Phytochemistry, Vol. 25, No. 9, pp. 2089–2092, 1986.
Printed in Great Britain.

0031–9422/86 $3.00 + 0.00
Pergamon Journals Ltd.

BIOSYNTHESIS OF THE LIGNANS α- AND β-PELTATIN*

WIDAD M. KAMIL and PAUL M. DEWICK

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

(*Received* 9 *January* 1986)

Key Word Index—*Podophyllum peltatum*; *P. hexandrum*; Podophyllaceae; aryltetralin; lignans; biosynthesis; α-peltatin; β-peltatin; podophyllotoxin.

Abstract—Phytochemical analysis of the tumour-inhibitory aryltetralin lignans in *Podophyllum peltatum* has demonstrated that the major lignans α-peltatin and β-peltatin are subject to metabolic turnover. The peltatin content falls to an extremely low level during the growing season, maximum amounts being present during dormancy. Feeding experiments with labelled desoxypodophyllotoxin and 4′-demethyldesoxypodophyllotoxin indicated these compounds are the likely precursors of β- and α-peltatins respectively *via* aromatic hydroxylation at C-5, confirming suggestions made earlier. The transformation of desoxypodophyllotoxin into both β-peltatin and podophyllotoxin was also demonstrated in *P. hexandrum*.

INTRODUCTION

Species of *Podophyllum* contain a range of aryltetralin lignans [1–3] having valuable antitumour activity. The successful development of the anticancer drugs etoposide and teniposide [4] from natural podophyllotoxin (**4**) has focussed attention on *Podophyllum* as an economic source of lignans and has emphasized the need for detailed metabolic studies in these plants. Feeding experiments in Indian podophyllum (*Podophyllum hexandrum*) plants [5] have indicated that the major lignans podophyllotoxin (**4**) and 4′-demethylpodophyllotoxin (**3**) appear to be formed by oxidative coupling of two phenylpropane units containing the ferulic acid (4-hydroxy-3-methoxy) substitution pattern, although the sequence of events leading to the aryltetralin lactone skeleton has yet to be determined. Further feeding studies [6] demonstrated that the *Podophyllum* lignans may be subdivided into two groups which are biogenetically distinct. One group contains a 3,4,5-trimethoxy substituted pendent aromatic ring and is derived from desoxypodophyllotoxin (**2**), with podophyllotoxin (**4**) and podophyllotoxone (**6**) arising as shown in Scheme 1. Similarly, 4′-demethylpodophyllotoxin (**3**) and 4′-demethylpodophyllotoxone (**5**) are derived *via* 4′-demethyldesoxypodophyllotoxin (**1**) (Scheme 1) and are representatives of the group of lignans containing a 4-hydroxy-3,5-dimethoxy substituted pendent ring. Although no direct evidence was available at the time, the peltatins, α-peltatin (**7**) and β-peltatin (**8**) were suggested to arise from **1** and **2** respectively by hydroxylation in the aromatic ring (Scheme 1). This communication describes experiments supporting a 5-hydroxylation route for the biosynthesis of the peltatins.

