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BIOSYNTHESIS OF *PODOPHYLLUM* LIGNANS—I. CINNAMIC ACID PRECURSORS OF PODOPHYLLOTOXIN IN *PODOPHYLLUM HEXANDRUM*

DAVID E. JACKSON and PAUL M. DEWICK

Department of Pharmacy, University of Nottingham, Nottingham NG7 2RD, U.K.

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Key Word Index—*Podophyllum hexandrum*; Podophyllaceae; aryltetralin; lignans; biosynthesis; podophyllotoxin.

Abstract—Feeding experiments in *Podophyllum hexandrum* plants have established that phenylalanine, cinnamic acid and ferulic acid are good precursors of the two major aryltetralin lignans podophyllotoxin and 4′-demethylpodophyllotoxin. Sinapic and 3,4,5-trimethoxycinnamic acids were poorly utilized, showing that the substitution pattern of the pendent aryl ring is built up after coupling of the two phenylpropane units. Degradation studies on podophyllotoxin derived from [3-O^{14}CH$_3$] ferulic acid show that the two halves of the lignan molecule are equally labelled supporting a biosynthetic sequence involving oxidative coupling of two similar phenylpropane precursors having the substitution pattern of ferulic acid. Although 3,4-methylenedioxycinnamic acid was readily incorporated, degradative studies prove that this compound is not incorporated intact, but via a metabolic sequence in which the methylenedioxy carbon atom enters the C_1-pool and then labels the methylenedioxy and methoxyl substituents of podophyllotoxin. The rest of the skeleton is incorporated via ferulic acid, presumably by way of caffeic acid.

INTRODUCTION

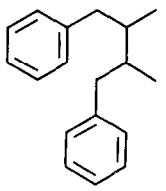
Despite the widespread occurrence of lignans in nature [1] and long-established hypotheses concerning their origins [2], the biosynthetic pathways to these compounds are poorly investigated and little definitive information is available. Most of the pathways proposed involve phenolic oxidative coupling of suitable C_6–C_3 monomers via free-radical or equivalent two electron processes in a manner analogous to those predicted in lignin biosynthesis [3]. However, the production of optically active lignan dimers rather than racemic products like lignin is indicative of enzyme-controlled reactions and not random free-radical couplings. From structural analysis, most lignans appear to be derived from the same C_6–C_3 monomer units coniferyl alcohol and/or sinapyl alcohol as are gymnosperm and dicotyledonous lignins, although there is little evidence that coupling occurs at the alcohol rather than acid, aldehyde or other oxidation level. Historically, the term lignan refers to a dimer in which the two C_6–C_3 units were linked initially by a β–β bond (**1**) [4], although the term has since been broadened to include dimers with different linkages [5]. More recent distinctions between lignans and neolignans [6] based on biosynthetic precursors of cinnamyl alcohols/cinnamic acids or propenyl/allyl phenols respectively are inappropriate in view of the almost total lack of biosynthetic data.

Specific radiochemical feeding experiments directed towards lignan biosynthesis are relatively few. Ayres initially reported the incorporation of labelled phenylalanine into podophyllotoxin (**2**) in *Podophyllum hexandrum* (syn. *emodi*) [7], and in later experiments [8] the incorporation of *p*-coumaric acid but not tyrosine or acetate. Partial degradation of podophyllotoxin from [β-^{14}C]phenylalanine feedings confirmed the incorporation of two C_6–C_3 monomers into the lignan dimer [8]. In a series of double-labelling experiments using *Forsythia suspensa* shoots [9], Stöckigt and Klischies were able to demonstrate the intact incorporation of ferulic acid, coniferyl alcohol and coniferyl aldehyde, fed as their glucose derivatives, into arctiin and phyllyrin. 3,4-Dimethoxycinnamic acid was not incorporated, in keeping with a phenolic oxidative coupling mechanism. These authors also briefly report that the primary alcohol function of coniferyl alcohol (in coniferin) is incorporated intact, suggesting that coupling occurs at the cinnamyl alcohol oxidation level. Lastly, Fujimoto and Higuchi reported incorporations of phenylalanine, ferulic acid and sinapyl alcohol into syringaresinol and its diglucoside liriodendrin in *Liriodendron tulipifera* [10], although coniferyl alcohol and sinapic acid were more poorly utilized. Coupling of sinapyl alcohol units was indicated.

The present, and following [11] paper describe the results of feeding experiments to elucidate the biosynthetic pathways to the aryltetralin lignans of *Podophyllum* [12], a series of compounds of considerable medicinal and commercial interest as precursors for clinically-useful anticancer drugs [13]. Indian Podophyllum, *P. hexandrum*, was used in the studies, and the origins of the two major lignans present, podophyllotoxin (**2**) and 4′-demethylpodophyllotoxin (**3**) were investigated using a variety of labelled cinnamic acid precursors.

RESULTS AND DISCUSSION

In earlier studies [7, 8], Ayres *et al.* had employed 5-year old *P. hexandrum* plants for biosynthetic experiments, wick-feeding precursors into the stem and achieving satisfactory incorporations (0.6–1.4%) for phenylalanine and *p*-coumaric acid. For the present work, younger plants (2/3-year old) were employed, for which wick-feeding was inappropriate and therefore precursors were fed by the roots. Pot-grown plants, cultivated under normal garden conditions, were used, the soil mixture



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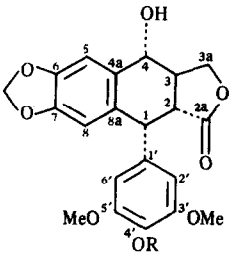
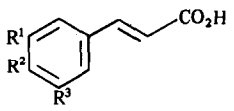


R
2 Me
3 H



4 R^1 = OMe, R^2 = OH, R^3 = H
5 R^1+R^2 = OCH_2O, R^3 = H
6 R^1 = R^3 = OMe, R^2 = OH
7 R^1 = R^2 = R^3 = OMe

being carefully washed off prior to feeding. The root system was placed in a suitable sized beaker, and aqueous solutions (*ca* 2 ml) of the precursors applied directly to the roots. The top of the beaker was covered over with a layer of Parafilm, and the plant was placed in a well-lit fume-cupboard to facilitate translocation of solution. At regular intervals, the drainings from the roots were re-applied via pipette, and uptake was usually complete after 6–8 hr. After absorption, the root system was covered with moist vermiculite, and the plant was grown on in a cool greenhouse for 7 days, prior to extraction and work-up.

