Biosynthesis of Canescin, a Metabolite of *Aspergillus malignus*: Incorporation of Methionine, Acetate, Succinate, and Isocoumarin Precursors, Labelled with Deuterium and Carbon-13

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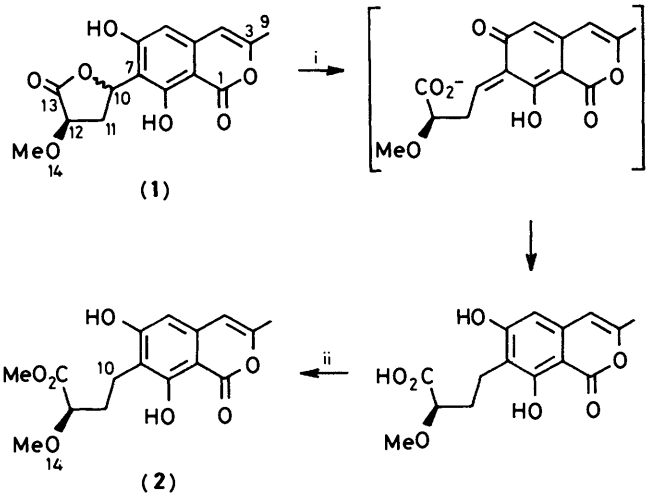
The biosynthesis of canescin (**1**), a metabolite of *Aspergillus malignus*, has been studied using [*Me*-$^{13}C^2H_3$]methionine, sodium [1,2-$^{13}C_2$]-and [1-^{13}C, 2-$^2H_3$]acetate, and sodium [2,3-$^{13}C_2$]- and [2-^{13}C, 2-$^2H_2$]-succinate as simple precursors, and deuterium labelled isocoumarins as potential advanced precursors. Analysis of the labelled products by both n.m.r. and mass spectrometry suggests that 6,8-dihydroxy-3,7-dimethylisocoumarin (**13**) is the first enzyme-free intermediate produced by the polyketide synthase, and the 7-formyl-6,8-dihydroxy-3-methylisocoumarin (**16**) is a later intermediate on the pathway. Incorporation into canescin of ^{13}C together with one deuterium atom from [$^{13}C^2H_3$]methionine proves that the formyl group of (**16**) is derived from this source and that it is not oxidised to the level of a carboxylic acid in subsequent steps. The ^{13}C labels in the succinate units are incorporated into the γ-lactone carbons (C-11 to C-13) of canescin, but with complete loss of the neighbouring deuterium label.

The isolation of canescin (**1**) from *Penicillium canescens* was first reported[1] in 1953, and the structure elucidated by Birch *et al.*,[2] using spectroscopic and chemical means. Independent work during the same period showed that the same compound was also produced by *Aspergillus malignus*. Birch's group also performed some experiments to determine the biosynthetic pathway to this metabolite.[3] The results suggested a mixed biosynthetic origin with the isocoumarin portion of the molecule being built up *via* the acetate/malonate pathway[4,5] but, surprisingly, C-10 appeared to be derived from methionine. This is unusual as it requires a formal alkylation of a methyl group originating from the C_1 pool, a sequence known in the biosynthesis of some steroids,[6] but in very few polyketides.[7,8] It is interesting to note that the stereochemistry about this carbon atom is a mixture of *R* and *S* forms, and therefore that the natural product is isolated as a mixture of two diastereoisomers.

We elected to study the biosynthesis of this unusual metabolite using stable isotopes and n.m.r. spectroscopy to determine sites of incorporation. Initial studies[9] using ^{13}C labelled precursors were hampered by the existence of the two diastereoisomers, which have slightly different chemical shifts for corresponding carbon atoms. For many of the sites the chemical shift difference is sufficiently large for the duplicate signals to be resolved, especially in the area of the γ-lactone ring. This difficulty was circumvented by treatment of canescin with sodium borohydride, followed by *O*-methylation, to give the derivative (**2**); the reduction step in this transformation is thought to procede *via* a ring-opened quinone-methide intermediate which is trapped by hydride to reform the aromatic system; the resulting acid is isolated and treated with diazomethane (Scheme 1). The derivative is a crystalline solid, highly soluble in chloroform (in contrast to canescin itself) and hence very suitable for n.m.r. experiments. More important still, the offending chiral centre of the natural product has been destroyed, resulting in an uncomplicated ^{13}C n.m.r. spectrum, which was unambigously assigned using straightforward chemical-shift arguments and single-frequency proton-decoupling experiments (Table 1).

Biosynthetic Studies.—In his pioneering study, Birch showed that C-10 and C-14 of canescin are derived from methionine. In the present investigation [$^{13}C^2H_3$]methionine, prepared by



Scheme 1. *Reagents:* i, $NaBH_4$; ii, CH_2N_2

standard methods,[10] was administered to *A. malignus*, batchwise over 5 days, starting at the onset of canescin production (monitored by u.v. spectroscopy of the culture medium). After 2 more days of incubation, the medium was extracted to give canescin, which was converted into the derivative (**2**). The ^{13}C n.m.r. spectrum of this material, with simultaneous proton and deuterium decoupling, showed α-shifted peaks[11] for C-10 and C-14, the magnitudes of the shifts being consistent with the retention of one and three deuteriums respectively. The corresponding spectrum with proton coupling showed a doublet and a singlet consistent with these assignments. The retention of three deuteriums at C-14 is not surprising as the intact transfer of methyl groups to oxygen is well documented,[5,11] but the result at C-10 deserves comment. That one deuterium is retained (the maximum possible) demonstrates that this carbon is not oxidised at any stage to the carboxylic acid level, as is the case for the equivalent residue in citrinin[12] and ochratoxin.[13]

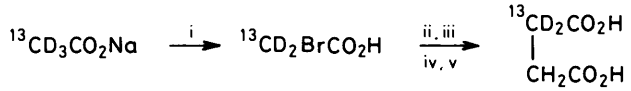
In the next experiment, sodium [1,2-$^{13}C_2$]acetate was used as precursor. In the ^{13}C n.m.r. spectrum of the resulting sample of (**2**), 12 of the peaks were flanked by doublets due to ^{13}C-^{13}C coupling. The coupling constants could be paired up unam-

Table 1. ^{13}C N.m.r. data[a] for canescin derivatives

Carbon	δ (p.p.m.)	$J(^{13}C-^{13}C)$[b] (Hz)	α-Shift[c] (p.p.m.)
13	173.1	61.8[d]	
1	166.5	73.4	
6	164.5	67.6	
8	160.3	72.3	
3	153.8	53.5	
4a	137.6	54.6	
7	115.6	72.2	
4	104,7	54.4	
8a	100.0	73.4	
5	97.0	67.9	
12	80.6	61.9[d]	
14	58.0		0.9
ArOMe	55.6		
CO_2Me	51.6		
11	31.5		
9	19.3	53.5	
10	18.7		0.3

[a] Relative to Me_4Si in a $CDCl_3$ solution. [b] In (**2**) derived from sodium [1,2-$^{13}C_2$]acetate. [c] In (**2**) derived from [*Me*-$^{13}C^2H_3$]methionine. [d] These satellites were of lower relative intensity than the others.

tricarboxylic acid (TCA) cycle before incorporation. In an attempt to determine which particular acid of the TCA cycle is involved directly as a canescin precursor, sodium [2-^{13}C, 2-$^2H_2$]succinate was synthesized (Scheme 2) and fed. Although



Scheme 2. *Reagents:* i, CF_3CO_2D, $(CF_3CO_2)_2O$, Br_2; ii, CH_2N_2; iii, $NaCH(CO_2Et)_2$; iv, H^+/H_2O; v, Heat

this was incorporated, as shown by the increased peak height for C-11 and C-12 in the ^{13}C n.m.r. spectrum of the derivative (**2**), no α-shifted peaks could be detected, indicating that no deuterium had been retained (tests with deuteriated solvent had shown that none of the relevant protons underwent exchange during the derivation sequence). The most likely explanation of this result is that oxaloacetic acid is the compound removed from the TCA cycle, because at this stage total exchange of the hydrogen label with the medium could occur by reversible enolisation of the ketone carbonyl group; repeated interconversion of succinate and fumarate at a previous stage of the cycle is an alternative, but less likely, explanation.

