Chavicol formation in sweet basil (*Ocimum basilicum*): cleavage of an esterified C9 hydroxyl group with NAD(P)H-dependent reduction

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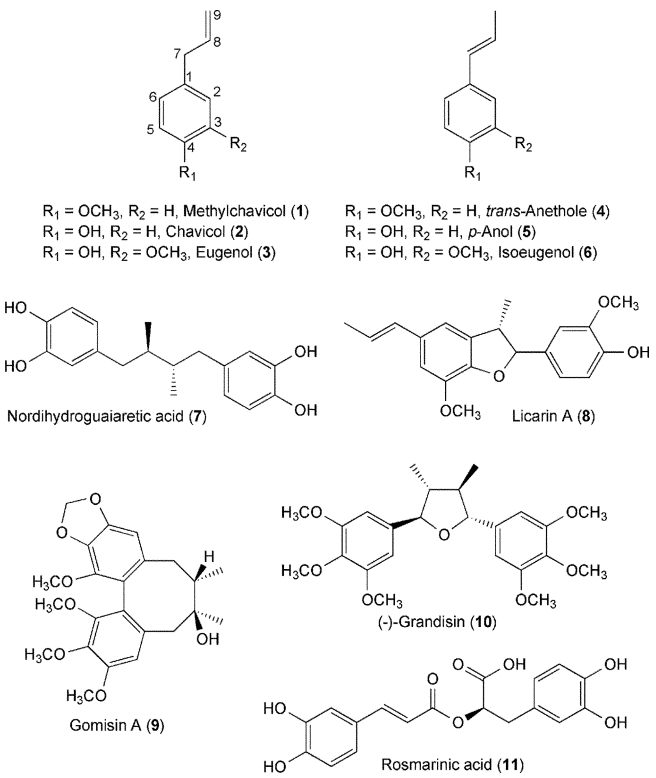
Propenyl- and allyl-phenols, such as methylchavicol, *p*-anol and eugenol, have gained importance as flavoring agents and also as putative precursors in the biosynthesis of 9,9′-deoxygenated lignans, many of which have potential medicinal applications. In spite of several decades of investigation, however, the complete biosynthetic pathway to a propenyl/allylphenol had not yet been reported. We have subjected a Thai basil variety accumulating relatively large amounts of the simplest volatile allylphenol, methylchavicol, to *in vivo* administration of radiolabeled precursors and assays of protein preparations *in vitro*. Through these experiments, the biosynthesis of chavicol was shown to occur *via* the phenylpropanoid pathway to *p*-coumaryl alcohol. Various possibilities leading to deoxygenation of the latter were examined, including reduction of the side-chain double bond to form *p*-dihydrocoumaryl alcohol, followed by dehydration to afford chavicol, as well as formation of *p*-methoxycinnamyl alcohol, with further side-chain modification to afford methylchavicol. A third possibility studied was activation of the side-chain alcohol of *p*-coumaryl alcohol, *e.g. via* esterification, to form a more facile leaving group *via* reductive elimination. The latter was shown to be the case using *p*-coumaryl esters as potential substrates for a NAD(P)H-dependent reductase to afford chavicol, which is then *O*-methylated to afford methylchavicol.

Introduction

The plant natural products methylchavicol (**1**), its regioisomer *trans*-anethole (**4**), eugenol (**3**) and isoeugenol (**6**), as well as nordihydroguaiaretic acid (**7**) and gomisin A (**9**), have attracted much interest due to their flavor/fragrance properties[1,2] and potential medicinal applications, respectively.[3] All are apparently biochemically related, being allyl/propenylphenol derived, and lack the oxygenated functionality at C9 characteristic of most other phenylpropanoids. Of these, methylchavicol (**1**) is a hypotensive[4] and acaricidal agent[5] as well as being partly responsible for the anise-like flavor of several spices, whereas eugenol (**3**) is a major essential oil component and flavoring agent, with a characteristic clove/cinnamon aroma. Eugenol (**3**) also has good antimicrobial activity against many pathogenic bacteria, fungi and nematodes, while being relatively harmless to plants and humans.[2] and refs. cited therein On the other hand, derivatives of the dimeric lignan nordihydroguaiaretic acid (**7**), which is abundant in the creosote bush (*Larrea tridentata*),[6–8] are promising antitumor agents.[9–11] Other lignans such as gomisin A (**9**) (isolated from *Schizandra chinensis*) and its analogs have excellent antioxidant properties, being especially used in traditional Chinese medicine to protect the liver.[12,13] In contrast, licarin A (**8**) from *Aristolochia pubescens*[14,15] and *Machilus thunbergii* induces apoptosis of cancer cells,[16] and (−)-grandisin (**10**) from *Litsea grandis*[17] and *Piper solmsianum*[18] has promising activity against the parasite that causes Chagas' disease.[19]



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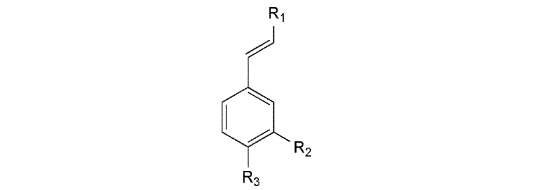
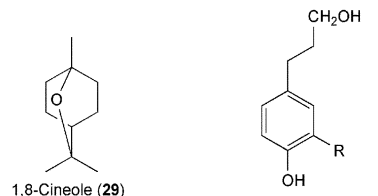
In terms of their biosynthetic roles, the monomeric propenyl/allylphenols are envisaged to serve as substrates for protein-controlled coupling reactions that generate, for example, the dimeric 9,9′-deoxygenated lignans, such as the aforementioned nordihydroguaiaretic acid (**7**), licarin A (**8**), gomisin A (**9**), and (−)-grandisin (**10**).[3,7,8,20] Various studies have also been directed towards explicitly establishing how the allyl/propenyl side-chain moiety is modified to result in loss of the C9 oxygenated functionality.[2,21–25] The biosynthetic pathways envisaged, however, generally include steps common to the known phenylpropanoid pathway (Fig. 1). For example, this could involve reduction of *p*-coumaroyl (**17**)/feruloyl (**19**) esters, aldehydes (**20**/**21**), or monolignols (**22**/**23**) to afford the corresponding dihydromonolignols (**30**/**31**) with subsequent dehydration, *e.g.* of *p*-dihydrocoumaryl alcohol (**30**), to give chavicol (**2**).[2] Alternatively, modification of the



R_1 = COOH, R_2 = R_3 = H, Cinnamic acid (**12**)
R_1 = COOH, R_2 = H, R_3 = OH, *p*-Coumaric acid (**13**)
R_1 = COOH, R_2 = OCH_3, R_3 = OH, Ferulic acid (**14**)
R_1 = COOH, R_2 = H, R_3 = OAc, *p*-Acetoxycinnamic acid (**15**)
R_1 = COCl, R_2 = H, R_3 = OAc, *p*-Acetoxycinnamoyl chloride (**16**)
R_1 = COX, R_2 = H, R_3 = OH, X = CoA, quinate, etc. (**17**)
R_1 = $COOCH_3$, R_2 = H, R_3 = OH, *p*-Coumaric acid methyl ester (**18**)
R_1 = COX, R_2 = OCH_3, R_3 = OH, X = CoA, quinate, etc. (**19**)
R_1 = CHO, R_2 = H, R_3 = OH, *p*-Coumaryl aldehyde (**20**)
R_1 = CHO, R_2 = OCH_3, R_3 = OH, Coniferyl aldehyde (**21**)
R_1 = CH_2OH, R_2 = H, R_3 = OH, *p*-Coumaryl alcohol (**22**)
R_1 = CH_2OH, R_2 = OCH_3, R_3 = OH, Coniferyl alcohol (**23**)
R_1 = CH_2OH, R_2 = H, R_3 = OCH_3, *p*-Methoxycinnamyl alcohol (**24**)
R_1 = CH_2OH, R_2 = H, R_3 = OTBS, *p*-Coumaryl alcohol TBS derivative (**25**)
R_1 = CH_2OH, R_2 = H, R_3 = ODNB, *p*-Coumaryl alcohol DNB derivative (**26**)
R_1 = CH_2OAc, R_2 = H, R_3 = OH, *p*-Coumaryl acetate (**27**)
R_1 = CH_2OAc, R_2 = OCH_3, R_3 = OH, Coniferyl acetate (**28**)



1,8-Cineole (**29**)

R = H, Dihydro-*p*-coumaryl alcohol (**30**)
R = OCH_3, Dihydroconiferyl alcohol (**31**)

allylic hydroxymethyl group functionality can also be considered to occur in order to provide a more facile leaving group at C9 with concomitant reduction either directly or *via* a putative quinone methide intermediate. A third and less likely possibility for allylphenol formation has been proposed, which would involve substitution of the terminal oxygenated side-chain carbon with a reduced carbon donated from, presumably, Met,[21] with this potentially involving a cyclopropyl intermediate.[2]

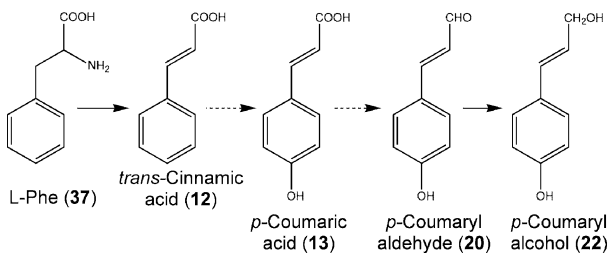
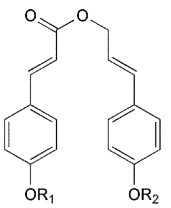


Fig. 1 Phenylpropanoid pathway, simplified.

There is some literature precedence in support of involvement of either of the first two postulated pathways in the production of chavicol (**2**): first, we have described the genes encoding a 7,8-allylic double bond reductase[26] in phenylpropanoid metabolism which would afford the saturated propanol side-chain, and, second, the proposed (displacement) mechanism could possibly occur directly or involve a quinone methide intermediate analogous to that envisaged for pinoresinol-lariciresinol, phenylcoumaran benzylic ether and isoflavone reductases (PLR, PCBER and IFR) catalyzed transformations.[27–29] Among the potential intermediates with suitable leaving groups for allyl/propenylphenol formation, there are several reports of both acetylated and hydroxycinnamoyl phenylpropanoids, *e.g.* coniferyl acetate (**28**)[30] and *p*-coumaryl coumarate (**32**).[31]



R_1 = R_2 = H, *p*-Coumaryl coumarate (**32**)
R_1 = Ac, R_2 = DNB, Protected *p*-coumaryl coumarate (**33**)

In this study, we now describe the delineation of the biochemical pathway to the simplest allylphenol, chavicol (**2**), in basil (*Ocimum basilicum*) using both isotopic labeling and enzymatic transformations.