As a preliminary experiment (exp i), L-[U-^{14}C] phenylalanine was fed to a single plant. Podophyllotoxin and 4′-demethylpodophyllotoxin were isolated, purified and quantified by UV absorption, and where necessary, diluted with carrier material prior to derivatization and purification to constant specific activity. Podophyllotoxin was acetylated by heating under reflux with acetic anhydride to yield the readily crystallizable podophyllotoxin acetate (**8**). 4′-Demethylpodophyllotoxin was methylated with ethereal diazomethane in acetone–methanol solution (other solvents such as ether, dioxan, methanol–dioxan being less suitable) to give podophyllotoxin, which was then also acetylated. The two compounds **2** and **3** are usually present in *P. hexandrum* in a ratio of *ca* 10:1 [12], although in some plants (see data from individual experiments) much higher levels of **3** are recorded. After counting, the following incorporation data were calculated: podophyllotoxin, 39.8 mg, incorporation 0.25%, dilution 7.76×10^3; 4′-demethylpodophyllotoxin, 20.5 mg, incorporation 0.05%, dilution 2.07×10^4. These incorporations were sufficiently high to permit a general technique of root feeding over 7 days for other precursor compounds.

Since *P. hexandrum* plants are herbaceous perennials, starting their growth cycle in the U.K. about April–May, the foliage dying down in late September, a series of experiments (exp ii) to ascertain the best feeding time was undertaken. Aliquots of DL-[1-^{14}C]phenylalanine were fed at monthly intervals to plants from a selected batch of similar specimens. Incorporations into podophyllotoxin were measured, and are presented in Table 1. The September-fed plant had been subjected to artificially-lengthened daylight hours to delay senescence, in order to perhaps extend the relatively short annual lifetime of the plants, and thus the period available for experimental studies. In the event, maximum incorporation was observed during the midseason period, July, when the fruits are developing. Whenever possible, feedings were thus conducted during this phase.

The aryltetralin lignans of *Podophyllum* may be subdivided into two groups according to the substitution pattern in the pendent aryl ring, which may carry 3,4,5-trimethoxy or 4-hydroxy-3,5-dimethoxy substituents [12]. The other aromatic ring in general contains only 3,4-methylenedioxy substitution (disregarding the C-1/C-8a linkage), although an extra 2-hydroxy is observed in the peltatins. It is probable that the substitution patterns of both rings are derived from the 4-hydroxy-3-methoxy of ferulic acid, although whether the two phenylpropane units coupling during the lignan biosynthesis are alike or different has yet to be determined. To investigate the nature, with respect to substitution pattern, of the phenylpropane units involved, a series of feeding experiments with suitably-substituted cinnamic acids was conducted. Those chosen were [β-^{14}C]cinnamic acid itself, to complement Ayres' data [8] on the incorporation of phenylalanine and *p*-coumaric acid, and the [α-^{14}C] labelled compounds ferulic acid (**4**), 3,4-methylenedioxycinnamic acid (**5**), sinapic acid (**6**) and 3,4,5-trimethoxycinnamic acid (**7**). Although **5** and **7** contain the same substitution patterns as the two aromatic rings of podophyllotoxin,

Table 1. Incorporation of DL-[1-^{14}C]phenylalanine into podophyllotoxin in *Podophyllum hexandrum*

Date fed	Podophyllotoxin isolated (mg)	% Incorporation	Dilution
12 May	12.0	0.04	5.15×10^5
12 June	12.9	0.13	1.69×10^5
14 July	22.6	1.19	3.37×10^4
12 August	2.8	0.08	6.13×10^4
15 September*	57.3	0.13	7.77×10^5

*Delayed senescence, see Results.

they lack the necessary phenolic groups required for postulated oxidative coupling mechanisms. However, ferulic and sinapic acids do contain this functional group. The [α-^{14}C] labelled cinnamic acids were synthesized by Knoevenagel condensation [14] of [2-^{14}C]malonic acid with the appropriate aldehyde in aniline–pyridine solution. The acids, as their sodium salts, were fed (exps iii, iv and v) via the roots to *P. hexandrum* plants as described above. As far as possible, plants of similar age and vigour were employed, and some repeat feedings were carried out the following year. Incorporations into podophyllotoxin and 4′-demethylpodophyllotoxin were measured as listed in Table 2.

The results demonstrate satisfactory incorporations into both lignans of cinnamic acid, ferulic acid and 3,4-methylenedioxycinnamic acid, but not sinapic acid or 3,4,5-trimethoxycinnamic acid. The non-incorporation of **6** and **7** would suggest the substitution pattern of the pendent ring is built up after initial coupling rather than before. However, of particular significance was the incorporation of 3,4-methylenedioxycinnamic acid, and at an extremely high level (1.34%) in the repeat feeding, into the lignans. Its incorporation is not explicable if a phenolic oxidative coupling mechanism is operative, since no free phenolic group is present. Other mechanisms, e.g. reductive coupling [15] could thus be functioning, or alternatively, a degradative sequence prior to incorporation was occurring. The specificity of labelling was partially checked by degradation (Scheme 1) of podophyllotoxin. Alkaline permanganate oxidation [8] of the acetate (**8**) gave two acids **9** and **10**, both essentially inactive (Table 3), thus demonstrating the incorporation of label from [α-^{14}C]methylenedioxycinnamic acid into, at most, any of the four carbons 3,3a,2 or 2a, in agreement with prediction, and excluding any substantial randomization. Thus, any degradative sequence would probably result in the formation of labelled ferulic or caffeic acids which could then be specifically incorporated.

