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BIOSYNTHESIS OF *PODOPHYLLUM* LIGNANS—II. INTERCONVERSIONS OF ARYLTETRALIN LIGNANS IN *PODOPHYLLUM HEXANDRUM*

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

Abstract—Feeding experiments in *Podophyllum hexandrum* plants with labelled aryltetralin lignans have established much of the biosynthetic interrelationships existing amongst *Podophyllum* lignans. Thus, desoxypodophyllotoxin is converted into podophyllotoxin, which in turn is oxidized to podophyllotoxone, although this latter step appears to be reversible. A similar sequence is proposed for the corresponding 4′-demethyl derivatives. Although 4′-demethyldesoxypodophyllotoxin is readily converted into 4′-demethylpodophyllotoxin, neither compound is incorporated into lignans of the 4′-methyl series such as podophyllotoxin. The *Podophyllum* lignans may be subdivided biogenetically into two groups, those with 3,4,5-trimethoxy substitution in the pendent aryl ring, and those with a 4-hydroxy-3,5-dimethoxy substituted pendent ring, although these probably arise from a common precursor. A biogenetic scheme interrelating all of the known *Podophyllum* aryltetralin lignans is proposed.

INTRODUCTION

From the results of feeding experiments in Indian Podophyllum (*Podophyllum hexandrum*) plants [1], the major aryltetralin lignans [2] podophyllotoxin (**7**) and 4′-demethylpodophyllotoxin (**3**) appear to be formed via oxidative coupling of two phenylpropane units containing the ferulic (4-hydroxy-3-methoxy) substitution pattern. However, the nature of this dimer, and the sequence of events leading to formation of the aryltetralin lactone structure, have yet to be determined. Structural analysis of the range of *Podophyllum* lignans encountered in *P. hexandrum* and *P. peltatum* has indicated that two groups of lignans are present, those containing 3,4,5-trimethoxy substitution in the pendent aromatic ring, and those with a 4-hydroxy-3,5-dimethoxy substituted pendent ring [2]. Further feeding experiments as described in this paper suggest these two groups are biogenetically distinct, although probably arising from a common precursor. The sequence of reactions resulting in the oxygenation at C-4 of the lignans has also been established. These results have been reported briefly in abstract form [3, 4].

RESULTS AND DISCUSSION

Feeding experiments

Three of the major aryltetralin lignans of *P. hexandrum* are podophyllotoxin (**7**), desoxypodophyllotoxin (**6**) and podophyllotoxone (**8**) [2, 5]. These compounds are chemically related, therefore, by the oxidation level at C-4, and may be expected to be related biosynthetically by simple oxidation/reduction processes. Most chemical interconversions of these compounds are readily achieved [6, 7]. To test the biosynthetic relationships existing among these three compounds, [4′-$OCH_2{}^3H$]podophyllotoxin, [4′-$OCH_2{}^3H$]desoxypodophyllotoxin and [4′-$OCH_2{}^3H$]podophyllotoxone were synthesized and fed via the roots to *P. hexandrum* plants as described previously [1], solubilizing the precursors in a 2-methoxyethanol/Tween 20/H_2O mixture. Following a 7-day metabolism period, **6**, **7** and **8** were isolated from the plant material using TLC [2] together with HPLC to separate the **6** and **8** mixture [1, 5]. The lignans were then diluted as necessary before purification to constant specific activity. Podophyllotoxin was converted into its acetate to assist purification [1]. The data from the feeding experiments are summarized in Table 1.

The results demonstrate the significant incorporation of desoxypodophyllotoxin into podophyllotoxin, of podophyllotoxin into podophyllotoxone but not desoxypodophyllotoxin, and of podophyllotoxone into podophyllotoxin but not desoxypodophyllotoxin. From these data, the sequence **6** → **7** → **8** can be assumed, although the last reaction, the alcohol → ketone conversion would appear to be reversible. The incorporation of desoxypodophyllotoxin into podophyllotoxone (exp i) cannot be regarded as significant, although a positive result may have been expected. The low figure is probably due to the small amount of **8** isolated, together with the increased dilution of activity resulting from the relatively large pool of inactive podophyllotoxin present in the plant.

