Lignans and Related Phenols. Part 16.[1] The Biogenesis of Podophyllotoxin

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The incorporation of DL-[β-^{14}C]phenylalanine into podophyllotoxin (1) has been demonstrated. The subsequent stepwise degradation to 3',4'-methylenedioxy-3,4,5-trimethoxybenzophenone bearing a ^{14}C-carbonyl label establishes the *in vivo* synthesis of the carbon skeleton of this lignan.

THE widely held view [2] of lignan biogenesis is based by chemical analogy upon the dimerisation of cinnamic acid units, or their biogenetic equivalents. Support for this assumption is to be found in the numerous examples [3] of the incorporation of these C_6–C_3 unit precursors into lignin. However, this can only be regarded as indirect evidence since polymeric plant lignins are randomly structured and racemic, whereas the lignans occur typically as discrete optically active molecules. Our initial attempts [4] to test this mechanism experimentally by feeding possible labelled precursors such as [1-^{14}C]-acetate and L-[U-^{14}C]tyrosine to *Podophyllum emodi* plants were inconclusive. With [2-^{14}C]-*p*-coumaric acid the incorporation into the resin was 0.5% (Table 1) but this figure is not regarded as firm as it depends on a count rate only 75% above background.

Specific incorporation into podophyllotoxin was established by repeating the *P. emodi* feed with DL-[β-^{14}C]phenylalanine. This led to a level of incorporation in the resin of 1.17% with 85% of this in the lignan. This figure should probably be doubled as it is likely that only the L-form of the amino-acid was available to the plant.

In the absence of any procedure for the controlled oxidative degradation of podophyllotoxin we used the classical permanganate method.[8] This gives poor yields of the key product, 3,4-methylenedioxy-6-(3,4,5-trimethoxybenzoyl)benzoic acid (2) because this compound is labile under the reaction conditions, as also are the oxybenzoic acid fragments. The procedure did, however, have the merit that the keto-acid (2) was readily separated from other acidic fragments and it was characterised by its melting point and by its u.v. and i.r. spectra. Recrystallised material retained 86% of the activity of the purified podophyllotoxin (Table 2). On decarboxylation radioactive carbon dioxide was emitted to afford 3',4'-methylenedioxy-3,4,5-trimethoxybenzophenone (3) which retained effectively half the activity of the parent keto-acid (2). These results show that two C_6–C_3

TABLE 1

Activity of *Podophyllum* resin

Entry [a]	Precursor	Total activity fed/μCi	Feed time/ days	Resin yield/mg	Resin count over background/ mg s^{-1}	% Incorporation corrected for quenching
1	Na [1-^{14}C]Acetate	7.0	12	44	1.52	0.042
2	L-[U-^{14}C]Tyrosine HCl	2.8	21	31	0.46	0.023
3	[2-^{14}C]-*p*-Coumaric acid	1.0	21	200	1.72 [b]	0.57
4	L-[U-^{14}C]Phenylalanine	5.0	8	67.5	23.0 (54.2) [c]	1.40
5	DL-[β-^{14}C]Phenylalanine	5.0	8	103	12.5 (33.6) [c]	1.17

