The Biosynthesis of Fungal Metabolites: Sclerin, a Plant Growth Hormone from *Sclerotinia sclerotiorum*

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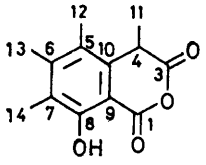
Sclerin, a plant-growth hormone from *Sclerotinia sclerotiorum*, incorporates five intact acetate units from [1,2-$^{13}C_2$]-acetate. The hydrogen atom at C-4 was shown not to be acetate-derived by a ^2H n.m.r. study with [$^2H_3$]acetate. The specific incorporations of 6,8-dihydroxy-3,4,5,7-tetramethylisocoumarin (10) and 3,6,8-trihydroxy-3,4,5,7-tetramethyl-3,4-dihydroisocoumarin (2) support the proposal that sclerotinin A (2) is an intermediate in sclerin biosynthesis. A ^2H n.m.r. investigation showed that 4,6-dihydroxy-3-[$^2H_1$]methyl-5-methyl-2-(1-methyl-2-oxopropyl)benzaldehyde (11) is not involved in the formation of sclerin.

Sclerotinia sclerotiorum, also called *Sclerotinia libertiana*, is a phytopathogenic fungus with a wide host range. Sclerin (1) was first isolated as a lipase-stimulating component [1] in 1965 and assigned the structure 8-hydroxy-4,5,6,7-tetramethylisochroman-1,3-dione after extensive chemical and spectroscopic investigation.[2] It was first synthesised by Kubota *et al.* in 1967; [3] since then, several syntheses have been reported.[4,5]



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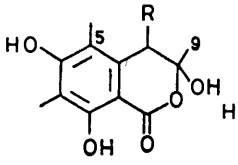
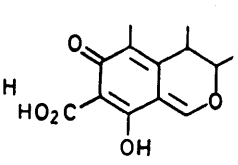
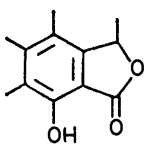


(2) R = Me
(3) R = H



(4)



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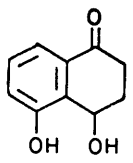

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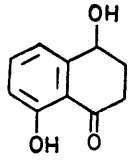
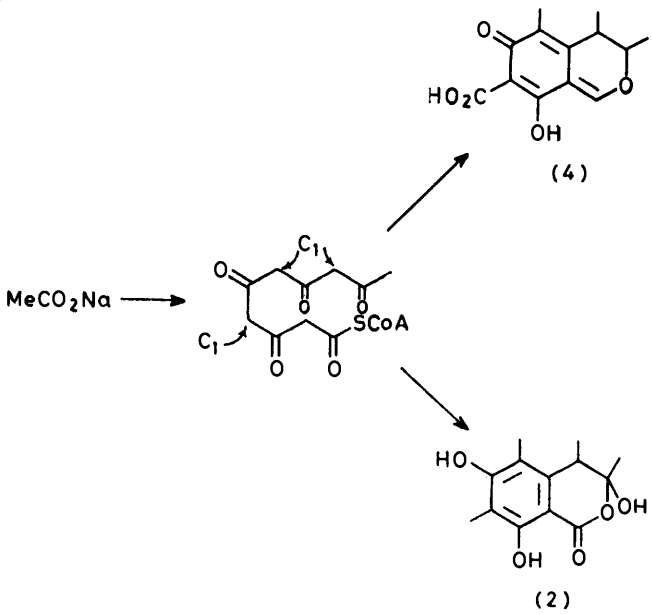


(7)

There is considerable interest in the physiological properties of sclerin. It functions as a plant-growth hormone at concentrations in the range 5—10 p.p.m. by stimulating enzyme activity.[1,6,7] Both promotion of root formation and increase in dry weight per unit shoot-length occur in plant seedlings and the combined use of sclerin with gibberellin brings about a synergistic effect. Sclerin also regulates the activity of enzymes involved in the maturation and pigmentation of fungal sclerotia, an effect linked to the control of carbohydrate metabolism.[8]

Five other metabolites of *S. sclerotiorum* have been described; all are highly oxygenated. Sclerotinins A (2) [7,9,10] and B (3),[9,11] which differ only in the presence of a methyl group at C-4, both reveal the alternating oxygenation pattern characteristic of fungal polyketides. Both are closely related to citrinin (4); [12] interestingly, *Penicillium citrinum* which produces citrinin also produces sclerotinin A.[13] Sclerolide (5) [2,3,14] possesses the same unusual methylation pattern as sclerin, while sclerone (6) [15] and isosclerone (7) [16] are naphthalenes and completely lack methyl substituents. All these minor metabolites possess useful physiological properties.