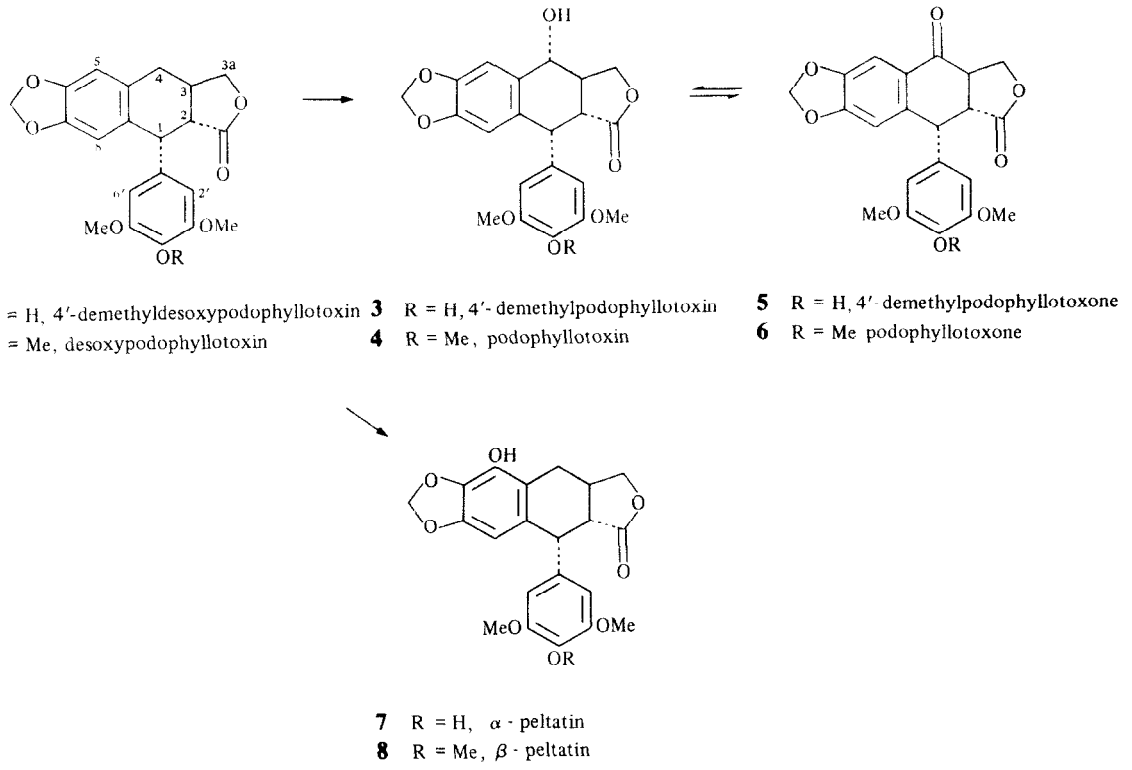
*Part 3 in the series "Biosynthesis of *Podophyllum* Lignans". For Part 2 see ref. [6].

RESULTS AND DISCUSSION

Feeding experiments

Detailed analysis of root material of Indian podophyllum (*P. hexandrum*) [2] had indicated that although α- and β-peltatins were present, the amounts were quite small (*ca* 1 mg/10 g dried root). Accordingly, these materials were not isolated and analysed in the earlier feeding experiments. The related American species (*P. peltatum*) however, contains the same range of lignans but in different proportions to *P. hexandrum* [2] and produces podophyllotoxin, α-peltatin and β-peltatin in roughly equal amounts. Preliminary feeding experiments in intact *P. peltatum* plants were thus carried out during midsummer, using techniques as described for *P. hexandrum* [5]. Subsequent work-up of the plants and analysis gave quite unexpected results: hardly any peltatins were present, and podophyllotoxin and 4′-demethylpodophyllotoxin were the major lignans observed [7]. In fact, the lignan pattern was almost analogous to that of *P. hexandrum*.

Detailed analysis of *P. peltatum* plants raised in the U.K. and also the U.S.A. has confirmed these early observations. Variation in lignan pattern between individual plants is noted, but in general, dormant plants contain podophyllotoxin, α-peltatin and β-peltatin, with β-peltatin usually predominating (cf. [2]). Subsequent analysis of root specimens excised from plants during the growing season (March–August) showed the peltatin content rapidly disappeared, and by May only traces could be detected. Podophyllotoxin was now the main constituent. Towards the end of July, some peltatin content could again be observed in many plants, coinciding with the onset of senescence. Dormant plants were then shown to have regained the normal lignan pattern. The loss of the peltatins during growth of *P. peltatum* is almost certainly due to metabolic turnover. There was no



1 R = H, 4′-demethyldesoxypodophyllotoxin
2 R = Me, desoxypodophyllotoxin
3 R = H, 4′-demethylpodophyllotoxin
4 R = Me, podophyllotoxin
5 R = H, 4′-demethylpodophyllotoxone
6 R = Me podophyllotoxone

7 R = H, α-peltatin
8 R = Me, β-peltatin

Scheme 1. Proposed biosynthetic interrelationships for *Podophyllum* lignans [6].

evidence for transport to other parts of the plant, e.g. leaves, nor storage as glycosides. Material in the dormant plant appears to be metabolized during growth, then resynthesized during senescence. Podophyllotoxin levels are not affected in the same way. It is perhaps possible that α- and β-peltatins may be utilized for lignin biosynthesis (compare ref. [8]), the phenolic grouping allowing further oxidative coupling processes which are denied to podophyllotoxin. Changes in lignan patterns in *P. peltatum*, particularly in relation to lower levels observed during periods of maximum growth, have previously been noted in Russian studies [9].