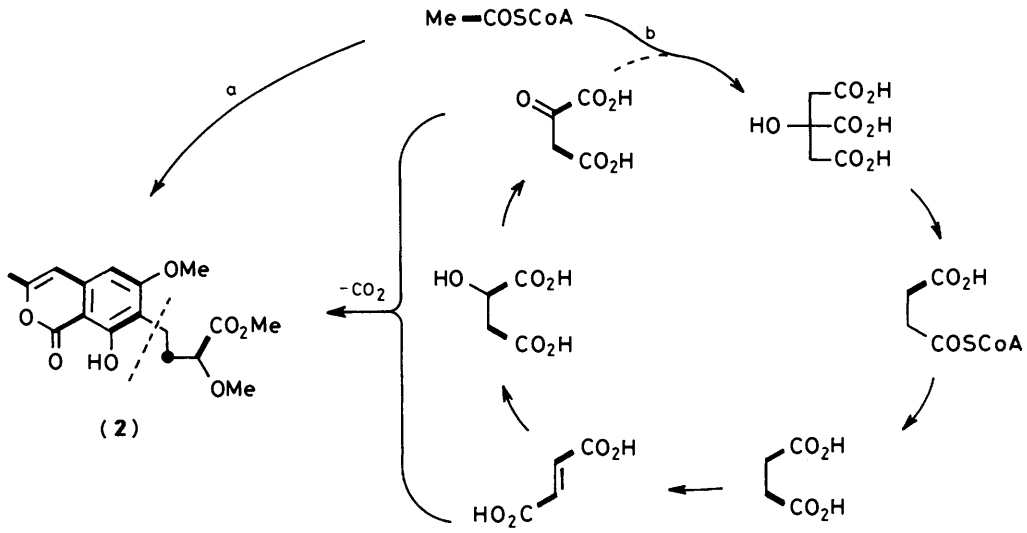


Fig. 1. Incorporation of ^{13}C from [$^{13}C_2$]acetate into the canescin derivative (**2**); a, *via* acetate/malonate pathway; b, *via* TCA cycle

biguously, thus demonstrating the existence of six intact acetate units. As expected, five of these make up the isocoumarin portion of the molecule, but the sixth pair belong to C-12 and C-13. Further examination showed that the singlet for C-11 was enhanced in intensity over natural abundance (by 20%) suggesting that C-11 is part of an acetate unit that has been cleaved. This pattern of enrichment is consistent with derivation of the three-carbon segment C-11 to C-13 from one of the tricarboxylic acid cycle intermediates, as was suggested by Birch.[3] The Figure shows how doubly enriched C_4 units can be generated, which would account for these labelling patterns.

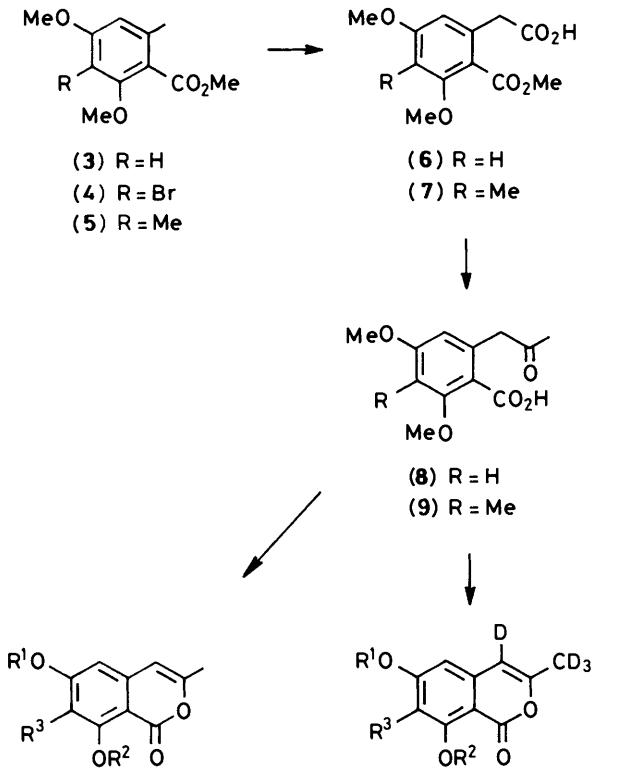
To test this hypothesis further, we first administered sodium [2,3-$^{13}C_2$]succinate[14] to the growing organism. This gave a derivative whose spectrum showed ^{13}C-^{13}C doublets consistent with an intact incorporation of doubly labelled C_2 units into C-11 and C-12, and also to a lesser extent, into C-12 and C-13. Intact ^{13}C-^{13}C units at these latter positions could arise from material that has been carried around a complete turn of the

Returning to the biosynthesis of the isocoumarin residue, it is not clear from the preceding evidence if the methionine-derived carbon, C-10, is attached first to the segment destined to become the lactone, or to the isocoumarin portion of canescin. Therefore, two isocoumarins (**12**) and (**13**) were considered as possible precursors, and these were synthesised multiply labelled with dueterium as indicated in (**19**) and (**20**). These sites were chosen for labelling because they are easily distinguished in the canescin derivative by n.m.r., and are easy to label synthetically *via* the keto acids (**8**) and (**9**). The synthetic route used is shown in Scheme 3. Ethyl orsellinate dimethyl ether (**3**) is readily available by the method of Anker and Cook.[15] A variation[16,17] of this route gives the 3-bromo compound (**4**) which by metallation and treatment with methyl iodide gives (**5**).[17] The corresponding acids are simply converted[18] into the homophthalic acids (**6**) and (**7**), which can be acylated and decarboxylated to give the required keto acids (**8**) and (**9**). Exchange with sodium deuteroxide in D_2O, and ring closure

Table 2. Incorporation experiments with ^2H labelled isocoumarin precursors

Expt.	Precursor	Incorporation Level (%)[b]	Amounts of ^2H at indicated sites[a]		
			Precursor[c]		Derivative (2)[d]
1	(20)	6	0.90	0	0.78
2	(23)	1	0.48	0.39	0.54
3	(19)	<0.1	0.88	0	
4	$CD_3{}^{13}CO_2Na$	0.6			

[a] Relative to 9-H position (=3); in the isocoumarin precursors deuteriation of the 9-H position was essentially complete. [b] Based on mass spectrometry. [c] Based on ^1H n.m.r. data. [d] Based on ^2H n.m.r. data.



Scheme 3.

followed by deprotection gives the potential precursors in labelled form as (**19**) and (**20**).

In each case, the deuterium distribution was determined from the ^1H n.m.r. spectrum; the methyl position (9-H) was almost totally deuteriated, and the vinyl position (4-H) was approximately 90% labelled (Table 2).