The stereospecific benzylic hydroxylation of desoxypodophyllotoxin is mechanistically acceptable [8], and the sequence proposed is supported by the observed distribution of the three lignans in nature. Thus desoxypodophyllotoxin has been isolated from a whole range of higher plants [9] whereas podophyllotoxin has a much more restricted distribution [9] and podophyllotoxone has only been found in *Podophyllum* species [5]. The hydroxylation of **6** does, however, invalidate a number of hypotheses [10] for the biosynthesis of podophyllotoxin and related compounds, in which the 4-hydroxyl is introduced soon after oxidative coupling of the phenylpropane units by addition to a quinone methide inter-

Table 1. Interconversions of labelled lignans in *Podophyllum hexandrum*

Lignan fed*	Exp	Desoxypodophyllotoxin			Podophyllotoxin			Podophyllotoxone		
		mg	% Incorporation	Dilution	mg	% Incorporation	Dilution	mg	% Incorporation	Dilution
Desoxypodophyllotoxin (**6**)	i	0.35	2.6	13	95	0.81	1.08×10^4	0.21	0.0073	2.67×10^3
Podophyllotoxin (**7**)	ii†	3.2	0.007	3.69×10^3	1104	22	706	5.5	2.47	32
Podophyllotoxone (**8**)	iii	0.14	0.0014	1.09×10^4	23	2.16	1.07×10^3	0.10	0.84	12

*[4′-$OCH_2{}^3H$].
†A large plant of *P. hexandrum* var. *majus* was used in this experiment.

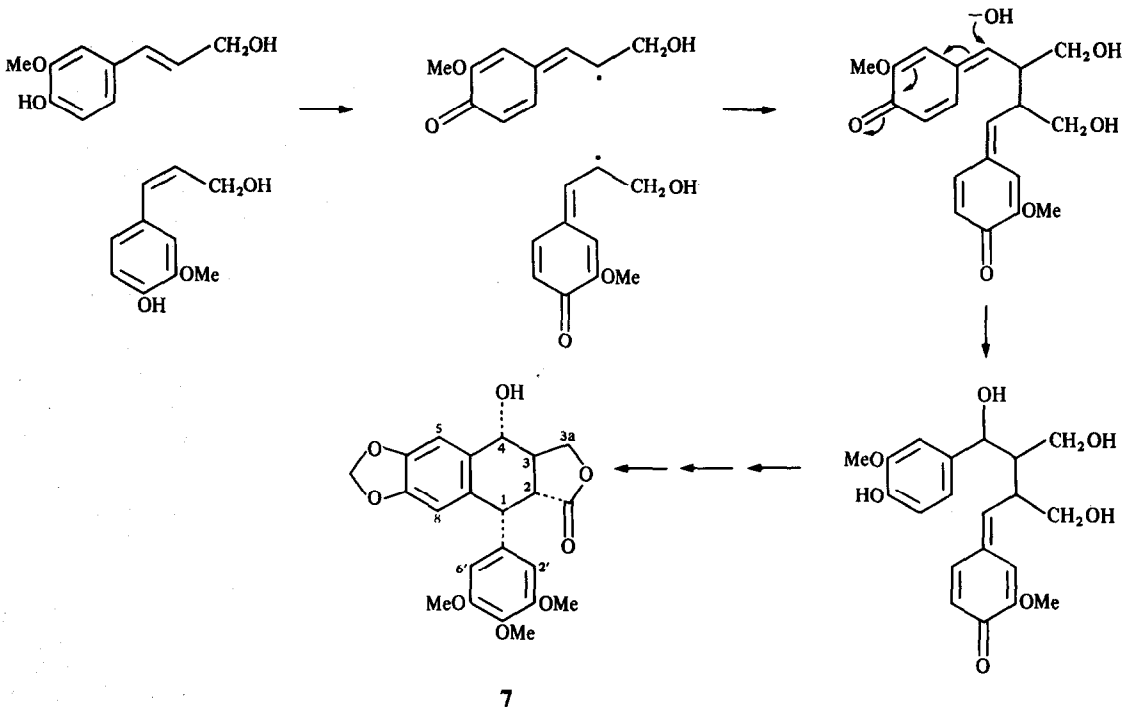
mediate, e.g. Scheme 1. Such schemes do not reflect the close biosynthetic relationship observed, but would suggest the production of 4-desoxy and 4-oxy aryltetralin lignans diverged at an early stage.