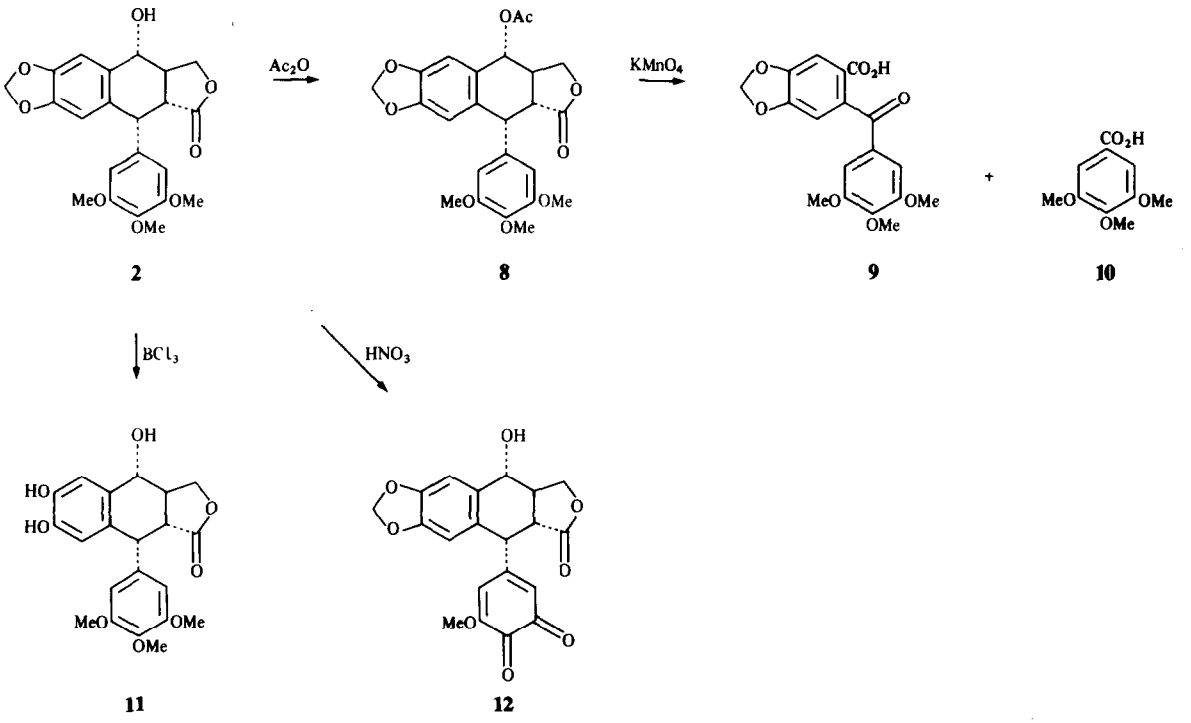
The incorporation of ferulic acid into podophyllotoxin was checked by the feeding of [3-O^{14}CH$_3$]ferulic acid, followed by degradation of this lignan. The lower levels of 4′-demethylpodophyllotoxin present precluded its degradation in these experiments. [3-O^{14}CH$_3$]Ferulic acid was synthesized by [^{14}C]methyl iodide methylation of methyl 4-mesylcaffeate followed by deprotection. The crucial reaction in the degradation sequence was the specific demethyleneation of podophyllotoxin to the quinol (**11**) using boron trichloride [16] (Scheme 1), thus allowing activity in the methylenedioxy group to be estimated. Label in the pendent ring methyls was assessed by oxidation as before to 3,4,5-trimethoxybenzoic acid (**10**). Although the two feedings conducted were of necessity performed very late in the season (October), satisfactory incorporation levels into podophyllotoxin were obtained: exp vi, 41.8 mg, incorporation 0.06%, dilution 1.55×10^4; exp vii, 44.8 mg, incorporation 0.25%, dilution 3.36×10^3. After degradation, the data in Table 4 were obtained, demonstrating in both cases that essentially equivalent amounts of label were present in the methylenedioxy group and the benzoic acid fragment containing the pendent ring. This evidence suggests very strongly that during the biosynthesis of podophyllotoxin, coupling occurs between two like units containing the 4-hydroxy-3-methoxy substitution pattern of ferulic acid. Any further substitution in either ring prior to coupling would have distorted the proportions of labelling from the 1:1 observed. Therefore, the non-incorporation of sinapic and 3,4,5-trimethoxycinnamic acids is now explicable, but the incorporation of 3,4-methylenedioxycinnamic acid as an intact unit would seem less likely.

The incorporation of **5** was examined by feeding [O^{14}CH$_2$O]3,4-methylenedioxycinnamic acid and partially degrading the labelled podophyllotoxin, again observing activity in the aromatic ring substituents. [O^{14}CH$_2$O]3,4-Methylenedioxycinnamic acid was synthesized by methyleneation of methyl caffeate with [^{14}C]methylene iodide followed by base hydrolysis. [^{14}C]Methylene iodide was conveniently prepared from [1,3-^{14}C]acetone via [^{14}C]iodoform according to published procedures [17]. In the degradation sequence (Scheme 1), a further valuable reaction was the nitric acid oxidation of podophyllotoxin to yield the quinone (**12**) [18], thus removing two of the methyl groups. Coupled with the other reactions, this allowed the activities of all three methoxyls and the methylenedioxy to be established. Incorporation data for podophyllotoxin were good:

Table 2. Incorporation of [^{14}C] labelled cinnamic acids into *Podophyllum hexandrum* lignans