Results and discussion

Many of the propenyl/allylphenol natural products accumulate on (or are exuded from) leaf surfaces, and several are volatile, serving as defensive signals and/or conferring important biological properties to the plants that accumulate them, *e.g.* as antimicrobial agents or as flavors/fragrances. In basil varieties, the two main classes of such volatile compounds include the terpenoids and allyl/propenylphenols, with both being prevalent within their essential oils and hence defining or contributing to the characteristics of their aromatic spices.

A member of the mint family (Lamiaceae), sweet basil (*Ocimum basilicum*) is one of the most cultivated herbaceous plants and confers remarkable flavors to popular culinary dishes. Different basil varieties accumulate distinctive combinations of volatile allylphenol compounds,[32] with eugenol (**3**) and methylchavicol (**1**) being the most abundant. Among the varieties readily available to our laboratory (lettuce-leaf, Thai and Italian broad-leaf from different sources), the sweet Thai basil variety accumulated higher levels of methylchavicol (**1**) and was thus chosen as a means to potentially simplify the biochemical reactions under study. The MeOH extracts of leaves of this basil variety, upon HPLC analysis, showed predominance of two UV-absorbing compounds, namely methylchavicol (**1**) and rosmarinic acid (**11**), based on comparison to chromatographic, UV absorption and NMR/mass spectroscopic properties with authentic standards, in addition to the terpenoid 1,8-cineole (**29**) (data not shown).

Most of the biosynthetic processes leading to formation of the essential oil components in basil (and several other Lamiaceae) are believed to occur in specialized structures called peltate glandular trichomes.[2] These consist of one stalk cell that protrudes out of the leaf surface, atop which sits a disc of four cells that secrete the terpenoids and allyl/propenylphenols into an oil sac isolated from the environment by a thin cuticular membrane. As the leaf and the glandular trichomes mature, these oil sacs are filled with the essential oil components, which can then be released upon physical rupture of the cuticle.

Young developing plant tissues are often sites with highest production of defensive compounds, and thus they help to defend against herbivores and pathogens until the leaf reaches maturity. Based on the higher densities of glandular trichomes and accumulated essential oil observed in developing basil leaves,[33] and an apparently higher propensity towards formation of the final *O*-methylated allylphenols,[34] we performed a preliminary analysis of methylchavicol (**1**) content in sweet Thai basil leaves of different ages. These measurements indicated that the youngest leaves produced and accumulated higher levels of methylchavicol (**1**) than the older leaves, in contrast to observed rosmarinic acid (**11**) contents which increased in amount as the leaves matured (Fig. 2). This was confirmed further by [U-^{14}C]-Phe (**37**) (18.4 kBq, 18.4 GBq mmol^{-1}) administration to leaf pairs of different ages, in which the apical pair of leaves gave highest incorporation (*circa* 4% by 7 h) of radiolabel into methylchavicol (**1**) (data not shown).

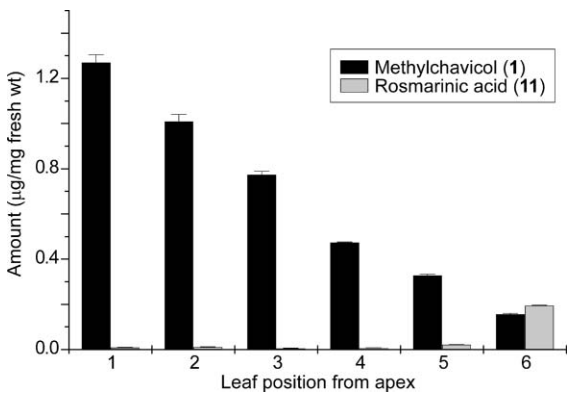


Fig. 2 Methylchavicol (**1**) and rosmarinic acid (**11**) contents in leaves of different ages.

For these reasons, the youngest apical leaves were generally employed in all further experiments. Accordingly, pairs of apical leaves were carefully detached from young basil stems and offered radioisotopically labeled compounds suspected to be pathway precursors in *in vivo* administration experiments; these tissues also served as a source of peltate glandular trichome cell suspensions from which crude protein extracts were prepared and used in *in vitro* assays as follows.

In vivo conversion of *p*-coumaryl alcohol (22) and phenylpropanoid intermediates into methylchavicol (1)

In vivo administration experiments using potential radiolabeled precursors were first carried out, at different time intervals, in order to identify satisfactory conditions for uptake and metabolism into methylchavicol (**1**). Thus, in accordance with previous observations regarding allylphenol biosynthesis,[23,35] [U-^{14}C]-Phe (**37**) (36.7 kBq, 18.4 GBq mmol^{-1}) administration to sets of 2–3 apical pairs of basil leaves resulted in its intact conversion into [^{14}C]-methylchavicol (**1**), and, to a smaller degree into [^{14}C]-rosmarinic acid (**11**) (Fig. 3a). Specifically, the radiolabel incorporation increased with increasing times of metabolism, *i.e.* to reach about 3% into [^{14}C]-methylchavicol (**1**) by 4 h, and to about 1% into rosmarinic acid (**11**). The latter observation was, therefore, consistent with the reported rosmarinic acid (**11**) biosynthetic pathway,[36–38] which utilizes Phe (**37**) to give, specifically, the caffeoyl moiety of rosmarinic acid (**11**). To verify that the radiolabel was indeed due to incorporation into [^{14}C]-methylchavicol (**1**),

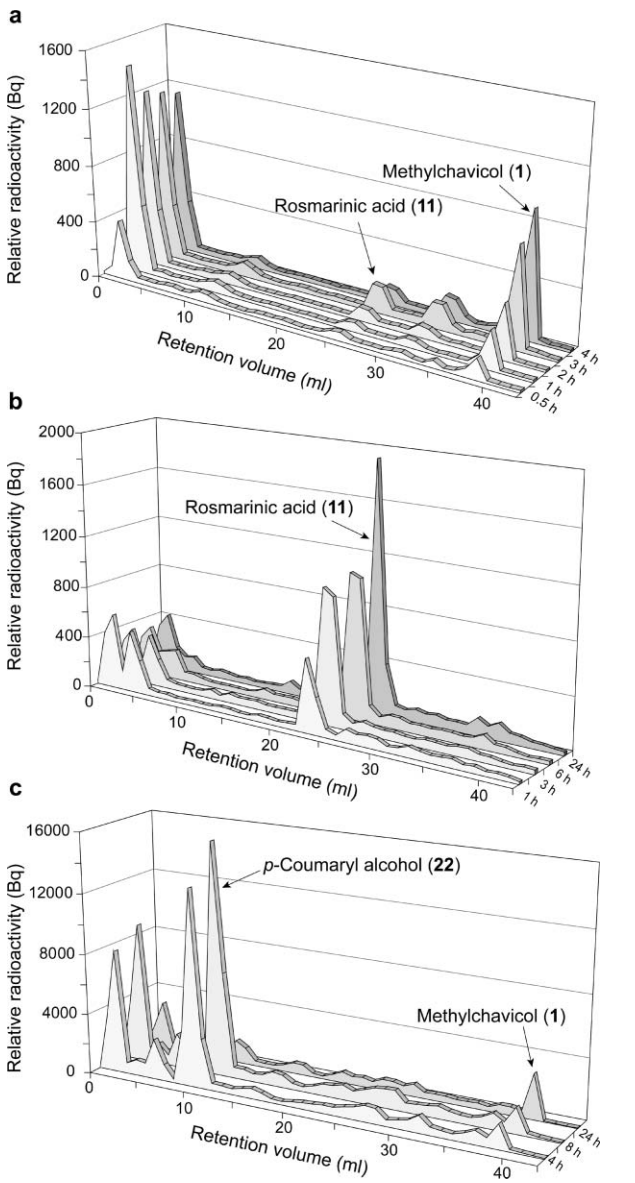
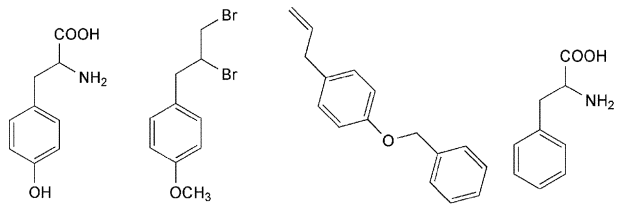


Fig. 3 Uptake and metabolism of various potential phenylpropanoid pathway intermediates into [^{14}C]-methylchavicol (**1**) and [^{14}C]-rosmarinic acid (**11**) at different time intervals. Administration and metabolism of (**a**) [U-^{14}C]-Phe (**37**) into [^{14}C]-methylchavicol (**1**) and [^{14}C]-rosmarinic acid (**11**); (**b**) [U-^{14}C]-Tyr (**34**) into [^{14}C]-rosmarinic acid (**11**); (**c**) [9-^{3}H]-*p*-coumaryl alcohol (**22**) into [9-^{3}H]-methylchavicol (**1**).

the latter was converted into its 8,9-dibromo derivative (**35**) by reaction using $TBABr_3$ (tetrabutylammonium tribromide) in $CHCl_3$. As expected, the radiolabel was also present in the [^{14}C]-8,9-dibromomethylchavicol (**35**), thereby indicating that [U-^{14}C]-Phe (**37**) had been intactly converted into methylchavicol (**1**).

