Metabolic analysis of the cinnamate/monolignol pathway in *Carthamus tinctorius* seeds by a stable-isotope-dilution method

Norikazu Sakakibara,†[a] Tomoyuki Nakatsubo,[a] Shiro Suzuki,‡[a] Daisuke Shibata,[b] Mikio Shimada§[a] and Toshiaki Umezawa*[a]

Received 15th November 2006, Accepted 8th January 2007
First published as an Advance Article on the web 29th January 2007
DOI: 10.1039/b616705e

The present study established a system for comprehensive metabolic analysis of the cinnamate/monolignol and lignan pathways by the use of a stable-isotope-dilution method. The system was successfully applied to characterization of the pathways in *Carthamus tinctorius* cv. Round-leaved White maturing seeds in combination with administration of stable-isotope-labelled precursors. Experimental results obtained using this technique strongly suggested the intermediacy of ferulic acid in lignan biosynthesis in the plant.

Introduction

The cinnamate/monolignol pathway, in which monolignols are biosynthesized from cinnamic acids, supplies precursors for various phenylpropanoid compounds such as lignins, lignans, neolignans, norlignans, flavonoids, and stilbenes.[1–3] As illustrated in Fig. 1, many parallel routes can be envisaged for this pathway, and it is very difficult to determine the physiologically important routes among the various possibilities.

For example, the pathway towards sinapyl alcohol (**15**) *via* ferulic acid (**3**), sinapic acid (**5**), and sinapaldehyde (**10**) had long been proposed for angiosperm syringyl lignin biosynthesis.[4] This was based mostly on the fact that administration of ^{14}C-labelled *p*-hydroxycinnamic acids such as *p*-coumaric (**1**), caffeic (**2**), ferulic (**3**), 5-hydroxyferulic (**4**) and sinapic (**5**) acids into some plants, especially wheat (*Triticum vulgare* var. *tharcher*), resulted in the formation of ^{14}C-labelled syringyl lignin.[5,6] Recently, however, this pathway was challenged and a novel pathway *via* caffeic acid (**2**), caffeoyl CoA, feruloyl CoA, coniferaldehyde (**8**), 5-hydroxyconiferaldehyde (**9**), sinapaldehyde (**10**) and sinapyl alcohol (**15**) was proposed for syringyl lignin biosynthesis in angiosperm trees.[7–9] Later, similar pathways were also proposed for herbaceous model plants, arabidopsis (*Arabidopsis thaliana*)[10] and alfalfa (*Medicago sativa*).[11]

The discovery of the novel pathway *via* 5-hydroxydroxyconiferyldehyde (**9**) was based on kinetic analysis of recombinant caffeic acid *O*-methyltransferases (CAOMTs) and ferulic acid 5-hydroxylase (F5H).[7,8] Thus, individual incubation of caffeic acid (**2**), 5-hydroxyferulic acid (**4**), and 5-hydroxyconiferaldehyde (**9**) with CAOMT in the presence of *S*-adenosyl-L-methionine gave rise to the corresponding methylation products, ferulic acid (**3**), sinapic acid (**5**), and sinapaldehyde (**10**), respectively. Ferulic acid (**3**) and coniferaldehyde (**8**) were hydoxylated efficiently to afford 5-hydroxyferulic acid (**4**) and 5-hydroxyconiferaldehyde (**9**), respectively, when incubated individually with F5H. However, 5-hydroxyconiferaldehyde (**9**) was found to inhibit the CAOMT-catalyzed methylation of caffeic acid (**2**) and 5-hydroxyferulic acid (**4**), while coniferaldehyde (**8**) inhibited the F5H-catalyzed 5-hydroxylation of ferulic acid (**3**), indicating that 5-hydroxyconiferaldehyde (**9**) and coniferaldehyde (**8**) are preferred substrates of CAOMT and F5H, respectively.[7,8] Therefore CAOMT and F5H were renamed as 5-hydroxyconiferaldehyde OMT (CAldOMT) and coniferaldehyde 5-hydroxylase (CAld5H), respectively.[7–9] The novel pathway accords in all respects with the recent findings of lignin characters in transgenic plants where caffeoyl CoA *O*-methyltransferase (CCoAOMT) and/or CAldOMT (CAOMT) were downregulated. The suppression of CCoAOMT resulted in reduction of lignin content with unchanged syringyl/guaiacyl (S/G) ratio, whereas the downregulation of CAldOMT (CAOMT) caused a significant decrease in S/G ratio with essentially no change in lignin content and the appearance of 5-hydroxyguaiacyl unit.[11–22] Thus, the current view of the cinnamate/monolignol pathway involves the 5-hydroxyconiferaldehyde pathway for syringyl lignin biosynthesis.[3,7–11]

However, the conventional pathway *via* sinapic acid (**5**), sinapaldehyde (**10**), and sinapyl alcohol (**15**) towards syringyl lignin was re-proposed based on administration of labelled ferulic (**3**) and sinapic (**5**) acids to black locust (*Robinia pseudoacacia*) and oleander (*Nerium indicum*).[23,24] Recently, a new and redundant route for 3-hydroxylation of the *p*-hydroxyphenyl moiety has been proposed,[25,26] which involved the ester exchange catalyzed by hydroxycinnamoyl CoA:shikimate hydroxyxinnamoyl transferase (HCT) and the subsequent 3-hydroxylation of the formed *p*-coumaroyl ester giving rise to the corresponding caffeoyl ester, followed by its conversion to caffeoyl CoA by HCT (Fig. 1).[25–28]

These results suggest that physiologically important routes in the cinnamate/monolignol pathway towards lignin biosynthesis might vary with plant species. In addition, it seems plausible that the physiologically important routes in the pathway and

[a]*Research Institute for Sustainable Humanosphare, Kyoto University, Uji, Kyoto, 611-0011, Japan. E-mail: tumezawa@rish.kyoto-u.ac.jp; Fax: +81 774 38 3682; Tel: +81 774 38 3625*
[b]*Kazusa DNA Research Institute, 2-6-7 Kazusa-kamatari, Kisarazu, Chiba, 292-0818, Japan*
† Present address: Faculty of Pharmaceutical Sciences at Kagawa Campus, Tokushima Bunri University, 1314-1 Shido, Sanuki City, Kagawa, 769-2193, Japan
‡ Present address: Institute of Sustainability Science, Kyoto University, Uji, Kyoto 611-0011, Japan
§ Present address: Fukui University of Technology, 3-6-1 Gakuen, Fukui 910-8505, Japan

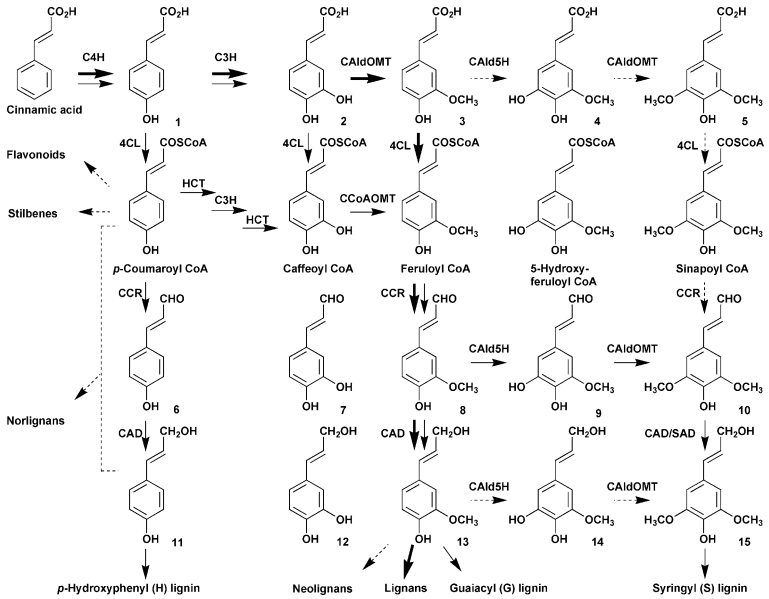


Fig. 1 The cinnamate/monolignol pathway. **1**, *p*-coumaric acid; **2**, caffeic acid; **3**, ferulic acid; **4**, **5**-hydroxyferulic acid; **5**, sinapic acid; **6**, *p*-coumaraldehyde; **7**, caffealdehyde; **8**, coniferaldehyde; **9**, 5-hydroxyconiferaldehyde; **10**, sinapaldehyde; **11**, *p*-coumaryl alcohol; **12**, caffeyl alcohol; **13**, coniferyl alcohol; **14**, 5-hydroxyconiferyl alcohol; and **15**, sinapyl alcohol. Thick solid arrow: a lignan biosynthetic pathway in *Carthamus tinctorius* proposed in this study, solid arrow: currently accepted lignin biosynthetic pathways, broken arrow: other routes in the cinnamate/monolignol pathway. C4H, cinnamate 4-hydroxylase; C3H, *p*-coumarate 3-hydroxylase; CAldOMT, 5-hydroxyconiferaldehyde *O*-methyltransferase: 4CL, 4-hydroxycinnamate CoA ligase; HCT, hydroxycinnmamoyl CoA :shikimate hydroxycinnamoyl transferase; CCoAOMT, caffeoyl CoA *O*-methyltransferase; CCR, cinnamoyl CoA reductase; CAld5H, coniferaldehyde 5-hydroxylase; CAD, cinnmamyl alcohol dehydrogenase; SAD, sinapyl alcohol dehydrogenase.

genes for lignin synthesis in normal cell wall formation in development might be different from those for biosynthesis of other phenylpropanoid compounds, such as lignan, norlignan, and neolignan and even from those for defense lignin formation after fungal attack or physical wounding.

Thus, many questions remain unsolved in this field, and in the post-genomic era, functional genomics including the identification of the roles of each gene from different gene families and their assignment to the appropriate step of metabolism is indispensable towards answering these questions. One of the major concerns associated with functional genomics is comprehensive analyses of expressed mRNAs (transcriptomics), proteins (proteomics) and metabolites (metabolomics), together with the integration of omics data.[29] However, no single technology for metabolomics, such as a DNA sequencer for genomics or DNA arrays for transcriptomics, is available.[30,31] Practically, for comprehensive analysis of metabolites, two strategies can be adopted.

One is non-targeted and high-throughput analysis with Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS) which can cover a larger number of compounds than any other type of mass spectrometry. However, this technique is very weak at quantitation, and is used for metabolic fingerprinting purposes, but not for quantitative metabolic analysis.[30,31] The other concentrates on rather limited but still significant numbers of metabolites through the use of gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) *etc.*, which allow us an accurate and comprehensive quantitative analysis, or metabolic profiling,[31,32] for the target compounds.

Recently, taking the latter strategy with GC-MS, we have established a pathway towards a dibenzylbutyrolactone lignan, yatein, from matairesinol in cow parsley (*Anthriscus sylvestris*).[33] Such a metabolic analysis of the lignan pathway was conducted in combination with administration of stable-isotope-labelled precursors.[33]

We have expanded this technique in this study to examine the upstream lignan pathway, that is, the formation of matairesinol and its congeners from coniferyl alcohol and the cinnamate/monolignol pathway which supplies coniferyl alcohol, and established a system to comprehensively quantify metabolic intermediates of such pathways by GC-MS. This new system has been successfully applied to safflower (*Carthamus tinctorius*) seeds to characterize both lignin and lignan biosynthesis (Figs. 1 and 2).

Results

Lignans, *p*-hydroxycinnamates, *p*-hydroxycinnamaldehydes and *p*-hydroxycinnamyl alcohols in *C. tinctorius* seeds

GC-MS analysis of the methyl alcohol extracts of *Carthamus tinctorius* seeds from 12 days after flowering (DAF) indicated the presence of *p*-coumaric acid (**1**), caffeic acid (**2**), ferulic acid (**3**), sinapic acid (**5**), coniferyl alcohol (**13**), sinapyl alcohol (**15**), secoisolariciresinol (**18**), matairesinol (**19**), arctigenin (**20**), and trachelogenin (**21**). These compounds were identified by comparison of their mass spectra and retention times on GC with those of authentic samples [*p*-coumaric acid (**1**) (TMS ether): MS *m/z* (EI) 308 (M^+, 41.8%), 293 (56.0), 249 (37.4), 219 (46.7), 179 (15.2), t_R = 8.1 min; caffeic acid (**2**) (TMS ether): MS *m/z* (EI) 396 (M^+, 41.5%), 381 (13.1), 307 (6.9), 219 (53.4), 191 (9.9), t_R = 9.4 min; ferulic acid (**3**) (TMS ether): MS *m/z* (EI) 338 (M^+, 81.3%), 322 (53.5), 308 (48.8), 293 (30.2), 249 (34.9), t_R = 9.3 min; sinapic acid (**5**) (TMS ether): MS *m/z* (EI) 368 (M^+, 83.7%), 353

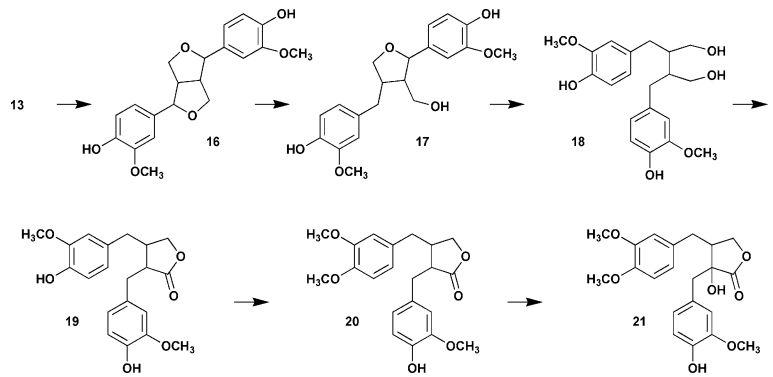


Fig. 2 The lignan pathway in *Carthamus tinctorius*. **16**, pinoresinol; **17**, lariciresinol; **18**, secoisolariciresinol; **19**, matairesinol; **20**, arctigenin; and **21**, trachelogenin.