		Podophyllotoxin			4′-Demethylpodophyllotoxin		
Cinnamic acid	Exp*	mg Isolated	% Incorporation	Dilution	mg Isolated	% Incorporation	Dilution
Cinnamic†	iii§	32.2	0.17	5.27×10^4	10.4	0.04	7.72×10^4
Ferulic‡	iv	34.0	0.053	1.10×10^4	3.4	0.053	1.14×10^3
3,4-Methylenedioxycinnamic‡	iv	5.5	0.016	6.37×10^3	9.6	0.017	1.54×10^4
	v	398	1.34	1.47×10^4		ND	
Sinapic‡	iv	25.3	0.00064	7.66×10^5	4.6	0.00016	5.69×10^5
	v	33	0.00245	1.09×10^6		ND	
3,4,5-Trimethoxycinnamic‡	iv	7.4	0.00039	3.86×10^5	7.4	0.00005	2.95×10^6
	v	104	0.00763	4.16×10^5		ND	

*Dates of experiments—iii: 11.9.79; vi: 10.6.80; v: 10.7.81.
†[β-^{14}C].
‡[α-^{14}C].
§Minor lignans were also shown to be labelled: podophyllotoxone (0.0008%), desoxypodophyllotoxin (0.0047%).
ND, Not determined.



Scheme 1. Degradation of podophyllotoxin.

Table 3. Degradation of podophyllotoxin from [α-^{14}C]3,4-methylenedioxycinnamic acid feeding (exp v)

Compound	Specific activity (dpm/mM)	Relative specific activity
Podophyllotoxin acetate (**8**)	1.42×10^5	1.00
9	7.78×10^2	0.006
10	4.73×10^2	0.003

exp viii, 360 mg, incorporation 0.43%, dilution 2.12×10^4; exp ix, 455 mg, incorporation 0.70% dilution 1.87×10^4. The degradative information is presented in Table 5, with a summary of the results in Fig. 1. These results now clarify the incorporation of **5**. The presence of over 70% of the activity in the methoxyls means intact incorporation of **5** does not occur. Since the two halves of the molecule were not equally labelled, incorporation of **5** by ring opening to ferulic acid is also excluded, and the likely explanation is demethyleneation of **5** to caffeic acid and labelled formaldehyde or formic acid, which could enter the C_1-pool and re-emerge as a labelled methyl in S-adenosylmethionine (SAM). Thus, [O^{14}CH$_2$O]3,4-methylenedioxycinnamic acid could be transformed into [3-O^{14}CH$_3$]ferulic acid plus [S-^{14}CH$_3$]SAM which are then incorporated to give the observed labelling pattern. Consistent with this is the similar degree of labelling in the methylenedioxy and the 3′ (or 5′) methoxyl (cf. incorporation of [3-O^{14}CH$_3$]ferulic acid), and then the imbalance between 4′ and 5′ levels might indicate that the 5′-*O*-methyl is introduced before the 4′-*O*-methyl. The relatively high incorporation of **5** via degradation is noteworthy, though the reasons are not clear.

It would appear, therefore, that podophyllotoxin arises

Table 4. Degradation of podophyllotoxin from [3-O^{14}CH$_3$] ferulic acid feedings (exps vi and vii)

Compound	Exp vi		Exp vii	
	Specific activity (dpm/mM)	Relative specific activity	Specific activity (dpm/mM)	Relative specific activity
Podophyllotoxin acetate (**8**)	3.25×10^4	1.00	1.57×10^5	1.00
Podophyllotoxin (**2**)	3.19×10^4	0.98	1.60×10^5	1.02
6,7-Demethylenepodophyllotoxin (**11**)	1.67×10^4	0.51	8.07×10^4	0.51
. ·. Methylenedioxy group		0.49		0.49
3,4,5-Trimethoxybenzoic acid (**10**)	1.69×10^4	0.52	7.08×10^4	0.45

Table 5. Degradation of podophyllotoxin from [O^{14}CH$_2$O]methylenedioxycinnamic acid feedings (exps viii and ix)

Compound	Exp viii		Exp ix	
	Specific activity (dpm/mM)	Relative specific activity	Specific activity (dpm/mM)	Relative specific activity
Podophyllotoxin acetate (**8**)	2.76×10^4	1.00	3.13×10^4	1.00
Podophyllotoxin (**2**)	2.80×10^4	1.01	3.11×10^4	0.99
6,7-Demethylenepodophyllotoxin (**11**)	2.04×10^4	0.74	2.24×10^4	0.72
∴ Methylenedioxy group		0.26		0.28
3,4,5-Trimethoxybenzoic acid (**10**)	1.99×10^4	0.72	ND	
Quinone (**12**)	1.59×10^4	0.58	1.79×10^4	0.57

ND, Not determined.

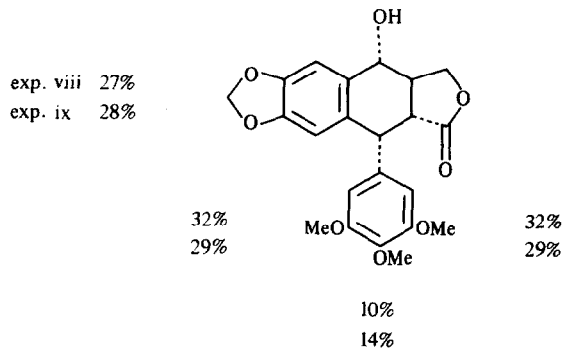


Fig. 1. Distribution of label from [O^{14}CH$_2$O]methylenedioxycinnamic acid in podophyllotoxin (exp. viii—upper fig; exp. ix—lower fig).