Next, [8-^{14}C]-cinnamic (**12**) and *p*-coumaric (**13**) acids were individually administered to intact leaf tissues as above. In this way, [8-^{14}C]-cinnamic acid (**12**) was intactly incorporated into [^{14}C]-methylchavicol (**1**), albeit in a smaller amount (*circa* 1.5% of radiolabel in 4 h, 4% in 24 h) relative to [U-^{14}C]-Phe (**37**) (data not shown). This comparatively lower conversion may result from both its less efficient transport *in planta*, as well as its lower solubility in aqueous solution. Verification that the radiolabel was indeed due to [^{14}C]-methylchavicol (**1**) was again demonstrated by conversion into the [^{14}C]-8,9-dibromo derivative (**35**). By contrast, administration of [8-^{14}C]-*p*-coumaric acid (**13**) was unsuccessful, possibly due to the toxicity of this precursor, as suggested by noticeable browning and wilting of the leaf samples upon uptake and metabolism.



L-Tyr (**34**) 8,9-Dibromo-methylchavicol (**35**) Benzylchavicol (**36**) L-Phe (**37**)

We also examined whether [U-^{14}C]-tyrosine (**34**) could be converted into methylchavicol (**1**), even though deamination of tyrosine (**34**) into *p*-coumaric acid (**13**) is generally considered restricted to monocots, and not generally present in dicots. Thus, the administration of [U-^{14}C]-Tyr (**34**) (18.3 kBq, 18.3 MBq mmol^{-1}) did not result in the formation of the corresponding radiolabeled methylchavicol (**1**), as expected (Fig. 3b). On the other hand, it was efficiently incorporated into rosmarinic acid (**11**) (>4% radiolabel in 24 h), *i.e.* as expected from its known biosynthetic pathway,[36–38] in which Tyr (**34**) is metabolized specifically to form the dihydroxyphenyllactate moiety of rosmarinic acid (**11**). This observation is thus consistent with a monofunctional PAL enzyme, with Phe (**37**) and Tyr (**34**) metabolism occurring separately with biochemically distinct phenylpropanoid pools within the glandular trichomes.

It was next instructive to determine if *p*-coumaryl alcohol (**22**) served as a precursor. The latter was synthesized in [9-^{3}H]-radiolabeled form and administered (262.5 kBq, 1.68 GBq mmol^{-1}) to intact leaf tissues as before (Fig. 3c). This resulted in its relatively low incorporation into [^{3}H]-methylchavicol (**1**) (<1% radiolabel in 4 h, *ca.* 1.3% in 24 h), which may result from less efficient transport of the precursor *in planta*, and/or its lower solubility in aqueous media, and perhaps toxicity as well. Conversion of the putative [9-^{3}H]-methylchavicol (**1**) into [9-^{3}H]-8,9-dibromomethylchavicol (**35**) verified that it had been intactly incorporated. Taken together, these data thus suggested that methylchavicol (**1**) resulted from metabolism within the phenylpropanoid pathway to give *p*-coumaryl alcohol (**22**) which was then further converted into methylchavicol (**1**). These precursor metabolism data were thus in agreement with a previous study by Klischies *et al.*[24] regarding the biogenesis of eugenol (**3**), which was considered to involve coniferyl alcohol (**23**) as an intermediate.

We therefore next assessed whether dihydro-*p*-coumaryl alcohol (**30**) underwent dehydration *via* action of a dehydratase to form chavicol (**2**) (Fig. 4, pathway A). Thus, [9-^{3}H]-dihydro-*p*-coumaryl alcohol (**30**) was prepared by catalytic (Pd/C) hydrogenation of [9-^{3}H]-*p*-coumaryl alcohol (**22**) with H_2 in MeOH.[26] In subsequent *in vivo* administration experiments as before, however, no conditions were identified for its conversion into either [9-^{3}H]-chavicol (**2**) or [9-^{3}H]-methylchavicol (**1**). Methylchavicol (**1**) from administered samples was derivatized with $TBABr_3$ as before and yielded unlabeled 8,9-dibromomethylchavicol (**35**) (data not shown). These experiments thus suggested that the pathway to chavicol (**2**)/methylchavicol (**1**) utilized some other intermediate(s) derived from *p*-coumaryl alcohol (**22**). Pathway B was also eliminated from consideration since chavicol (**2**) has previously been demonstrated to undergo *O*-methylation in sweet basil to give methylchavicol (**1**),[34] thus indicating that methylation is the final step in its biosynthetic pathway.

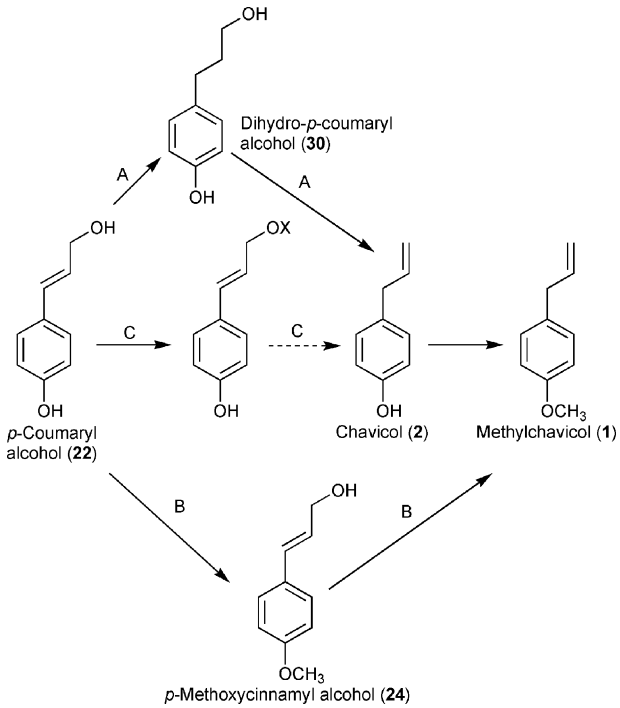


Fig. 4 Possible biosynthetic pathways to chavicol (**2**) and methylchavicol (**1**). (X = facile leaving group.)

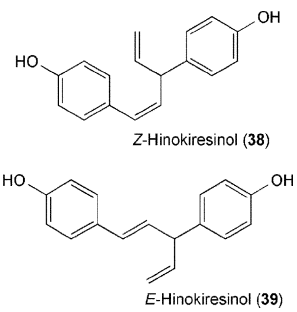
In vitro conversion of *p*-coumaryl alcohol derivatives into chavicol (2)

Attention was next directed to investigating whether cell-free extracts obtained from glandular trichomes in the young leaf tissue were able to convert *p*-coumaryl alcohol (**22**), or derivatives thereof, into either chavicol (**2**) or methylchavicol (**1**), respectively. The cell-free extracts were obtained by selective extraction of peltate glandular trichomes from the surface of young Thai basil leaves by a protocol similar to that of Gang *et al.*,[2] using a bead-beater apparatus and minute glass beads, followed by sonication of the resulting cell suspension.

Various *in vitro* experiments employing crude, cell-free, enzymatic extracts from young apical leaves, however, failed to demonstrate any direct conversion of [9-^{3}H]-*p*-coumaryl alcohol (**22**) into either [9-^{3}H]-chavicol (**2**) or [9-^{3}H]-methylchavicol (**1**) in the presence or absence of common enzymatic cofactors such as ATP and NAD(P)H. On the other hand, all phenylpropanoid pathway enzymes assayed could readily be detected (*e.g.* phenylalanine ammonia lyase, 4-coumarate CoA-ligase, and cinnamyl alcohol dehydrogenase; data not shown).

The next possible mechanism to consider was *via* modification of the terminal hydroxyl group of *p*-coumaryl alcohol (**22**) to form an activated (more facile) leaving group, thus facilitating enzymatic displacement of the oxygenated moiety (Fig. 4, pathway C) to form chavicol (**2**).

Among the potential leaving groups at C9 to afford chavicol (**2**), the possibility of ester formation through conjugation to an (activated) acid which then acts as a leaving group was next considered. Of possible ester functionalities, both *p*-coumaryl coumarate (**32**)[31] and coniferyl acetate (**28**)[30] have been reported as plant natural products. In this regard, *p*-coumaryl coumarate (**32**) has also been reported recently[39–41] to serve as a substrate in *Z*/*E*-hinokiresinol (**38**/**39**) biosynthesis, through the action of a yet to be described enzyme that apparently requires no co-substrate/cofactor. Two potential mechanisms can be considered



to account for *Z*/*E*-hinokiresinol (**38**/**39**) formation, *i.e.* involving either a concerted intramolecular rearrangement with decarboxylation (Fig. 5a, pathway A), or *via* cleavage to form the putative quinone methide intermediate shown with subsequent cyclization, decarboxylation and aromatization (Fig. 5a, pathway B). Neither mechanism, however, would require a reducing cofactor such as NAD(P)H. On the other hand, a comparable enzymatic displacement of an activated ester moiety for chavicol (**2**) or *p*-anol (**5**) formation could be envisaged to occur either directly following hydride attack at either C7 or C9, respectively (not shown), or alternatively *via* intermediacy of a quinone methide (Fig. 5b, pathways C and D). In either case, the reductions would be considered to require a cofactor such as NAD(P)H. *p*-Coumaryl coumarate (**32**) was thus synthesized from *p*-coumaric acid (**13**) and *p*-coumaryl alcohol (**22**) through protection of their phenolic groups, activation of the carboxylic acid with $SOCl_2$ and esterification, followed by deprotection using piperazine. Its structure was unambiguously identified based on exhaustive 2D NMR spectroscopic experiments which showed, among others, the diagnostic correlation H9–C10 (δ_H 4.79–δ_C 167.38 ppm) in the HMBC spectrum, thus eliminating the possibility of a phenol ester bond.