(46.5), 338 (88.4), 323 (27.9), t_R = 11.2 min; coniferyl alcohol (**13**) (TMS ether): MS *m/z* (EI) 324 (M^+, 41.9%), 309 (14.9), 293 (34.9), 235 (16.3), 204 (25.6), t_R = 8.0 min; sinapyl alcohol (**15**) (TMS ether): MS *m/z* (EI) 354 (M^+, 56.9%), 323 (32.6), 293 (11.6), 265 (15.1), 234 (18.6), t_R = 9.0 min; secoisolariciresinol (**18**) (TMS ether): MS *m/z* (EI) 650 (M^+, 12.5%), 560 (27.6), 470 (14.6), 261 (56.1), 209 (100), t_R = 11.1 min; matairesinol (**19**) (TMS ether): MS *m/z* (EI) 502 (M^+, 51.2%), 209 (100), 179 (30.1), t_R = 16.1 min; arctigenin (**20**) (TMS ether): MS *m/z* (EI) 444 (M^+, 60.9%), 209 (100), 179 (35.4), 151 (32.2), t_R = 16.5 min; and trachelogenin (**21**) (TMS ether): MS *m/z* (EI) 532 (M^+, 34.4%), 396 (17.1), 245 (82.9), 209 (100), 151 (80.5), t_R = 14.3 min].

The other possible metabolic intermediates shown in Figs. 1 and 2, namely, 5-hydroxyferulic acid (**4**), *p*-coumaraldehyde (**6**), caffealdehyde (**7**), coniferaldehyde (**8**), 5-hydroxyconiferaldehyde (**9**), sinapaldehyde (**10**), *p*-coumaryl alcohol (**11**), caffeyl alcohol (**12**), 5-hydroxyconiferyl alcohol (**14**), pinoresinol (**16**), and lariciresinol (**17**) were not detected in GC-MS analysis of methyl alcohol extracts from the seeds of 5–15 DAF even after searching using the corresponding authentic samples. The CoA esters (*p*-coumaroyl, caffeoyl, feruloyl, 5-hydroxyferuloyl, and synapoyl CoAs) were not analyzed because of their insufficient volatility and stability for GC-MS measurement.

Quantitation of *p*-hydroxycinnamic acids, *p*-hydroxycinnamaldehydes, *p*-hydroxycinnamyl alcohols, and lignans in *C. tinctorius* seeds

Quantitation of the compounds of the cinnamate/monolignol and lignan pathways was conducted by a stable-isotope-dilution method with GC-MS. Deuterium-labelled and unlabelled standards for the fifteen phenylpropanoid monomers [*p*-hydroxycinnamic acids (**1**)–(**5**), *p*-hydroxycinnamaldehydes (**6**)–(**10**), *p*-hydroxycinnamyl alcohols (**11**)–(**15**)] and two lignans [arctigenin (**20**) and trachelogenin (**21**)] were synthesized by organic chemical techniques, as summarized in Schemes 1 and 2. The standards for four lignans (pinoresinol (**16**), lariciresinol (**17**), secoisolariciresinol (**18**), and matairesinol (**19**)) were prepared previously.[34–36] Using these standards, calibration curves for the targets were established, and used for their quantitation. The phenylpropanoid monomers were analyzed all at once by a single injection to GC-MS, while the lignan fraction was analyzed with a shorter GC column. The quantitative analysis was conducted for the seeds of 5–15 DAF, and the time-course data are summarized in Fig. 3.

Analysis of stable-isotope incorporation after administration of labelled precursors

The six stable-isotope-labelled compounds, [U-*ring*-$^{13}C_6$]phenylalanine, *p*-[U-*ring*-$^{13}C_6$]coumaric acid (**1**-$^{13}C_6$), [2,5-$^2H_2$, OC^2H_3]-ferulic acid (**3**-d_5), [2,5,7-$^2H_3$]caffeic acid (**2**-d_3), [3-OC^2H_3]-coniferaldehyde (**8**-d_3), and [3-OC^2H_3]sinapaldehyde (**10**-d_3), were administered individually to 7 and 13 DAF seeds of *C. tinctorius* (0.4 mg of each stable-isotope-labelled compound to 200 mg of seeds). The methyl alcohol extracts obtained following the administration were submitted to analysis of stable-isotope incorporation into the *p*-hydroxycinnamic acids (**1**)–(**5**), *p*-hydroxycinnamaldehydes (**6**)–(**10**), *p*-hydroxycinnamyl alcohols (**11**)–(**15**) by GC-MS after β-glucosidase treatment (Table 1). In addition, the stable-isotope incorporation into matairesinol (**19**) was examined (Table 1).

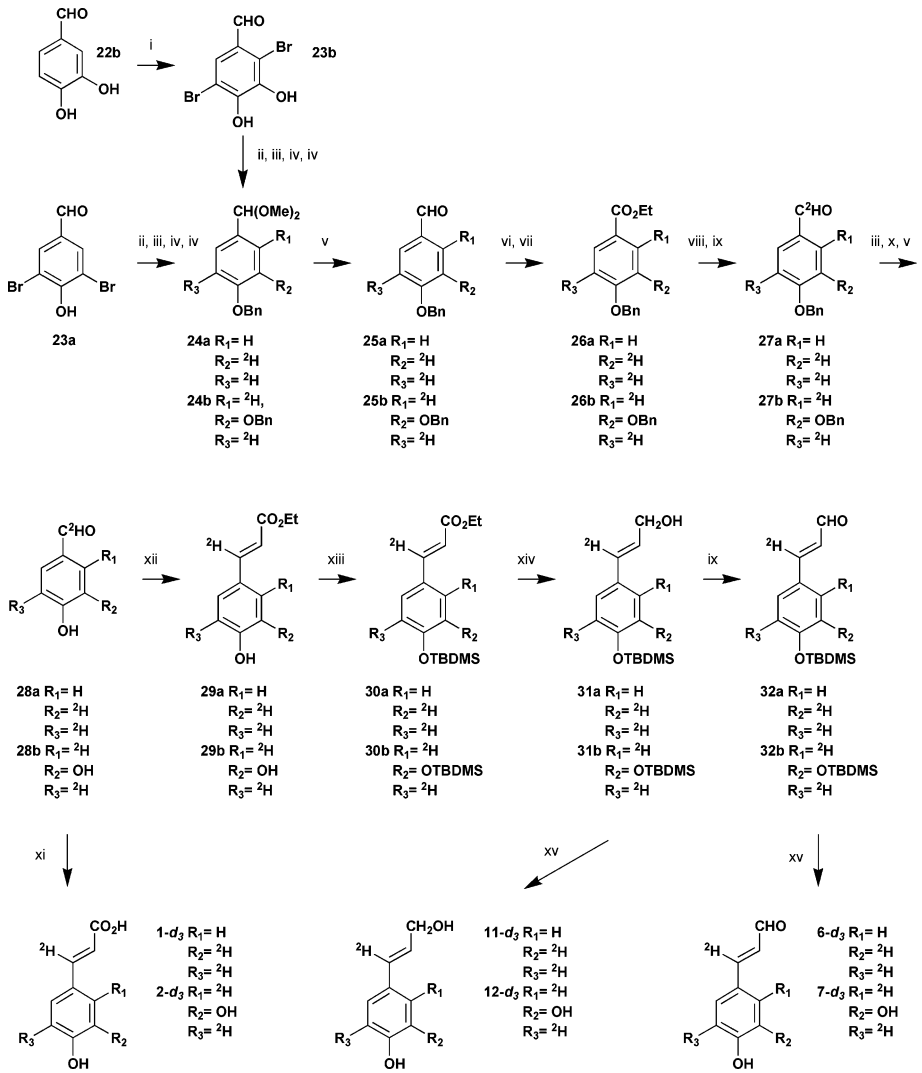
In summary, the following conversions shown in Scheme 3 were observed, while incorporation of ^{13}C or ^2H into the other cinnmamic acids, cinnamaldehydes and cinnamyl alcohols shown in Fig. 1 was not detected.

Simultaneous administration

Three sets of simultaneous administration experiments with (a) [2,5-$^2H_2$, 3-OC^2H_3]ferulic acid (**3**-d_5) and [U-*ring*-$^{13}C_6$]phenylalanine (**Phe**-$^{13}C_6$); (b) [2,5-$^2H_2$, 3-OC^2H_3]ferulic acid (**3**-d_5) and *p*-[U-*ring*-$^{13}C_6$]coumaric acid (**1**-$^{13}C_6$); and (c) [2,5-$^2H_2$, 3-OC^2H_3]ferulic acid (**3**-d_5) and [2,5,7-$^2H_3$]caffeic acid (**2**-d_3) were conducted. Stable isotope incorporation into coniferyl alcohol (**13**) and matairesinol (**19**) was examined by GC-MS. As shown in Table 2, formation of [$^2H_5$]coniferyl alcohol (**13**-d_5) and [$^2H_5$]matairesinol (**19**-d_5) was observed in all cases, and the deuterium incorporation into matairesinol (**19**) (0.020–0.0073 nmol mg^{-1}) was not reduced, but slightly increased, compared with those in the sole administration of [2,5-$^2H_2$, 3-OC^2H_3]ferulic acid (**3**-d_5) [0.0039 nmol mg^{-1}, (Table 1)].

Lignin analysis

Wiesner color reaction[37,38] with phloroglucinol in hydrochloric acid indicated clear red–purple color in the seed hulls but not



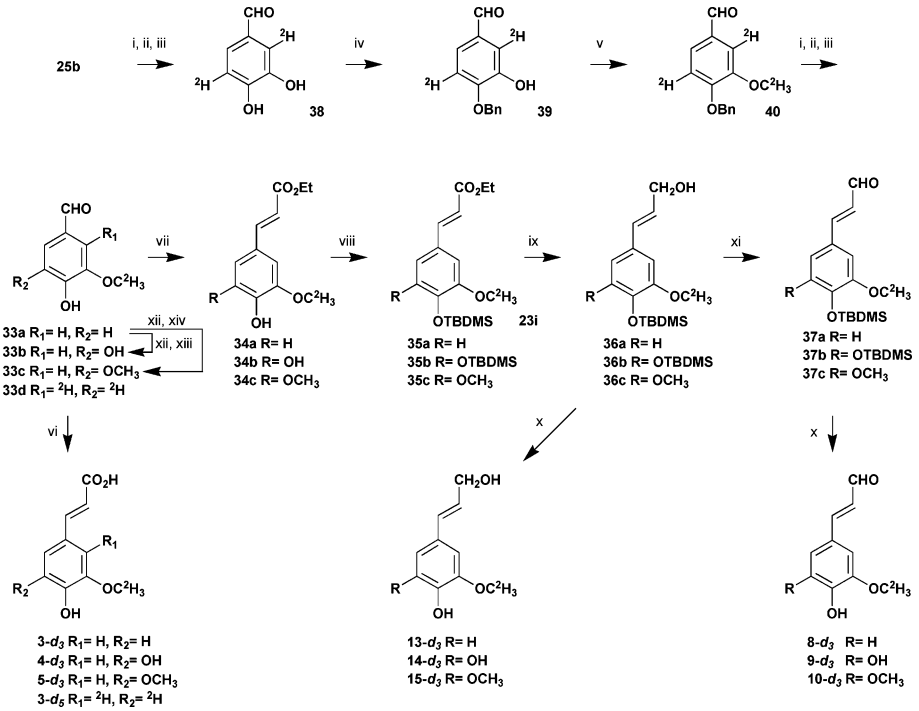
Scheme 1 Synthetic routes for *p*-[3,5,7-$^2H_3$]coumaric acid (**1**-d_3), [2,5,7-$^2H_3$]caffeic acid (**2**-d_3), *p*-[3,5,7-$^2H_3$]coumaraldehyde (**6**-d_3), [2,5,7-$^2H_3$]-caffealdehyde (**7**-d_3), *p*-[3,5,7-$^2H_3$]coumaryl alcohol (**11**-d_3), and [2,5,7-$^2H_3$]caffeyl alcohol (**12**-d_3). *Reagents and conditions*: i, Br_2, $CHCl_3$, 60 °C, 18 h; ii, BnBr, K_2CO_3, KI, DMF, 55 °C, 3 h; iii, $CH(OMe)_3$, *p*-TsOH, MeOH, 0 °C, 10 min; iv, *n*-BuLi, MeO^2H, Et_2O, −35 °C, 0.5 h; v, 1 N HCl, acetone, 0 °C, 5 min; vi, Ag_2O, EtOH, 60 °C, 10 min; vii, CH_3CHN_2, Et_2O, rt, 15 min; viii, $LiAl^2H_4$, Et_2O, −35 °C, 0.5 h; ix, MnO_2, CH_2Cl_2, rt, 28 h; x, H_2, 10% Pd–C, THF, MeOH, rt. 6 min; xi, malonic acid, pyridine, piperidine, aniline, 65 °C, 12 h; xii, monoethyl malonate, pyridine, piperidine, aniline, 60 °C, 11 h; xiii, TBDMSCl, imidazole, DMF, rt, 4 h; xiv, $LiAlH_4$, Et_2O, −35 °C, 0.5 h; xv, *n*-Bu_4NF, THF, rt, 10 min.

in the inside, of matured *C. tinctorius* seeds (Fig. 4), suggesting lignification in the seed hulls. This was confirmed by chemical degradation analysis. Thus, the seed hulls from commercial matured seeds were submitted to thioacidolysis.[39] GC-MS analysis of the thioacidolysis products indicated the presence of trithioethyl phenylpropane compounds (Fig. 5) which are derived specifically from β-*O*-4 substructures in lignin polymers. Both guaiacyl and syringyl monomers derived from guaiacyl and syringyl lignins, respectively, were detected, indicating the *Carthamus* seed hull lignin is guaiacyl-syringyl lignin. This was further confirmed by nitrobenzene oxidation,[40] which gave vanillin and syringaldehyde from the seed hulls (Fig. 6), whereas *p*-hydroxybenzaldehyde was not detected (data not shown).