The canescin derivative (**2**) produced after incorporation of (**20**) showed in its mass spectrum a peak at *m/z* 340 ($M^+ + 4$) *ca.* 15% of the size of that at *m/z* 336 (M^+), whilst that at *m/z* 339 was *ca.* 2%. Taking into account the amount of the precursor consumed (74 mg), and the amount of derivative (**2**) isolated (68 mg), we calculate that at least 6% of the labelled precursor was incorporated intact into the metabolite (**1**), a level which strongly supports the status of (**13**) as a true biosynthetic intermediate. The location of the ^2H labels was confirmed by the ^2H n.m.r. spectrum which showed two singlets corresponding to the methyl (9-H) and vinyl (4-H) positions (the latter overlapped with the signal from the 5-H position in the ^2H n.m.r. spectrum); the isotopic ratio for the two sites, as measured by the relative intensities of the two resonances, was almost identical with that of the precursor (expt. 1, Table 2).

For comparison, a control experiment (expt. 4) with $CD_3{}^{13}CO_2Na$ as precursor was carried out: 9-H of the resultant derivative (**2**) was labelled (^2H n.m.r. spectrum and the β-shift technique[19]), but not the other methyl derived sites 4-H, and 5-H, presumably because those sites suffered loss of deuterium by exchange with the medium in early intermediates of the pathway. In view of this observation, we suggest that the change in the isotopic ratio in favour of the 9-H position in the first experiment may be attributed to a small degree of competing indirect incorporation of ^2H, resulting from breakdown of the precursor to produce ^2H labelled acetate. A similar effect was observed in a study of citrinin biosynthesis.[20]

In contrast with the above result, the derivative (**2**) isolated from the experiment with (**19**) gave no evidence for intact incorporation in either the mass spectrum or the ^2H n.m.r. spectrum, at the limits of detection of those techniques (expt. 3).

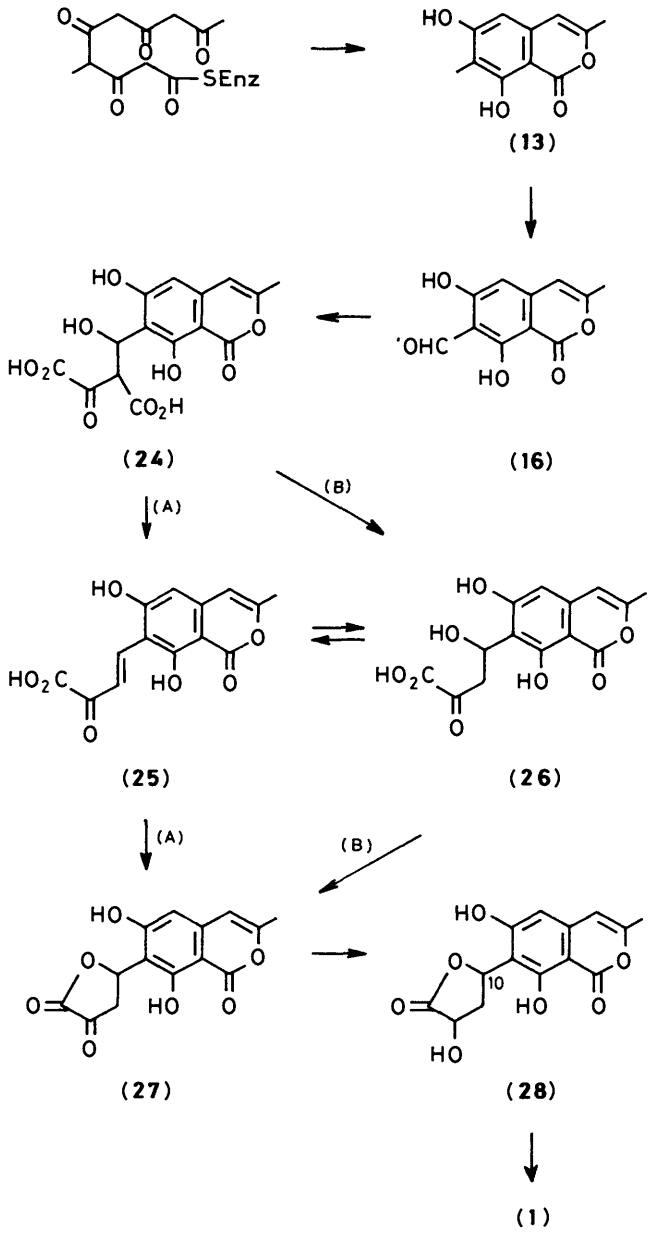
Next we decided to test the intermediacy of the aldehyde (**16**), suggested but not verified by Birch.[3] The required labelled precursor (**23**) could not be obtained directly from (**19**) in good yield so an alternative synthesis was devised. Partial demethylation of (**17**) using Me_3SiI with a D_2O work-up gave (**21**), although under the acidic conditions of the work-up some deuterium was incorporated at an additional site, 5-H. This material was formylated[21] to give the aldehyde (**22**) which was demethylated with BBr_3 to yield (**23**). Again, ^1H n.m.r. spectroscopy confirmed the extent of the deuterium labelling (Table 2, expt. 2).

The feeding experiment was carried out as before, and the canescin derivative found to give a ^2H n.m.r. spectrum very similar to that of the administered compound, suggesting that the material was incorporated intact. In the mass spectrum of the derivative peaks could be seen for *m/z* 341 ($M^+ + 5$), 340 and 339 confirming the intact incorporation (in this case 5-H was labelled in addition to 4-H). Applying the same analysis as before shows that more than 1% of the material available to the organism had been taken onto canescin, a biosynthetically significant amount.

An interesting point arising from this experiment is that although the peak heights in the mass spectrum for material containing five or four deuterium atoms are in the same ratio (2.2:1) as in the administered aldehyde, that for $M^+ + 3$ is larger than expected. A comparison of the integrals in the

deuterium n.m.r. spectra of the precursor and derivative, shows that the excess of deuterium is located at the methyl position, H-9 (expt. 2, Table 2). Again we attribute this to a partial breakdown of the administered compound to deuteriated acetate, which is then incorporated into canescin. As demonstrated previously, deuterium from labelled acetate will only survive at 9-H. This experiment shows that deuterium labelled precursors can give reliable biosynthetic results even when competing degradation and reincorporation is prevalent, providing the resulting material is analysed by *both* n.m.r. and mass spectrometry.

On the basis of these results, we propose in Scheme 4 that the isocoumarin (**13**) is the product released from the polyketide synthase. Oxidation of one of the methyl groups of (**13**) gives the aldehyde (**16**), which then undergoes an aldol condensation with oxaloacetate to give (**24**). In subsequent steps decarboxylation and lactonisation take place. Different mechanisms are possible for both of these reactions, and therefore alternative pathways (A) and (B) are suggested. A further degree of uncertainty is created by the existence of a possible cross-link between potential intermediates (**25**) and (**26**) of the two pathways, as indicated. In later steps, the ketone group of (**27**) is reduced to produce the alcohol (**28**) which is then *O*-methylated to give canescin (**1**). The alcohol, demethylcanescin (**28**), has been isolated (as a mixture of diastereoisomers) from the culture medium, which suggests that methylation is a late reaction in the biosynthetic sequence.



Scheme 4.

The formation of two diastereoisomers of canescin, with alternative configurations at C-10, could be explained on the basis of Scheme 4 either by a spontaneous enzyme-free ring closure in the conversion of (**25**) into (**27**), or by repeated chemical interconversion of (**26**) with (**25**) prior to lactonisation. Alternatively, a single enantiomer of canescin produced by either pathway (A) or (B) could undergo spontaneous epimerisation at C-10 *via* a reversible *O*-alkyl cleavage of the lactone residue to form a quinone methide intermediate, as indicated in Scheme 1. We have separated canescin into two components by h.p.l.c., and found that they rapidly revert to the original equilibrium mixture. The reversion was too rapid for the individual components to be characterised, but it seems likely that they were the two separate diastereoisomers, and therefore that canescin is indeed configurationally labile.