Feeding experiments were subsequently conducted during the period of peltatin biosynthesis, namely just prior to the onset of senescence in the plants. Unfortunately, the declining vigour of the plants made them poor subjects for biosynthetic experiments, and this is almost certainly reflected in the rather low incorporation figures obtained. Four pot-grown plants were selected after analysis of root specimens had confirmed the presence of sufficient levels of α- and β-peltatins. Soil was carefully washed off, and labelled precursors were fed in aqueous solution to the root system as in earlier experiments [5, 6]. Two compounds, [3′-*O*-methyl-^{14}C]-4′-demethyldesoxypodophyllotoxin (**1**) and [4′-*O*-methyl-^{14}C]-desoxypodophyllotoxin (**2**) were tested as precursors of α- and β-peltatins as well as podophyllotoxin. After 7 days these metabolites were isolated from the plant extract, quantified, diluted with carrier material, then converted into easily purified acetate or diacetate derivatives. After rigorous purification to constant specific activity, the incorporation data in Table 1 were obtained.

Good incorporations of desoxypodophyllotoxin into podophyllotoxin were observed, in agreement with earlier studies in *P. hexandrum* [6]. Incorporations into β-peltatin were also recorded, but at much lower levels than into podophyllotoxin. The β-peltatin isolated was, however, significantly more active than α-peltatin. Demethyldesoxypodophyllotoxin was not a good precursor of podophyllotoxin, again in agreement with the *P. hexandrum* studies, but it did prove to be a better precursor of α-peltatin than for either β-peltatin or podophyllotoxin, though incorporation levels were again rather low. These low positive incorporations of desoxypodophyllotoxin into β-peltatin but not into α-peltatin, and of 4′-demethyldesoxypodophyllotoxin into α-peltatin but not into β-peltatin, indicate that the proposed C-5 hydroxylation process is probably operative. The low incorporations observed were probably the result of low rates of synthesis of the peltatins during the feeding period, although podophyllotoxin synthesis during this time was obviously quite substantial.

For confirmation of the desoxypodophyllotoxin to β-peltatin conversion, a further experiment was carried out, this time using *P. hexandrum*. Individual plants of both *P. peltatum* and *P. hexandrum* may show quite significant variations in lignan patterns produced, and it was possible to select an actively growing *P. hexandrum* plant producing enough β-peltatin for analysis in a biosynthetic experiment. [4′-*O*-methyl-^{3}H]Desoxypodophyllotoxin was fed to this plant, and subsequent work-up gave the data in Table 2. The plant yielded about 15 times as much podophyllotoxin as β-peltatin, but the specific activity of the latter compound was almost twice the level for podophyllotoxin. This demonstrates the conversion of desoxypodophyllotoxin

Table 1. Incorporation data from feeding experiments in *Podophyllum peltatum*

Lignan fed	Expt	Podophyllotoxin mg	Podophyllotoxin % Incorporation	Podophyllotoxin Dilution	α-Peltatin mg	α-Peltatin % Incorporation	α-Peltatin Dilution	β-Peltatin mg	β-Peltatin % Incorporation	β-Peltatin Dilution
Desoxypodophyllotoxin (**2**)*	(i)	11.0	0.33	2.96×10^3	2.77	0.0059	4.35×10^4	5.50	0.023	2.09×10^4
	(ii)	15.9	0.82	1.68×10^3	4.23	0.0053	7.10×10^4	7.15	0.021	2.87×10^4
4'-Demethyldesoxypodophyllotoxin (**1**)†	(iii)	5.05	0.0076	1.37×10^5	2.71	0.035	1.66×10^4	5.10	0.0029	3.55×10^5
	(iv)	5.67	0.0078	1.45×10^5	1.41	0.016	1.91×10^4	6.42	0.0067	1.93×10^5

*[3'-*O*-methyl-^{14}C].
†[4'-*O*-methyl-^{14}C].