Next, seed lignin amounts were measured by the acetyl bromide method.[41] The seed lignin content increased from 9 DAF to 12 DAF, and there was no significant difference between 12 and 15 DAF seeds (Fig. 6). The final lignin amounts, 125 μg mg^{-1}, correspond to *ca.* 700 nmol of coniferyl alcohol unit mg^{-1}. In addition, nitrobenzene oxidation showed that lignin accumulation started at 6 DAF. Taking these results together, the accumulation of seed lignin was found to start about 6 DAF and terminated at around 12 DAF.

Discussion

To quantify precisely and comprehensively the compounds in the cinnamate/monolignol and lignan pathways, we employed a stable-isotope-dilution method using the corresponding deuterium-labelled compounds as the internal standards. This technique has several advantages. First, the loss of the target compounds during extraction and derivatization can be ignored due to the presence of the labelled internal standards. Second,



Scheme 2 Synthetic routes for [3-OC^{2}H$_3$]ferulic acid (**3**-d_3), [2,5-^{2}H$_2$, 3-OC^{2}H$_3$]ferulic acid (**3**-d_5), [3-OC^{2}H$_3$]-5-hydroxyferulic acid (**4**-d_3), [3-OC^{2}H$_3$]sinapic acid (**5**-d_3), [3-OC^{2}H$_3$]coniferaldehyde (**8**-d_3), [3-OC^{2}H$_3$]-5-hydroxyconiferaldehyde (**9**-d_3), [3-OC^{2}H$_3$]sinapaldehyde (**10**-d_3), [3-OC^{2}H$_3$]coniferyl alcohol (**13**-d_3), [3-OC^{2}H$_3$]-5-hydroxyconiferyl alcohol (**14**-d_3), and [3-OC^{2}H$_3$]sinapyl alcohol (**15**-d_3). *Reagents and conditions*: i, CH(OMe)$_3$, *p*-TsOH, MeOH, 0 °C, 10 min; ii, H_2, 10% Pd–C, THF, MeOH, rt, 1 h; iii, 1 N HCl, acetone, 0 °C, 3 min; iv, BnBr, K_2CO_3, KI, DMF, rt, 12 h; v, C^{2}H$_3$I, DMF, rt, 8 h; vi, malonic acid, pyridine, piperidine, aniline, 65 °C, 12 h; vii, monoethyl malonate, pyridine, piperidine, aniline, 65 °C, 11 h; viii, TBDMSCl, imidazole, DMF, rt, 4 h; ix, $LiAlH_4$, ether, −35 °C, 0.5 h; x, *n*-Bu_4NF, THF, rt, 10 min; xi, MnO_2, CH_2Cl_2, rt, 23 h; xii, 1 N NaOH, I_2, KI, H_2O, rt, 8 h; xiii, $CuSO_4 \cdot 5H_2O$, 4 N NaOH, 145 °C, 4 h; xiv, Cu, Na, MeOH, 128 °C, 3 h.

incomplete chromatographic separation can be overcome by mass separation. Third, ion suppression in mass spectrometry due to a significant reduction of the ion intensity of the target compound by the coexisting compounds can be neglected. Fourth, this technique, employing mass spectrometry, is highly sensitive. In fact, when the deuterium-labelled internal standards were added to the MeOH extracts of *C. tinctorius* seeds after β-glucosidase treatment, measured amounts of sinapyl alcohol (**15**) decreased about 2 orders of magnitude compared with the value obtained when the standards were added in the initial stage of the extraction (data not shown). This was not a surprising result, because it is well-known among lignin chemists that sinapyl alcohol (**15**) is a very unstable compound.[42] This result clearly demonstrates the effectiveness of the stable-isotope-dilution method, especially for quantitation of unstable metabolites.

However, this technique requires a stable-isotope-labelled internal standard as well as an unlabelled authentic sample for each target compound. In most cases, these labelled and unlabelled compounds are not commercially available, and therefore, need to be synthesized. We have chemically synthesized the required labelled and unlabelled compounds. Schemes 1 and 2 outline the synthesis of the deuterium-labelled compounds. Using the labelled compounds together with the corresponding unlabelled ones, we prepared calibration curves for the target compounds and established a metabolite quantitation system for the cinnamate/monolignol and lignan pathways.

Next, we tested the applicability of the system to metabolic analysis of the pathways. For this purpose, plants which have time-dependently varying metabolic flows towards monolignols, lignins, and lignans are the most suitable systems.

Members of tribe Cynareae of family Asteraceae such as edible burdock (*Arctium lappa*) were characterized for their wide distribution of 4′-*O*-methyldibenzylbutyrolactone lignans (*e.g.* arctiin = arctigenin glucoside), especially in their seeds.[43–49] Recently, when we analyzed the stereochemical properties of lignans,[48,50,51] we found that the accumulation of matairesinol (**19**) and arctigenin (**20**) in *A. lappa* seeds started at about 6 DAF (data not shown). In addition, histochemical analysis with Wiesner reagent (phloroglucinol–HCl) showed the purple coloration in the seed hulls, strongly suggesting lignin deposition (data not shown). Therefore, the seeds, where the biosynthesis of lignan and most probably lignin is taking place during the seed maturation phase, seem to be an attractive system to test the applicability of the stable-isotope-dilution-method-based quantitation system to the biosynthesis of monolignol and lignan. However, flowering in this species often occurs more than one year after germination, meaning it is not be feasible for our purpose. Instead, *C. tinctorius* which is another member of the tribe and also produces dibenzylbutyrolactone lignans in the seeds[44–47] has been chosen in the present work, because it flowers at about 2 months after germination.

Preliminary GC-MS analysis of β-glucosidase-treated methyl alcohol extracts from *C. tinctorius* seeds of 12 DAF indicated the presence of several lignans and the phenylpropanoid monomers of the cinnamate/monolignol pathway. Thus, *p*-coumaric (**1**), caffeic (**2**), ferulic (**3**) and sinapic acids (**5**), and coniferyl (**13**) and sinapyl

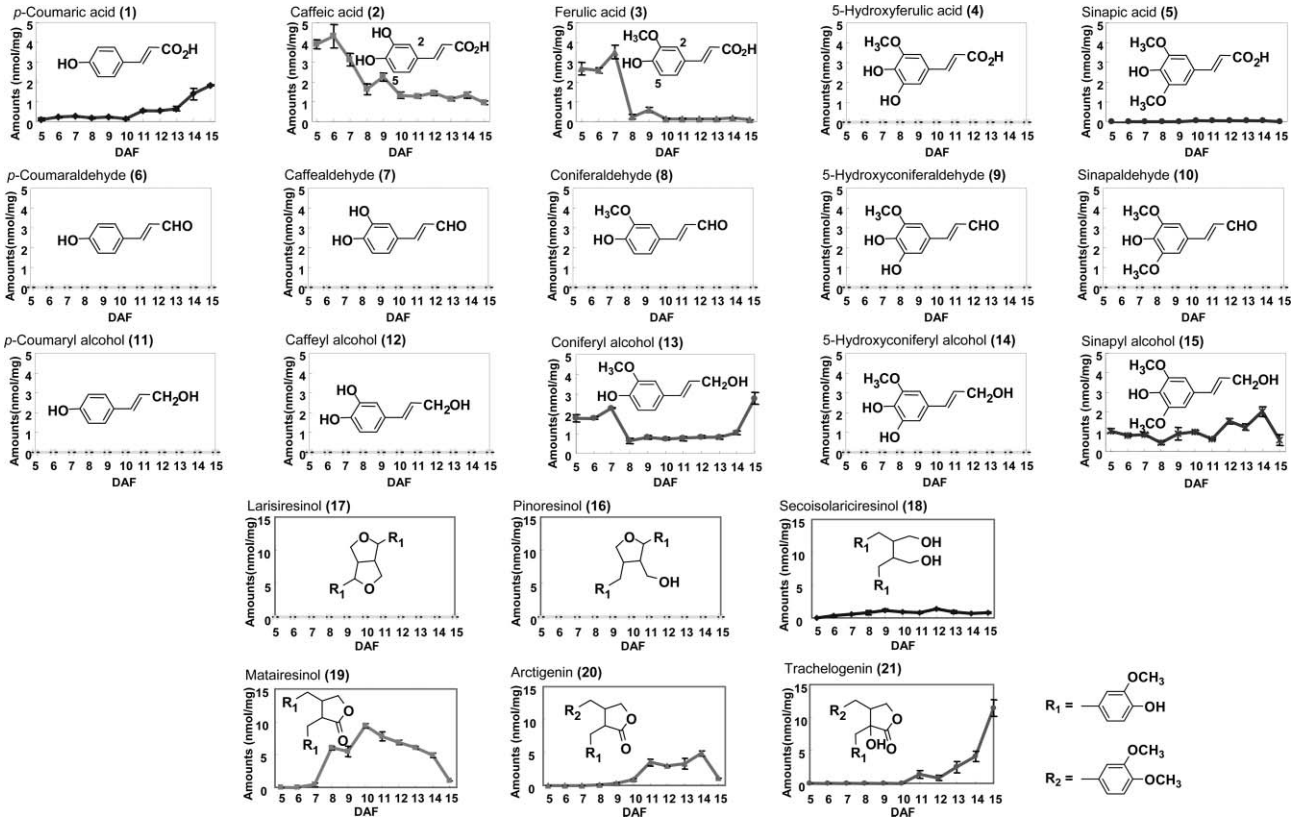


Fig. 3 Time course (from 5 to 15 day after flowering) of accumulation of *p*-hydroxycinnamates (**1–5**), *p*-hydroxycinnamaldehydes (**6–10**), *p*-hydroxycinnamyl alcohols (**11–15**) and lignans (**16–21**). Each value is the average of three independent experiments. Standard deviations are indicated by vertical bars. The panels are laid out according to the biosynthetic pathways (Figs. 1 and 2).

alcohols (**15**), as well as secoisolariciresinol (**18**), matairesinol (**19**), arctigenin (**20**), and trachelogenin (**21**) were detected. In contrast, other monomeric compounds, such as coniferaldehyde (**8**) and sinapaldehyde (**10**) were not detected. The CoA esters (*p*-coumaroyl, caffeoyl, feruloyl, 5-hydroxyferuroyl, and sinapoyl CoAs), which are not sufficiently volatile and or stable to be analyzed by GC-MS, were not pursued in the present study. In addition, semi-quantitative GC-MS analysis of the methyl alcohol extracts from 5 to 15 DAF *C. tinctorius* seeds showed rapid increases in the amounts of dibenzylbutyrolactone lignans after 8 DAF, as in the case of *A. lappa*, indicating the drastic metabolic change in the cinnamate/monolignol pathway as seeds mature.

Like the *Arctium* seeds, histochemical detection of cinnamaldehyde groups in lignins with Wiesner reagent showed the red–purple coloration in *C. tinctorius* matured seed hulls (Fig. 4), strongly suggesting lignin deposition. This was also confirmed by chemical degradation of lignins. Thioacidolysis and nitrobenzene oxidation of the powdered seed hulls after organic solvent extraction gave the phenylpropanoid monomers, 1-guaiacyl-1,2,3-trithioethylpropane and 1-syringyl-1,2,3-trithioethylpropane in thioacidolysis (Fig. 5) and vanillin and syringaldehyde in nitrobenzene oxidation, respectively, which are diagnostic of the presence of guaiacyl and syringyl lignins.

Having the preliminary data in hand, the quantitation system was used for the metabolic analysis of the cinnamate/monolignol and lignan pathways in *C. tinctorius* maturing seeds. This was done by measuring the accumulation of the cinnamate/monolignol pathway compounds and lignans at various time intervals. As shown in Fig. 3, the detected compounds from 5 to 15 DAF seeds were again *p*-coumaric (**1**), caffeic (**2**), ferulic (**3**) and sinapic acids (**5**), and coniferyl (**13**) and sinapyl alcohols (**15**), secoisolariciresinol (**18**), matairesinol (**19**), arctigenin (**20**), and trachelogenin (**21**). On the other hand, none of the other compounds, pinoresinol (**16**), lariciresinol (**17**), and 5-hydroxyferulic acid (**4**), *p*-coumaraldehyde (**6**), caffealdehyde (**7**), coniferaldehyde (**8**), 5-hydroxyconiferaldehyde (**9**), sinapaldehyde (**10**), *p*-coumaryl alcohol (**11**), caffeyl alcohol (**12**), 5-hydroxyconiferyl alcohol (**14**) were detected throughout the GC-MS measurement of the 5–15 DAF samples.

The amounts of matairesinol (**19**) reached the maximum at 10 DAF, which is followed by the accumulation peak of arctigenin (**20**) (14 DAF) (Fig. 3). This is in good accordance with their metabolic sequence; *O*-methyltransferase catalyzing methylation of matairesinol (**19**) to afford arctigenin (**20**) was detected in the seeds and a cDNA encoding the enzyme has been cloned.[52] The accumulation of arctigenin (**20**) was followed by that of trachelogenin (**21**) (Fig. 3), strongly suggesting that trachelogenin (**21**) is formed by hydroxylation of arctigenin (**20**).

Three phenylpropanoid monomers, caffeic (**2**) and ferulic acids (**3**) and coniferyl alcohol (**13**) exhibited a transient accumulation around 5–7 DAF (Fig. 3), while the start of matairesinol (**19**) accumulation occurs just after this 5–7 DAF period. Lignin deposition also started around 6 DAF, as determined by nitrobenzene oxidation analysis (Fig. 6). The amounts (2–4 nmol mg^{-1}) of the

Table 1 Amounts of ^{13}C- or ^{2}H-labelled compounds obtained following administration of labelled precursors into *C. tinctorius* (nmol mg^{-1})

Compound[c]	Phe-$^{13}C_6$[a]		(1-$^{13}C_6$)[a]		(2-d_3)[a]		(3-d_5)[a]		(8-d_3)[a]		(10-d_3)[a]	
	7DAF[b]	13DAF[b]	7DAF[b]	13DAF[b]	7DAF[b]	13DAF[b]	7DAF[b]	13DAF[b]	7DAF[b]	13DAF[b]	7DAF[b]	13DAF[b]
1	0.0028 ± 0.0038	0	—	—	0	0	0	0	0	0	0	0
2	0	0	0.81 ± 0.11	0.050 ± 0.043	—	—	0	0	0	0	0	0
3	0	0	0.40 ± 0.40	0.014 ± 0.0033	12.70 ± 5.26	0.041 ± 0.0086	—	—	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	—	—	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	—	—
11	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0
13	0.051 ± 0.047	0.076 ± 0.058	0.073 ± 0.022	0.016 ± 0.0063	14.22 ± 1.75	0.29 ± 0.066	79.05 ± 11.89	5.74 ± 0.35	43.33 ± 12.48	47.41 ± 1.83	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0
15	0	0.021 ± 0.014	0	0	0	0	0	0	0.10 ± 0.020	0	27.75 ± 3.37	18.84 ± 0.14
19	0.011 ± 0.010	0.067 ± 0.041	0.0015 ± 0.0011	0	0.0087 ± 0.0015	0.0016 ± 0.0022	0.0039 ± 0.00035	0.0085 ± 0.0066	0.026 ± 0.040	0.15 ± 0.062	N.D.	N.D.