Experimental

M.p.s were determined on a Reichert hot-stage apparatus and are uncorrected. I.r. spectra were recorded on a Perkin-Elmer 297 spectrometer, and are in chloroform solution unless noted otherwise. ^{1}H N.m.r. spectra were usually obtained in deuteriochloroform solution at 80 MHz, different solvents or operating frequencies are noted. Instruments used were a Varian EM-360A, Bruker WR-80, Bruker WM-250, or Bruker WH-400. ^{2}H N.m.r. spectra were obtained either on the WM-250 running unlocked, or on the WH-400 using internal C_6F_6 and an ^{19}F lock. Experiments requiring deuterium decoupling were run under the latter conditions. ^{13}C Spectra were obtained on either of the two highfield instruments. Chemical shifts are given as p.p.m. downfield of tetramethylsilane. Mass spectra were obtained on an AEI MS30 or MS50 fitted with a fast atom bombardment (f.a.b.) ionisation system.

Flash chromatography refers to the method of Still *et al.*[22] Preparative t.l.c. was carried out on 20 cm^2 plates coated with Merck Kieselgel GF_{254}. Organic solutions were dried over anhydrous sodium sulphate, ether refers to diethyl ether and light petroleum to that fraction boiling at 40—60 °C.

Carbon-13 labelled precursors were obtained from Amersham International.

Growth of Aspergillus malignus *and Isolation of Canescin* (**1**).—*Aspergillus malignus* (C.M.I. 89980) was grown on agar slopes at 25 °C for 4 days and then stored at 4 °C. Spores were introduced to Raulin Thom medium and grown as a stationary culture. At daily intervals an aliquot of the medium was removed, diluted to 1% with distilled water, and the u.v. spectrum measured. Administration of precursors was started when canescin could be detected in the medium, usually on the 6th day. Simple precursors (100 mg per flask) were dissolved in distilled water (10 ml) and 1 ml of solution injected *via* a long needle into the growth medium, every 12 h. Advanced precursors were dissolved in aqueous sodium hydroxide (0.1M; 2 ml) and injected on the 7th day, with sulphuric acid (0.1M; 1 ml) injected directly afterwards. On the 13th day the medium was filtered off, the mycelium thoroughly washed with more water, and the combined aqueous solution extracted with an equal volume of ether (× 4). The organic layers were combined, dried, and evaporated down to a brown solid, or in some cases, a dark brown gum. Washing with cold ethanol (1 ml) gave a light

brown solid (typically 100—200 mg) which was usually derivatised without further purification. (Higher yields of canescin were possible if the harvesting took place on the 20th day). If necessary canescin could be purified by repeated recrystallisation from aqueous ethanol to give a beige solid as needles, m.p. 189—192 °C (lit.,[2] 200—201 °C) (Found: C, 59.0; H, 4.60. Calc. for $C_{15}H_{14}O_7$: C, 58.8; H, 4.6%), $\nu_{max.}$(Nujol) 3 500—2 700 (OH), 1 780 (lactone C=O), 1 680 (C=O) and 1 620 cm^{-1} (C=C); $\lambda_{max.}(H_2O)$ 246 and 328 nm; $\delta_H(CD_3OD)$ 6.36 (1 H, s, ArH), 6.33 (1 H, d, *J* 1 Hz, =CH), 6.27—5.97 (1 H, m, ArCH), 4.5—4.3 (1 H, m, CH_2CH), 3.6 (3 H, d*, OCH_3) 2.9—2.3 (2 H, m, CH_2), and 2.27 (3 H, d, *J* 1 Hz, CCH_3); *m/z* (f.a.b.) 307 (100%, $M + H^+$) and 275 (80, $M - OCH_3$).

The two isomers of canescin were separated by h.p.l.c. on two 30 cm Waters μBondapak CN columns mounted in series, eluting with isopropyl alcohol–hexane (1:4) at a flow rate of 2.25 ml min^{-1}. Two peaks were detected by u.v. absorption at 280 nm, with R_t 15 and 21 min (ratio of areas 2:1). The two fractions were collected and evaporated to dryness (water bath temp. below 50 °C); on re-injection both recovered samples gave a trace similar to that of the natural product.

8-Hydroxy-6-methoxy-7-(3-methoxy-3-methoxycarbonylpropyl)-3-methylisocoumarin (**2**).—Sodium borohydride (105 mg, 2.88 mmol) was added to a solution of canescin (**1**) (245 mg, 0.8 mmol) in 95% ethanol (50 ml) at room temperature. The mixture was stirred for 1 h after which the solvent was removed under reduced pressure and the residue dissolved in saturated aqueous sodium hydrogen carbonate (50 ml). The aqueous solution was washed with ethyl acetate (2 × 50 ml) and then acidified and extracted with ethyl acetate (4 × 50 ml). The combined extracts were dried and evaporated to provide a gum which was dissolved in methanol (25 ml) and treated with diazomethane in ether (80 ml) for 1 h, before addition of a little glacial acetic acid to destroy the excess of reagent. The solvent was removed, the resulting gum dissolved in ethyl acetate (50 ml), and the solution washed with aqueous sodium hydrogen carbonate (50 ml) and dilute hydrochloric acid (50 ml), before drying and evaporation to leave a solid. Purification by preparative t.l.c. eluting with methanol–dichloromethane (1:19) and recrystallisation from dichloromethane–ether gave the *derivative* (**2**) (145 mg, 54%) as needles, m.p. 127—128 °C, $[\alpha]_D^{20}$ −1° (*c* 4 in dichloromethane) (Found: C, 60.6; H, 6.00. $C_{17}H_{20}O_7$ requires C, 60.7; H, 6.30%); $\nu_{max.}$ 3 400—2 900 (OH), 1 740 (ester C=O), 1 680 (C=O), and 1 640 cm^{-1} (C=C); δ_H (400 MHz) 11.2 (1 H, s, OH), 6.27 (1 H, s, ArH), 6.20 (1 H, q, *J* 0.9 Hz, =CH), 3.9 (3 H, s, $ArOCH_3$), 3.8 (1 H, m, CH_2CH), 3.7 (3 H, s, CO_2CH_3), 3.4 (3 H, s, $COCH_3$), 2.8 (2 H, m, $ArCH_2$), 2.2 (3 H, d, *J* 0.9 Hz, $=CCH_3$), and 1.9 (2 H, m, CH_2); *m/z* 336 (15%, M^+), 233 (65, $M - C_4H_7O_3$), and 219 (100, $M - C_5H_9O_3$).

[2-^{13}C, 2-$^2H_2$]*Bromoacetic Acid.*—Fused [2-^{13}C, 2-$^2H_3$]sodium acetate (1.51 g, 17.4 mmol) was cooled to −15 °C and deuteriotrifluoracetic acid (10 ml) added slowly, followed by trifluoroacetic anhydride (5.5 ml), red phosphorus (20 mg), and thionyl chloride (eight drops). The mixture was allowed to warm with stirring until all the solid had dissolved; it was then refluxed for 1 h. Bromine (0.9 ml, 17.5 mmol) was added dropwise over 20 min until the red colour did not rapidly fade, and this was followed by D_2O (2 ml). Most of the solvent was distilled off, the remainder extracted with ether, and the combined organic extracts filtered through Celite and allowed to evaporate at room temperature. Bulb-to-bulb distillation of the residue at 80 °C/20 mmHg gave the acid (370 mg, 15%), δ_D (38 MHz; $CHCl_3$) 3.8 (d, *J* 23 Hz, $^{13}CD_2$) (Found: M^+, 140.9502. $^{13}C^2H_2{}^{79}BrCO_2H$ requires 140.9476).