Table 2. Incorporation of [4'-*O*-methyl-^{3}H]desoxypodophyllotoxin in *Podophyllum hexandrum*

	Podophyllotoxin	β-peltatin
mg isolated	62.0	4.13
Sp. act (dpm/mM)	4.81×10^4	7.89×10^4
% Incorporation	0.17	0.019
Dilution	3.70×10^4	2.26×10^4

into both podophyllotoxin and β-peltatin, confirming that hydroxylation may occur at both C-4 and C-5 respectively.

Synthesis of labelled compounds

In previous syntheses [6] of labelled *Podophyllum* lignans, two approaches have been utilized. 4'-*O*-Methylation using labelled diazomethane offered a satisfactory route from 4'-demethyl compounds to labelled compounds in the 3',4',5'-trimethoxy series. However, labelling of the 4'-demethyl compounds themselves involved a tedious exchange procedure for hydrogen at C-2 and required the use of protecting groups. In the present studies, a convenient sequence giving labelled lignans of both groups in acceptable yields was employed.

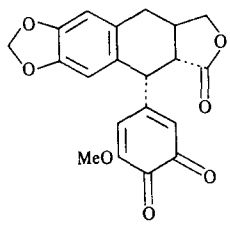
Oxidation of desoxypodophyllotoxin using nitric acid in acetic acid [3, 5, 10] gave the quinone (**9**), which was smoothly reduced to the quinol, 3',4'-didemethyldesoxypodophyllotoxin (**10**) using sodium dithionite. Partial methylation of **10** using $^{14}CH_2N_2$ generated from [*N*-methyl-^{14}C]-*N*-nitroso-*N*-methylurea gave a mixture of [4'-*O*-methyl-^{14}C]-3'-demethyldesoxypodophyllotoxin (**11**) and [3'-*O*-methyl-^{14}C]-4'-demethyldesoxypodophyllotoxin (**1**), together with traces of [3',4'-*O*-methyl-^{14}C]desoxypodophyllotoxin. These compounds were readily resolved by TLC. Methylation of **11** with inactive diazomethane gave [4'-*O*-methyl-^{14}C]desoxypodophyllotoxin (**2**).

EXPERIMENTAL

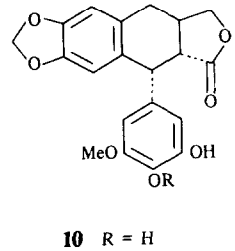
General. Techniques were as previously described [5].

Plant material, feeding techniques and isolation of lignans. The procedures utilized were as described earlier [5, 6], the labelled lignan precursors being administered in a 2-methoxyethanol (0.2 ml) + Tween 20 (1 drop) + H_2O (1 ml) soln. The isolated lignans were separated by TLC ($CHCl_3$–MeOH, 25:1), quantified by UV absorption [2] and then treated as described below.

Podophyllotoxin was purified further by TLC (hexane–Me_2CO, 1:1; $CHCl_3$–*iso*-PrOH, 10:1), then diluted with carrier material (to 20 mg) if the amount isolated was less



9



10 R = H
11 R = Me

than 20 mg. This was acetylated by heating under reflux with Ac_2O (0.5 ml) for 30 min. The reaction mixture was diluted with H_2O (2 ml) and EtOH (10 ml), then evaporated to dryness under red. pres. at 50°. The labelled podophyllotoxin acetate was then purified by TLC ($CHCl_3$–*iso*-PrOH, 10:1) and recrystallized × 3 from EtOH. Unlabelled podophyllotoxin acetate had mp 208–209° (lit. [11] 210–211°).

β-Peltatin was purified by TLC [Me_2CO–petrol (60–80°), 1:1; hexane–Me_2CO, 2:1], diluted to *ca* 20 mg and acetylated in a similar manner to produce β-peltatin acetate. This was purified by TLC (hexane–Me_2CO, 2:1), and recrystallized × 3 from EtOH. Unlabelled β-peltatin acetate had mp 226–228° (lit. [12] 227–229°).

α-Peltatin was purified by TLC [hexane–Me_2CO, 1:1; Me_2CO–petrol (60–80°), 1:1], diluted to *ca* 20 mg, and acetylated as above. This was purified further by TLC [Me_2CO–petrol (bp 60–80°), 1:1] and recrystallized × 3 from EtOH. Unlabelled α-peltatin diacetate had mp 218–220° (lit. [12] 229–231°).