Each value (average ± standard deviations) represents the average of three independent experiments.[a] Administered compounds: Phe-$^{13}C_6$, [U-*ring*-$^{13}C_6$]phenylalanine; **1**-$^{13}C_6$, *p*-[U-*ring*-$^{13}C_6$]coumaric acid; **2**-d_3, [2,5,7-$^2H_3$]caffeic acid; **3**-d_5, [2,5-$^2H_2$,3-OC$^2H_3$]ferulic acid; **8**-d_3, [3-OC$^2H_3$]coniferaldehyde; **10**-d_3, [3-OC$^2H_3$]sinapaldehyde. [b] DAF, days after flowering. [c] Labelled compound formed from the labelled precursor: **1**, *p*-coumaric acid; **2**, caffeic acid; **3**, ferulic acid; **4**, 5-hydroxyferulic acid; **5**, sinapic acid; **6**, *p*-coumaraldehyde; **7**, caffealdehyde; **8**, coniferaldehyde; **9**, 5-hydroxyconiferaldehyde; **10**, sinapaldehyde; **11**, *p*-coumaryl alcohol; **12**, caffeyl alcohol; **13**, coniferyl alcohol; **14**, 5-hydroxyconiferyl alcohol; **15**, sinapyl alcohol; **19**, matairesinol. N.D., not determined.

[U-*ring*-$^{13}C_6$]phenylalanine →
p-[$^{13}C_6$]coumaric acid (**1**-$^{13}C_6$)
[$^{13}C_6$]coniferyl alcohol (**13**-$^{13}C_6$)
[$^{13}C_6$]sinapyl alcohol (**15**-$^{13}C_6$)
[$^{13}C_6$]matairesinol (**19**-$^{13}C_6$)
p-[U-*ring*-$^{13}C_6$]coumaric acid (**1**-$^{13}C_6$) →
[$^{13}C_6$]caffeic acid (**2**-$^{13}C_6$)
[$^{13}C_6$]ferulic acid (**3**-$^{13}C_6$)
[$^{13}C_6$]coniferyl alcohol (**13**-$^{13}C_6$)
[$^{13}C_6$]matairesinol (**19**-$^{13}C_6$)
[2,5,7-$^2H_3$]caffeic acid (**2**-d_3) →
[$^2H_3$]ferulic acid (**3**-d_3)
[$^2H_3$]coniferyl alcohol (**13**-d_3)
[$^2H_3$]matairesinol (**19**-d_3)
[2,5-$^2H_2$, 3-OC$^2H_3$]ferulic acid (**3**-d_5) →
[$^2H_5$]coniferyl alcohol (**13**-d_5)
[$^2H_5$]matairesinol (**19**-d_5)
[3-OC$^2H_3$]coniferaldehyde (**8**-d_3) →
[$^2H_3$]coniferyl alcohol (**13**-d_3)
[$^2H_3$]sinapyl alcohol (**15**-d_3)
[$^2H_3$]matairesinol (**19**-d_3)
[3-OC$^2H_3$]sinapaldehyde (**10**-d_3) →
[$^2H_3$]sinapyl alcohol (**15**-d_3)

Scheme 3

Fig. 4 Phloroglucinol staining of *C. tinctorius* seed section. (a) before staining, (b) after staining.

Fig. 5 Typical thioacidolysis monomers obtained from *Catrhamum tinctorius* seed hulls.

monomers are comparable to those of lignans (*e.g.* 10 nmol mg^{-1} for matairesinol (**19**)) (Fig. 3). In sharp contrast, lignin content in the 14 DAF seeds was *ca.* 125 μg mg^{-1} (*ca.* 700 nmol of coniferyl alcohol unit mg^{-1}), which is two orders of magnitude greater than the amounts of the cinnamate/monolignol pathway compounds (Fig. 3). Obviously, the metabolic intermediate pools in the cinnamate/monolignol pathway in *C. tinctorius* maturing seeds are much smaller than the amounts of lignins.

Table 2 Amounts of ^{13}C- or ^{2}H-labelled compounds obtained following simultaneous administration of labelled precursors into *C. tinctorius* (nmol mg^{-1})

	(**3**-d_5) + (Phe-$^{13}C_6$)[a]				(**3**-d_5) + (**1**-$^{13}C_6$)[a]				(**3**-d_5) + (**2**-d_3)[a]			
	From (**3**-d_5)		From Phe-[$^{13}C_6$]		From (**3**-d_5)		From (**1**-$^{13}C_6$)		From (**3**-d_5)		From (**2**-d_3)	
Compound	7DAF[b]	13DAF[b]	7DAF[b]	13DAF[b]	7DAF[b]	13DAF[b]	7DAF[b]	13DAF[b]	7DAF[b]	13DAF[b]	7DAF[b]	13DAF[b]
1	0	0	0	0	—	—	—	—	0	0	0	0
2	0	0	0	0	0	0	0	0	—	—	—	—
3	—	—	—	—	—	—	—	—	—	—	—	—
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0
13	28.95 ± 3.64	2.47 ± 0.61	0	0	26.72 ± 3.50	0.74 ± 0.16	0	0.081 ± 0.0058	18.97 ± 3.19	4.60 ± 1.35	6.44 ± 0.89	1.35 ± 0.22
14	0	0	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0	0	0
19	0.0058 ± 0.0019	0.012 ± 0.0079	0	0	0.0073 ± 0.00094	0.032 ± 0.011	0	0	0.020 ± 0.0037	0.080 ± 0.040	0.0014 ± 0.0012	0

Each value (average ± standard deviations) represents the average of three independent experiments.[a] Administered compounds: **Phe**-$^{13}C_6$, [U-*ring*-$^{13}C_6$]phenylalanine; **1**-$^{13}C_6$, *p*-[U-*ring*-$^{13}C_6$]coumaric acid; **2**-d_3, [2,5,7-$^2H_3$]caffeic acid; **3**-d_5, [2,5-$^2H_2$,3-OC$^2H_3$]ferulic acid. [b] DAF, days after flowering. [c] Labelled compounds formed from the labelled precursors: **1**, *p*-coumaric acid; **2**, caffeic acid; **3**, ferulic acid; **4**, 5-hydroxyferulic acid; **5**, sinapic acid; **6**, *p*-coumaraldehyde; **7**, caffealdehyde; **8**, coniferaldehyde; **9**, 5-hydroxyconiferaldehyde; **10**, sinapaldehyde; **11**, *p*-coumaryl alcohol; **12**, caffeyl alcohol; **13**, coniferyl alcohol; **14**, 5-hydroxyconiferyl alcohol; **15**, sinapyl alcohol; **19**, matairesinol.

Fig. 6 Amounts of lignin and nitrobenzene oxidation products: ◇: vanillin; □: syringaldehyde; ■: lignin content measured by acetyl bromide method.

Clearly, these results suggest that the monomers [*i.e.* caffeic (**2**) and ferulic acids (**3**) and coniferyl alcohol (**13**)] accumulated transiently during the 5–7 DAF are converted to lignans, but probably not to lignins. In addition, because there is almost no doubt that in *C. tinctorius* seeds guaiacyl lignin is biosynthesized by the polymerization of coniferyl alcohol (**13**) as in other plants,[4,53] the biosynthesis of coniferyl alcohol (**13**) used for lignan is probably regulated differently from that towards guaiacyl lignin biosynthesis.

In contrast to coniferyl alcohol (**13**), the current view of lignin biosynthesis does not involve ferulic acid (**3**) as a precursor.[3,7–11] Hence, we conducted feeding experiments with labelled precursors to confirm the intermediacy of ferulic acid (**3**) in lignan biosynthesis. Administration of [2,5-$^2H_2$, 3-OC$^2H_3$]ferulic acid (**3**-d_5) to the seeds followed by comprehensive analysis of deuterium incorporation indicated that the deuterium atoms were incorporated into matairesinol (**19**) as well as coniferyl alcohol (**13**), while no deuterium incorporation into other phenylpropanoid monomers (**1**, **2**, **4–12**, **14**, **15**) could be detected (Table 1). Similarly, when *p*-[U-*ring*-$^{13}C_6$]coumaric acid (**1**-$^{13}C_6$) was administered, ^{13}C incorporation into caffeic (**2**) and ferulic (**3**) acids, coniferyl alcohol (**13**) and matairesinol (**19**) was observed (Table 1). Furthermore, the administration of [2,5,7-$^2H_3$]caffeic acid (**2**-d_3) resulted in deuterium incorporation into ferulic acid (**3**), coniferyl alcohol (**13**), and matairesinol (**19**) (Table 1). These results further suggest that ferulic acid (**3**) is a precursor to lignans.

However, it should be noted that even compounds that are not in the physiological metabolic route can be converted to the pathway compounds, when administered exogenously, as pointed out in our previous work.[33] While this artifactual process has been shown to take place,[33] we have also demonstrated in our previous study of the biosynthesis of a norlignan, *cis*-hinokiresinol,[54] that simultaneous or competitive administration of a true pathway precursor together with the tested compound can inhibit the non-physiological conversion. Since there is almost no doubt that phenylalanine is a physiological precursor for lignans, we carried out the simultaneous administration of [2,5-$^2H_2$, 3-OC$^2H_3$]ferulic acid (**3**-d_5) and [U-*ring*-$^{13}C_6$]phenylalanine (Table 2). In addition, because *p*-coumaric (**1**) and caffeic acids (**2**) are possible precursors of ferulic acid (**3**), and because the sole administration of *p*-[U-*ring*-$^{13}C_6$]coumaric acid (**1**-$^{13}C_6$) and [2,5,7-$^2H_3$]caffeic acid (**2**-d_3) resulted in the incorporation of ^{13}C and ^{2}H, respectively,

into ferulic acid (Table 1), two additional simultaneous administration experiments were carried out: one with *p*-[U-*ring*-$^{13}C_6$]coumaric acid (**1**-$^{13}C_6$) and [2,5-$^2H_2$, 3-OC$^2H_3$]ferulic acid (**3**-d_5) and the other with [2,5,7-$^2H_3$]caffeic acid (**2**-d_3) and [2,5-$^2H_2$, 3-OC$^2H_3$]ferulic acid (**3**-d_5) (Table 2). The results showed that these simultaneous or competitive administrations did not lead to the reduction of deuterium incorporation from [2,5-$^2H_2$, 3-OC$^2H_3$]ferulic acid (**3**-d_5) into matairesinol (**19**) (Table 2). Taken together, these results reveal that ferulic acid (**3**) is a precursor of lignans in *C. tinctorius* seeds.

Using the described metabolite quantitation system, we demonstrate in this study the difference between the metabolic flow towards lignans and that towards lignins. The fact that the three monomeric precursors for lignans, caffeic (**2**) and ferulic acids (**3**) and coniferyl alcohol (**13**), show similar accumulation profiles indicates the fast conversion of caffeic acid (**2**) to coniferyl alcohol (**13**), which is in accordance with no accumulation of coniferaldehyde (**8**). However, the conversion of these transiently accumulated monomers to matairesinol (**19**) proceeded within a time span of a few days, which hold for further conversion of matairesinol (**19**) to arctigenin (**20**) and trachelogenin (**21**) (Fig. 3). This time span suggests a slow metabolism for lignans relative to the lignin metabolic rate as follows.

Compared with the amounts of the lignins formed (125 μg mg^{-1}, correspond to 700 nmol of coniferyl alcohol unit mg^{-1}), the accumulation of metabolic intermediates in the cinnamate/monolignol pathway is insignificant (2–4 nmol mg^{-1}) and some, such as sinapaldehyde (**10**), which is a precursor specific to syringyl lignin but not lignans, are not even detectable (Fig. 3). In addition, β-glucosidase treatment of the methyl alcohol extracts from the seeds eliminates the possibility of the monolignol accumulation as their glycosides, namely coniferin and syringin. This indicates that once formed, the monomeric intermediates for lignins are converted rapidly to their products, lignin polymers. Together, these results strongly suggest a rapid metabolic flow for the biosynthesis of lignin.

In our study of the pathway for sinapyl alcohol (**15**), the precursor of syringyl lignin, administration of [3-OC$^2H_3$]-coniferaldehyde (**8**-d_3), [3-OC$^2H_3$]sinapaldehyde (**10**-d_3), and [U-*ring*-$^{13}C_6$]phenylalanine resulted in the formation of [$^2H_3$]sinapyl alcohol (**15**-d_3) and [$^{13}C_6$]sinapyl alcohol (**15**-$^{13}C_6$), respectively. As importantly, deuterium incorporation from [3-OC$^2H_3$]ferulic acid (**3**-d_3) into sinapyl alcohol (**15**) was completely absent, though it was incorporated into coniferyl alcohol (**13**) very efficiently. These results are in excellent agreement with the current view that syringyl lignin is formed from sinapyl alcohol (**15**) *via* coniferaldehyde (**8**), 5-hydroxyconiferaldehyde (**9**), and sinapaldehyde (**10**), but not from ferulic acid (**3**).[7–11] Furthermore, this accords well with the kinetic properties of recombinant *C. tinctorius* CAldOMT; a cDNA encoding CtCAldOMT was functionally expressed in *Escherichia coli* and 5-hydroxyconiferaldehyde (**9**) was found to be the best substrate of the recombinant CtCAldOMT among the possible substrates of the cinnamate/monolignol pathway. In addition, 5-hydroxyconiferaldehyde (**9**) inhibits methylation of other substrates, such as caffeic acid (**2**) and 5-hydroxyferulic acid (**4**) (T. Nakatsubo *et al.*, unpublished), like sweetgum (*Liquidambar styraciflua*) and aspen (*Populus tremuloides*) CAldOMTs.[8] Recently, Nair *et al.* proposed that in *A. thaliana*, sinapic acid (**5**) was formed from sinapaldehyde (**10**).[55] However, our [3-OC$^2H_3$]sinapaldehyde (**10**-d_3) administration did not result in deuterium incorporation into sinapic acid (**5**), though it was efficiently reduced to [$^2H_3$]sinapyl alcohol (**15**-d_3).