by coupling of two C_6–C_3 units possessing the ferulic substitution pattern. The necessity for the *p*-hydroxyl function suggests its involvement in the coupling process, either by a stereocontrolled free-radical or two electron process. The oxidation level of the C_6–C_3 units which couple is still unknown, since incorporation of cinnamic acids could have taken place at the aldehyde or alcohol oxidation level. Combination of two aldehyde units, followed by a Cannizzaro reaction would lead to the alcohol and acid functions necessary for lactone formation. Direct combination of an acid and alcohol unit giving rise to the lactone directly is rendered less likely by the equal incorporation of ferulic acid into both halves of the lignan. Further support for the coupling of two similar units comes from the range of aryltetralin lignans encountered in *Polygala polygama* [19, 20], where podophyllotoxin, 4′-demethylpodophyllotoxin and desoxypodophyllotoxin co-occur with other lignans such as polygamain and polygamatin containing two disubstituted aromatic rings. This whole range of lignans can be imagined as arising from a common precursor derived from the coupling of two C_6–C_3 units possessing the ferulic substitution pattern. In *P. hexandrum*, the two groups of lignans distinguished by the substitution patterns in the pendent ring [12] are obviously derived from a common pathway, but further experiments, as described in the following paper [11] indicate that divergence from the common pathway probably occurs before the aryltetralin skeleton is constructed.

EXPERIMENTAL

General. TLC was carried out using 0.5 mm layers of silica gel (Merck TLC-Kiesel gel 60GF$_{254}$), and Me_2CO (Analar) was used for elution of TLC zones. Radioactive samples were counted in dioxan-based liquid scintillator (BDH 19228) and counting efficiencies were determined by the use of [^{14}C]toluene int. standards.

Plant material, feeding techniques and isolation of lignans. P. hexandrum plants were purchased from Jack Drake, Aviemore, and were grown under normal garden conditions in 6-inch plastic pots, the pots being sunken into the ground to facilitate moisture retention. Plants for feeding were removed from the pots and the soil mixture was carefully removed by washing. The root system was gently eased into a beaker of suitable size, keeping the total vol. occupied to a minimum. Radioactive precursors (1–3 mg) were dissolved in H_2O or 0.1% aq. NaOH (1–2 ml) as appropriate, and the soln applied directly to the root system by pipette. Drainings were reapplied at regular intervals. During the feeding, the root system was enclosed by stretching a layer of Parafilm over the beaker and the plant was kept in a well-lit fume-cupboard to ensure a satisfactory airflow over the leaf system. When uptake of precursor was complete (6–8 hr), the root system was covered with moist Vermiculite and the plant grown on for 7 days in a cool greenhouse, watering as necessary.

The plant was removed from the Vermiculite, cut into small pieces, then homogenized with a little H_2O using ground glass in a mortar. The mixture was then extracted with hot EtOH, partitioned and separated by TLC ($CHCl_3$–MeOH, 25:1) as described for the dried plant material [12]. Podophyllotoxin and 4′-demethylpodophyllotoxin were quantified by UV absorption [12].

Podophyllotoxin was purified further by TLC ($CHCl_3$–*iso*-PrOH, 10:1; Et_2O–CH_2Cl_2, 6:1), diluted with unlabelled material (*ca* 20 mg) where necessary, converted into the acetate, and rechromatographed (Et_2O–CH_2Cl_2, 6:1). The resulting podophyllotoxin acetate was recrystallized to constant sp. act. from EtOH.

4′-Demethylpodophyllotoxin was purifed further by TLC (Me_2CO–petrol (60–80°), 1:1; $CHCl_3$–MeOH, 9:1), converted into podophyllotoxin using CH_2N_2–Et_2O soln, diluted where necessary with unlabelled material (*ca* 20 mg), then purified as above as podophyllotoxin acetate.

Podophyllotoxin acetate (**8**). Podophyllotoxin (50 mg) was dissolved in Ac_2O (1 ml) and heated under reflux for 30 min. After cooling, the reaction mixture was diluted with HOAc (1 ml), poured into H_2O (50 ml) and extracted with EtOAc (3 × 50 ml). The combined extracts were washed with H_2O (2 ×), evapd to dryness and podophyllotoxin acetate recrystallized from EtOH.

Yield 49 mg, mp 207–209° (lit. [21] 210–211°); $[\alpha]_D$ −143° (c = 0.943, $CHCl_3$) (lit. [21] −143°); UV λ_{max}^{EtOH} nm: 288 (log ε 3.60), 291 (3.61), 296 sh; IR ν_{max}^{KBr} cm^{-1}: 1781, 1761, 1729, 1589, 1509; ^1H NMR (250 MHz, $CDCl_3$, TMS): δ 6.78 (1H, *s*, H-5), 6.55 (1H, *s*, H-8), 6.39 (2H, *s*, H-2′, H-6′), 6.00 (1H, *d*, J = 1.2 Hz, OCH_2O), 5.99 (1H, *d*, J = 1.2 Hz, OCH_2O), 5.89 (1H, *d*, J = 8.8 Hz, H-4), 4.61 (1H, *d*, J = 4.0 Hz, H-1), 4.39 (1H, *dd*, J = 9.5, 6.7 Hz, H-3aα); 4.21 (1H, *t*, J = 9.9 Hz, H-3aβ), 3.81 (3H, *s*, 4′-OMe), 3.77 (6H, *s*, 3′,5′-OMe), 2.94 (1H, *dd*, J = 14.6, 4.0 Hz, H-2), 2.83 (1H, *m*, H-3), 2.19 (3H, *s*, OAc).

Methylation of 4′-demethylpodophyllotoxin. 4′-Demethylpodophyllotoxin (30 mg) was dissolved in dry Me_2CO–MeOH (1:5, 3 ml), an excess of CH_2N_2–Et_2O soln added and the mixture left at room temp for 24 hr. The reaction mixture was evapd to dryness and podophyllotoxin isolated by TLC ($CHCl_3$–MeOH, 25:1) and recrystallized from EtOH. Yield 20 mg, mp 182–184°, identical to natural material [12].

