percentage of labelled scopoletin was 3.7%.

**Feeding experiments under an atmosphere of $^{18}O_2$**: Cassava roots (0.38 kg, cv MNGA 2) were peeled (0.30 kg) and divided into three equal groups (85 g), the first and second groups were both fed with (*E*)-[$D_7$]cinnamic acid (20 mg) dissolved in aq. 4% $Na_2CO_3$ (2 mL); no exogenous substrate was added to the third group. The first and third groups were placed in a vacuum desiccator, which was immediately evacuated by using a vacuubrand PC 2001 VARIO vacuum pump, with a vacuubrand cvc 2 pressure monitor, until a final pressure of 10–20 mbar was reached, and was then back-flushed with anhydrous nitrogen. This procedure was repeated twice. After further evacuation, the desiccator was filled with anhydrous nitrogen to a pressure of only 800 mbar and then taken to 1000 mbar with $^{18}O_2$ to afford an atmosphere of approximately $N_2$/

$^{18}O_2$ 4:1 (*v*/*v*). The second group was allowed to deteriorate under normal aerobic conditions. All three groups were allowed to deteriorate at 20 °C for 5 days, and then all were extracted according to the general procedure. A representative sample of the EtOH extract (2 g) was then purified by HPLC. HR MS data of the HPLC peak at 24.7 min are listed in Table 13 for $^{18}O_3$-scopoletin (fed under $^{18}O_2$, group 3) and 14 for $^{18}O_3$-[$D_3$]scopoletin (fed with [$D_7$]cinnamic acid under $^{18}O_2$, group 1). The results from group 2 (fed with [$D_7$]cinnamic acid under normal air) were as reported above, and typical of the results in Table 10.

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[1] M. Petersen, D. Strack, U. Matern in *Biochemistry of Plant Secondary Metabolism* (Ed.: M. Wink), Academic Press, Sheffield, **1999**, pp. 151–185.
[2] F. Bourgaud, A. Hehn, R. Larbat, S. Doerper, E. Gontier, S. Kellner, U. Matern, *Phytochem. Rev.* **2006**, *5*, 293–308.
[3] H. Buschmann, M. X. Rodriguez, J. Tohme, J. R. Beeching, *Ann. Bot.* **2000**, *86*, 1153–1160.
[4] K. Reilly, R. Gomez-Vasquez, H. Buschmann, J. Tohme, J. R. Beeching, *Plant Mol. Biol.* **2003**, *53*, 669–685.
[5] K. Reilly, D. Bernal, D. F. Cortes, R. Gomez-Vasquez, J. Tohme, J. R. Beeching, *Plant Mol. Biol.* **2007**, *64*, 187–203.
[6] K. Kai, B. Shimizu, M. Mizutani, K. Watanabe, K. Sakata, *Phytochemistry* **2006**, *67*, 379–386.
[7] S. A. Brown, D. E. A. Rivett, H. J. Thompson, *Z. Naturforsch. C: Biosci.* **1984**, *39*, 31–37.
[8] S. A. Brown, R. E. March, D. E. A. Rivett, H. J. Thompson, *Phytochemistry* **1988**, *27*, 391–395.
[9] S. A. Brown, *Can. J. Biochem. Cell Biol.* **1985**, *63*, 292–295.
[10] V. C. Runeckles, *Can. J. Biochem. Physiol.* **1963**, *41*, 2259–2267.
[11] B. Fritig, L. Hirth, G. Ourisson, *Phytochemistry* **1970**, *9*, 1963–1975.
[12] K. Kai, M. Mizutani, N. Kawamura, R. Yamamoto, M. Tamai, H. Yamaguchi, K. Sakata, B.-I. Shimizu, *Plant J.* **2008**, *55*, 989–999.
[13] S. A. L. Bayoumi, M. G. Rowan, I. S. Blagbrough, J. R. Beeching, *Phytochemistry* **2008**; DOI: 10.1016/j.phytochem.2008.09.023.
[14] C. A. Bunton, G. W. Kenner, M. J. Robinson, B. R. Webster, *Tetrahedron* **1963**, *19*, 1001–1010.
[15] U. Matern, *Planta Med.* **1991**, *57*, S15–S20.
[16] H. Grisebach, W. D. Ollis, *Experientia* **1961**, *17*, 4.
[17] H. L. Schmidt, R. A. Werner, W. Eisenreich, C. Fuganti, G. Fronza, G. Remaud, R. J. Robins, *Phytochemistry* **2006**, *67*, 1094–1103.
[18] B. E. Ellis, N. Amrhein, *Phytochemistry* **1971**, *10*, 3069–3072.
[19] J. Shah, *Curr. Opin. Plant Biol.* **2003**, *6*, 365–371.
[20] J. Catinot, A. Buchala, E. Abou-Mansour, J. P. Metraux, *FEBS Lett.* **2008**, *582*, 473–478.
[21] J. A. Pallas, N. L. Paiva, C. Lamb, R. A. Dixon, *Plant J.* **1996**, *10*, 281–293.
[22] K. T. Watts, P. C. Lee, C. Schmidt-Dannert, *ChemBioChem* **2004**, *5*, 500–507.
[23] C. Hertweck, A. P. Jarvis, L. K. Xiang, B. S. Moore, N. J. Oldham, *ChemBioChem* **2001**, *2*, 784–786.
[24] J. Leon, V. Shulaev, N. Yalpani, M. A. Lawton, I. Raskin, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 10413–10417.
[25] R. J. Robbins, W. F. Schmidt, *J. Labelled Compd. Radiopharm.* **2004**, *47*, 797–806.
[26] R. Ji, Z. X. Chen, P. F. X. Corvini, A. Kappler, A. Brune, K. Haider, A. Schaffer, *Chemosphere* **2005**, *60*, 1169–1181.
[27] D. Williams, I. Fleming, *Spectroscopic Methods in Organic Chemistry*, 6th ed., McGraw–Hill, Maidenhead, **2008**, pp. 162–163.
[28] W. Koźmiński, D. Nanz, *J. Magn. Reson. Ser. A* **1996**, *122*, 245–247.
[29] B. Reif, M. Kock, R. Kerssebaum, J. Schleucher, C. Griesinger, *J. Magn. Reson. Ser. B* **1996**, *112*, 295–301.
[30] T. N. Pham, K. E. Kover, L. Jin, D. Uhrin, *J. Magn. Reson.* **2005**, *176*, 199–206.
[31] G. B. Ma, S. E. Hayes, *J. Labelled Compd. Radiopharm.* **2004**, *47*, 895–901.
[32] M. A. Bernards, W. D. Fleming, D. B. Llewellyn, R. Priefer, X. Yang, A. Sabatino, G. L. Plourde, *Plant Physiol.* **1999**, *121*, 135–145.
[33] T. Sato, F. Kiuchi, U. Sankawa, *Phytochemistry* **1982**, *21*, 845–850.
[34] S. Chandrasekaran, W. D. Wilson, D. W. Boykin, *Org. Magn. Reson.* **1984**, *22*, 757–760.
[35] G. Kollenz, H. Sterk, G. Hutter, *J. Org. Chem.* **1991**, *56*, 235–239.

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