Taken together, these results demonstrate unequivocally that the metabolite quantitation system coupled with administration of stable-isotope-labelled precursors is a powerful tool for the characterization of complex metabolic pathways or metabolic profiling.

Experimental

Plant materials

Seeds of *Carthamus tinctorius* L. cv. Round-leaved White (Asteraceae) were purchased from Takii Seed, Co. Ltd. The seeds were germinated and the plants were maintained in a controlled-environment chamber (1.2 m × 1.2 m × 2.0 m; Koito-tron KG50HLA; Koito Industries, Co., Ltd.) at 24 °C under a 14–10 h light–dark regime.

Synthesis of monolignols and lignans

Synthesis of 5-hydroxyferulic acid (4), *p*-hydroxycinnamaldehydes (6)–(10), and *p*-hydroxycinnamyl alcohols (11)–(15). 5-Hydroxyferulic acid (**4**),[7] *p*-coumaraldehyde (**6**),[9] caffealdehyde (**7**),[9] coniferaldehyde (**8**),[56] 5-hydroxyconiferaldehyde (**9**),[7] *p*-coumaryl alcohol (**11**),[9] caffeyl alcohol (**12**),[9] coniferyl alcohol (**13**),[34,57] and 5-hydroxyconiferyl alcohol (**14**)[9] were prepared as previously. Sinapaldehyde (**10**) and sinapyl alcohol (**15**) were prepared from syringaldehyde in a similar manner to the synthesis of 5-hydroxyconiferaldehyde (**9**)[7] and 5-hydroxyconiferyl alcohol (**14**).[9]

5-Hydroxyferulic acid (**4**): δ_H (acetone-d_6) 7.52 (1 H, d, *J* 15.9), 6.90 (1 H, d, *J* 2.0), 6.82 (1 H, d, *J* 1.8), 6.32 (1 H, d, *J* 15.9), 3.88 (3 H, s); *m*/*z* (EI) 210.0553 (M$^+$, 100%. $C_{10}H_{10}O_5$ requires 210.0528), 195 (40.9), 168 (33.6), 166 (36.2), 128 (74.5), 114 (59.7).

p-Coumaraldehyde (**6**): δ_H ($CDCl_3$) 9.65 (1 H, d, *J* 7.7), 7.49 (2 H, d, *J* 7.4), 7.42 (1 H, d, *J* 15.9), 6.89 (2H, d, *J* 7.2), 6.61 (1 H, dd, *J* 15.8 and 7.8); *m*/*z* (EI) 148.0538 (M$^+$, 100%. $C_9H_8O_2$ requires 148.0525), 131 (35.6), 119 (47.0), 107 (13.8), 91 (54.4).

Caffealdehyde (**7**): δ_H (acetone-d_6) 9.62 (1 H, d, *J* 7.7), 7.52 (1 H, d, *J* 15.8), 7.21 (1 H, d, *J* 2.1), 7.12 (1 H, dd, *J* 8.2 and 1.8), 6.90 (1 H, d, *J* 8.2), 6.53 (1 H, dd, *J* 15.8 and 7.7); *m*/*z* (EI) 164.0448 (M$^+$, 100%. $C_9H_8O_3$ requires 164.0474), 147 (47.6), 136 (36.9), 110 (36.5), 97 (34.5).

Coniferaldehyde (**8**): δ_H 9.64 (1 H, d, *J* 7.8), 7.40 (1 H, d, *J* 15.6), 7.13 (1 H, dd, *J* 7.8, 2.0), 7.06 (1 H, d, *J* 1.9), 6.97 (1 H, d, *J* 8.0), 6.59 (1 H, dd, *J* 15.8 and 7.8), 3.91 (3 H, s); *m*/*z* (EI) 178.0629 (M$^+$, 100%. $C_{10}H_{10}O_3$ requires 178.0629), 161 (11.5), 147 (24.4), 135 (33.3), 107 (15.2).

5-Hydroxyconiferaldehyde (**9**): δ_H ($CDCl_3$) 9.65 (1 H, d, *J* 7.6), 7.34 (1 H, d, *J* 15.6), 6.87 (1 H, d, *J* 2.0), 6.70 (1 H, d, *J* 2.0), 6.58 (1 H, dd, *J* 15.7 and 7.7), 3.90 (3 H, s); *m*/*z* (EI) 194.0601 (M$^+$, 100%. $C_{10}H_{10}O_4$ requires 194.0583), 177 (14.3), 166 (52.9), 151 (98.5), 123 (32.3).

Sinapaldehyde (**10**): δ_H ($CDCl_3$) 9.65 (1 H, d, *J* 7.6), 7.38 (1 H, d, *J* 15.8), 6.80 (2 H, s), 6.60 (1 H, dd, *J* 15.8 and 7.8), 3.93 (6 H, s); *m*/*z* (EI) 208.0719 (M$^+$, 100%. $C_{11}H_{12}O_4$ requires 208.0736), 180 (80.0), 165 (98.0), 149 (31.4), 137 (61.1).

p-Coumaryl alcohol (**11**): δ_H ($CDCl_3$) 7.28 (2 H, d, *J* 8.6), 6.78 (2 H, d, *J* 8.6), 6.55 (1 H, d, *J* 15.9), 6.23 (1 H, dt, *J* 15.8 and 6.0), 4.30 (2 H, d, *J* 6.0); *m*/*z* (EI) 150.0670 (M^+, 54.3%. $C_9H_{10}O_2$ requires 150.0681), 147 (100), 131 (38.5), 121 (22.8), 107 (88.6), 94 (37.7).

Caffeyl alcohol (**12**): δ_H (acetone-d_6) 6.93 (1 H, d, *J* 1.8), 6.73–6.77 (2 H, m), 6.43 (1 H, d, *J* 15.8), 6.14 (1 H, dt, *J* 15.8 and 5.6), 4.18 (2 H, d, *J* 5.6); *m*/*z* (EI) 166.0625 (M^+, 100%. $C_9H_{10}O_3$ requires 166.0630), 148 (34.3), 138 (58.6), 123 (95.6), 110 (100), 101 (65.7).

Coniferyl alcohol (**13**): δ_H ($CDCl_3$) 6.83–6.95 (3 H, m), 6.53 (1 H, d, *J* 15.9), 6.23 (1 H, dt, *J* 15.8 and 5.8), 4.29 (2 H, d, *J* 5.7), 3.83 (3 H, s); *m*/*z* (EI) 180.0789 (M^+, 100%. $C_{10}H_{12}O_3$ requires 180.0787), 162 (10.4), 137 (92.6), 124 (61.1), 119 (37.0), 91 (33.3).

5-Hydroxyconiferyl alcohol (**14**): δ_H (acetone-d_6) 6.60 (1 H, d, *J* 1.9), 6.58 (1 H, d, *J* 1.9), 6.43 (1 H, d, *J* 15.8), 6.19 (1 H, dt, *J* 15.8 and 5.6), 4.18 (2 H, d, *J* 5.5), 3.82 (3 H, s); *m*/*z* (EI) 196.0753 (M^+, 100%. $C_{10}H_{12}O_4$ requires 196.0736), 178 (14.3), 167 (49.1), 153 (97.6), 140 (35.1), 107 (18.6).

Sinapyl alcohol (**15**): δ_H ($CDCl_3$) 6.63 (2 H, s), 6.53 (1 H, d, *J* 15.9), 6.24 (1 H, dt, *J* 15.8 and 5.8), 4.31 (2 H, dd, *J* 5.8 and 5.1), 3.90 (6 H, s); *m*/*z* (EI) 210.0911 (M^+, 100%. $C_{11}H_{14}O_4$ requires 210.0892), 194 (22.9), 182 (44.6), 167 (82.9), 154 (35.5), 149 (52.3), 128 (42.6).

Synthesis of *p*-[3,5,7-$^2H_3$]coumaric acid (1-d_3), [2,5,7-$^2H_3$]-caffeic acid (2-d_3), *p*-[3,5,7-$^2H_3$]coumaraldehyde (6-d_3), [2,5,7-$^2H_3$]-caffealdehyde (7-d_3), *p*-[3,5,7-$^2H_3$]coumaryl alcohol (11-d_3), and [2,5,7-$^2H_3$]caffeyl alcohol (12-d_3). 2,5-Dibromoprotocatechualdehyde (**23b**) was prepared by bromination of protocatechualdehyde (**22b**).[58] Phenolic hydroxyl and formyl groups of 3,5-dibromobenzaldehyde (**23a**) (Tokyo Chemical Industry, Co., Ltd.) and (**23b**) were protected, and resulting derivatives were deuterated with *n*-butyllithium and methyl alcohol-d_1 to give dimethyl acetals (**24a** and **b**), respectively. The acetals (**24a** and **b**) were hydrolyzed with 1 N HCl to give [$^2H_2$]benzyloxybenzaldehydes (**25a** and **b**). Then, **25a** and **25b** were oxidized with Ag_2O, and esterified to give ethyl [$^2H_2$]benzyloxybenzoates (**26a** and **b**). The esters were reduced with $LiAl^2H_4$ (Aldrich, ^2H: 98 atom%), and subsequent oxidation afforded [$^2H_3$]benzyloxybenzaldehydes (**27a** and **b**). Then, **27a** and **27b** were debenzylated to give [$^2H_3$]hydroxybenzaldehydes (**28a** and **b**). The deuterium-labelled benzaldehydes **28a** and **28b** were converted to **1**-d_3 or **2**-d_3 by Knoevenagel reaction[57] with malonic acid. Similarly, condensation of **28a** and **28b** with monoethyl malonate afforded ethyl cinnamates (**29a** and **b**). The esters were protected with *tert*-buthyldimethylsilyl chloride to give **30a** and **30b**, which were then reduced with $LiAlH_4$ to the corresponding alcohols (**31a** and **b**). The cinnamyl alcohols, **31a** and **31b**, were oxidized and deprotected to afford deuterium labelled cinnamaldehydes **6**-d_3 and **7**-d_3, while **31a** and **31b** were deprotected directly to give **11**-d_3 and **12**-d_3, respectively. The overall outline of the synthesis is displayed in Scheme 1.

2,5-Dibromoprotocatechualdehyde (**23b**): δ_H (acetone-d_6) 10.12 (1 H, s), 7.63 (1 H, s).

[3,5-$^2H_2$]-4-Benzyloxybenzaldehyde dimethyl acetal (**24a**): δ_H ($CDCl_3$) 7.36 (2 H, s), 7.28–7.50 (5 H, m), 5.35 (1 H, s), 5.07 (2 H, s), 3.31 (6 H, s).

[2,5-$^2H_2$]-3,4-Dibenzyloxybenzaldehyde dimethyl acetal (**24b**): δ_H ($CDCl_3$) 7.24–7.50 (10 H, m), 6.95 (1 H, s), 5.29 (1 H, s), 5.17 (2 H, s), 5.16 (2 H, s), 3.28 (6 H, s).

[3,5-$^2H_2$]-4-Benzyloxybenzaldehyde (**25a**): δ_H ($CDCl_3$) 9.89 (1 H, s), 7.84 (2 H, s), 7.28–7.50 (5 H, m), 5.15 (2 H, s).

[2,5-$^2H_2$]-3,4-Dibenzyloxybenzaldehyde (**25b**): δ_H ($CDCl_3$) 9.81 (1 H, s), 7.26–7.50 (11 H, m), 5.26 (2 H, s), 5.22 (2 H, s).

Ethyl [3,5-$^2H_2$]-4-benzyloxybenzoate (**26a**): δ_H ($CDCl_3$) 7.99 (2 H, s), 7.27–7.50 (5 H, m), 5.11 (2 H, s), 4.33 (2 H, q, *J* 7.1), 1.37 (3 H, t, *J* 7.2).

Ethyl [2,5-$^2H_2$]-3,4-dibenzyloxybenzoate (**26b**): δ_H ($CDCl_3$) 7.63 (1 H, s), 7.27–7.50 (10 H, m), 5.22 (2 H, s), 5.20 (2 H, s), 4.43 (2 H, q, *J* 7.2), 1.36 (3 H, t, *J* 7.2).

[3,5-$^2H_2$]-4-Benzyloxybenz(^2H)aldehyde (**27a**): δ_H ($CDCl_3$) 7.84 (2 H, s), 7.32–7.50 (5 H, m), 5.15 (2 H, s).

[2,5-$^2H_2$]-3,4-Dibenzyloxybenz(^2H)aldehyde (**27b**): δ_H ($CDCl_3$) 7.28–7.50 (11 H, m), 5.26 (2 H, s), 5.22 (2 H, s).

[3,5-$^2H_2$]-4-Hydroxybenz(^2H)aldehyde (**28a**): δ_H ($CDCl_3$) 7.81 (2 H, s).

[2,5-$^2H_2$]Protocatechu(^2H)aldehyde (**28b**): δ_H (acetone-d_6) 7.33 (1 H, s).

Ethyl *p*-[3,5,7-$^2H_3$]coumarate (**29a**): δ_H ($CDCl_3$) 7.42 (2 H, s), 6.28 (1 H, s), 4.27 (2 H, q, *J* 7.2), 1.33 (3 H, t, *J* 7.2).