Radiochemicals. L-[U-^{14}C]Phenylalanine (10 mCi/mM), DL-[1-^{14}C]phenylalanine (61 mCi/mM), [β-^{14}C]cinnamic acid (57 mCi/mM), sodium [2-^{14}C]malonate (55.8 mCi/mM), [^{14}C] methyl iodide (58 mCi/mM) and [1,3-^{14}C]acetone (25 mCi/mM) were purchased (Amersham).

[α-^{14}C]*Cinnamic acids* (**4**–**7**). 0.025 M H_2SO_4 (0.14 ml) was added to sodium [2-^{14}C]malonate (250 μCi) to liberate the free acid. After evapn, the acid was dissolved in Me_2CO (0.4 ml) and divided into two portions which were placed in individual Reactivials (1 ml), and the Me_2CO carefully removed in a stream of N_2. The vials were then dried overnight in a desiccator over P_2O_5. A second 250 μCi of sodium [2-^{14}C]malonate was treated similarly.

Malonic acid (12.8 mg), dry pyridine (50 μl), freshly dist aniline (0.25 μl) and the appropriate aldehyde [vanillin (20 mg), piperonal (20 mg), syringaldehyde (25 mg) or 3,4,5-trimethoxybenzaldehyde (25 mg)] were placed in one of the above vials, which was sealed and heated at 55° for 16 hr. After this time the mixture was acidified by treating with 10% HCl (2 ml for **5**, 0.1 ml for **6**), or by pouring into 10% HCl (25 ml for **4** and **7**). The products were collected by filtration (**5** and **6**), or by extraction (EtOAc, 3 × 25 ml) for **4** and **7**, and were recrystallized to constant sp. act. from aq. EtOH.

[β-^{14}C]*Ferulic acid* (**4**). Yield 22 mg, sp. act. 2.39 × 10^9 dpm/mM; unlabelled material had mp 168–171° (lit. [22] 174°); UV λ_{max}^{EtOH} nm: 295 sh, 320 (log ε 4.25); ^1H NMR (60 MHz, DMSO-d_6, TMS): δ 7.55 (1H, *d*, J = 16 Hz, H-β), 7.15 (1H, *d*, J = 2 Hz, H-2), 7.08 (1H, *dd*, J = 8, 2 Hz, H-6), 6.82 (1H, *d*, J = 8 Hz, H-5), 6.27 (1H, *d*, J = 16 Hz, H-α), 3.90 (3H, *s*, OMe).

[β-^{14}C]3,4-*Methylenedioxycinnamic acid* (**5**). Yield 21 mg, sp. act. 2.02 × 10^9 dpm/mM; unlabelled material had mp 240–242° (lit. [22] 247°); UV λ_{max}^{EtOH} nm: 285 (log ε 4.05), 321 (4.16); ^1H NMR (60 MHz, DMSO-d_6, TMS): δ 7.50 (1H, *d*, J = 16 Hz, H-β), 7.20 (1H, *d*, J = 2 Hz, H-2), 7.10 (1H, *dd*, J = 8, 2 Hz, H-6), 6.83 (1H, *d*, J = 8 Hz, H-5), 6.30 (1H, *d*, J = 16 Hz, H-α), 6.05 (2H, *s*, OCH_2O).

[β-^{14}C]*Sinapic acid* (**6**). Yield 20 mg, sp. act. 1.60 × 10^9 dpm/mM; unlabelled material had mp 197–198° (lit. [23] 192°; UV λ_{max}^{EtOH} nm: 320 (log ε 4.25); ^1H NMR (60 MHz, acetone-d_6, TMS): δ 7.63 (1H, *d*, J = 16 Hz, H-β), 7.03 (2H, *s*, H-2, H-6), 6.40 (1H, *d*, J = 16 Hz, H-α), 3.92 (6H, *s*, 3,5-OMe).

[β-^{14}C]3,4,5-*Trimethoxycinnamic acid* (**7**). Yield 21 mg, sp. act. 2.05 × 10^9 dpm/mM; unlabelled material had mp 126–127° (lit. [24] 125.5–127°); UV λ_{max}^{EtOH} nm: 299 (log ε 4.25); ^1H NMR (60 MHz, DMSO-d_6, TMS): 7.55 (1H, *d*, J = 16 Hz, H-β), 6.78 (2H, *s*, H-2, H-6), 6.30 (1H, *d*, J = 16 Hz, H-α), 3.90 (6H, *s*, 3,5-OMe), 3.85 (3H, *s*, 4-OMe).

[3-O^{14}CH_3]*Ferulic acid.* 4-Mesylprotocatechualdehyde [25] (100 mg), malonic acid (50 mg), dry pyridine (100 μl) and freshly distilled aniline (10 μl) were heated together in a Reactivial at 55° for 16 hr. After cooling, the reaction mixture was poured into 10% HCl (50 ml) and extracted with EtOAc (3 × 50 ml). The combined extracts were washed with H_2O (100 ml) and evapd to dryness. 3-Hydroxy-4-mesyloxycinnamic acid was recrystallized from aq. EtOH. Yield 107 mg, mp 225–227°. ^1H NMR (60 MHz, DMSO-d_6–D_2O, TMS): δ 7.42 (1H, *d*, J = 16 Hz, H-β), 7.15 (1H, *d*, J = 9 Hz, H-5), 7.10 (1H, *d*, J = 2 Hz, H-2), 6.90 (1H, *dd*, J = 9, 2 Hz, H-6), 6.22 (1H, *d*, J = 16 Hz, H-α), 3.23 (3H, *s*, OSO_2Me).