[2-^{13}C, 2-$^2H_2$]*Succinic Acid.*—Diazomethane in ether was added slowly to the labelled bromoacetic acid (370 mg, 2.60 mmol) until the yellow colour did not immediately fade after which the ether was carefully evaporated at reduced pressure and low temperature to leave the ester as an oil.

Diethyl malonate (0.54 ml, 3.55 mmol) was dissolved in a solution of sodium ethoxide in ethanol (0.55M; 6.5 ml, 3.58 mmol) at 0 °C and stirred for 30 min. The labelled ester was added, washing in with dry ethanol (1 ml), after which the cooling bath was removed and the mixture stirred for 2 h. Dilute hydrochloric acid (10 ml) was added and the mixture extracted with dichloromethane (3 × 15 ml); the combined organic layers were then dried and evaporated to give an oil. Dilute hydrochloric acid (1M; 10 ml) was added, the mixture heated under reflux for 8 h, and the solvent evaporated to give a solid, which was heated at 150 °C for 1 h under a stream of nitrogen. Sublimation at 160 °C/0.1 mmHg and recrystallisation from acetone gave [2-^{13}C, 2-$^2H_2$]*succinic acid* (80 mg, 26%), m.p. 184—188 °C; δ_H [$(CD_3)_2CO$] 2.6 (multiplet flanked by small doublet, *J* 126 Hz, from incompletely deuteriated compound); δ_C [100 MHz; $(CD_3)_2CO$] 29.21 (s, CH_2), 28.95 (t, J_{CD} 20 Hz, CHD), and 28.68 (quintet, J_{CD} 20 Hz, CD_2); *m/z* (f.a.b.) 122 (58%, $M + H^+$) and 104 (100%, $M + H^+ - H_2O$). Analysis of peak heights for incompletely deuteriated material gave a figure of 55% deuteriation at each site. The acid was converted into the disodium salt for the incorporation experiments by titration with 1M sodium hydroxide, and stored at −10 °C.

Ethyl 3-Bromo-2,4-dihydroxy-6-methylbenzoate.[17]—A solution of bromine (1.0 ml, 19 mmol) in glacial acetic acid (8 ml) was slowly added to ethyl dihydro-orsellinate[15] (3.8 g, 19 mmol) in glacial acetic acid (10 ml), so that the temperature stayed below 20 °C. After being stirred at room temperature for 15 h the mixture was poured into ice-cold water (50 ml) and the resulting precipitate filtered off, washed with cold water (with a few drops of acetic acid added), and dried *in vacuo*. Column chromatography on silica gel (200 g) eluting with dichloromethane, and recrystallisation gave the bromo compound (1.2 g, 23%), m.p. 142—143 °C (from ether) (lit.,[17] 142—144.5 °C) (Found: C, 43.5; H, 4.30; Br, 28.8. $C_{10}H_{11}BrO_4$ requires C, 43.7; H, 4.05; Br 29.0%); $\nu_{max.}$ 3 500 (OH), 1 640 (C=O), and 1 600 cm^{-1}; δ_H 10.8† (1 H, s, OH), 6.4 (1 H, s, ArH), 5.9† (1 H, s, OH), 4.4 (2 H, q, *J* 7 Hz, CH_2), 2.5 (3 H, s, $ArCH_3$), and 1.4 (3 H, t, *J* 7 Hz, CH_2CH_3); *m/z* 274/276 (20%, M^+) and 228/230 (100, $M - C_2H_5OH$).

Ethyl 3-Bromo-2,4-dimethoxy-6-methylbenzoate[17] (**4**).—A mixture of the above dihydroxy compound (700 mg, 2.54 mmol), anhydrous potassium carbonate (1.4 g), and dimethyl sulphate (1.0 g, 8.2 mmol) in acetone (15 ml) was refluxed for 19 h under argon. The mixture was then cooled and aqueous ammonia (2 ml) added to destroy the excess of reagent. The solid was filtered off and washed carefully with acetone. The combined organic extracts were evaporated to give an oil which was taken up in ether (50 ml) and the solution washed twice with dilute aqueous sodium hydroxide. The organic solution was dried and evaporated to give the dimethyl ether[17] (**8**) as an oil (700 mg, 91%) spectroscopically pure, b.p. 148—151 °C/0.1 mmHg; $\nu_{max.}$(neat) 2 970, 1 720 (C=O), and 1 590 cm^{-1}; δ_H(400 MHz) 6.5 (1 H, s, ArH), 4.3 (2 H, q, *J* 7 Hz, CH_2), 3.88 (3 H, s, OCH_3), 3.87 (3 H, s, OCH_3), 2.3 (3 H, s, $ArCH_3$), and 1.3 (3 H, t, *J* 7 Hz, CH_2CH_3) (Found: M^+, 302.0153. $C_{12}H_{15}{}^{79}BrO_4$ requires *M*, 302.0153); *m/z* 302/304 (50%, M^+), 257/259 (100, $M - OC_2H_5$).

* Two peaks due to diastereoisomers.

† These shifts vary with concentration.

Ethyl 2,4-*Dimethoxy*-3,6-*dimethylbenzoate*[23] (**5**).—Butyl-lithium (1.9M solution in hexane; 2.0 ml, 3.8 mmol) was added to a solution of the bromo compound (**4**) (1.2 g, 3.9 mmol) in dry tetrahydrofuran (THF) under nitrogen at −78 °C, and this was followed after a few minutes stirring, by an excess of methyl iodide. The mixture was allowed to warm and the solvent evaporated. The residual oil was redissolved in dichloromethane and the solution washed with water, dried, and evaporated to give an oil. Flash chromatography eluting with ethyl acetate–light petroleum (1:3) and preparative t.l.c. of mixed fractions gave unchanged starting material (310 mg), R_F (ether–hexane, 3:1) 0.57, ethyl 2,4-dimethoxy-6-methylbenzoate (**3**) (110 mg, 11% based on unrecovered starting material) identical with authentic material,[15] R_F 0.50 and the *dimethyl compound*[23] (**5**) (520 mg, 74%) as an oil b.p. 119—123 °C/0.1 mmHg (Found: C, 65.5; H, 7.85. $C_{13}H_{18}O_4$ requires C, 65.5; H, 7.60%), R_F 0.63; $\nu_{max.}$ 2 930 (CH), 1 700 (C=O) and 1 600 cm^{-1}; δ_H 6.44 (1 H, s, ArH), 4.36 (2 H, q, *J* 7 Hz, CH_2), 3.80 (3 H, s, OCH_3), 3.74 (3 H, s, OCH_3), 2.29 (3 H, s, $ArCH_3$), 2.09 (3 H, s, $ArCH_3$), and 1.37 (3 H, t, *J* 7 Hz, CH_2CH_3); *m/z* 238 (13%, M^+), 193 (27, $M - OC_2H_5$), and 142 (100).