Ethyl [2,5,7-$^2H_3$]caffeate (**29b**): δ_H (acetone-d_6) 7.03 (1 H, s), 6.25 (1 H, s), 4.17 (2 H, q, *J* 7.1), 1.26 (3 H, t, *J* 7.1).

Ethyl [3,5,7-$^2H_3$]-4-*O*-*tert*-butyldimethylsilyl-*p*-coumarate (**30a**): δ_H ($CDCl_3$) 7.41 (2 H, s), 6.28 (1 H, s), 4.25 (2 H, q, *J* 7.1), 1.32 (3 H, t, *J* 7.2), 0.98 (9 H, s), 0.21 (6 H, s).

Ethyl [2,5,7-$^2H_3$]-3,4-bis(*O*-*tert*-butyldimethylsilyl)caffeate (**30b**): δ_H ($CDCl_3$) 7.01 (1 H, s), 6.22 (1 H, s), 4.25 (2 H, q, *J* 7.2), 1.33 (3 H, t, *J* 7.2), 0.99 (9 H, s), 0.98 (9 H, s), 0.22 (6 H, s), 0.21 (6 H, s).

[3,5,7-$^2H_3$]-4-*O*-*tert*-Butyldimethylsilyl-*p*-coumaryl alcohol (**31a**): δ_H ($CDCl_3$) 7.26 (2 H, s), 6.23 (1 H, t, *J* 5.8), 4.28 (2 H, d, *J* 5.8), 0.97 (9 H, s), 0.19 (6 H, s).

[2,5,7-$^2H_3$]-3,4-Bis(*O*-*tert*-butyldimethylsilyl)caffeyl alcohol (**31b**): δ_H ($CDCl_3$) 6.85 (1 H, s), 6.17 (1 H, t, *J* 5.9), 4.29 (2 H, dd, *J* 5.9 and 5.8), 1.00 (9 H, s), 0.98 (9 H, s), 0.20 (6 H, s), 0.19 (6 H, s).

[3,5,7-$^2H_3$]-4-*O*-*tert*-Butyldimethylsilyl-*p*-coumaraldehyde (**32a**): δ_H ($CDCl_3$) 9.64 (1 H, d, *J* 7.8), 7.46 (2 H, s), 6.59 (1 H, d, *J* 7.8), 0.98 (9 H, s), 0.21 (6 H, s).

[2,5,7-$^2H_3$]-3,4-Bis(*O*-*tert*-butyldimethylsilyl)caffealdehyde (**32b**): δ_H ($CDCl_3$) 9.65 (1 H, d, *J* 7.8), 7.06 (1 H, s), 6.54 (1 H, d, *J* 8.0), 1.00 (9 H, s), 0.99 (9 H, s), 0.23 (6 H, s), 0.22 (6 H, s).

p-[3,5,7-$^2H_3$]Coumaric acid (**1**-d_3): δ_H (acetone-d_6) 7.54 (2 H, s), 6.32 (1 H, s); *m*/*z* (EI) 167.0657 (M^+, 100%. $C_9H_5{}^2H_3O_3$ requires 167.0662), 166 (40.3), 165 (8.4), 164 (1.5), 150 (33.4), 122 (23.3), 94 (20.0).

[2,5,7-$^2H_3$]Caffeic acid (**2**-d_3): δ_H (acetone-d_6) 7.03 (1 H, s), 6.25 (1 H, s); *m*/*z* (EI) 183.0613 (M^+, 100%. $C_9H_5{}^2H_3O_4$ requires 183.0611), 182 (44.1), 181 (13.6), 180 (1.0), 166 (26.8), 149 (20.1), 136 (33.6), 92 (20.1).

p-[3,5,7-$^2H_3$]Coumaraldehyde (**6**-d_3): δ_H ($CDCl_3$) 9.65 (1 H, d, *J* 7.7), 7.48 (2 H, s), 6.60 (1 H, d, *J* 7.8); *m*/*z* (EI) 151.0708 (M^+, 100%. $C_9H_5{}^2H_3O_2$ requires 151.0710), 150 (79.7), 149 (27.1), 148 (1.1), 134 (30.0), 122 (37.3), 94 (42.3).

[2,5,7-$^2H_3$]Caffealdehyde (**7**-d_3): δ_H (acetone-d_6) 9.61 (1 H, d, *J* 7.8), 7.11 (1 H, s), 6.52 (1 H, d, *J* 7.8); *m*/*z* (EI) 167.0654 (M^+,

100%. $C_9H_5{}^2H_3O_3$ requires 167.0662), 166 (39.0), 165 (14.2), 164 (1.4), 150 (40.3), 139 (30.2), 112 (23.5), 92 (24.2).

p-[3,5,7-$^2H_3$]Coumaryl alcohol (**11**-d_3): δ_H ($CDCl_3$) 7.27 (2 H, s), 6.22 (1 H, t, *J* 5.8), 4.30 (2 H, d, *J* 5.8); *m*/*z* (EI) 153.0855 (M^+, 59.7%. $C_9H_7{}^2H_3O_2$ requires 153.0869), 152 (13.6), 151 (7.8), 150 (1.2), 135 (23.5), 122 (10.1), 110 (100), 96 (50.3).

[2,5,7-$^2H_3$]Caffeyl alcohol (**12**-d_3): δ_H (acetone-d_6) 6.74 (1 H, s), 6.13 (1 H, t, *J* 5.6), 4.17 (2 H, d, *J* 5.6); *m*/*z* (EI) 169.0805 (M^+, 94.0%. $C_9H_7{}^2H_3O_3$ requires 169.0818), 168 (20.3), 167 (7.8), 166 (3.7), 151 (67.1), 126 (100), 112 (53.7), 94 (60.4).

Synthesis of [3-OC^{2}H$_3$]cinnamic acids (3-d_3)–(5-d_3), [2,5-$^2H_2$, 3-OC^{2}H$_3$]ferulic acid (3-d_5), [3-OC^{2}H$_3$]cinnamaldehydes (8-d_3)–(10-d_3), and [3-OC^{2}H$_3$]cinnamyl alcohols (13-d_3)–(15-d_3). [3-OC^{2}H$_3$]Cinnamic acids, [3-OC^{2}H$_3$]cinnamaldehydes, and [3-OC^{2}H$_3$]cinnamyl alcohols were prepared from protocatechualdehyde (**22b**), as outlined in Schemes 1 and 2.

Briefly, [3-OC^{2}H$_3$]vanillin (**33a**) was prepared from protocatechualdehyde (**22b**) as described previously,[33,57] and then transformed to [3-OC^{2}H$_3$]-5-hydroxyvanillin (**33b**)[33,59] and [3-OC^{2}H$_3$]syringaldehyde (**33c**)[33,60] by the methods reported previously. Similarly, [2,5-$^2H_2$, 3-OC^{2}H$_3$]vanillin (**33d**) was synthesized from [$^2H_2$]dibenzylprotocatechualdehyde (**25b**) (Scheme 2).

[3-OC^{2}H$_3$]Ferulic acid (**3**-d_3), [2,5-$^2H_2$, 3-OC^{2}H$_3$]ferulic acid (**3**-d_5), [3-OC^{2}H$_3$]-5-hydroxyferulic acid (**4**-d_3), [3-OC^{2}H$_3$]sinapic acid (**5**-d_3), [3-OC^{2}H$_3$]coniferaldehyde (**8**-d_3), [3-OC^{2}H$_3$]-5-hydroxyconiferaldehyde (**9**-d_3), [3-OC^{2}H$_3$]sinapaldehyde (**10**-d_3), [3-OC^{2}H$_3$]coniferyl alcohol (**13**-d_3), [3-OC^{2}H$_3$]-5-hydroxyconiferyl alcohol (**14**-d_3), and [3-OC^{2}H$_3$]sinapyl alcohol (**15**-d_3), were prepared from the corresponding labelled benzaldehydes {[3-OC^{2}H$_3$]vanillin (**33a**), [3-OC^{2}H$_3$]-5-hydroxyvanillin (**33b**), [3-OC^{2}H$_3$]syringaldehyde (**33c**), and [2,5-$^2H_2$, 3-OC^{2}H$_3$]vanillin (**33d**)} in the same manner as preparation of the corresponding unlabelled compounds and labelled *p*-hydroxyphnenyl and 3,4-dihydroxyphenyl compounds (Scheme 1).

[3-OC^{2}H$_3$]Ferulic acid (**3**-d_3): δ_H (acetone-d_6) 7.58 (1 H, d, *J* 15.8), 7.25 (1 H, d, *J* 2.0), 7.13 (1 H, dd, *J* 8.3, 1.9), 6.86 (1 H, d, *J* 8.0), 6.36 (1 H, d, *J* 16.1); *m*/*z* (EI) 197.0757 (M^+, 100%. $C_{10}H_7{}^2H_3O_4$ requires 197.0768), 196 (10.2), 195 (0.0), 194 (0.0), 179 (26.9), 151 (13.4), 133 (19.5), 105 (10.1).

[2,5-$^2H_2$, 3-OC^{2}H$_3$]Ferulic acid (**3**-d_5): δ_H (acetone-d_6) 7.59 (1 H, d, *J* 15.8), 7.13 (1 H, s), 6.37 (1 H, d, *J* 15.8); *m*/*z* (EI) 199.0883 (M^+, 72.1%. $C_{10}H_5{}^2H_5O_4$ requires 199.0892), 198 (41.4), 197 (15.2), 196 (1.5), 195 (0.0), 194 (0.0), 181 (19.2), 165 (13.5), 136 (50.0), 108 (91.3).

[3-OC^{2}H$_3$]-5-Hydroxyferulic acid (**4**-d_3): δ_H (acetone-d_6) 7.53 (1 H, d, *J* 15.9), 6.88 (1 H, d, *J* 2.0), 6.81 (1 H, d, *J* 2.0), 6.32 (1 H, d, *J* 15.8); *m*/*z* (EI) 213.0710 (M^+, 100%. $C_{10}H_7{}^2H_3O_5$ requires 213.0716), 212 (8.4), 211 (0.0), 210 (0.0), 195 (22.8), 167 (16.8), 149 (17.1), 121 (17.8).

[3-OC^{2}H$_3$]Sinapic acid (**5**-d_3): δ_H (acetone-d_6) 7.35 (1 H, d, *J* 15.9), 6.78 (2 H, s), 6.17 (1 H, d, *J* 15.8), 3.66 (3 H, s); *m*/*z* (EI) 227.0864 (M^+, 100%. $C_{11}H_9{}^2H_3O_5$ requires 227.0873), 226 (3.7), 225 (0.0), 224 (0.0), 212 (17.3), 181 (9.7), 149 (12.8), 121 (13.4).

[3-OC^{2}H$_3$]Coniferaldehyde (**8**-d_3): δ_H ($CDCl_3$) 9.65 (1 H, d, *J* 7.8), 7.40 (1 H, d, *J* 15.6), 7.12 (1 H, dd, *J* 8.0, 2.0), 7.06 (1 H, d, *J* 1.9), 6.96 (1 H, d, *J* 8.3), 6.59 (1 H, dd, *J* 15.8 and 7.8); *m*/*z* (EI) 181.0793 (M^+, 100%. $C_{10}H_7{}^2H_3O_3$ requires 181.0818), 180 (31.1), 179 (0.0), 178 (0.0), 164 (21.8), 147 (34.2), 135 (38.6), 107 (26.8).

[3-OC^{2}H$_3$]-5-Hydroxyconiferaldehyde (**9**-d_3): δ_H (acetone-d_6) 9.62 (1 H, d, *J* 7.7), 7.50 (1 H, d, *J* 15.8), 6.96 (1 H, d, *J* 1.6), 6.89 (1 H, d, *J* 1.7), 6.61 (1 H, dd, *J* 15.8 and 7.8); *m*/*z* (EI) 197.0759 (M^+, 100%. $C_{10}H_7{}^2H_3O_4$ requires 197.0768), 196 (7.8), 195 (1.0), 194 (0.0), 180 (14.1), 169 (37.9), 151 (62.4), 123 (31.5).

[3-OC^{2}H$_3$]Sinapaldehyde (**10**-d_3): δ_H ($CDCl_3$) 9.66 (1 H, d, *J* 7.6), 7.38 (1 H, d, *J* 15.6), 6.81 (2 H, s), 6.61 (1 H, dd, *J* 15.9 and 7.8), 3.94 (3 H, s); *m*/*z* (EI) 211.0935 (M^+, 100%. $C_{11}H_9{}^2H_3O_4$ requires 211.0924), 210 (7.9), 209 (0.4), 208 (0.0), 183 (43.6), 168 (33.6), 140 (22.5), 122 (14.4), 105 (14.1).

[3-OC^{2}H$_3$]Coniferyl alcohol (**13**-d_3): δ_H ($CDCl_3$) 6.85–6.97 (3 H, m), 6.53 (1 H, d, *J* 15.9), 6.22 (1 H, dt, *J* 15.6 and 5.8), 4.30 (2 H, d, *J* 5.6); *m*/*z* (EI) 183.0981 (M^+, 94.0%. $C_{10}H_9{}^2H_3O_3$ requires 183.0974), 182 (6.7), 181 (2.7), 180 (0.0), 165 (23.5), 140 (100), 127 (53.0), 119 (33.9), 91 (37.3).

[3-OC^{2}H$_3$]-5-Hydroxyconiferyl alcohol (**14**-d_3): δ_H ($CDCl_3$) 6.65 (1 H, d, *J* 1.7), 6.50 (1 H, d, *J* 1.7), 6.46 (1 H, d, *J* 15.8), 6.22 (1 H, dt, *J* 15.8 and 5.9), 4.28 (2 H, d, *J* 5.8); *m*/*z* (EI) 199.0915 (M^+, 100%. $C_{10}H_9{}^2H_3O_4$ requires 199.0923), 198 (10.5), 197 (3.4), 196 (0.0), 181 (48.0), 171 (51.0), 156 (86.5), 135 (50.3).