To include the biosynthetic relationships of the 4′-hydroxy-3′,5′-dimethoxy derivatives in the study, [2-^{3}H]4′-demethylpodophyllotoxin (**3**) and [2-^{3}H]4′-demethyldesoxypodophyllotoxin (**2**) were synthesized and fed. These compounds are both natural *P. hexandrum* metabolites [2]. Their incorporation into podophyllotoxin (**7**) and 4′-demethylpodophyllotoxin (**3**), the major representatives of the two lignan groups, were assessed, and the results are presented in Table 2. These results showed little incorporation of either compound into podophyllotoxin, but very good incorporation of 4′-demethyldesoxypodophyllotoxin into 4′-demethylpodophyllotoxin. Thus, methylation of a 4′-demethyl compound to give the corresponding compound in the trimethoxy series does not occur. However, 4-hydroxylation of **2** occurs, analogously to the observed conversion of desoxypodophyllotoxin to podophyllotoxin.

The chemical subdivision of the *Podophyllum* lignans into 3′,4′,5′-trimethoxy and 4′-hydroxy-3′,5′-dimethoxy groups [2] is thus also a biosynthetic subdivision, and the known transformations can be summarized as shown in Scheme 2. Using this as a basis, a proposed biosynthetic interrelationship for all of the *Podophyllum* aryltetralin lignan aglycones so far identified is given in Scheme 3. This comprises the two separate groups, emanating in each case from a 4-desoxy lignan. Presumably these two compounds, **2** and **6**, are produced from a common precursor, although this has yet to be identified. The position of the peltatins, α-peltatin (**1**) and β-peltatin (**10**), has also to be established, since their concentration in *P. hexandrum* is too low for biosynthetic experimentation [2]. In Scheme 3, a possible relationship has been indicated, by 5-hydroxylation of **2** or **6**, as opposed to the 4-hydroxylation giving podophyllotoxin derivatives **3** and **7**. Support for this tentative pathway is the lack of reports of any natural 4,5-dihydroxy aryltetralin lignans. The significant difference in proportions of constituents in *P. hexandrum* (principally podophyllotoxin and 4′-demethylpodophyllotoxin) and *P. peltatun* (principally α- and β-peltatins) [2] could then be accounted for by preferential hydroxylation at C-4 or C-5, respectively. '5'-Hydroxylation could, of course, occur earlier whilst the other substitution patterns in the two aromatic rings are being built up from the initial ferulic 4-hydroxy-3-methoxy pattern. The two ketones isopicropodophyllone (**9**) and 4′-demethylisopicropodophyllone (**5**) [2] are probably artefacts of extraction rather than natural products, arising by epimerizations at C-3 of **8** and **4**, respectively. As such, their status in Scheme 3 cannot be regarded as proven. This scheme now provides a working hypothesis for further experimentation.

Synthesis of labelled compounds

[4′-$OCH_2{}^3H$]Podophyllotoxin was synthesized by methylation of 4′-demethylpodophyllotoxin using ethereal CH_2N_2–^3H_2O in dioxan solution. Although dioxan is a less satisfactory solvent for this reaction than acetone–methanol [1], it was employed to maximize incorporation of label. [4′-$OCH_2{}^3H$]Desoxypodophyllotoxin was then obtained by catalytic hydrogenolysis [6] of the labelled podophyllotoxin, and [4′-$OCH_2{}^3H$]podo-

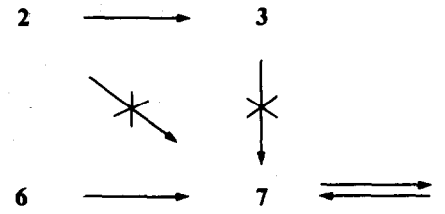


Scheme 1. Hypothetical scheme for biosynthesis of podophyllotoxin involving early '4'-hydroxylation.

Table 2. Incorporations of labelled lignans in *Podophyllum hexandrum*

		Podophyllotoxin			4'-Demethylpodophyllotoxin		
Lignan fed*	Exp	mg	% Incorporation	Dilution	mg	% Incorporation	Dilution
4'-Demethylpodophyllotoxin (3)	iv	78.7	0.058	1.27×10^5	11.4	12	93
4'-Demethyldesoxypodophyllotoxin (2)	v	92.5	0.012	5.94×10^5	14.4	3.1	373

*[2-^{3}H].



Scheme 2. Lignan interconversions demonstrated in *Podophyllum hexandrum.*

phyllotoxone by manganese dioxide oxidation [7].