2,4-*Dimethoxy*-3,6-*dimethylbenzoic Acid.*[24]—A solution of the ester (**5**) (1.79 g, 7.5 mmol) in dimethyl sulphoxide (10 ml) and 20% aqueous potassium hydroxide solution (10 ml) was refluxed for 5 h. The mixture was cooled and acidified with concentrated hydrochloric acid, and extracted with dichloromethane (3 × 25 ml). The combined organic layers were evaporated to give an oil which was taken up in ether (25 ml) and the solution washed with dilute hydrochloric acid (2 × 25 ml); it was then extracted with aqueous sodium hydroxide (2 × 25 ml). The alkaline solution was acidified and extracted with dichloromethane (3 × 30 ml). Drying and evaporation of the combined organic layers, followed by recrystallisation of the residue gave the acid (1.4 g, 86%), m.p. 100—101 °C (from benzene–light petroleum) (lit.,[24] m.p. 104—105 °C) (Found: C, 62.6; H, 6.70. Calc. for $C_{11}H_{14}O_4$: C, 62.8; H, 6.70%); $\nu_{max.}$ 3 600—2 800 (OH) 1 710 (C=O), 1 600, and 1 580 cm^{-1}; δ_H 6.5 (1 H, s, ArH), 5.9 (1 H, br s, CO_2H), 3.85 (3 H, s, OMe), 3.82 (3 H, s, OMe), 2.5 (3 H, s, $ArCH_3$), and 2.1 (3 H, s, $ArCH_3$); *m/z* 210 (75%, M^+) and 192 (100, $M - H_2O$).

(2-*Carboxy*-3,5-*dimethoxy*-4-*methylphenyl*)*acetic Acid* (**7**).—Butyl-lithium (1.6M solution in hexane; 16.0 ml, 25.6 mmol) was added to a cooled solution of di-isopropylamine (3.7 ml, 26.4 mmol) in dry THF (50 ml) under nitrogen. The mixture was cooled to −78 °C and a solution of the above benzoic acid (1.40 g, 6.66 mmol) and diethyl carbonate (1.6 ml, 13 mmol) in dry THF (30 ml) was slowly added to give an intense red colour. The cooling bath was removed and the mixture allowed to warm to room temperature; it was then stirred for 3 h. Water (15 ml) was then added and the mixture stirred vigorously overnight. The organic solvents were evaporated under reduced pressure and the resulting oil taken up in aqueous sodium hydrogen carbonate (80 ml). This was washed with ether (2 × 40 ml) and then acidified and extracted with ethyl acetate (3 × 100 ml). Drying and evaporation of the combined extracts gave a crude yellow solid which on treatment with charcoal and crystallisation from dichloromethane–hexane gave almost colourless needles. Recrystallisation from acetone gave the *title compound* (**7**) (1.46 g, 85%) as needles, m.p. 153—156 °C (Found: C, 56.6; H, 5.55. $C_{12}H_{14}O_6$ requires C, 56.7; H, 5.55%); $\nu_{max.}$ 3 500—2 500 (OH), 1 710 (C=O), 1 700 (conj. C=O) and 1 600 cm^{-1}; δ_H[400 MHz; $(CD_3)_2CO$], 7.3 (2 H, br s, OH), 6.78 (1 H, s, ArH), 3.87 (3 H, s, OCH_3), 3.78 (3 H, s, OCH_3), 3.77 (2 H, s, CH_2), and 2.09 (3 H, s, $ArCH_3$); *m/z* 254 (25%, M^+), 210 (60, $M - CO_2$), 208 (65, $M - HCO_2H$), and 192 (100, $M - H_2O - CO_2$).

2,4-*Dimethoxy*-3-*methyl*-6-(2-*oxopropyl*)*benzoic Acid* (**9**).—The homophthalic acid (**7**) (0.20 g, 0.79 mmol) was added in one lot to a well stirred mixture of acetic anhydride (1 ml) and dry pyridine (0.25 ml). When a precipitate started to form dry ether (2 ml) was added and stirring continued for 3 h. The mixture was then filtered and the solid washed with a little ether before being dissolved in dilute aqueous sodium hydroxide (10 ml) and allowed to stand for 30 min. The aqueous solution was acidified and extracted with ethyl acetate (3 × 10 ml) and the combined organic layers were then dried and evaporated to dryness. Recrystallisation from dichloromethane–hexane gave 2,4-*dimethoxy*-3-*methyl*-6-(2-*oxopropyl*)*benzoic acid* (**9**) (140 mg, 70%) as prisms, m.p. 120—122 °C (Found: C, 61.7; H, 6.45. $C_{13}H_{16}O_5$ requires C, 61.9; H, 6.40%); $\nu_{max.}$ 3 600—2 800 (OH), 1 725 (C=O), and 1 600 cm^{-1}; δ_H(250 MHz) 6.51 (1 H, s, ArH), 4.06 (2 H, s, CH_2), 3.87 (3 H, s, OCH_3), 3.86 (3 H, s, OCH_3), 2.32 (3 H, s, $ArCH_3$), and 2.15 (3 H, s, $COCH_3$); *m/z* 252 (10%, M^+), 234 (10, $M - H_2O$), and 192 (100, $M - CH_3CO_2H$).

2,4-*Dimethoxy*-6-(2-*oxopropyl*)*benzoic Acid*[25] (**8**).—2,4-Dimethoxyhomophthalic acid[18] (**6**) (4.0 g, 16.7 mmol) was added portionwise to a mixture of acetic anhydride (8 ml) and dry pyridine (2 ml) in a flask equipped with a close fitting mechanical stirrer, at such a rate that it all dissolved. After the mixture had been stirred for 5 min, when a thick precipitate had started to form, dry ether (15 ml) was added and stirring continued for 2 h. Aqueous sodium hydroxide (4M; 150 ml) was then slowly added and the mixture refluxed until all the solid material had dissolved. The solution was then cooled, washed with an equal volume of dichloromethane, and then cautiously acidified; gas was evolved. The acidic solution was extracted with ethyl acetate (3 × 200 ml) and the combined extracts were then dried and evaporated to provide a solid. Recrystallisation of this from acetone gave the keto acid[25] (**8**) (2.68 g, 67%), m.p. 137—141 °C (lit.,[25] 139—141 °C) (Found: C, 60.6; H, 5.85. Calc. for $C_{12}H_{14}O_5$: C, 60.5; H, 6.00%); $\nu_{max.}$ 3 700—2 900 (OH), 1 715 (ketone C=O), 1 690 (carboxyl C=O), and 1 600 cm^{-1}; δ_H 6.48 (1 H, d, *J* 2 Hz, ArH), 6.39 (1 H, d, *J* 2 Hz, ArH), 4.06 (1 H, s, OH), 3.99 (2 H, s, CH_2), 3.85 (6 H, s, 2 × OCH_3), and 2.29 (3 H, s, CCH_3); *m/z* 238 (10%, M^+), 220 (5%, $M - H_2O$), 196 (15, $M - CH_2CO$), and 178 (100, $M - CH_3CO_2H$).

6,8-*Dimethoxy*-3-*methylisocoumarin*[26–28] (**10**).—The keto acid (**8**) (450 mg, 1.89 mmol) was dissolved in a solution of acetic anhydride and perchloric acid in ethyl acetate (50 ml; made up by the method[25] of Edwards and Rao) and allowed to stand at room temperature for 10 min. The solution was then washed with aqueous sodium hydrogen carbonate (50 ml), dried, and evaporated to leave a brown solid. Flash chromatography of this on silica eluting with ethyl acetate–light petroleum (3:1), and recrystallisation gave the isocoumarin (**10**) (317 mg, 76%), m.p. 156—158 °C (from ethanol) (lit.,[28] m.p. 157—160 °C) (Found: C, 65.2; H, 5.40. Calc. for $C_{12}H_{12}O_4$: C, 65.4; H, 5.50%), $\nu_{max.}$ 1 710 (C=O), 1 660 (C=C), and 1 600 cm^{-1}; δ_H 6.41 (1 H, d, *J* 2 Hz, ArH), 6.27 (1 H, d, *J* 2 Hz, ArH), 6.05 (1 H, q, *J* 1 Hz, =CH), 3.94 (3 H, s, OCH_3), 3.86 (3 H, s, OCH_3), and 2.20 (3 H, d, *J* 1 Hz, $=CCH_3$); *m/z* 220 (100%, M^+), 191 (75, $M - HCO$), and 149 (96, $M - C_3H_3O_2$).