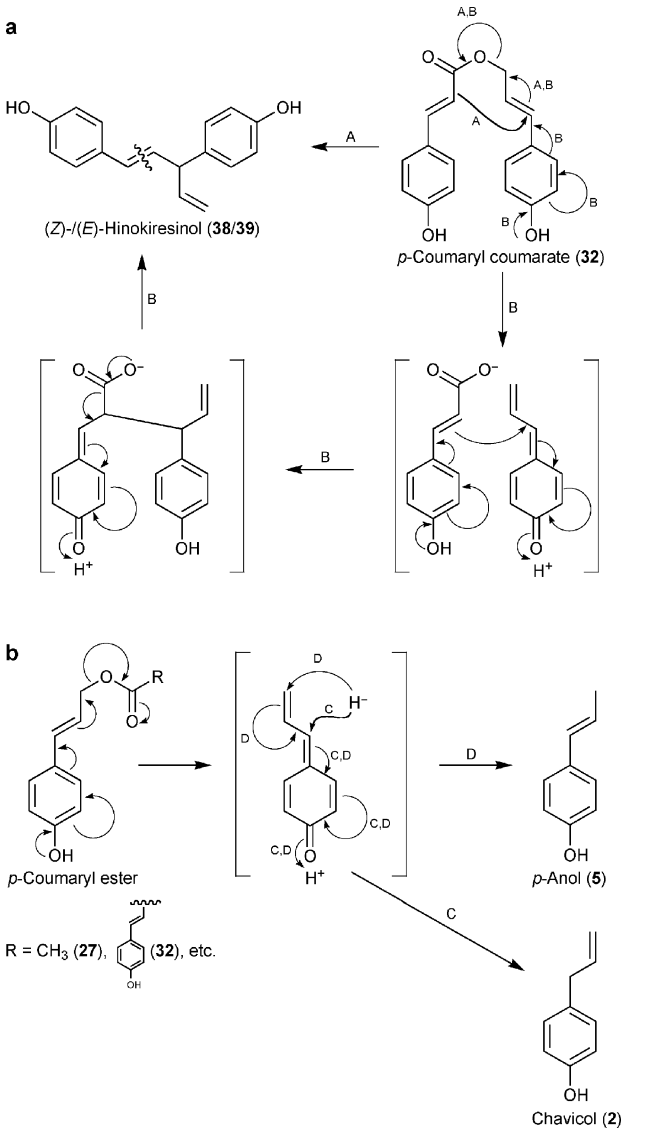


Fig. 5 Possible mechanisms for conversion of *p*-coumaryl esters into hinokiresinol (**38**/**39**) and chavicol (**2**). (**a**) (A) Concerted; (B) ester cleavage, followed by cyclization, decarboxylation and re-aromatization; (**b**) (C) and (D) ester displacement, putative quinone methide formation with subsequent reduction by hydride [from NAD(P)H] and re-aromatization to form (C) chavicol (**2**) and (D) *p*-anol (**5**). In (C) and (D), the acid moiety may be interchangeable. The reaction may also proceed through direct displacement of the ester moiety by the incoming hydride at carbons 7 and 9 (not shown).

[9-^{3}H]-*p*-Coumaryl coumarate (**32**, 24.7 MBq mmol^{-1}) was thus next prepared and incubated with the crude, cell-free preparation in the presence and absence of both NADPH and NADH as potential cofactors, respectively. An efficient conversion (*circa* 20% by 1 h, 30% by 2 h) of this substrate occurred into [9-^{3}H]-chavicol (**2**), but only in the presence of either NADPH or NADH. To further verify that the radioactivity was unambiguously associated with [9-^{3}H]-chavicol (**2**), the latter was purified and benzylated with benzyl chloride in MeOH using potassium iodide as catalyst, to afford radiochemically pure [9-^{3}H]-benzylchavicol (**36**). On the other hand, the crude enzyme preparation was unable to convert a mixture containing [9-^{3}H]-*p*-coumaryl alcohol (**22**), *p*-coumaric

acid (**13**) and/or NADPH as well (data not shown). The time-course for [9-^{3}H]-chavicol (**2**) formation from [9-^{3}H]-*p*-coumaryl coumarate (**32**) in the presence of either NADPH or NADH is shown in Fig. 6: in general, NADPH was better utilized.

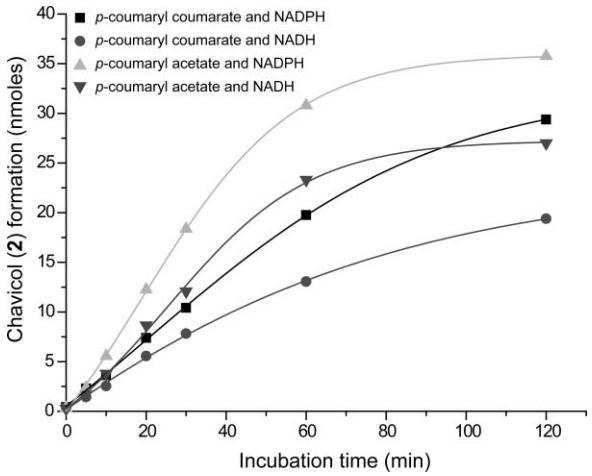


Fig. 6 Time-course formation of [9-^{3}H]-chavicol (**2**) from [9-^{3}H] *p*-coumaryl coumarate (**32**) and [9-^{3}H] *p*-coumaryl acetate (**27**) using cell-free extracts in the presence of NADPH and NADH.

It is noteworthy that *p*-coumaryl coumarate (**32**) is relatively unstable in aqueous solution, being rapidly hydrolyzed ($t_{1/2} < 2$ h) to form the acid **13** and alcohol **22**. It was also quite reactive towards the β-mercaptoethanol present in our initial protein preparations, but those difficulties were overcome by substitution with sodium ascorbate. Under such conditions, the enzymatic transformation occurred under both acidic and basic conditions (pH 6.25 and 8.0), although the latter favored much faster hydrolysis side-reactions, which therefore led to an unproductive substrate depletion. Generally, the enzymatic assays were terminated using 10% (v/v) glacial HOAc, and then immediately frozen. Subsequent manipulations, such as chromatographic analysis, used samples that had only been thawed for a few minutes to avoid experimental artifacts.

Similarly, the enzyme preparation was able to convert [9-^{3}H]-*p*-coumaryl acetate (**27**) into [9-^{3}H]-chavicol (**2**) in the presence of either NADPH or NADH (with slight preference for NADPH), as shown in Fig. 6. Enzymatic conversion of the acetate ester (**27**) was slightly faster than that of the *p*-coumarate ester (**32**), and *p*-coumaryl alcohol (**22**) resulting from hydrolysis was observed in all assays and controls as well.

Chavicol synthase activity was next unequivocally demonstrated using the deuterium-labeled [9-$^2H_2$]-*p*-coumaryl coumarate (**32**). This deuterated precursor was prepared from commercial *p*-coumaric acid (**13**) through esterification with MeOH in acidic solution, protection of the phenolic group and reduction with $LiAlD_4$ in MeOH to afford, after deprotection, [9-$^2H_2$]-*p*-coumaryl alcohol (**22**). The latter had an evident 2 Da increase in its mass relative to natural abundance *p*-coumaryl alcohol (**22**), and its ^{1}H NMR spectra clearly showed the disappearance of the 9-H_2 resonances at δ 4.17 ppm. The deuterated alcohol **22** so formed was then next protected and esterified to *p*-coumaroyl chloride as before, then deprotected to afford [9-$^2H_2$]-*p*-coumaryl coumarate (**32**). The latter was incubated with the crude enzyme preparation in the presence of NADPH for 2 h, with the assay terminated, the mixture extracted with Et_2O, and the organic solubles then silylated and subjected to GC-MS analysis. The mass spectral fragmentation patterns of enzymatically formed natural abundance and [9-$^2H_2$]-chavicol (**2**) as well as synthetic natural abundance chavicol (**2**) are displayed in Fig. 7. Natural abundance chavicol (**2**, silyl derivative, Fig. 7c) had a molecular ion (M^+, base peak) at m/z 206, with the next most abundant fragment [$M^+ - 15$] corresponding to the loss of a methyl group from the silyl derivative. Moreover, the fragmentation pattern of enzymatically synthesized (unlabeled) chavicol (**2**, silyl derivative) gave essentially

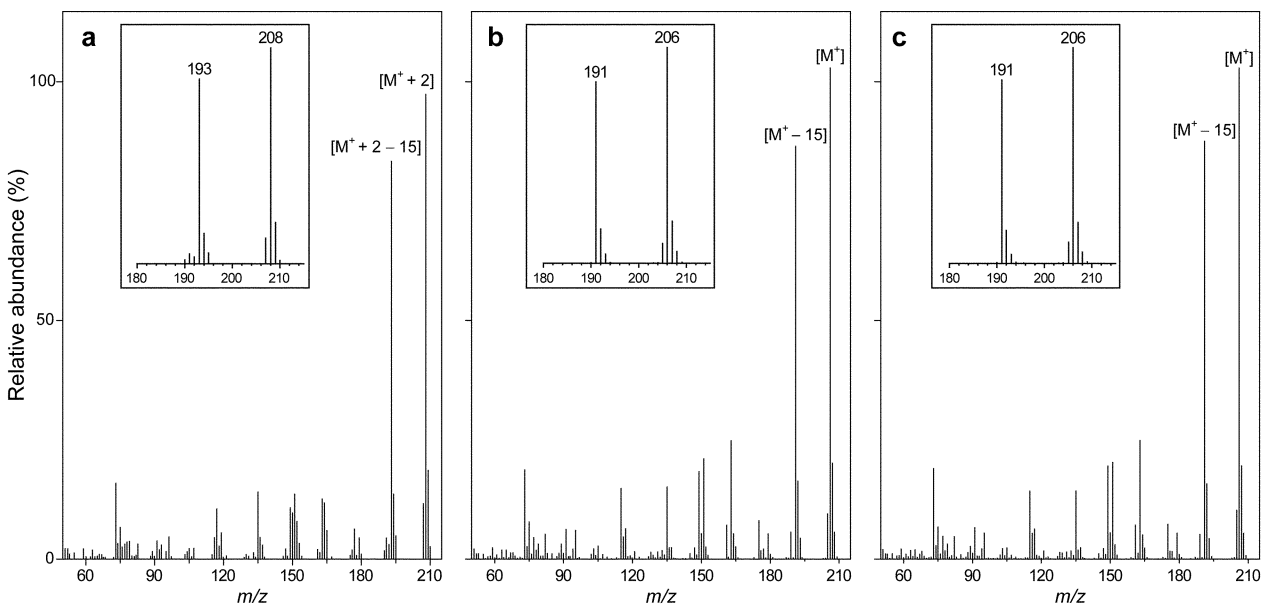


Fig. 7 Mass spectrometric fragmentation patterns of [9-$^2H_2$]-chavicol (**2**) and natural abundance chavicol (**2**) silyl derivatives. (**a**) Enzymatically formed [9-$^2H_2$]-chavicol (**2**); (**b**) enzymatically formed natural abundance chavicol (**2**); (**c**) synthetic chavicol (**2**).

an identical spectrum (Fig. 7b). That this was indeed the enzymatic product was confirmed by analysis of the enzymatically generated [9-$^2H_2$]-chavicol (**2**, silyl derivative, Fig. 7a): as expected, the molecular ion [M^+] and the [$M^+ - 15$] fragment revealed the presence of deuterium by [$M^+ + 2$] and [$M^+ + 2 - 15$], respectively. Thus, the chavicol synthase preparation unambiguously converted [9-$^2H_2$]-*p*-coumaryl esters into [9-$^2H_2$]-chavicol (**2**).