3-Hydroxy-4-mesyloxycinnamic acid (30 mg) in dry dioxan (1 ml) was treated with CH_2N_2–Et_2O soln. As soon as an excess of reagent was present, the reaction mixture was evapd to dryness to give methyl 3-hydroxy-4-mesyloxycinnamate with ^1H NMR (60 MHz, acetone-d_6, TMS): δ 7.45 (1H, *d*, J = 16 Hz, H-β), 7.25–6.95 (3H, *m*, H-2, H-5, H-6), 6.31 (1H, *d*, J = 16 Hz, H-α), 3.70 (3H, *s*, CO_2Me), 3.28 (3H, *s*, OSO_2Me). Without further purification, the sample was dried in a desiccator overnight and then dissolved in dry Me_2CO (10 ml) to which was added dry K_2CO_3 (1 g), MeI (5.5 μl) and $^{14}CH_3I$ (100 μCi). The mixture was stirred and heated under reflux for 2 hr, after which time an excess of MeI (10 μl) was added, and the reaction was continued for a further 1 hr. After cooling, the salts were filtered off, washed with Me_2CO (10 ml) and the combined filtrates evaporated to dryness to give methyl [3-O^{14}CH_3] 4-mesyloxy-3-methoxycinnamate which was purified by TLC ($CHCl_3$–*iso*-PrOH, 10:1). An unlabelled sample had ^1H NMR (60 MHz, acetone-d_6, TMS): δ 7.60 (1H, *d*, J = 16 Hz, H-β), 7.45 (1H, *d*, J = 2 Hz, H-2), 7.30–7.15 (2H, *m*, H-5, H-6), 6.52 (1H, *d*, J = 16 Hz, H-α), 3.99 (3H, *s*, 3-OMe), 3.75 (3H, *s*, CO_2Me), 3.30 (3H, *s*, OSO_2Me).

Without further purification, the labelled ester was dissolved in EtOH (2 ml) and hydrolysed by heating under reflux for 1 hr with KOH (0.5 g) in H_2O (10 ml). The mixture was cooled, acidified and extracted with EtOAc (3 × 20 ml). The combined extracts were evapd to dryness and [3-O^{14}CH_3]ferulic acid recrystallized (× 3) from aq. EtOH. Yield 15 mg, sp. act. 1.47 × 10^9 dpm/mM.

[O^{14}CH_2O]3,4-*Methylenedioxycinnamic acid.* Caffeic acid (350 mg) in MeOH (10 ml) was heated under reflux with conc H_2SO_4 (1 ml) for 1 hr. After cooling, and dilution with H_2O (100 ml), the mixture was extracted with $CHCl_3$ (2 × 80 ml), the combined extracts washed with H_2O and evapd to dryness. Methyl caffeate was recrystallized from aq. EtOH, yield 304 mg, mp 159–161° (lit. [26] 158–160°). ^1H NMR (60 MHz, acetone-d_6, TMS): 7.53 (1H, *d*, J = 16 Hz, H-β), 7.2–6.8 (3H, *m*, H-2, H-5, H-6), 6.20 (1H, *d*, J = 16 Hz, H-α), 3.74 (3H, *s*, CO_2Me).

Dry Me_2CO (30 μl, 23.7 mg) was mixed with H_2O (5 ml) containing [1,3-^{14}C]acetone (500 μCi) and the mixture cooled to 0°. A soln of KI (250 mg) in H_2O (1 ml) was then added. NaOCl soln (12%, 5 ml) was added dropwise with stirring at 0° over 10 min, after which the soln was stirred for a further 30 min. The resulting [^{14}C]iodoform (170 mg) was filtered off and dried in a desiccator for 2 hr. [^{14}C]Iodoform (170 mg) and sodium arsenite soln (0.5 ml, made from 0.5 g As_2O_3, 1 g NaOH and 5 ml H_2O) were vigorously stirred together in a Reactivial at 55° for 3 hr. After this time, the upper aq. layer was carefully removed by pipette from the lower layer of [^{14}C]diiodomethane, which was used directly without further purification to methyleneate the methyl caffeate produced above. Residual traces of H_2O did not appear to affect the methyleneation reaction.

Methyl caffeate (70 mg) was dissolved in dry, N_2-purged DMF (2 ml) containing dry K_2CO_3 (1 g) and CuO (50 mg). The [^{14}C]diiodomethane produced above was transferred to the flask using dry, N_2-purged DMF (2 × 0.5 ml) and the mixture was stirred vigorously and heated under reflux in a N_2 atmosphere for 1 hr. After cooling, the mixture was poured into 10% HCl (50 ml) and extracted with EtOAc (3 × 50 ml), the combined extracts washed with H_2O (2 × 100 ml) and evapd to dryness. Methyl

[$O^{14}CH_2O$]3,4-methylenedioxycinnamate was isolated by TLC (hexane–Me_2CO, 2:1), and without further purification hydrolysed by heating under reflux with KOH (0.5 g) in H_2O (10 ml) and EtOH (2 ml). After cooling, the reaction mixture was diluted with H_2O (30 ml), acidified and extracted with EtOAc (3 × 40 ml). The combined extracts were washed with H_2O (× 2), evapd to dryness and the residue of [$O^{14}CH_2O$]3,4-methylenedioxycinnamic acid recrystallized from aq. EtOH (× 3). Yield 15 mg, sp. act. 5.85×10^6 dpm/mM.

Degradation of podophyllotoxin acetate to 6-(3,4,5-*trimethoxybenzoyl*)*piperonylic acid* (**9**) *and* 3,4,5-*trimethoxybenzoic acid* (**10**). Labelled podophyllotoxin acetate (100 mg) was dissolved in *t*-BuOH (10 ml), treated with 3% $NaHCO_3$ (20 ml) and the soln stirred at 80° for 1 hr. Sufficient 3% aq. $KMnO_4$ (*ca* 15 ml) was added over a 20 min period to keep the soln purple. After cooling, the reaction mixture was acidified with conc HCl and sufficient $Na_2S_2O_5$ added to dissolve the MnO_2 ppt. The mixture was then extracted with $CHCl_3$ (4 × 50 ml), the combined extracts washed with H_2O and evapd to dryness. Compounds **9** and **10** were isolated by TLC (toluene–HCO_2H–HCO_2Et, 5:4:1).