6,8-*Dimethoxy*-3,7-*dimethylisocoumarin*[29] (**11**).—Treatment of the methylated keto acid (**9**) as above gave the isocoumarin, m.p. 151—154 °C (from dichloromethane–hexane) (lit.,[29] 153—156 °C) (Found: C, 66.7; H, 6.10. Calc. for $C_{13}H_{14}O_4$: C, 66.7; H, 6.00%); $\nu_{max.}$ 2 920 (CH), 1 715 (C=O), 1 660 (C=C) and 1 600 cm^{-1}; δ_H 6.45 (1 H, s, ArH), 6.15 (1 H, q, *J* 1 Hz, =CH), 3.90 (3 H, s, OCH_3), 3.85 (3 H, s, OCH_3), 2.21 (3 H, d, *J* 1 Hz, $=CCH_3$), and 2.18 (3 H, s, $ArCH_3$); *m/z* 234 (100%, M^+), 219 (80, $M - CH_3$), 189 (50, $M - CO_2H$), and 163 (45, $M - C_3H_3O_2$).

[4-^{2}H, 9-$^2H_3$]6,8-*Dimethoxy*-3-*methylisocoumarin* (**17**).—The keto acid (**8**) was dissolved in 2M NaOD–D_2O solution (5 ml) and refluxed gently for 30 min under N_2. The solution was evaporated to dryness after which fresh D_2O (5 ml) was added and the solution heated under reflux for a further 30 min; it was then cooled and acidified by addition of thionyl chloride in ethyl acetate. Extraction of the aqueous solution with ethyl acetate and treatment as above gave the labelled isocoumarin (**17**) δ_H 6.40 (1 H, d, *J* 2 Hz, ArH), 6.29 (1 H, d, *J* 2 Hz, ArH), 3.94 (3 H, s, OCH_3), and 3.82 (3 H, s, OCH_3); δ_D (38 MHz; $CHCl_3$) 6.0 (s) and 2.2 (s) (Found: M^+ 224.1036. $C_{12}H_8O_4D_4$ requires *M*, 224.0987); *m/z* 224 (100%, M^+), 195 (80, M − HCO), and 150 (80, $M - C_3O_2D_3$).

[4-^{2}H, 9-$^2H_3$]-6,8-*Dimethoxy*-3,7-*dimethylisocoumarin* (**18**).—The reaction was carried out as described above, starting with the methylated keto acid (**9**); δ_H 6.41 (1 H, s, ArH), 3.85 (3 H, s, OCH_3), 3.80 (3 H, s, OCH_3), and 2.11 (3 H, s, $ArCH_3$); δ_D ($CHCl_3$, 38 MHz) 6.1 (s) and 2.2 (s) (Found: M^+ 238.1193. $C_{13}H_{10}D_4O_4$ requires *M*, 238.1143); *m/z* 238 (55%, M^+), 223 (35, $M - CH_3$), 193 (30, $M - CO_2H$), and 164 (20, $M - C_3O_2D_3$).

6,8-*Dihydroxy*-3-*methylisocoumarin*[26] (**12**).—Boron tribromide (2 ml, 21 mmol) was injected into a solution of the dimethoxyisocoumarin (**10**) (540 mg, 2.45 mmol) in dry dichloromethane (50 ml) at −78 °C under nitrogen. The mixture was allowed to warm to room temperature after which it was stirred for 3 days and then poured slowly into water (150 ml). The layers were separated and the aqueous solution extracted with ethyl acetate (4 × 100 ml). The combined organic phases were dried and evaporated to give a crude brown solid which was continuously extracted with ether for 5 h. The organic solution was evaporated and the resulting solid sublimed at 140 °C/0.3 mmHg to give the dihydroxyisocoumarin (**12**) (448 mg, 95%), m.p. 245—248 °C (with sublimation) (lit.,[30] 250—253 °C); $\nu_{max.}$(Nujol) 3 250—2 925 (OH), 1 675 (C=O) 1 625 (C=C), and 1 570 cm^{-1}; δ_H[400 MHz; $(CD_3)_2CO$] 10.95 (1 H, s, OH), 10.82 (1 H, br s, OH), 6.46 (1 H, s, =CH), 6.33 (1 H, d, *J* 2 Hz, ArH). 6.29 (1 H, d, *J* 2 Hz, ArH), and 2.19 (3 H, s, CH_3) (Found: M^+, 192.0431. $C_{10}H_8O_4$ requires *M*, 192.0423); *m/z* 192 (100%, M^+), 177 (65, $M - CH_3$) and 121 (50, $M - CO_2CCH_3$).

[4-^{2}H, 9-$^2H_3$]-6,8-*Dihydroxy*-3-*methylisocoumarin* (**19**).—The reaction was carried out as above, starting from the labelled dimethoxyisocoumarin (**17**); δ_H[$(CD_3)_2SO$] 10.94 (1 H, s, OH), 10.73 (1 H, s, OH), 6.32 (1 H, d, *J* 2 Hz, ArH), and 6.30 (1 H, d, *J* 2 Hz, ArH) (Found: M^+, 196.0726. $C_{10}H_4D_4O_4$ requires 196.0674); *m/z* 196 (100%, M^+), 178 (55, $M^+ - CD_3$), 122 (45, $M^+ - CO_2CCD_3$).

6,8-*Dihydroxy*-3,7-*dimethylisocoumarin*[29] (**13**).—Boron tribromide (0.5 ml, 5.2 mmol) was injected into a solution of the dimethoxy compound (**11**) (85 mg, 0.36 mmol) in dry dichloromethane (7 ml) at −78 °C under nitrogen. The mixture was warmed to room temperature, stirred for 20 h, and then poured carefully into water (15 ml). The layers were separated, and the aqueous solution extracted with ethyl acetate (4 × 10 ml). The organic layers were combined, dried, and evaporated to give a brown solid. This was dissolved in ethyl acetate (30 ml) and the solution washed with buffer solution (pH 9.2; 3 × 10 ml). The combined aqueous layers were back extracted with more ethyl acetate (1 × 30 ml) and the combined organic solutions evaporated to give the dihydroxyisocoumarin (**13**) (45 mg, 60%) as needles, m.p. 235—240 °C (with sublimation) (lit.,[29] 230 °C with sublimation); $\nu_{max.}$(Nujol) 3 500—3 000 (OH), 1 680 (C=O), 1 620 (C=C) and 1 590 cm^{-1}; δ_H[$(CD_3)_2CO$] 11.4 (1 H, s, OH), 9.4 (1 H, br s, OH), 6.43 (1 H, s, ArH), 6.30 (1 H, q, *J* 1 Hz, =CH), 2.20 (3 H, d, *J* 1 Hz, $=CCH_3$), and 2.10 (3 H, s, $ArCH_3$) (Found: M^+ 206.0565. $C_{11}H_{10}O_4$ requires *M* 206.0579); *m/z* 206 (100%, M^+), 191 (25, $M - CH_3$), 163 (25, $M - OCCH_3$), and 135 (40, $M - CO_2CCH_3$).