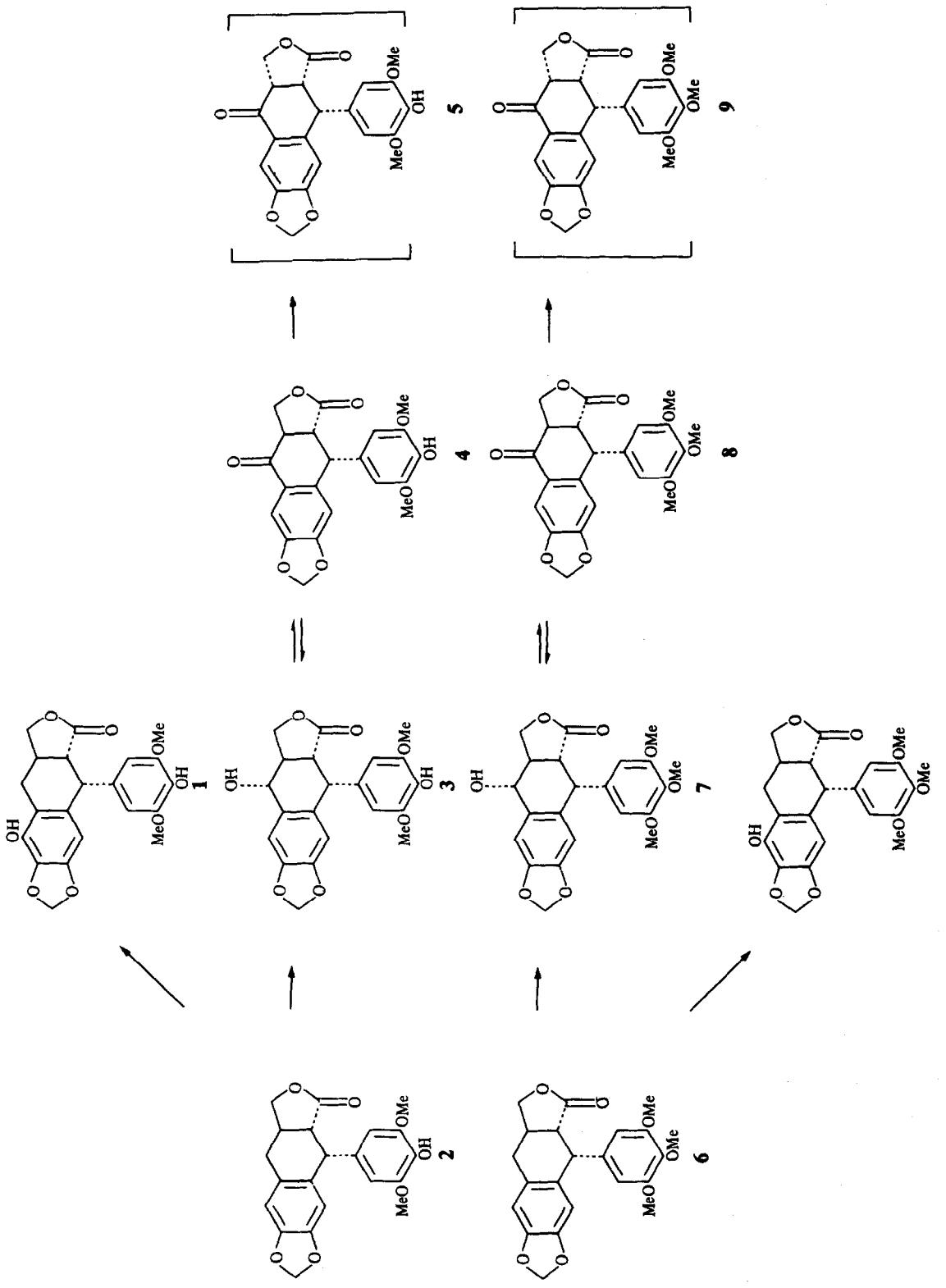
Exchange labelling of 4'-demethylpodophyllotoxin was achieved by base-catalysed enolization of the compound as its 4'-benzyl-4-tetrahydropyranyl ether, followed by quenching of the enolate with acetic acid–HCl–^3H_2O, in a series of reactions parallelling those of Gensler and Gatsonis [11]. This yielded a mixture of the C-2 epimers which were separated after removal of the protecting groups, as [2-^{3}H]4'-demethylpodophyllotoxin (3) and [2-^{3}H]4'-demethylpicropodophyllin (11). Catalytic hydrogenolysis of 3 gave [2-^{3}H]4'-demethyldesoxypodophyllotoxin (2). Mild base treatment of the *Podophyllum* lignans leads to C-2 epimerization, a reduction in ring-strain in the lactone system, and formation of the thermodynamically more stable *picro* compounds [12]. This would, of course, result in loss of the [2-^{3}H] label, and appropriate precautions were taken on all subsequent manipulations of the biosynthetic lignans.