Conclusions

The above data are thus consistent with activation of *p*-coumaryl alcohol (**22**) in an ester form to serve as the substrate for chavicol (**2**) synthesis. This study will be extended in the future to establish to what extent purified chavicol synthase displays substrate versatility and specificity, *e.g.* in terms of relative kinetic properties using, for example, *p*-coumaryl acetate (**27**) and *p*-coumaryl coumarate (**32**) as substrates, respectively. Other studies will help determine the substrate(s) being utilized *in vivo*, and whether they are either acetate, *p*-coumarate or some yet to be defined ester. Additionally, to account for chavicol (**2**) formation in some species and *p*-anol (**5**) in others, the incoming hydride would only need to attack either position 9 for *p*-anol (**5**), or position 7 for chavicol (**2**), with concomitant acid displacement, *i.e.* either directly or *via* quinone methide formation and reduction.

In summary, the findings herein establish the overall biochemical pathway from phenylalanine (**37**) to methylchavicol (**1**). In related work using a data mining approach, the pathway to eugenol (**3**) and isoeugenol (**6**) was also found to proceed *via* a coniferyl alcohol ester, these being catalyzed by eugenol synthase and isoeugenol synthase, respectively.[42] Both proteins are NAD(P)H-dependent reductases, showing good similarity to pinoresinol-lariciresinol reductases,[27,29,43] isoflavone reductases[29] and phenylcoumaran benzylic ether reductase family.[29,44] What relationship they have to chavicol/*p*-anol synthase will be the subject of further studies. In preliminary work (data not shown), the eugenol/isoeugenol synthases convert *p*-coumaryl acetate (**27**) less efficiently into either chavicol (**2**) or *p*-anol (**5**), relative to coniferyl alcohol esters. Further work will compare and contrast the enzymology of chavicol (**2**)/*p*-anol (**5**) formation with that of eugenol (**3**)/isoeugenol (**6**).

Materials and methods

Plant material

Basil (*Ocimum basilicum*, 'sweet Thai') seeds were purchased from Johnny's Selected Seeds (Winslow, ME), germinated in vermiculite and grown in Sunshine growth media #1 (SunGro Horticulture, Bellevue, WA) in Washington State University greenhouses. Plants were fertilized five times each week with 200 ppm nitrogen fertilizer and kept under 16-h days (high pressure Na supplemental lights, day-time temperature 26–28 °C, night-time 15–16 °C).

Materials

[U-^{14}C]-Phe (**37**), [U-^{14}C]-Tyr (**34**) and NaB^3H_4 were purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA), $LiAlD_4$ was obtained from Cambridge Isotope Laboratories (Andover, MA), and TBSCl and Pd/C were from Lancaster Synthesis (Pelham, NH). All solvents used, either HPLC or reagent grade, were purchased from Mallinckrodt Baker (Phillipsburg, NJ), with GC-MS derivatization reagent purchased from Supelco (Bellefonte, PA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Instrumentation

^{1}H and ^{13}C NMR spectra were acquired on a Varian Mercury 300 spectrometer, with chemical shifts δ given in ppm relative to Me_4Si (^{1}H) or solvent (^{13}C), and coupling constant values *J* given in Hz. Electrostatic ionization mass spectrometry (EIMS) was carried out on an Integrity LC/MS System (Waters, Milford, MA) using He as a carrier gas and an ion source temperature of 205 °C, whereas electrospray ionization mass spectrometry (ESIMS) employed an LCQ dual-octapole MS system (Finnigan, Waltham, MA) using N_2 as a carrier gas, with 3.5–5 kV spray voltage and a capillary temperature of 200 °C. The high resolution mass spectrometric analyses were performed on an Agilent Series 1100 SL equipped with an ESI source. All acquisitions were performed under positive ionization mode with a capillary voltage of +4000 V. Nitrogen was used as nebulizer gas (30 psig) as well as drying gas at 10 L min^{-1} at a drying gas temperature of 325 °C. Data acquisition and processing was done with the software AnalystTM QS (Agilent Technologies, Palo Alto, CA, USA). GC-MS analyses of silylated samples were carried out on a HP 6890 Series GC System equipped with a RESTEK-5Sil-MS (30 m × 0.25 mm × 0.25 μm) column and a HP 5973 MS detector (EI mode, 70 eV). Reversed-phase HPLC analyses employed a Waters Alliance 2690 HPLC system equipped with UV-Vis diode-array detection under a flow rate of 1 cm^3 min^{-1}, gradient solvent system A : B (CH_3CN–3% HOAc in H_2O), linear unless otherwise noted. Separations used Symmetry Shield RP_{18} and RP_8 columns (Waters; 150 × 3.9 mm inner diameter, 5 μm particle size). Radioactive samples were analyzed in 5 cm^3 biodegradable counting scintillant (Amersham Biosciences, Piscataway, NJ) and measured using a Packard Tri-carb 2100TR liquid scintillation counter.

Chromatography conditions

Gas chromatography separations were carried out with the following temperature gradient: 120 °C for 5 min, 120–280 °C at 10 °C min^{-1}, 280 °C for a further 10 min. HPLC: Detection at 280 nm; RP_{18}: gradient for analysis of chavicol synthase activity assays: 10 to 70% A in 40 min, 100% A from 41 to 43 min, then to 10% A at 44 min, 60 min total run time; RP_8: gradient for analysis of radiolabeled precursor uptake experiments, PAL and CAD activity assays: 5 to 15% A in 15 min, then to 40% A at 33 min, 80% A at 40 min, 95% A from 41 to 43 min, and 5% A at 44 min, 60 min total run time; gradient for analysis of benzylated chavicol derivatives: 20 to 35% A in 15 min, then to 85% A at 33 min, 100% A from 34 to 37 min, and 20% A at 38 min, 50 min total run time; gradient for analysis of dibrominated methylchavicol **35**: 40 to 50% A in 15 min, then to 70% A at 33 min, 85% A at 40 min, 95% A from 41 to 43 min, and 40% A at 44 min, 55 min total run time.

Chemical syntheses

[8-^{14}C]-Cinnamic acid (12). [8-^{14}C]-Cinnamic acid (**12**) was prepared by a Döbner–Knoevenagel condensation reaction based

on the general procedure of Mitra *et al*.[45] Benzaldehyde (12.8 mm^3, 0.13 mmol), [2-^{14}C]-malonic acid (10.4 mg, 0.1 mmol, 9.25 MBq) and piperidine (5 mm^3) were dissolved in pyridine (100 mm^3) with the whole microwaved (66 s at 700 W) using a commercial microwave oven. After cooling to room temperature, H_2O (5 cm^3) was added with the resulting mixture extracted with Et_2O (4 × 3 cm^3). The combined organic solubles were then concentrated and purified by silica gel pTLC using CH_2Cl_2–MeOH–HOAc (95.5 : 4 : 0.5) to afford [8-^{14}C]-cinnamic acid (**12**, 10.0 mg, 68 μmol, 68% yield, 68 MBq $mmol^{-1}$).

[9-$^2H_2$]-*p*-Coumaryl alcohol (22). [9-$^2H_2$]-*p*-Coumaryl alcohol (**22**) was prepared by modification of the method of Kim *et al*.[46] as follows: *p*-coumaric acid (**13**) (1.97 g, 12 mmol) was dissolved in dry MeOH (70 cm^3) containing conc. H_2SO_4 (800 mm^3), with the solution heated until reflux began. After 16 h, the reaction was cooled to room temperature and neutralized using sat. $NaHCO_3$. H_2O (20 cm^3) was then added, with the whole extracted with $CHCl_3$ (3 × 75 cm^3). The combined organic layers were next washed with brine and dried (Na_2SO_4) to yield, after evaporation to dryness *in vacuo*, the crude *p*-coumaric acid methyl ester (**18**, 2.11 g), which was used without further purification. An aliquot of the methyl ester (**18**, 997 mg, *ca.* 5.6 mmol) was dissolved in dry THF (40 cm^3) under N_2 on an ice-bath, to which were sequentially added imidazole (1.54 g, 22.7 mmol) and TBSCl (*tert*-butyl-dimethylsilyl chloride, 3.09 g, 20.5 mmol). The resulting mixture was then stirred for 6 h, following which sat. NH_4Cl (30 cm^3) was added, with the whole extracted with Et_2O (3 × 50 cm^3). The combined organic layers were next washed with 1% aqueous HCl (2 × 50 cm^3), brine (2 × 50 cm^3), then dried (Na_2SO_4) and concentrated *in vacuo* to give an oil. The latter was dissolved in dry THF (50 cm^3) and slowly added to an ice-cold, vigorously stirred, suspension of $LiAlD_4$ (1.1 g, 26 mmol) in dry THF (100 cm^3) under N_2. The resulting mixture was stirred for 4 h, following which EtOAc (100 cm^3) and 3% aqueous HCl (20 cm^3) were added, with the whole extracted with EtOAc (4 × 150 cm^3). The combined organic solubles were next washed with brine (2 × 75 cm^3), dried (Na_2SO_4), concentrated *in vacuo* and purified by silica gel column chromatography using $CHCl_3$–MeOH (98 : 2) as eluent to afford the crude *p*-coumaryl alcohol TBS derivative (**25**). The latter was dissolved in dry THF (10 cm^3) on an ice-bath under N_2. TBAF (tetrabutylammonium fluoride, 21.2 cm^3, 1 M solution in THF, 21.2 mmol) was then slowly added, with the resulting mixture stirred for 1 h, after which sat. NH_4Cl (50 cm^3) was added and the whole stirred for 15 min. The THF was next evaporated *in vacuo*, with the resulting mixture extracted with Et_2O (3 × 100 cm^3). The combined organic solubles were washed with sat. NH_4Cl (50 cm^3), H_2O (50 cm^3) and brine (2 × 50 cm^3), then dried (Na_2SO_4) and concentrated *in vacuo*. The resulting oil was purified by silica gel column chromatography using a stepwise elution with $CHCl_3$ (for elution of deprotected byproducts) and $CHCl_3$–CH_3CN (9 : 1) to afford [9-$^2H_2$]-*p*-coumaryl alcohol (**22**, 216 mg, 1.42 mmol, 25.4% yield). δ_H (300 MHz; Me_2CO-d_6; Me_4Si) 7.27 (2 H, d, $J_{2,3/5,6}$ 8.4 Hz, 2-H/6-H), 6.79 (2 H, d, $J_{2,3/5,6}$ 8.4 Hz, 3-H/5-H), 6.51 (1 H, d, $J_{7,8}$ 15.9 Hz, 7-H), 6.19 (1 H, d, $J_{7,8}$ 15.9 Hz, 8-H); δ_C (75 MHz; Me_2CO-d_6) 157.90 (C-4), 130.31 (C-7), 129.87 (C-1), 128.46 (C-2/C-6), 127.85 (C-8), 116.28 (C-3/C-5); *m/z* (EI) 152 (M^+, 68%), 135 (11, $M^+ - OH$), 134 (13, $M^+ - H_2O$), 133 (13), 108 (41), 107 (100).