[4-^{2}H, 9-$^2H_3$]-6,8-*Dihydroxy*-3,7-*dimethylisocoumarin* (**20**).—The reaction was carried out as described above, starting with the labelled dimethoxyisocoumarin (**18**); δ_H[$(CD_3)_2CO$] 11.4 (1 H, s, OH), 9.4 (1 H, br s, OH), 6.42 (1 H, s, ArH), and 2.10 (3 H, s, $ArCH_3$) (Found: M^+ 210.0899. $C_{11}H_6O_4D_4$ requires *M* 210.0830); *m/z* 210 (90%, M^+), 192 (20, $M - CD_3$), and 136 (25, $M - CO_2CCD_3$).

8-*Hydroxy*-6-*methoxy*-3-*methylisocoumarin*[26] (**14**).—A solution of the dimethoxyisocoumarin (**10**) (56 mg, 0.25 mmol) and trimethylsilyl iodide (0.1 ml; 0.7 mmol) in chloroform (15 ml) was heated to 50 °C under argon for 41 h. The mixture was poured into aqueous sodium hydrogen carbonate (10 ml); the layers separated, and the aqueous layer extracted with dichloromethane (2 × 10 ml). The combined organic layers were dried and evaporated to give a solid. Preparative t.l.c. eluting with ethyl acetate and recrystallisation gave the hydroxyisocoumarin (**14**) (43 mg, 82%), m.p. 124—127 °C (from dichloromethane) (lit.,[31] 127—128 °C), $\nu_{max.}$ 3 200—2 800 (OH), 1 685 (C=O), and 1 620 cm^{-1} (C=C); δ_H 11.1 (1 H, s, OH), 6.4 (1 H, d, *J* 2 Hz, ArH), 6.2 (1 H, d, *J* 2 Hz, ArH), 6.1 (1 H, q, *J* 1 Hz, =CH), 3.8 (3 H, s, OCH_3), and 2.2 (3 H, d, *J* 1 Hz, CCH_3) (Found: M^+, 206.0574. Calc. for $C_{11}H_{10}O_4$ *M*, 206.0579), *m/z* 206 (100%, M^+), 191 (40, $M - CH_3$), 177 (35, M − OCH), and 135 (75, $M - CO_2CCH_3$).

[4-^{2}H, 9-$^2H_3$]-8-*Hydroxy*-6-*methoxy*-3-*methylisocoumarin* (**21**).—The reaction was carried out as described above, starting with the deuteriated compound (**17**). The reaction was quenched by adding D_2O (1 ml) and then worked up as before, δ_H(60 Mz) 11.1 (1 H, s, OH), 6.4 (0.3 H, m, ArH), 6.2 (0.6 H, singlet superimposed on doublet, ArH), 6.1 (0.5 H, s, =CH), and 3.8 (3 H, s, OCH_3).

7-*Formyl*-8-*hydroxy*-6-*methoxy*-3-*methylisocoumarin* (**15**).—Dichloromethyl methyl ether (1.25 ml, 13.8 mmol) in dry dichloromethane (25 ml) was added dropwise over 1 h, to a solution of the hydroxyisocoumarin (**14**) (198 mg, 0.96 mmol) and titanium tetrachloride (0.22 ml; 2.0 mmol) in dichloromethane (50 ml) at 0 °C. The cooling bath was removed, the mixture stirred for a further 2 h, and then poured into water (20 ml). The layers were separated, the aqueous layer extracted with dichloromethane, and the combined organic phases dried and evaporated. Chromatography on silica, eluting with dichloromethane–methanol (from 40:1 to 12:1) gave firstly 5-*formyl*-8-*hydroxy*-6-*methoxy*-3-*methylisocoumarin* (44 mg, 19%) as needles from dichloromethane, m.p. 182—184 °C (Found: C, 61.7; H, 4.20. $C_{12}H_{10}O_5$ requires C, 61.5; H, 4.30%); $\nu_{max.}$ 3 200—2 750 (OH), 1 690 (aldehyde C=O), 1 670 (lactone C=O), and 1 645 cm^{-1} (C=C); δ_H 12.1 (1 H, s, OH), 10.4 (1 H, s, CHO), 7.9 (1 H, q, *J* 1 Hz, =CH), 6.4 (1 H, s, ArH), 3.9 (3 H, s, OCH_3), and 2.3 (3 H, d, *J* 1 Hz, CCH_3); *m/z* 234 (100%, M^+), 191 (60, $M - CH_3CO$), and 164 (65, $M - CO_2CCH_2$); and secondly 7-*formyl*-8-*hydroxy*-6-*methoxy*-3-*methylisocoumarin* (**15**) (140 mg, 62%) as a microcrystalline solid from aqueous methanol, decomposes ~240 °C; $\nu_{max.}$ 3 100—2 800br, 1 695 (lactone C=O), 1 675 (CHO), and 1 640 cm^{-1} (C=C); δ_H 13.0 (1 H, s, OH), 10.33 (1 H, s, CHO), 6.21 (1 H, s, ArH), 6.15 (1 H, q, *J* 0.8 Hz, =CH), 3.97 (3 H, s, OCH_3), and 2.26 (3 H, d, *J* 0.8 Hz, CCH_3) (Found: M^+, 234.0518. $C_{12}H_{10}O_5$ requires *M*, 234.0528); *m/z* 234 (45%, M^+), 206 (55, $M + H^+ - CHO$), and 205 (100, M − CHO).

7-Formyl-6,8-dihydroxy-3-methylisocoumarin (**16**).—Boron tribromide (1 ml, 10.5 mmol) was added to a solution of the methoxy aldehyde (**15**) (140 mg, 0.60 mmol) in dichloromethane (25 ml) at −78 °C; the mixture was allowed to warm to room temperature and then stirred for 28 h. A few drops of water were added cautiously and the dark solution evaporated to give a solid which on sublimation at 120 °C/0.5 mmHg, and recrystallisation from acetone, gave the *aldehyde* (**16**) (68 mg, 51%) as needles, m.p. >230 °C; $\nu_{max.}$ 3 200—2 800br (OH), 1 680 (C=O), and 1 645 cm^{-1} (C=C); δ_H[$(CD_3)_2CO$] 10.3 (1 H, s, CHO), 6.5 (1 H, q, *J* 1 Hz, =CH), 6.4 (1 H, s, ArH), and 2.3 (3 H, d, *J* 1 Hz, CH_3) (Found: M^+ 220.0368. $C_{11}H_8O_5$ requires *M*, 220.0372); *m/z* 220 (35%, M^+), 192 (95, *M* − CO), 149 (40, *M* − CO_2CCH_3), and 55 (100).

[4-^{2}H, 9-$^2H_3$]-*7-Formyl-6,8-dihydroxy-3-methylisocoumarin* (**20**).—The deuteriated material (**21**) was formylated and demethylated as above to give the labelled precursor (**23**); δ_H[$(CD_3)_2CO$] 12.3 (1 H, br, OH), 10.3 (1 H, s, CHO), 6.5 (0.52 H, s, =CH), and 6.4 (0.61 H, s, ArH) (Found: M^+, 224.0608. $C_{11}H_4O_5D_4$ requires 224.0622); *m/z* 224 (40%, M^+), 196 (100%, *M* − CO), and 179 (90, *M* − HCO_2H); ratio *m/z* 225:224:223 was 0.63:1.00:0.46.

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