EXPERIMENTAL

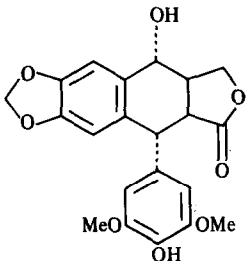
General. Techniques were as previously described [1].

Plant material, feeding techniques and isolation of lignans. The procedures utilized were as described in the earlier paper [1], the labelled lignan precursors being administered in a 2-methoxyethanol (0.2 ml) + Tween 20 (1 drop) → H_2O (1 ml) soln.

After isolation by TLC ($CHCl_3$–MeOH, 25:1), the desoxypodophyllotoxin (6)-podophyllotoxone (8) mixture [2, 5] was purified further by TLC (Et_2O–CH_2Cl_2, 6:1) and then separated by HPLC (Partisil-10 ODS2 column, 250 × 9.4 mm; solvent MeOH–H_2O, 3:2; flow rate 5.6 ml/min; UV detector 291 nm).



Scheme 3. Proposed biosynthetic interrelationships for *Podophyllum* lignans.



11

R_fs were 18.6 min for **6**, and 12.4 min for **8**. The separated lignans were quantified by UV absorption [5], diluted with unlabelled material (20 mg), then recrystallized to constant sp. act. (aq. EtOH).

Radiochemicals. ^3H_2O (5 Ci/ml) was purchased (Amersham).

[4'-$OCH_2{}^3H$]*Podophyllotoxin* (**7**). 4'-Demethylpodophyllotoxin (30 mg) was dissolved in dry dioxan (1 ml) and ^3H_2O (100 mCi, 0.02 ml) was added. After 1.5 hr, an excess of CH_2N_2–Et_2O soln was added and the mixture left at room temp for 5 days. The reaction mixture was then washed with H_2O (3 × 10 ml), evapd to dryness, and [4'-$OCH_2{}^3H$]podophyllotoxin isolated by TLC ($CHCl_3$–MeOH, 25:1). Further TLC purification gave labelled podophyllotoxin (22.4 mg) which was diluted with unlabelled podophyllotoxin (14.0 mg), and divided into three equal portions. One portion was purified further by TLC (hexane–EtOAc–MeOH, 6:4:1; Me_2CO–petrol (60–80°), 1:1) to give [4'-$OCH_2{}^3H$]podophyllotoxin (10.4 mg), sp. act. 1.02×10^{10} dpm/mM.

[4'-$OCH_2{}^3H$]*Desoxypodophyllotoxin* (**6**). A second portion of [4'-$OCH_2{}^3H$] podophyllotoxin from above was dissolved in HOAc (1 ml) and Pd–C catalyst (10%, 15 mg) added. The reaction mixture was heated to 95° and a slow stream of H_2 was bubbled through for 2 hr. After cooling, the reaction mixture was diluted with EtOH–H_2O (1:1, 30 ml) and evapd to dryness. The residue, including catalyst, was applied to TLC plates, developed with $CHCl_3$–*iso*-PrOH, 10:1, and labelled desoxypodophyllotoxin eluted. Further TLC purification (hexane–EtOAc–MeOH, 6:4:1) gave [4'-$OCH_2{}^3H$]desoxypodophyllotoxin (6.6 mg), sp. act. 1.02×10^{10} dpm/mM.

[4'-$OCH_2{}^3H$]*Podophyllotoxone* (**8**). The third portion of [4'-$OCH_2{}^3H$]podophyllotoxin from above was dissolved in $CHCl_3$ (1 ml), treated with freshly prepared MnO_2 (50 mg), and heated under reflux for 1 hr. After cooling, labelled podophyllotoxone was isolated by TLC ($CHCl_3$–MeOH, 25:1) and purified further by TLC ($CHCl_3$–*iso*-PrOH, 10:1). Yield 8.0 mg, sp. act. 1.02×10^{10} dpm/mM.