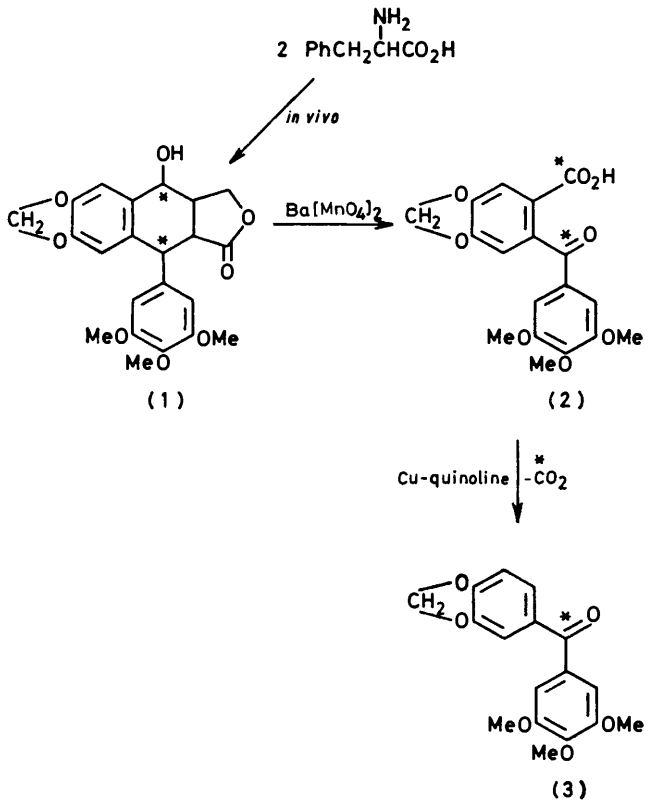
[a] Entries 1—3 are for counting with the Panax unit giving a background in the range 1.8—2.2 disint. s^{-1}. Entries 4 and 5 are for counting with the Nuclear Enterprises counter with background 0.3 disint. s^{-1}. [b] This measurement for a 4.9 mg sample, background 2.27 disint. s^{-1}. [c] Figures in parentheses refer to the podophyllotoxin fraction of this resin.

The early experiments did, however, establish that mature five-year-old plants grown in open ground were satisfactory, provided that they were transferred to large pots well in advance of the experiments. Autoradiography of harvested leaves established that wick-feeding of the plants in pots was the best method of introducing the sample and that it was most readily translocated during May–June. Later experiments [5] under these conditions with L-[U-^{14}C]-phenylalanine showed it to be an effective precursor, since the level of incorporation in the podophyllum resin was 1.4% of which 84% was present in the podophyllotoxin fraction. The remaining 16% of the activity is largely accounted for by co-occurring 4'-demethylpodophyllotoxin [6] (2%) and by the flavonols quercetin and kaempferol (*ca.* 9%), which were not isolated but which are expected [7] to incorporate a cinnamate residue.

TABLE 2

Activities of compounds (1)—(3)

Compound	
(1)	Relative activity 100% 33.6 disint. mg^{-1} s^{-1} at 80% efficiency 17 380 disint. mmol^{-1} s^{-1}
(2)	Relative activity 86% 14 900 disint. mmol^{-1} s^{-1} at 86% efficiency
(3)	Relative activity 42% to (2) 36% to (1) 6 213 disint. mmol^{-1} s^{-1} at 88% efficiency



units are necessary and sufficient for the biosynthesis of the podophyllotoxin skeleton in plants.

The recently discovered [9,10] mammalian lignans which bear only *meta*-oxy-substituents may well form part of a group with related structures to those previously characterised in plants. It is also possible that their biogenesis is initiated by the same free-radical coupling process, but if so the essential *para*-hydroxy-group must be shifted directly or indirectly during a later stage. The union of the two C_6–C_3 residues could occur through their initial combination as cinnamyl esters or cinnamic anhydrides, and this mechanism would clearly be independent of the ring substitution pattern.

EXPERIMENTAL

M.p.s were determined on a Köfler hot-stage microscope. I.r. and u.v. spectra were recorded with Perkin-Elmer 237 and Unicam SP 800 instruments respectively. Radioactive counting initially (Table) was with a Panax coincidence unit 2032 and liquid measuring head 2022; latterly a Nuclear Enterprises 6500 liquid scintillation counter was used.

Method of Feeding Labelled Sybstances to Podophyllum emodi.—The mature plants established in pots the previous autumn were wick fed.[11] A length of unmercerised cotton (*ca.* 15 cm) was threaded with a fine needle along the length of the stem * so that 2—3 cm were in contact with the plant tissue. The points of entry and of emergence were taped to prevent tearing the plant and the ends of the wick were immersed in a solution of the substrate dissolved in water (*ca.* 2 ml), which was replenished as uptake proceeded during the 8 day feeding period.