Semiquantitative analysis of P. peltatum. Portions of root material were excised, dried and powdered. Samples (200 mg) from each plant were extracted with EtOH (0.4 ml) by heating in a Reactivial at 60° for 30 min. Aliquots (20 μl) were then taken from each extract and applied as 1.5 cm streaks on 200 × 200 × 0.25 mm silica gel TLC plates. The plates were then developed using $CHCl_3$–MeOH (25:1). R_f values of the separated lignans were compared with standard materials and relative concns were assessed from the size of the bands and their relative intensities when viewed under UV light.

Radiochemicals. [4′-*O*-methyl-^{3}H]Desoxypodophyllotoxin (0.80 mCi/mM) was available from earlier studies [2]. [*N*-methyl-^{14}C]-*N*-Nitroso-*N*-methylurea (17.9 mCi/mM) was purchased (Amersham).

3′,4′-Didemethyldesoxypodophyllotoxin (**10**). Desoxypodophyllotoxin (0.55 g) in glacial HOAc (6.5 ml) was added dropwise to a stirred mixture of conc. HNO_3 (3 ml) and HOAc (10 ml) at 0°. The reaction was allowed to proceed for 20 min, then was diluted with H_2O and extracted with $CHCl_3$ (3 × 100 ml). The combined extracts were washed with H_2O (3 × 200 ml), dried over $MgSO_4$ and evaporated to dryness. A portion of the residue (1/20) was purified by TLC [Me_2CO–petrol (60–80°), 1:1] to give the red solid quinone (12 mg), 3′,4′-dioxo-3′,4′-dihydro-3′,4′-didemethoxydesoxypodophyllotoxin (**9**), mp 182–184°; IR ν_{max}^{KBr} cm^{-1}: 1770, 1700, 1665, 1220; ^{1}H NMR (90 MHz, $CDCl_3$, TMS): δ6.65 (1H, *s*, H-5), 6.5 (2H, *br s*, H-8, H-6′), 6.02 (2H, *s*, OCH_2O), 5.40 (1H, *br s*, H-2′), 4.4–4.0 (3H, *m*, H-3aα, H-3aβ, H-1), 3.90 (3H, *s*, 5′-OMe), 2.9–2.7 (4H, *m*, H-2, H-3, H-4α, H-4β); EIMS (probe) 70 eV, *m/z* (rel. int.): 370 [M + 2]$^+$ (27%), 368 [M]$^+$ (1), 149 (30), 78 (100).

The remainder of the residue was dissolved in EtOH–H_2O (6:1, 21 ml) and solid sodium dithionite (*ca* 150 mg) was added gradually until the deep red colour disappeared. The mixture was then filtered, concd under red. pres., diluted with H_2O (50 ml) and extracted with EtOAc (3 × 100 ml). The combined extracts were washed with H_2O (2 × 200 ml) and evaporated to dryness. The product was purified by TLC [Me_2CO–petrol (60–80°), 1:1] and recrystallized from MeOH to give 3′,4-didemethyldesoxypodophyllotoxin (**10**, 200 mg), mp 240–242°; UV λ_{max}^{EtOH} nm: 289, 293, 297 sh; IR ν_{max}^{KBr} cm^{-1}: 3560, 3500, 3320, 1770, 1610, 1470–1520, 1220; ^{1}H NMR (90 MHz, $CDCl_3$, TMS): δ7.3 (1H, *br s*, OH), 7.15 (1H, *br s*, OH), 6.75 (1H, *s*, H-5), 6.52 (2H, *br s*, H-8, H-6′), 6.05 (1H, *d*, *J* = 1.8 Hz, H-2′), 5.9 (2H, *s*, OCH_2O), 4.5–3.9 (3H, *m*, H-1, H-3aα, H-3aβ), 3.75 (3H, *s*, 5′-OMe), 2.8–2.6 (4H, *m*, H-2, H-3, H-4α, H-4β); EIMS (probe) 70 eV, *m/z* (rel. int): 370 [M]$^+$ (100%), 185 (20), 173 (14), 153 (13).