[9-^{3}H]-*p*-Coumaryl alcohol (22). [9-^{3}H]-*p*-Coumaryl alcohol (**22**) was prepared based on the procedure of Kim *et al*.[46] with the following modifications: *p*-coumaryl aldehyde (**20**, 20 mg, 135 μmol) was dissolved in dry MeOH (2 cm^3) under He in an ice-bath. NaB^3H_4 (5.5 mg, 145.5 μmol, 925 MBq) was then added with the resulting solution stirred for 45 min. Next, 1 M aqueous HCl was added until *ca.* pH 6, following which H_2O (500 mm^3) was added, with the resulting mixture stirred for another 20 min. The whole was then extracted with Et_2O (4 × 10 cm^3), with the combined organic solubles dried (Na_2SO_4) and concentrated *in vacuo*. The resulting material was re-suspended in a minimal amount of Et_2O/MeOH and purified by silica gel pTLC using CH_2Cl_2–MeOH (9 : 1) as eluent to afford [9-^{3}H]-*p*-coumaryl alcohol (**22**, 17.6 mg, 116 μmol, 85% yield, 1.68 GBq $mmol^{-1}$).

***p*-Acetoxycinnamoyl chloride (16).** *p*-Acetoxycinnamoyl chloride (**16**) was prepared based on the procedure of Helm *et al*.[47] with the following modifications: *p*-coumaric acid (**13**) (1.25 g, 7.62 mmol) was dissolved in pyridine (2.25 cm^3), to which freshly distilled Ac_2O (2 cm^3) was added, and the whole left unstirred for 4 h at room temperature. Next, the reaction mixture was added to ice-cold H_2O (50 cm^3), then stirred for 5 min, with the resulting white suspension filtered *in vacuo*, washed with cold H_2O (200 cm^3) and air-dried to afford *p*-acetoxycinnamic acid (**15**, 1.48 g), which was used without further purification. An aliquot of the latter (1.03 g, *ca.* 5 mmol) was dissolved in benzene (15 cm^3) containing $SOCl_2$ (2.8 cm^3, 38 mmol) and heated until reflux began. After 2 h, the whole was concentrated *in vacuo*, then toluene (75 cm^3) was added and evaporated *in vacuo* for two consecutive times. Recrystallization from hot toluene (20 cm^3) yielded *p*-acetoxycinnamoyl chloride (**16**, 905.6 mg, 3.77 mmol, 75% yield).

***p*-Coumaryl alcohol-2,4-dinitrophenyl ether (26).** *p*-Coumaryl alcohol (**22**) was converted into its DNB (dinitrobenzene) derivative according to the protocol of Grabber *et al*.[48] with the following modifications: *p*-coumaryl alcohol (**22**, 75 mg, 0.5 mmol) was dissolved in Me_2CO (600 mm^3) on an ice-bath, and $NaHCO_3$ (84 mg, 1 mmol in 1.5 cm^3 H_2O) was added to give a cloudy solution to which DNFB (2,4-dinitrofluorobenzene, 103 mg, 0.55 mmol, 70 mm^3 in 600 mm^3 Me_2CO) was added, with the resulting mixture stirred for 24 h at room temperature in the dark. Next, cold 3% aqueous HCl (9 cm^3) was added, with the whole stirred for 2 h, after which the suspension was filtered *in vacuo*, the precipitate washed with H_2O (50 cm^3), Et_2O (50 cm^3) and purified by silica gel pTLC using $CHCl_3$–MeOH (96 : 4) as eluent to afford *p*-coumaryl alcohol-2,4-dinitrophenyl ether (**26**, 68 mg, 0.21 mmol, 43% yield).

***p*-Coumaryl coumarate (32).** *p*-Coumaryl alcohol-2,4-dinitrophenyl ether (**26**, 68 mg, 0.21 mmol) was dissolved in freshly distilled CH_2Cl_2 (2 cm^3) on an ice-bath, then *p*-acetoxycinnamoyl chloride (**16**, 58 mg, 0.26 mmol) and DMAP (dimethylaminopyridine, 35 mg, 0.28 mmol) were sequentially added. The resulting solution was warmed to room temperature, with the whole stirred for 75 min. Next, CH_2Cl_2 (8 cm^3) was added, with the whole sequentially washed with cold aqueous 3% HCl (4 × 5 cm^3), cold brine (4 × 5 cm^3), and the organic solubles dried (Na_2SO_4). After concentration under N_2 flow, purification by silica gel column chromatography using $CHCl_3$–MeOH

(98 : 2) as eluent afforded the protected *p*-coumaryl coumarate (**33**, 89.9 mg, 0.175 mmol). An aliquot of the latter (87.2 mg, 0.17 mmol) was dissolved in dry THF (2 cm^3), with piperazine (146 mg, 1.7 mmol in 2 cm^3 dry THF) added at room temperature under N_2. Following stirring for 2 h, the mixture was diluted with $CHCl_3$ (3 cm^3) and EtOAc (25 cm^3), with the whole washed with sat. NH_4Cl solution (8 × 15 cm^3) to remove excess piperazine. The organic solubles were dried (Na_2SO_4), concentrated *in vacuo* and subjected to column chromatography using deactivated silica gel (pre-treatment with EtOH–HOAc, 99 : 1) and $CHCl_3$–EtOAc (1 : 1) as eluent, to afford *p*-coumaryl coumarate (**32**, 48.2 mg, 0.16 mmol, 80% yield) as a pale yellow solid. δ_H (300 MHz; Me_2CO-d_6; Me_4Si) 7.66 (1 H, d, $J_{11,12}$ 16.0 Hz, 12-H), 7.57 (2 H, d, $J_{14,15/17,18}$ 7.3 Hz, 14-H/18-H), 7.35 (2 H, d, $J_{2,3/5,6}$ 7.0 Hz, 2-H/6-H), 6.91 (2 H, d, $J_{14,15/17,18}$ 7.3 Hz, 15-H/17-H), 6.83 (2 H, d, $J_{2,3/5,6}$ 7.0 Hz, 3-H/5-H), 6.68 (1 H, d, $J_{7,8}$ 15.9 Hz, 7-H), 6.40 (1 H, d, $J_{11,12}$ 16.0 Hz, 11-H), 6.24 (1 H, m, H8), 4.79 (2 H, d, $J_{8,9}$ 6.5 Hz, 9-H); δ_C (75 MHz; Me_2CO-d_6) 167.38 (C-10), 160.64 (C-16), 158.45 (C-4), 145.55 (C-12), 134.71 (C-7), 131.04 (C-14/C-18), 129.02 (C-1), 128.89 (C-2/C-6), 127.05 (C-13), 121.54 (C-8), 116.76 (C-15/C-17), 116.38 (C-3/C-5), 115.58 (C-11), 65.64 (C-9); *m/z* (ESI) 294.7 (14%, M − 1^-), 177.1 (26), 163.0 (100, *p*-coumarate), 145.1 (16), 119.0 (43, *p*-coumarate − CO_2). NMR assignments were confirmed by exhaustive 2D NMR experiments (300 MHz ^1H–^1H COSY, 500 MHz HMBC and HMQC in a Varian Inova 500 spectrometer).

[9-$^2H_2$]-*p*-Coumaryl coumarate (32). [9-$^2H_2$]-*p*-Coumaryl alcohol (**22**, 203 mg, 1.34 mmol) was converted into [9-$^2H_2$]-*p*-coumaryl coumarate (**32**, 1.17 mmol, 348.1 mg, 87% overall yield) as described above. δ_H (300 MHz; Me_2CO-d_6; Me_4Si) 7.64 (1 H, d, $J_{11,12}$ 16.0 Hz, 12-H), 7.57 (2 H, d, $J_{14,15/17,18}$ 8.5 Hz, 14-H/18H), 7.35 (2 H, d, $J_{2,3/5,6}$ 8.5 Hz, 2-H/6-H), 6.90 (2 H, d, $J_{14,15/17,18}$ 8.5 Hz, 15-H/17-H), 6.83 (2 H, d, $J_{2,3/5,6}$ 8.5 Hz, 3-H/5-H), 6.68 (1 H, d, $J_{7,8}$ 15.9 Hz, 7-H), 6.39 (1 H, d, $J_{11,12}$ 16.0 Hz, 11-H), 6.24 (1 H, d, $J_{7,8}$ 15.9 Hz, 8-H); δ_C (75 MHz; Me_2CO-d_6) 167.34 (C-10), 160.66 (C-16), 158.50 (C-4), 145.54 (C-12), 134.86 (C-7), 131.06 (C-14/C-18), 129.06 (C-1), 128.91 (C-2/C-6), 127.10 (C-13), 121.45 (C-8), 116.76 (C-15/C-17), 116.39 (C-3/C-5), 115.64 (C-11); *m/z* (EI) 298 (M^+, 6%), 253 (3, M − CO_2), 192 (17), 164 (100, *p*-coumaric acid), 147 (97), 136 (58), 134 (94), 133 (95), 120 (53, *p*-coumaric acid − CO_2), 119 (53), 117 (28), 107 (38), 106 (60), 105 (82), 103 (28). *m/z* (ESI-HRMS) 321.1025 ([M + Na] requires 321.1072).