Feeding by dipping the leaves or a portion of the rhizome was found to give less efficient translocation of labelled precursors. Autoradiography was used to monitor the efficiency of translocation. Sections of leaf were taken with a cork borer (No. 4), dried for 4 h between filter papers, then held in contact with an *X*-ray plate (Ilford Industrial B) in a lead-backed cassette for 7 days. After the removal of plant material the plate was developed with Ilford ID 19 solution and fixed with I.F.9 solution. This method was satisfactory for recording translocation in whole leaves or stems provided that the drying time was extended to 3 days.

Isolation of Podophyllum Resin and of Podophyllotoxin from the Roots and Rhizomes of Podophyllum emodi.—The method was adapted for small-scale working from those of Chatterjee [12] and Hartwell *et al.*[13] The material was air-dried (typical weight 12.4 g) for 5 days, ground (pestle and mortar), and continuously extracted with ethanol. Concentration of the extract and slow addition to ice–hydrochloric acid (1M) gave a firm yellow resin that was readily filtered and desiccated. This material was taken up in a little ethanol, reprecipitated, and dried before counting. The roots and rhizomes of 5-year-old plants afforded up to 6% of their dry weight as resin.

In a typical chromatographic separation podophyllum resin (313 mg) yielded podophyllotoxin (133 mg, 43%) which was further purified by recrystallisation, m.p. 115 °C (from EtOH). On t.l.c. [Kieselgel; elution with benzene–ethanol (5 : 1 v/v)] this material had the same R_F value (0.68) as an authentic sample of podophyllotoxin; its i.r. spectrum and o.r.d. and c.d. curves [14] were also characteristic.

Before determining the radioactivity of podophyllotoxin it was freed from solvent by heating at 100 °C for 3 h *in vacuo* (*ca.* 10^{-3} mmHg).

Oxidation of Podophyllotoxin.—This was carried out by a modification of the original procedure [8] by the addition of barium permanganate (1.5 g, 6.0 mmol) to podophyllotoxin (240 mg, 0.52 mmol) dissolved in boiling acetone (30 ml). On disappearance of the permanganate colour the solvent was evaporated off and the residue clarified by the passage of sulphur dioxide through a suspension in water (30 ml). Alkali-soluble material was then treated by the method of Späth *et al.*[8] to yield 2-(3,4,5-trimethoxybenzoyl)piperonylic acid [15] (48 mg, 23%). After recrystallisation from methanol the product had the same m.p., mixed m.p. (215 °C), and i.r. spectrum as an authentic sample kindly supplied by Professor W. J. Gensler.

Decarboxylation of 2-(3,4,5-*Trimethoxybenzoyl*)*piperonylic Acid.*—This was carried out as before [8,15] with quinoline–copper on a recrystallised sample which had been mixed with twice its weight of inactive material (90 mg total). This afforded 3,4,5-trimethoxy-3′,4′-methylenedioxybenzophenone (62 mg, 73%), m.p. 125 °C (from MeOH), identical (m.p., mixed m.p., and i.r. spectrum) with an authentic sample.[15]

Counting Procedure.—The compounds were counted by the addition of ethanolic solutions (typically 0.2 ml of a solution containing *ca.* 10 mg ml^{-1}) to toluene phosphor [10 ml containing 30 mg of *p*-terphenyl and 1 mg of 1,4-bis-2-(5-phenyloxazolyl)benzene]. Quenching curves were obtained by counting a standard solution of [^{14}C]hexadecane (12.4 mg, activity 1.02 μCi g^{-1} ≡ 468 counts per second) in the presence of aliquots of the substrate in

* A structural drawing of the closely related plant *P. peltatum* L. is given on p. 85 of ref. 6.

ethanol. The resin quenching was relatively high, reaching 33% efficiency for a 4.0 mg sample; quenching for the other substances was in the range of 80—90% efficiency for 1.0 mg samples.

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