[3-OC^{2}H$_3$]Sinapyl alcohol (**15**-d_3): δ_H ($CDCl_3$) 6.63 (2 H, s), 6.52 (1 H, d, *J* 15.6), 6.24 (1 H, dt, *J* 15.6 and 5.9), 4.32 (2 H, d, *J* 5.7), 3.90 (3 H, s); *m*/*z* (EI) 213.1093 (M^+, 100%. $C_{11}H_{11}{}^2H_3O_4$ requires 213.1080), 212 (6.4), 211 (1.7), 210 (0.0), 195 (39.6), 185 (43.6), 170 (60.4), 157 (35.2), 149 (46.0).

Synthesis of *p*-[U-*ring*-$^{13}C_6$]coumaric acid. *p*-[U-*ring*-$^{13}C_6$]-Coumaric acid (**1**-$^{13}C_6$) was prepared by Knoevenagel reaction of [U-*ring*-$^{13}C_6$]-4-hydroxybenzaldehyde (CIL, 99 atom% ^{13}C) and malonic acid, as previously described.[54,57]

p-[U-*ring*-$^{13}C_6$]Coumaric acid (**1**-$^{13}C_6$): δ_H (acetone-d_6) 7.60 (1 H, ddt, *J* 16.0, 5.3 and 1.8), 7.29–7.78 (2 H, m), 6.64–7.14 (2 H, m), 6.33 (1 H, dd, *J* 16.0 and 5.2); *m*/*z* (EI) 172 (0.9%), 171 (6.0), 170.0705 (M^+, 100, $C_3{}^{13}C_6H_8O_3$, requires 170.0675), 169 (44.0), 168 (2.2), 167 (1.3), 166 (0.7), 165 (0.2), 164 (3.3), 125 (28.3), 124 (20.9), 113 (12.1), 96 (17.8).

Synthesis of lignans. (±)-Pinoresinols (**16**),[34] (±)-[9,9,9′,9′-$^2H_4$]pinoresinols (**16**-d_4)[61], (±)-lariciresinols (**17**),[35] (±)-[9,9,9′,9′-$^2H_4$]lariciresinols (**17**-d_4),[61] (±)-secoisolariciresinols (**18**),[34] (±)-[3-OC^{2}H$_3$]secoisolariciresinols (**18**-d_3),[33] (±)-matairesinols (**19**),[36] (±)-[3-OC^{2}H$_3$]matairesinols (**19**-d_3),[33] and (±)-arctigenins (**20**)[36] were prepared previously. (±)-[3-OC^{2}H$_3$]Arctigenins (**20**-d_3) were prepared in the same manner as the preparation of (±)-arctigenins (**20**)[36] but with [3-OC^{2}H$_3$]-4-benzyloxy-3-methoxybenzyl bromide.[33] (±)-Trachelogenins (**21**) were prepared in the same manner as the synthesis of wikstromol[62,63] but with (±)-β-(3,4-dimethoxybenzyl)-γ-butyrolactones prepared as previously described,[64] while (±)-[3-OC^{2}H$_3$]trachelogenins (**21**-d_3) were prepared exactly as that of the unlabelled (**21**) but with [3-OC^{2}H$_3$]-4-benzyloxy-3-methoxybenzyl bromide.[33]

(±)-[3-OC^{2}H$_3$]Arctigenins (**20**-d_3): δ_H ($CDCl_3$) 6.81 (1 H, d, *J* 7.8), 6.74 (1 H, d, *J* 8.3), 6.57–6.66 (2 H, m), 6.54 (1 H, dd, *J* 8.0 and 2.0), 6.46 (1 H, d, *J* 2.0), 4.13 (1 H, dd, *J* 9.2 and 7.2), 3.87 (1 H, dd, *J* 9.2 and 7.2), 3.84 (3 H, s), 3.80 (3 H, s), 2.95 (1 H, dd, *J* 14.2 and 5.2), 2.88 (1 H, dd, *J* 14.2 and 6.6), 2.42–2.68 (4 H, m); *m*/*z* (EI) 375.1787 (M^+, 42.8%. $C_{21}H_{21}{}^2H_3O_6$ requires 375.1761), 374 (0.0), 373 (0.6), 372 (0.0), 337 (9.1), 224 (10.0), 177 (25.7), 151 (100), 140 (88.6), 107 (15.7).

(±)-Trachelogenins (**21**): δ_H ($CDCl_3$) 6.85 (1 H, d, *J* 8.0), 6.79 (1 H, d, *J* 8.0), 6.60–6.74 (4 H, m), 3.98–4.07 (2 H, m), 3.86 (3 H, s), 3.85 (3 H, s), 3.85 (3 H, s), 3.10 (1 H, d, *J* 13.9), 2.93 (1 H, d, *J* 13.6), 2.90–3.03 (1 H, m), 2.46–2.57 (2 H, m); *m/z* (EI) 388.1497 (M^+, 88.6%. $C_{21}H_{24}O_7$ requires 388.1522), 337 (16.0), 356 (8.6), 151 (100), 138 (88.0), 122 (33.4), 107 (28.6).

(±)-[3-OC^2H_3]Trachelogenins (**21**-d_3): δ_H ($CDCl_3$) 6.85 (1 H, d, *J* 8.0), 6.79 (1 H, d, *J* 8.0), 6.60–6.73 (4 H, m), 3.98–4.07 (2 H, m), 3.86 (3 H, s), 3.85 (3 H, s), 3.10 (1 H, d, *J* 13.7), 2.93 (1 H, d, *J* 13.9), 2.90–3.02 (1 H, m), 2.47–2.59 (2 H, m); *m/z* (EI) 391.1691 (M^+, 100%. $C_{21}H_{21}{}^2H_3O_7$ requires 391.1710), 390 (0.0), 389 (0.0), 388 (0.0), 337 (9.7), 256 (18.6), 151 (78.9), 141 (87.1), 122 (31.4), 108 (40.0).

Chemicals

p-Coumaric acid (**1**), caffeic acid (**2**), ferulic acid (**3**), and sinapic acid (**5**) were purchased from Tokyo Chemical Industry, Co., Ltd.; Nakalai Tesque, Co., Ltd.; Nakalai Tesque, Co., Ltd.; Tokyo Chemical Industry, Co., Ltd. respectively. [U-*ring*-$^{13}C_6$]Phenylalanine (99 atom% ^{13}C), [U-*ring*-$^{13}C_6$]-4-hydroxybenzaldehyde (99 atom% ^{13}C), CH_3O^2H, $LiAl^2H_4$ (98 atom% ^2H), and C^2H_3I (99.5 atom% ^2H) were purchased from ICON, CIL, CIL, Aldrich, and Aldrich, respectively. The chemicals used were reagent grade unless otherwise stated.

Quantitative analysis of *p*-hydroxycinnamic acids, *p*-hydroxycinnamaldehydes, *p*-hydroxycinnamyl alcohols, and lignans in *C. tinctorius*

Preparation of standard curves. Standard curves for phenylpropanoid monomers (**1–15**) and lignans (**16–21**) were prepared using unlabelled (**1–21**) and labelled standards (**1**-d_3–**15**-d_3, **16**-d_4, **17**-d_4, **18**-d_3–**21**-d_3). Typically, 1.00 nmol of *p*-[3,5,7-$^2H_3$]coumaric acid (**1**-d_3) as internal standard was added to each of four 1 ml glass microtubes (Maruemu Co. Ltd.) containing certain amounts (0.25, 0.50, 1.25, 1.80 nmol, respectively) of unlabelled *p*-coumaric acid (**1**). After evaporation to dryness, each sample was dissolved in *N*,*O*-bis(trimethylsilyl)acetamide (8.0 μl), heated at 60 °C for 45 min, and an aliquot (0.8 μl) was subjected to GC-MS measurement with selected monitoring of ions arising from TMS ethers of *p*-coumaric acid (**1**) (*m/z* 308) and *p*-[3,5,7-$^2H_3$]coumaric acid (**1**-d_3) (*m/z* 311). Peaks at the appropriate GC retention times were integrated, and the ratio of the ion current at *m/z* 308 was divided by that at *m/z* 311. This ratio was a linear function of the amount of unlabelled *p*-coumaric acid (**1**) initially added to each microtube over the 0.25–1.80 nmol range: $Y = 1.30X + 0.31$, X = *p*-coumaric acid (**1**) (nmol), Y = ion current ratio of *p*-coumaric acid (**1**)/*p*-[3,5,7-$^2H_3$]coumaric acid (**1**-d_3). Standard curves of the other compounds (**2–21**) were prepared as above; caffeic acid (**2**), $Y = 0.38X + 0.07$, 0.22–1.66 nmol range; ferulic acid (**3**), $Y = 1.02X + 0.04$, 0.21–1.54 nmol range; 5-hydroxyferulic acid (**4**), $Y = 2.31X + 0.31$, 0.03–0.28 nmol range; sinapic acid (**5**), $Y = 1.04X + 0.32$, 0.03–0.26 nmol range; *p*-coumaraldehyde (**6**), $Y = 1.04X + 0.08$, 0.05–0.40 nmol range; caffealdehyde (**7**), $Y = 1.09X + 0.11$, 0.05–0.37 nmol range; coniferaldehyde (**8**), $Y = 0.92X + 0.03$, 0.05–0.34 nmol range; 5-hydroxyconiferaldehyde (**9**), $Y = 0.75X + 0.04$, 0.04–0.31 nmol range; sinapaldehyde (**10**), $Y = 0.83X + 0.07$, 0.08–0.58 nmol range; *p*-coumaryl alcohol (**11**), $Y = 0.76X + 0.07$, 0.05–0.40 nmol range; caffeyl alcohol (**12**), $Y = 1.44X + 0.07$, 0.05–0.36 nmol range; coniferyl alcohol (**13**), $Y = 0.26X + 0.03$, 0.44–3.32 nmol range; 5-hydroxyconiferyl alcohol (**14**), $Y = 3.96X + 0.73$, 0.04–0.31 nmol range; sinapyl alcohol (**15**), $Y = 0.72X + 0.07$, 0.04–0.29 nmol range; pinoresinol (**16**), $Y = 2.71X + 0.73$, 0.28–2.09 nmol range; lariciresinol (**17**), $Y = 1.31X + 0.36$, 0.28–2.08 nmol range; secoisolariciresinol (**18**), $Y = 0.87X + 0.14$, 2.20–16.56 nmol range; matairesinol (**19**), $Y = 1.09X + 0.15$, 2.23–16.75 nmol range; arctigenin (**20**), $Y = 1.22X + 0.16$, 2.15–16.12 nmol range; trachelogenin (**21**), $Y = 0.93X + 0.33$, 2.15–16.21 nmol range; where X represents the amounts of each unlabelled compound (**2–21**) (nmol), and Y represents the ion current ratio of unlabelled compounds (**2–21**)/deuterium-labelled compounds (**2**-d_3–**15**-d_3, **16**-d_4, **17**-d_4, **18**-d_3–**21**-d_3), respectively.

Quantitation of *p*-hydroxycinnamic acids, *p*-hydroxycinnamaldehydes, *p*-hydroxycinnamyl alcohols, and lignans. Maturing seeds of *C. tinctorius* were harvested and washed successively with tap and distilled water. Four seeds (*ca.* 0.2 g, each of 5–15 DAF) were frozen (liquid N_2), freeze-dried, powdered using a mortar and pestle, and extracted with hot MeOH (3.0 ml). Then deuterium-labelled internal standards [15 phenylpropanoid monomers (**1**-d_3–**15**-d_3) and 6 lignans (**16**-d_4, **17**-d_4, **18**-d_3–**21**-d_3)] were added to the MeOH solution. The MeOH solution thus obtained was extracted with hexane, and the hexane layer was removed. Solvents of the resultant MeOH solution were evaporated off, and the MeOH extracts thus obtained were treated with β-glucosidase (from almonds, SIGMA G-0395, 68 units in 1.0 ml of NaOAc buffer at pH 5.0) for 24 h at 37 °C. The reaction mixture was extracted with AcOEt (0.5 ml × 3), and the solvent was evaporated off. The extracts were dissolved in 8 μl of *N*,*O*-bis(trimethylsilyl)acetamide (BSA), heated for 45 min at 60 °C[34] and subjected to GC-MS measurement, so that phenylpropanoid monomers and lignans present in the extracts were identified and quantified by the use of the standard curves.

Administration experiments

Administration of ^{13}C- or deuterium-labelled compounds. [U-*ring*-$^{13}C_6$]Phenylalanine, *p*-[U-*ring*-$^{13}C_6$]coumaric acid (**1**-$^{13}C_6$), [2,5,7-$^2H_3$]caffeic acid (**2**-d_3), [2,5-$^2H_3$, 3-OC^2H_3]ferulic acid (**3**-d_5), [3-OC^2H_3]coniferaldehyde (**8**-d_3), and [3-OC^2H_3]sinapaldehyde (**10**-d_3) (0.4 mg each) were dissolved in 0.1 M KPB buffer (pH 7.0, 400.0 μl), and incubated individually with four maturing seeds (7 and 13 DAF) for 24 h. Then, the seeds were freeze-dried, powdered using a mortar and pestle, and extracted with hot MeOH. The MeOH extracts were submitted to GC-MS analysis exactly as above. The peaks on GC corresponding to phenylpropanoid monomers [(**1**)–(**15**)], and matairesinol (**19**) were analyzed for ^{13}C or deuterium incorporation.

Simultaneous administration of two distinct labelled compounds. Three sets of simultaneous administration of two distinct compounds {(i) [2,5-$^2H_2$, 3-OC^2H_3]ferulic acid (**3**-d_5) and [U-*ring*-$^{13}C_6$]phenylalanine, (ii) [2,5-$^2H_2$, 3-OC^2H_3]ferulic acid (**3**-d_5) and *p*-[U-*ring*-$^{13}C_6$]coumaric acid (**1**-$^{13}C_6$), and (iii) [2,5-$^2H_2$, 3-OC^2H_3]ferulic acid (**3**-d_5) and [2,5,7-$^2H_3$]caffeic acid (**2**-d_3)} were conducted. Thus, each mixture of the two compounds (0.2 mg each) was dissolved in 0.1 M KPB buffer (pH 7.0, 400.0 μl), and the resultant solution was incubated with four maturing

seeds of *C. tinctorius* (7 and 13 DAF) for 24 h. Then, the seeds were submitted to GC-MS analysis exactly as above.