[9-^{3}H]-*p*-Coumaryl coumarate (32). [9-^{3}H]-*p*-Coumaryl alcohol (**22**, 37.5 mg, 0.25 mmol) was converted into [9-^{3}H]-*p*-coumaryl coumarate (**32**, 45.2 mg, 0.15 mmol, 60% yield, 24.7 MBq $mmol^{-1}$) as described above.

***p*-Coumaryl acetate (27).** *p*-Coumaryl alcohol (**22**, 9.4 mg, 62.7 μmol) was dissolved in pyridine (150 mm^3) containing a catalytic amount of DMAP (dimethylaminopyridine, *ca.* 1 mg) and freshly distilled Ac_2O (150 mm^3), with the whole left unstirred at room temperature for 3 h. Next, the mixture was added to 3% aqueous HCl (10 cm^3) and extracted with Et_2O (50 cm^3), with the organic solubles washed with 3% aqueous HCl (4 × 10 cm^3), sat. $NaHCO_3$ solution (2 × 10 cm^3) and brine (10 cm^3), then dried (Na_2SO_4) and concentrated *in vacuo*. The resulting oil was dissolved in pyrrolidine (750 mm^3) and left unstirred for 10 min, then added to 3% aqueous HCl (15 cm^3), with the whole extracted with EtOAc (30 cm^3). The organic solubles were washed with 3% aqueous HCl (2 × 15 cm^3), sat. NH_4Cl solution (2 × 15 cm^3), brine (15 cm^3), dried (Na_2SO_4) and concentrated *in vacuo*. The resulting product was fractionated by pTLC using hexane–Me_2CO (2 : 1) as eluent to afford *p*-coumaryl acetate (**27**, 3.79 mg, 19.7 μmol, 31.5% yield). δ_H (300 MHz; Me_2CO-d_6) 7.32 (2 H, d, $J_{2,3/5,6}$ 8.4 Hz, 2-H/6-H), 6.82 (2 H, d, $J_{2,3/5,6}$ 8.4 Hz, 3-H/5-H), 6.62 (1 H, d, $J_{7,8}$ 15.9 Hz, 7-H), 6.16 (1 H, dt, $J_{7,8}$ 15.9, $J_{8,9}$ 6.6 Hz, 8-H), 4.66 (2 H, d, $J_{8,9}$ 6.6 Hz, 9-H), 2.02 (3 H, s, OAc).

[9-^{3}H]-*p*-Coumaryl acetate (27). [9-^{3}H]-*p*-Coumaryl alcohol (**22**, 15.0 mg, 100 μmol) was dissolved in pyridine (200 mm^3) and Ac_2O (200 mm^3) containing a catalytic amount of DMAP as above, and left unstirred for 4 h. The reaction mixture was added to Et_2O (50 cm^3), then extracted with 3% aqueous HCl (3 × 15 cm^3), sat. NH_4Cl solution (2 × 15 cm^3), brine (2 × 15 cm^3), and dried (Na_2SO_4) before concentration *in vacuo*. The resulting material was dissolved in pyrrolidine (500 mm^3) and left unstirred for 5 min, with the whole then added to Et_2O and washed with 3% aqueous HCl (20 cm^3), sat. NH_4Cl solution (20 cm^3), brine (20 cm^3), and dried (Na_2SO_4) before concentration *in vacuo*. Purification by pTLC using hexane–Me_2CO (2 : 1) as eluent afforded [9-^{3}H]-*p*-coumaryl acetate (**27**, 8.5 mg, 44.4 μmol, 53.4 MBq $mmol^{-1}$).

Chavicol (2). Chavicol (**2**) was prepared following the procedure of Agharahimi and LeBel[49] with the following modifications: methylchavicol (**1**, 5 cm^3, 32.6 mmol) was dissolved in CH_2Cl_2 (100 cm^3) in an acetone–dry-ice-bath, then BBr_3 (35 cm^3 1 M soln in CH_2Cl_2, 35 mmol) was slowly added, with the whole then warmed to room temperature and stirred for 80 min. Next, the solution was cooled with an ice-bath and H_2O (50 cm^3) was added, with the resulting mixture extracted with CH_2Cl_2 (3 × 40 cm^3). The combined organic solubles were washed with brine, concentrated *in vacuo* and fractionated by silica gel column chromatography using hexane–EtOAc (9 : 1) as eluent, to afford chavicol (**2**, 3.71 g, 85% yield). δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.04 (2 H, d, $J_{2,3/5,6}$ 8.6, 2-H/6-H), 6.77 (2 H, d, $J_{2,3/5,6}$ 8.6, 3-H/5-H), 5.94 (1 H, m, 8-H), 5.06 (1 H, m, 9-H_a), 5.02 (1 H, m, 9-H_b), 3.31 (2 H, d, $J_{7,8}$ 6.6 Hz, 7-H); δ_C (75 MHz; $CDCl_3$) 153.81 (C-4), 137.81 (C-8), 131.95 (C-1), 129.59 (C-2/C-6), 115.32 (C-9), 115.25 (C-3/C-5), 39.25 (C-7).

Benzylchavicol (36). Chavicol (**2**, 230 mg, 1.7 mmol, 250 mm^3) and BzCl (1.6 cm^3, 14 mmol) were dissolved in MeOH (20 cm^3) containing K_2CO_3 (4 g) and a catalytic amount of KI, and heated until reflux began. After 25 h, the mixture was filtered, H_2O (80 cm^3) was added to the filtrate and the whole extracted with $CHCl_3$ (3 × 100 cm^3), with the combined organic solubles washed with brine (100 cm^3) and dried (Na_2SO_4). After concentration *in vacuo*, purification with silica gel column chromatography using $CHCl_3$ as eluent afforded benzylchavicol (**36**, 168 mg, 0.75 mmol, 44% yield) as a volatile oil. δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.35 (5 H, m, 2′-6′-H), 7.08 (2 H, d, $J_{2,3/5,6}$ 8.7 Hz, 2-H/6-H), 6.89 (2 H, d, $J_{2,3/5,6}$ 8.7 Hz, 3-H/5-H), 5.93 (1 H, m, 8-H), 5.06 (1 H, m, 9-H_a), 5.01 (1 H, m, 9-H_b), 5.00 (2 H, s, 7′-H), 3.30 (2 H, d, $J_{7,8}$ 6.7 Hz, 7-H); δ_C (75 MHz; $CDCl_3$) 157.13 (C-4), 137.76 (C-8), 137.10 (C-1′), 132.28 (C-1), 129.46 (C-2/C-6), 128.48 (C-3′/C-5′), 127.81 (C-4′), 127.39 (C-2′/C-6′), 115.41 (C-9), 114.71 (C-3/C-5), 69.92 (C-7′), 39.30 (C-7).

8,9-Dibromomethylchavicol (35). 8,9-Dibromomethylchavicol (**35**) was prepared based on the general procedure of Berthelot

et al.[50] Methylchavicol (**1**, 29.6 mg, 0.2 mmol, 30.67 mm^3) and $TBABr_3$ (tetrabutylammonium tribromide, 97 mg, 0.2 mmol) were mixed together at room temperature in $CHCl_3$ (5 cm^3) to give a red solution which was then sonicated for 45 min, after which it became nearly colorless. The resulting solution was then sequentially extracted with H_2O (10 cm^3), 5% aqueous $Na_2S_2O_3$ (2 × 10 cm^3), and brine (10 cm^3), with the organic solubles dried (Na_2SO_4) and concentrated *in vacuo* to afford the crude 8,9-dibromomethylchavicol (**35**, 55.8 mg), which was used without further purification. δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.20 (2 H, d, $J_{2,3/5,6}$ 8.7 Hz, 2-H/6-H), 6.87 (2 H, d, $J_{2,3/5,6}$ 8.7 Hz, 3-H/5-H), 4.32 (1 H, m, 8-H), 3.80 (3 H, s, OCH_3), 3.80 (1 H, dd, $J_{9a,9b}$ 10.4, $J_{8,9a}$ 4.2 Hz, 9-H_a), 3.60 (1 H, dd, $J_{9a,9b}$ 10.4, $J_{8,9b}$ 8.9 Hz, 9-H_b), 3.41 (1 H, dd, $J_{7a,7b}$ 14.6, $J_{7a,8}$ 4.9 Hz, 7-H_a), 3.09 (1 H, dd, $J_{7a,7b}$ 14.6, $J_{7b,8}$ 7.5 Hz, 7-H_b); δ_C (75 MHz; $CDCl_3$) 158.97 (C-4), 130.82 (C-2/C-6), 128.98 (C-1), 114.10 (C-3/C-5), 55.49 (OMe), 53.15 (C-8), 41.23 (C-7), 36.19 (C-9); *m/z* (ESI) 310.7, 308.7, 306.7 (47%, 100, 49, M + 1), 229.0, 227.0 (13, 13, M − Br + 1).

Administration of radiolabeled precursors

General. Young apical pairs of Thai basil leaves were excised under water to avoid air plug formation, with the stems immediately immersed in solutions containing the radiolabeled compound. When uptake was near completion (a few mm^3 left, before complete drying, typically *ca.* 30–60 min), a small volume (*ca.* 20–50 mm^3) of a solution of the unlabeled compound was added (time = 0) and subsequently replenished as needed to avoid complete drying of the administered solution. Samples were collected, immediately frozen in liquid N_2, ground in a mortar, and the fine powder transferred to a glass vial containing MeOH. After extraction, each sample was filtered through a 0.45 μm membrane of a syringe disc filter and 80 mm^3 aliquots were analyzed by HPLC without further treatment, with minute-long fractions being collected and individually subjected to liquid scintillation counting.

[U-^{14}C]-Phenylalanine (37) administration to leaves of different ages. The first (apical), second and third pairs of leaves of two different Thai basil shoot apexes (counted from the top) were carefully excised, with the petioles immediately immersed in an aqueous [U-^{14}C]-phenylalanine (**37**) solution (125 mm^3, 18.4 kBq, 18.4 GBq $mmol^{-1}$). When uptake was near completion, 5 mM aqueous phenylalanine (**37**, *ca.* 50 mm^3) was added (time = 0) and replenished as needed. Samples were collected at 7 h, extracted with MeOH (2 cm^3) overnight then processed and fractionated by HPLC with liquid scintillation counting as described above.