[2-^3H]4'-*Demethylpodophyllotoxin* (**3**). A mixture of 4'-demethylpodophyllotoxin (100 mg), dry DMF (2 ml), dry K_2CO_3 (500 mg), dry KI (50 mg) and BzCl (40 μl) was stirred vigorously at 60° for 1.5 hr. After cooling, the solids were filtered off, 60° for 1.5 hr. After cooling, the solids were filtered off, washed with Me_2CO (2 ml) and the combined filtrates evapd to dryness. The resulting 4'-benzyl-4'-demethylpicropodophyllin (105 mg) was recrystallized from EtOH, mp 230–235°; UV λ_{max}^{EtOH} nm: 286; IR ν_{max}^{KBr} cm^{-1}: 3700–3200, 1773, 1590, 1505; ^1H NMR (60 MHz, DMSO-d_6, TMS): δ 7.45 (5H, *m*, PhCH_2O), 7.13 (1H, *s*, H-5), 6.67 (2H, *s*, H-2', H-6'), 6.05 (1H, *s*, H-8), 5.98 (2H, *s*, OCH_2O), 4.99 (2H, *s*, PhCH_2O), 4.65–3.90 (4H, *m*, H-1, H-3aα, H-3aβ, H-4), 3.83 (6H, *s*, 3',5'-OMe), 3.40 (1H, *m*, H-2), 2.70 (1H, *m*, H-3).

4'-Benzyl-4'-demethylpicropodophyllin (80 mg) in dry CH_2Cl_2 (10 ml) was treated with dihydropyran (10 ml) and conc HCl (2 drops). The mixture was stirred at room temp overnight, poured into $CHCl_3$ (100 ml), washed with $NaHCO_3$ (2%, 100 ml) and then with H_2O. After evapn of $CHCl_3$, the residue was recrystallized from EtOH to give a mixture of the epimeric tetrahydropyran derivatives (63 mg), mp 220–223°; IR ν_{max}^{KBr} cm^{-1}: 1764, 1589, 1503.

Triphenylmethyl chloride (200 mg) was dissolved in dry THF (3 ml) and to this was added Na amalgam (5%, 800 mg). The mixture was shaken overnight at room temp in a Reactivial under a N_2 atmosphere, giving a deep red soln of triphenylmethyl Na. The above mixture of epimeric tetrahydropyran ethers (20 mg) was added to the vial and the contents shaken for 2 min, after which unreacted amalgam and Hg were separated to the bottom of the vial by centrifugation. The red supernatant liquid was removed by pipette and added dropwise to a stirred soln of HOAc (5 ml) containing conc HCl (5 drops) and ^3H_2O (100 mCi, 0.02 ml). On quenching with acid, the red colour changed to yellow and MeOH (5 ml) and H_2O (5 ml) were added. The mixture was then heated under reflux for 1 hr, cooled, poured into EtOAc (50 ml) and washed with H_2O (5 × 50 ml). After removal of solvent, the residue was separated by TLC ($CHCl_3$–*iso*-PrOH, 10:1) to give a mixture of [2-^3H]4'-benzyl-4'-demethylpodophyllotoxin and [2-^3H]4'-benzyl-4'-demethylpicropodophyllin. Without further purification, this mixture of epimers was dissolved in HOAc (5 ml) and hydrogenated under slight positive pressure with Pd–C catalyst (10%, 20 mg) for 1 hr. After dilution with EtOH–H_2O (1:1, 50 ml), the reaction mixture was evapd to dryness and applied to TLC plates, including the catalyst. After development ($CHCl_3$–*iso*-PrOH, 10:1), [2-^3H]4'-demethylpodophyllotoxin (2.2 mg) was isolated separately from the *picro* derivative and diluted with unlabelled material (8.0 mg). A 4 mg portion was purified further by TLC ($CHCl_3$–MeOH, 9:1, Me_2CO–petrol (60–80°), 1:1) to give [2-^3H]4'-demethylpodophyllotoxin (3.2 mg), sp. act. 2.12×10^8 dpm/mM.

[2-^3H]4'-*Demethyldesoxypodophyllotoxin* (**2**). The remaining portion of [2-^3H]4'-demethylpodophyllotoxin (6 mg) was dissolved in HOAc (1 ml) then hydrogenated for 1.5 hr at 95° in a slow stream of H_2 gas using a Pd–C catalyst (10%, 10 mg). After cooling, the mixture was diluted with EtOH–H_2O (1:1, 30 ml), evapd to dryness and the total residue with catalyst applied to TLC plates. After development (hexane–EtOAc–MeOH, 6:4:1), [2-^3H]4'-demethyldesoxypodophyllotoxin was obtained and purified further by TLC ($CHCl_3$–MeOH, 50:1; hexane–Me_2CO, 2:1). Yield 2.7 mg, sp. act. 1.90×10^8 dpm/mM.

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