6-(3,4,5-Trimethoxybenzoyl)piperonylic acid (**9**) was recrystallized (× 4) from MeOH, yield 4 mg. An unlabelled sample had mp 209–214° (lit. [8] 215°); IR ν_{max}^{KBr} cm^{-1}: 3300–3000, 1718, 1642, 1583, 1503, 1420; ^1H NMR (60 MHz, acetone-d_6, TMS): δ 7.35 (1H, *s*, H-2), 6.96 (2H, *s*, H-2′, H-6′), 6.88 (1H, *s*, H-5), 6.18 (2H, *s*, OCH_2O), 3.76 (9H, *s*, 3′,4′,5′-OMe); EIMS (probe) 70 eV, *m/z* (rel. int.): 360 ([M]$^+$, 100%), 316 (42), 285 (58), 195 (97), 193 (50), 149 (34).

3,4,5-Trimethoxybenzoic acid (**10**) was purified (× 4) by sublimation (160°/0.1 mm), then recrystallization from aq. EtOH, yield 2.5 mg. An unlabelled sample had mp 169–172° (lit. [27] 169°), and was identical to authentic material.

Degradation of podophyllotoxin to 6,7-*demethylenepodophyllotoxin* (**11**) *and* 3,4,5-*trimethoxybenzoic acid* (**10**). A soln of labelled podophyllotoxin (93 mg) in dry CH_2Cl_2 (5 ml) was added dropwise over 20 min to a stirred soln of BCl_3 (1 M in CH_2Cl_2, 1 ml), cooled to −50°. The mixture was stirred for a further 2 hr, keeping the temp between −40° and −60°, then pipetted onto a mixture of satd aq. $KHCO_3$ (50 ml) and ice (50 g) and left at room temp for 30 min. This was then extracted with EtOAc (2 × 80 ml), the combined extracts washed with H_2O (× 2) and evapd to dryness. The residue was dissolved in Me_2CO (10 ml) and a suspension of $CaCO_3$ (0.83 g) in H_2O (10 ml) added. The mixture was stirred under reflux for 2 hr, cooled, acidified with dil HCl and extracted with EtOAc (3 × 50 ml). The combined extracts were washed with H_2O (× 2), evapd to dryness and divided into two portions of *ca* one-third and two-thirds.

The first portion (1/3) was purified by TLC (C_6H_6–EtOAc–HOAc, 5:4:1) to give 6,7-demethylenepodophyllotoxin and unreacted podophyllotoxin. 6,7-Demethylenepodophyllotoxin (**11**) was recrystallized (× 3) from aq. EtOH, yield 10.5 mg. Unlabelled material had mp 206–209° (lit. [16] 229–231°); IR ν_{max}^{KBr} cm^{-1}: 3535, 3425, 1775, 1588, 1515; ^1H NMR (250 MHz, acetone-d_6/D_2O, TMS): δ 7.18 (1H, *s*, H-5), 6.47 (3H, *s*, H-2′, H-6′, H-8), 4.76 (1H, *d*, J = 9.6 Hz, H-4), 4.53 (2H, *m*, H-1, 3aα), 4.15 (1H, *dd*, J = 10.4, 8.8 Hz, H-3aβ), 3.70 (6H, *s*, 3′,5′-OMe), 3.68 (3H, *s*, 4′-OMe), 3.03 (1H, *dd*, J = 14.3, 5.0 Hz, H-2), 2.81 (1H, *m*, H-3); EIMS (probe) 70 eV, *m/z* (rel. int.): 402 ([M]$^+$, 27%), 386 (32), 385 (24), 384 (100), 309 (11), 181 (13), 169 (18), 168 (100), 153 (39).

The second portion (2/3), containing **11** and unreacted podophyllotoxin, was combined with the podophyllotoxin from the first portion, and was treated with alkaline permanganate as described above. 3,4,5-Trimethoxybenzoic acid was isolated by direct sublimation of the oxidation residue, and purified by further sublimation (× 3) and recrystallization. Yield 1 mg.

Degradation of podophyllotoxin to 3′,4′-*demethyl*-3′,4′-*dioxopodophyllotoxin* (**12**). A soln of labelled podophyllotoxin (100 mg) in HOAc (5 ml) was added dropwise to a stirred soln of HNO_3 (3 ml) and HOAc (10 ml) at 0°. The resulting deep red soln was then stirred for a further 5 min, diluted with H_2O (100 ml) and extracted with $CHCl_3$ (3 × 80 ml). The combined extracts were washed with H_2O (× 2) and evapd to dryness to give **12** which was recrystallized from C_6H_6. Yield 40 mg. Unlabelled material had mp 184–188° (lit. [18] 190–192°); IR ν_{max}^{KBr} cm^{-1}: 3480, 1778, 1700, 1665, 1630; ^1H NMR (60 MHz, acetone-d_6, TMS): δ 7.18 (1H, *s*, H-5), 6.60 (1H, *s*, H-8), 6.45 (1H, *m*, H-6′), 5.99 (2H, *s*, OCH_2O), 5.38 (1H, *br s*, H-2′), 4.9–4.0 (4H, *m*, H-1, H-3aα, H-3aβ, H-4), 3.75 (3H, *s*, 5′-OMe), 3.6–2.8 (2H, *m*, H-2, H-3); EIMS (probe) 70 eV, *m/z* (rel. int.): 386 ([M + 2]$^+$, 100%), 384 ([M]$^+$, 95), 366 (35), 339 (47), 201 (7), 140 (45).

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