Lignin analysis

Seed hulls of 30 seeds of *C. tinctorius* cv. Round-leaved White were pulverized. The powder thus obtained was extracted three times (1 h × 2, 2 h × 1) with 10 ml of ethyl ether at room temperature, and then four times (1 h × 2, 2 h × 1 and overnight) with 10 ml of MeOH at room temperature. The extractive-free seed hull samples were characterized by acetyl bromide,[41] nitrobenzene oxidation,[40] and thioacidolysis[39] methods. Phloroglucinol-HCl histochemical lignin staining was done as previously described.[37,38]

Instrumentation

^{1}H NMR spectra were taken with a JAM-LA400MK FT NMR System (JEOL). Chemical shifts and coupling constants (J) were given in δ and Hz, respectively. GC-MS measurement of lignans (**16–21**) was performed on a JMS-DX303HF mass spectrometer (JEOL) equipped with a Hewlett-Packard 5890J gas chromatograph and a JMA-DA5000 mass data system [electron-impact mode, 70 eV; gas chromatographic column, Shimazu HiCup CBP-10 M25-025 (5 m × 0.22 mm); temperature, 40 °C at t = 0–2 min, then to 230 °C at 30 °C min^{-1}; carrier gas, He; splitless injection], while that of phenylpropanoid monomers (**1–15**) was done with a GC-MS QP5050A mass spectrometer (Shimadzu) equipped with a Shimadzu GC-17A gas chromatograph [electron-impact mode 70 eV; gas chromatographic column, Shimazu HiCup CBP-10 M25-025 (20 m × 0.22 mm); temperature, 40 °C at t = 0–2 min, then to 240 °C at 40 °C min^{-1}; carrier gas, He; splitless injection]. Ultraviolet (UV) spectra were recorded on a Shimadzu UV-1600PC spectrometer.

Conclusions

In conclusion, the present study established a comprehensive and quantitative system for the cinnamate/monolignol and lignan pathways. This system, coupled with the administration of stable-isotope-labelled pathway intermediates, was successfully applied to the characterization of these pathways in *C. tinctorius* seeds, indicating that ferulic acid (**3**) is a precursor of lignans in this species. The results also lead to a proposition that the metabolic flow in the cinnamate/monolignol pathway towards lignan is much slower than that for lignin in *C. tinctorius* and perhaps in plants in general.

Acknowledgements

This research was partly supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (nos. 12660150, 16380116, and 18658069) and by a grant from the New Energy and Industrial Technology Development Organization (Development of Fundamental Technologies for Controlling the Process of Material Production of Plants). The authors thank Professor Vincent L. Chiang, North Carolina State University, USA, for discussions. The authors are grateful to Professor Fumiaki Nakatsubo, Kyoto University, Japan, for his suggestion in nitrobenzene oxidation and acetyl bromide methods, and to Professor Kazuhiko Fukushima, Nagoya University, Japan, for his suggestion in thioacidolysis method.

References

1 T. Umezawa, *Regul. Plant Growth Dev.*, 2001, **36**, 57–67.
2 T. Umezawa, *Phytochem. Rev.*, 2003, **2**, 371–390.
3 T. Umezawa, *Kagaku to Seibutsu*, 2005, **43**, 461–467.
4 T. Higuchi, Biosynthesis of lignin, in *Biosynthesis and Biodegradation of Wood Components*, ed. T. Higuchi, Academic, Orlando, 1985, pp. 141–160.
5 T. Higuchi and S. A. Brown, *Can. J. Biochem. Physiol.*, 1963, **41**, 613–620.
6 S. A. Brown and A. C. Neish, *J. Am. Chem. Soc.*, 1959, **81**, 2419–2424.
7 K. Osakabe, C. C. Tsao, L. Li, J. L. Popko, T. Umezawa, D. T. Carraway, R. H. Smeltzer, C. P. Joshi and V. L. Chiang, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 8955–8960.
8 L. Li, J. L. Popko, T. Umezawa and V. L. Chiang, *J. Biol. Chem.*, 2000, **275**, 6537–6545.
9 L. Li, X. F. Cheng, J. Leshkevich, T. Umezawa, S. A. Harding and V. L. Chiang, *Plant Cell*, 2001, **13**, 1567–1585.
10 J. M. Humphreys, M. R. Hemm and C. Chapple, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 10045–10050.
11 J. M. Marita, J. Ralph, R. D. Hatfield, D. Guo, F. Chen and R. A. Dixon, *Phytochemistry*, 2003, **62**, 53–65.
12 U. N. Dwivedi, W. H. Campbell, J. Yu, R. S. S. Datla, R. C. Bugos, V. L. Chiang and G. K. Podila, *Plant Mol. Biol.*, 1994, **26**, 61–71.
13 J. Van Doorsselaere, M. Baucher, E. Chognot, B. Chabbert, M.-T. Tollier, M. Petit-Conil, J.-C. Leplé, G. Pilate, D. Cornu, B. Monties, M. Van Montagu, D. Inzé, W. Boerjan and L. Jouanin, *Plant J.*, 1995, **8**, 855–864.
14 C.-J. Tsai, J. L. Popko, M. R. Mielke, W.-J. Hu, G. K. Podila and V. L. Chiang, *Plant Physiol.*, 1998, **117**, 101–112.
15 R. Atanassova, N. Favet, F. Martz, B. Chabbert, M.-T. Tollier, B. Monties, B. Fritig and M. Legrand, *Plant J.*, 1995, **8**, 465–477.
16 R. Zhong, W. H. Morrison, III, J. Negrel and Z.-H. Ye, *Plant Cell*, 1998, **10**, 2033–2045.
17 R. Zhong, W. H. Morrison, III, D. S. Himmelsbach, F. L. Poole, II and Z.-H. Ye, *Plant Physiol.*, 2000, **124**, 563–577.
18 H. Meyermans, K. Morreel, C. Lapierre, B. Pollet, A. De Bruyn, R. Busson, P. Herdewijn, B. Devreese, J. Van Beeumen, J. M. Marita, J. Ralph, C. Chen, B. Burggraeve, M. Van Montagu, E. Messens and W. Boerjan, *J. Biol. Chem.*, 2000, **275**, 36899–36909.
19 G. Pinçon, S. Maury, L. Foffmann, P. Geoffroy, C. Lapierre, B. Pollet and M. Legrand, *Phytochemistry*, 2001, **57**, 1167–1176.
20 J. M. Marita, J. Ralph, C. Lapierre, L. Jouanin and W. Boerjan, *J. Chem. Soc., Perkin Trans. 1*, 2001, 2939–2945.
21 F. Chen, M. S. S. Reddy, S. Temple, L. Jackson, G. Shadle and R. A. Dixon, *Plant J.*, 2006, **48**, 113–124.
22 J. Ralph, C. Lapierre, J. M. Marita, H. Kim, F. Lu, R. D. Hatfield, S. Ralph, C. Chapple, R. Franke, M. R. Hemm, J. Van Doorsserlaere, R. R. Sederoff, D. M. O'Malley, J. T. Scott, J. J. MacKay, N. Yahiaoui, A.-M. Boudet, M. Pean, G. Pilate, L. Jouanin and W. Boerjan, *Phytochemistry*, 2001, **57**, 993–1003.
23 K. Yamauchi, S. Yasuda and K. Fukushima, *J. Agric. Food Chem.*, 2002, **50**, 3222–3227.
24 K. Yamauchi, S. Yasuda, K. Hamada, Y. Tsutsumi and K. Fukushima, *Planta*, 2003, **216**, 496–501.
25 G. Schock, S. Goepfert, M. Morant, A. Hehn, D. Neyer, P. Ullmann and D. Werck-Reichhart, *J. Biol. Chem.*, 2001, **276**, 36566–36574.
26 R. Franke, M. R. Hemm, J. W. Denault, M. O. Ruegger, J. M. Humphreys and C. Chapple, *Plant J.*, 2002, **30**, 47–59.
27 R. Niggeweg, A. J. Nichael and C. Martin, *Nat. Biotechnol.*, 2004, **22**, 746–754.
28 J. Ralph, T. Akiyama, H. Kim, F. Lu, P. F. Schatz, J. M. Marita, S. A. Ralph, M. S. S. Reddy, F. Chen and R. A. Dixon, *J. Biol. Chem.*, 2006, **281**, 8843–8853.
29 T. Tokimatsu, N. Sakurai, H. Suzuki, H. Ohta, K. Nishitani, T. Koyama, T. Umezawa, N. Misawa, K. Saito and D. Shibata, *Plant Physiol.*, 2005, **138**, 1289–1300.
30 K. M. Oksman-Caldentey and K. Saito, *Curr. Opin. Biotechnol.*, 2005, **16**, 174–179.
31 K. Saito, *Tanpakushitsu Kakusan Koso*, 2003, **48**, 2199–2204.

32 O. Fiehn, *Plant Mol. Biol.*, 2002, **48**, 155–171.
33 N. Sakakibara, S. Suzuki, T. Umezawa and M. Shimada, *Org. Biomol. Chem.*, 2003, **1**, 2474–2485.
34 T. Umezawa, H. Kuroda, T. Isohata, T. Higuchi and M. Shimada, *Biosci., Biotechnol., Biochem.*, 1994, **58**, 230–234.
35 T. Umezawa and M. Shimada, *Mokuzai Gakkaishi*, 1994, **40**, 231–235.
36 T. Umezawa, T. Isohata, H. Kuroda, T. Higuchi and M. Shimada, Chiral HPLC and LS-MS analysis of several lignans, in *Biotechnology in pulp and paper industry*, ed. M. Kuwahara and M. Shimada, Uni, Tokyo, 1994, pp. 507–512.
37 T. Umezawa and T. Higuchi, Analysis of lignin degradation intermediates by thin layer chromatography and gas chromatography/mass spectrometry, in *Methods in Enzymology*, ed. W. A. Wood and S. T. Kellogg, Academic, San Diego, 1988, vol. 161, pp. 200–211.
38 T. Umezawa and T. Higuchi, Methods used in chemistry of lignin biodegradation, in *Modern Methods of Plant Analysis*, New Series, ed. H. F. Linskens and J. F. Jackson, Springer-Verlag, Berlin, 1989, vol. 10, pp. 161–185.
39 K. Hamada, Y. Tsutsumi, K. Yamauchi, K. Fukushima and T. Nishida, *J. Wood Sci.*, 2003, **49**, 333–338.
40 R. Katahira and F. Nakatsubo, *J. Wood Sci.*, 2001, **47**, 378–382.
41 S. Hirosawa, R. Katahira and F. Nakatsubo, *J. Wood Sci.*, 2002, **48**, 46–50.
42 H. Kim and J. Ralph, *J. Agric. Food Chem.*, 2005, **53**, 3693–3695.
43 von R. Hänsel, H. Schulz and C. Leuckert, *Z. Naturforsch., B: Anorg. Chem., Org. Chem., Biochem., Biophys.*, 1964, **19**, 727–734.
44 R. Palter and R. E. Lundin, *Phytochemistry*, 1970, **9**, 2407–2409.
45 R. Palter, W. F. Haddon and R. E. Lundin, *Phytochemistry*, 1971, **10**, 1587–1589.
46 S. Nishibe, A. Sakushima, S. Hisada and I. Inagaki, *Phytochemistry*, 1972, **11**, 2629.
47 R. Palter, R. E. Lundin and W. F. Haddon, *Phytochemistry*, 1972, **11**, 2871–2874.
48 S. Suzuki, T. Umezawa and M. Shimada, *Biosci., Biotechnol., Biochem.*, 2002, **66**, 1262–1269.
49 T. Umezawa, *Wood Res. (Kyoto, Jpn.)*, 2003, **90**, 27–110.
50 T. Umezawa and M. Shimada, *Biosci., Biotechnol., Biochem.*, 1996, **60**, 736–737.
51 S. Suzuki, T. Umezawa and M. Shimada, *Biosci., Biotechnol., Biochem.*, 1998, **62**, 1468–1470.
52 T. Umezawa, L. Li, S. Suzuki, N. Sakakibara, T. Nakatsubo and V. L. Chiang, *Proceedings of the 49th Lignin Symposium (Tsukuba, Japan)*, 2004, pp. 33–36.
53 K. Freudenberg, *Science*, 1965, **148**, 595–600.
54 S. Suzuki, T. Umezawa and M. Shimada, *J. Chem. Soc., Perkin Trans. 1*, 2001, **24**, 3252–3257.
55 R. B. Nair, K. L. Bastress, M. O. Ruegger, J. W. Denault and C. Chapple, *Plant Cell*, 2004, **16**, 544–554.
56 Y. Nakamura, F. Nakatsubo and T. Higuchi, *Wood Res. (Kyoto, Jpn.)*, 1974, **56**, 1–6.
57 T. Umezawa, L. B. Davin and N. G. Lewis, *J. Biol. Chem.*, 1991, **266**, 10210–10217.
58 L. Lundgren, K. Olsson and O. Theander, *Acta Chem. Scand., Ser. B*, 1979, **33**, 105–108.
59 S. K. Banerjee, M. Manolopoulo and J. M. Pepper, *Can. J. Chem.*, 1962, **40**, 2175–2177.
60 J. M. Pepper and J. A. MacDonald, *Can. J. Chem.*, 1952, **31**, 476–483.
61 T. Okunishi, T. Umezawa and M. Shimada, *J. Wood Sci.*, 2000, **46**, 234–242.
62 T. Umezawa and M. Shimada, *Mokuzai Gakkaishi*, 1996, **42**, 180–185.
63 T. Okunishi, N. Takaku, P. Wattanawikkit, N. Sakakibara, S. Suzuki, F. Sakai, T. Umezawa and M. Shimada, *J. Wood Sci.*, 2002, **48**, 237–241.
64 E. Brown and A. Daugan, *Heterocycles*, 1987, **26**, 1169–1172.