[3′-O-methyl-^{14}C]-4′-Demethyldesoxypodophyllotoxin (**1**) *and [4′-O-methyl-^{14}C]-3′-demethyldesoxypodophyllotoxin* (**11**). [^{14}C]Diazomethane was generated from [*N*-methyl-^{14}C]-*N*-nitroso-*N*-methylurea (17.9 mCi/mM, 50 μCi, 0.29 mg) and inactive *N*-nitroso-*N*-methylurea (24 mg, 0.23 mM) using the Aldrich apparatus for small scale CH_2N_2 preparation. The ethereal CH_2N_2 soln was then pipetted onto a soln of 3′,4′-didemethyldesoxypodophyllotoxin (18 mg, 0.05 mM) in dry THF (1 ml). The mixture was left at room temp. overnight, treated with Me_2CO (1 ml), then evaporated to dryness. The residue was purified by TLC [Me_2CO–petrol (60–80°), 1:1] to give [3′-*O*-methyl-^{14}C]-4′demethyldesoxypodophyllotoxin (1.8 mg), [4′-*O*-methyl-^{14}C]-3′-demethyldesoxypodophyllotoxin (3.2 mg) and a small amount (0.6 mg) of [3′,4′-*O*-methyl-^{14}C] desoxypodophyllotoxin.

Unlabelled 3′-demethyldesoxypodophyllotoxin (**11**) (recrystallized from aq. MeOH) had mp 170–172°; UV λ_{max}^{EtOH} nm: 289, 293, 297 sh; IR ν_{max}^{KBr} cm^{-1}: 3420, 1770, 1590, 1505–1490, 1230; ^{1}H NMR (250 MHz, $CDCl_3$, TMS): δ6.73 (1H, *d*, *J* = 1.9 Hz, H-6′), 6.64 (1H, *s*, H-5), 6.49 (1H, *s*, H-8), 5.93 (3H, *s*, OCH_2O, H-2′), 5.68 (1H, *br s*, OH), 4.56 (1H, *d*, *J* = 3.3 Hz, H-1), 4.44 (1H, *dd*, *J* = 8.4, 6.2 Hz, H-3aα), 3.92 (1H, *m*, H-3aβ), 3.86 (2 × 3H, *s*, 4′-OMe, 5′-OMe), 3.06 (1H, *m*, H-4α), 2.74 (3H, *m*, H-2, H-3, H-4β); EIMS (probe) 70 eV, *m/z* (rel. int): 384 [M]$^+$ (100%), 185 (15), 173 (16), 167 (24). The other two products were identical to authentic specimens [2].

Acknowledgements—We thank Dr. D. E. Jackson for helpful discussions and assistance, and the Iraqi Government for financial support (to W.M.K.).

REFERENCES

1. Dewick, P. M. and Jackson, D. E. (1981) *Phytochemistry* **20**, 2277.
2. Jackson, D. E. and Dewick, P. M. (1984) *Phytochemistry* **23**, 1147.
3. Jackson, D. E. and Dewick, P. M. (1985) *Phytochemistry* **24**, 2407.
4. Issell, B. F., Muggia, F. M. and Carter, S. K. (1984) *Etoposide (VP-16)-Current Status and New Developments*. Academic Press, Orlando.
5. Jackson, D. E. and Dewick, P. M. (1984) *Phytochemistry* **23**, 1029.
6. Jackson, D. E. and Dewick, P. M. (1984) *Phytochemistry* **23**, 1037.
7. Jackson, D. E. (1983) Ph.D. Thesis, University of Nottingham.
8. Fujimoto, H. and Higuchi, T. (1977) *Wood Res.* **62**, 1.
9. Kuznetsova, G. A. and Bogdanova, V. P. (1970) *Rast. Resur.* **6**, 240; (1971) *Chem. Abstr.* **74**, 1085w.
10. Ayres, D. C. and Lim, C. K. (1982) *Cancer Chemother. Pharmacol.* **7**, 99.
11. Hartwell, J. L. and Schrecker, A. W. (1958) *Fortschr. Chem. Org. Naturst.* **15**, 83.
12. Hartwell, J. L. and Detty, W. E. (1950) *J. Am. Chem. Soc.* **72**, 246.