[U-^{14}C]-Phenylalanine (37) administration. Five Thai basil shoot apexes bearing 2–3 pairs of young leaves were excised and individually administered an aqueous solution (125 mm^3) containing [U-^{14}C]-phenylalanine (**37**, 36.7 kBq, 18.4 GBq $mmol^{-1}$). When uptake was near completion, 5 mM aqueous phenylalanine (**37**, *ca.* 50 mm^3) was added (time = 0) and replenished as needed. Samples were collected at 0.5, 1, 2, 3 and 4 h, extracted with MeOH (3 cm^3) for 10 min then processed and fractionated by HPLC with liquid scintillation counting as described above.

[U-^{14}C]-Tyrosine (34) administration. Four Thai basil shoot apexes bearing 2–3 pairs of young leaves were excised and individually administered an aqueous solution (125 mm^3) containing [U-^{14}C]-tyrosine (**34**, 18.3 kBq, 18.3 GBq $mmol^{-1}$) as above, followed by 5 mM aqueous tyrosine (**34**, *ca.* 50 mm^3) as needed. Samples were collected at 1, 3, 6 and 24 h, extracted with MeOH (4 cm^3) for 16 h then processed and fractionated by HPLC with liquid scintillation counting as described above.

[8-^{14}C]-Cinnamic acid (12) administration. Three pairs of the youngest (<1 cm) apical leaves of Thai basil shoots were carefully excised, with the individual petioles immediately immersed in a H_2O–DMSO (30 : 2.5 mm^3) solution containing [8-^{14}C]-cinnamic acid (**12**, 11.1 kBq). When uptake was near completion, 5 mM aqueous cinnamic acid (**12**, *ca.* 20 mm^3, with a minimal amount of 5 mM KOH added for dissolution) was added (time = 0) and replenished as needed. Samples were collected at 4, 8 and 24 h, individually extracted with MeOH (0.5 cm^3) overnight then processed and fractionated by HPLC with liquid scintillation counting as described above.

[9-^{3}H]-*p*-Coumaryl alcohol (22) administration. Three pairs of the youngest (<1 cm) apical leaves of Thai basil shoots were carefully excised, with the individual petioles immediately immersed in a H_2O (30 mm^3) solution containing [9-^{3}H]-*p*-coumaryl alcohol (**22**, 262.5 kBq). When uptake was near completion, a 2.5 mM solution of *p*-coumaryl alcohol (**22**, *ca.* 20 mm^3) in H_2O–DMSO–MeOH–5 M aqueous KOH (95 : 2.5 : 2.5 : 0.02) was added (time = 0) and replenished as needed. Samples were collected at 4, 8 and 24 h, individually extracted with MeOH (0.5 cm^3) overnight then processed and fractionated by HPLC with liquid scintillation counting as described above.

Crude cell-free preparations

Extraction of Thai basil glandular trichomes followed a protocol slightly modified from Gang *et al*.[2] Very young (<1 cm long) apical leaves of greenhouse-grown Thai basil were hand harvested and soaked in ice-cold 5 mM BisTris propane buffer (pH = 7.5) for *ca.* 15 min. Batches of leaves (12.5–15 g) were abraded in a Bead Beater (BioSpec Products, Bartlesville, OK) 300 cm^3 polycarbonate chamber containing glass beads (50 g, 0.5 mm diameter) and filled with a pH 7 buffer consisting of 50 mM Tris-HCl, 200 mM D-sorbitol, 20 mM sucrose, 10 mM KCl, 10 mM sodium ascorbate, 5 mM $mgCl_2$, 5 mM succinic acid, 1 mM EGTA, 0.5 mM KH_2PO_4, 1% w/v PVP (M_r = 360 000) and 0.6% w/v methylcellulose (M_r = 63 000). The mixture was abraded with 3 × 1 min pulses with 1 min cooling intervals on ice, then sequentially filtered through nylon meshes with pore diameters of 350, 105 and 40 μm, using the same buffer lacking PVP and methylcellulose for washing. The combined glandular trichomes retained on the 40 μm mesh were collected and the suspension allowed twice to settle on ice for 20 min, when empty oil sacs and floating cellular debris were removed and new buffer (*ca.* 10 cm^3) supplied. Packed oil gland yield was about 200 mm^3 per batch. The washing buffer was then removed and substituted for a protein extraction buffer (2–3 : 1, v/v, 50 mM BisTris, 10 mM sodium ascorbate, 10% (w/v) glycerol, pH = 8), with the glands ruptured by sonication with 3 × 20 s pulses with 40 s cooling intervals on ice. The resulting suspension (analyzed for disruption by microscopy) was immediately used as the enzyme extract for assays.

Enzyme assays

Phenylalanine ammonia-lyase assays were performed as previously described in Cochrane *et al.*[51] with the following modifications: assays were performed in 250 mm^3 of a buffered solution (100 mM potassium phosphate, pH = 8.1) containing 1 mM [U-^{14}C]-phenylalanine (**37**, *ca.* 3.7 kBq) and enzyme extract (10 mm^3), with the mixture incubated for 35 min at 30 °C and the assay terminated by addition of glacial HOAc (10 mm^3). Negative controls used boiled enzyme extracts. 80 mm^3 aliquots were then analyzed by HPLC, with minute-long fractions collected and individually analyzed for radioactivity.

4-Coumarate CoA-ligase assays were performed and analyzed as previously described in Costa *et al.*[52] with the following modifications: assays were performed in 250 mm^3 of a buffered solution (100 mM Tris, pH = 7.5) containing 2.5 mM ATP, 2.5 mM $mgCl_2$, 0.4 mM CoA, 0.5 mM *p*-coumaric acid (**13**), and enzyme extract (10 mm^3), with the mixture incubated for 35 min at 30 °C and the assay terminated by addition of glacial HOAc (10 mm^3). Negative controls included the use of boiled enzyme extracts and omission of substrates. Aliquots of 80 mm^3 each were then analyzed by HPLC as described.

Cinnamyl alcohol dehydrogenase assays were performed as previously described in Kim *et al.*[46] with the following modifications: assays were performed in 250 mm^3 of a buffered solution (100 mM BisTris, pH = 6.25) containing 1 mM NADPH, 0.5 mM *p*-coumaryl aldehyde (**20**), and enzyme extract (10 mm^3), with the mixture incubated for 30 min at 30 °C and the assay terminated by addition of glacial HOAc (10 mm^3). Negative controls included boiled enzyme extracts and omission of substrates. Aliquots of 80 mm^3 were then analyzed by HPLC.

Chavicol synthase assays were performed in 250 mm^3 of a buffered solution (100 mM potassium phosphate, pH = 6.25) containing 1 mM NAD(P)H, 0.4 mM [9-^{3}H]-*p*-coumaryl coumarate (**32**, *ca.* 2.7 kBq) or [9-^{3}H]-*p*-coumaryl acetate (**27**, *ca.* 6.5 kBq), and enzyme extract (50 mm^3). Assays were run for up to 120 min at 30 °C and terminated by addition of glacial HOAc (25 mm^3) and freezing; controls included both boiled enzyme extracts and omission of substrates.

Assays involving unlabeled and [9-$^2H_2$]-*p*-coumaryl coumarate (**32**) were performed by incubation in 2.5 cm^3 of the buffered solution as above containing 1 mM NADPH, 0.4 mM natural abundance or [9-$^2H_2$]-*p*-coumaryl coumarate (**32**) and enzyme extract (500 mm^3) for 2 h at 30 °C. Assays were terminated by addition of glacial HOAc (250 mm^3) and brine (125 mm^3), with the resulting mixture extracted with Et_2O (3 × 500 mm^3). An aliquot (50 mm^3) of the combined ethereal layer was derivatized with *N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA, 50 mm^3) in pyridine (20 mm^3) and analyzed by GC-MS.

Benzylation of [9-^{3}H]-chavicol (2)

Enzymatically synthesized [9-^{3}H]-chavicol (**2**) was isolated from a buffered solution (2.5 cm^3, 100 mM potassium phosphate, pH = 6.25) containing 1 mM NADPH, 0.4 mM [9-^{3}H]-*p*-coumaryl coumarate (**32**, *ca.* 27 kBq) and enzyme extract (500 mm^3). After incubation for 2 h at 30 °C, the reaction was stopped by addition of glacial HOAc (250 mm^3), unlabeled chavicol (**2**) (10 mm^3) was added as a carrier, and the mixture was extracted with Et_2O (3 × 5 cm^3) with the combined organic solubles dried (Na_2SO_4). [9-^{3}H]-Chavicol (**2**) was purified by silica gel pTLC using $CHCl_3$–CH_3CN (85 : 15) and benzylated to give (**36**) as before. [9-^{3}H]-Benzylchavicol (**36**) was further identified based both on HPLC retention time and UV spectrum in comparison to the authentic unlabeled sample.

Dibromination of radiolabeled methylchavicol (1)

Unlabeled methylchavicol (**1**, 10 mm^3) was added as a carrier to the MeOH extract of a sample administered [U-^{14}C]-Phe (**37**), and [U-^{14}C]-methylchavicol (**1**) was isolated by silica gel pTLC using $CHCl_3$ as eluent and dibrominated to give (**35**) as before. [U-^{14}C]-8,9-Dibromomethylchavicol (**35**) was further identified based both on HPLC retention time and UV spectrum in comparison to the authentic unlabeled sample.

[8-^{14}C]-Methylchavicol (**1**) was purified from the MeOH extract of a sample administered [8-^{14}C]-cinnamic acid (**12**), to which unlabeled methylchavicol (**1**, 10 mm^3) was added as a carrier, and dibrominated exactly as described above to give [8-^{14}C]-8,9-dibromomethylchavicol (**35**), which was further identified as before.

[9-^{3}H]-Methylchavicol (**1**) was co-purified with unlabeled methylchavicol (**1**, 5 mm^3) as a carrier from the MeOH extract of a sample administered [9-^{3}H]-*p*-coumaryl alcohol (**22**) as above, and dibrominated to give [9-^{3}H]-8,9-dibromomethylchavicol (**35**), which was identified as before.

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