The isolation of sclerin poses an intriguing biosynthetic problem in view of its unusual structure. Although an acetate–polymalonate pathway is suggested by the oxygenated nature of the carbon skeleton, the common pentaketide † cyclisation (Scheme 1) established for



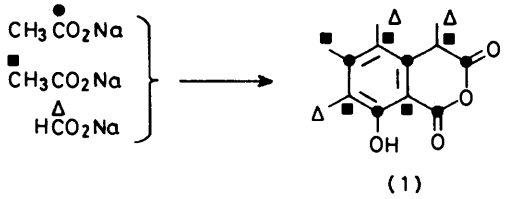
SCHEME 1

several fungal metabolites, including citrinin,[17,18] is unlikely since this would require methylation at a ketonic site in the case of sclerin. On the other hand, the biosyntheses of sclerotinins A and B can easily be rationalised as proceeding *via* a pathway of this type.

Kubota *et al.* studied the origin of the carbon skeleton of sclerin using ^{14}C-labelled precursors.[20] They verified that sclerin is acetate-derived and showed that three of the four methyl groups originate from the one carbon pool

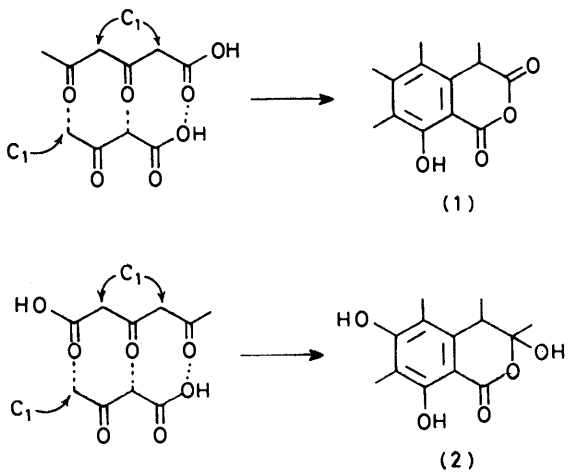
† The prefix indicates the number of acetate units involved (ref. 19).

(Scheme 2). They considered that sclerin is formed by direct condensation of a triketide with an acetoacetyl unit (Scheme 3); these two polyacetyl chains, orientated



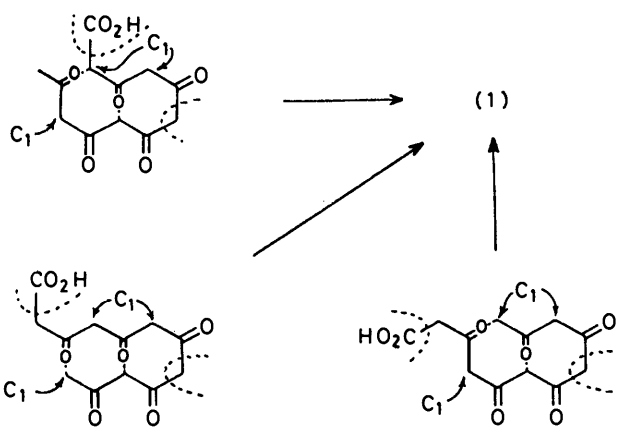
SCHEME 2

in opposite fashion, can also lead to the formation of sclerotinins A and B. The labelling pattern does not exclude the possibility that naphthalenes may be intermediates in sclerin biosynthesis. Three folding patterns



SCHEME 3

of a linear hexaketide chain which could generate suitable polyhydroxynaphthalenes are indicated in Scheme 4; cleavage of one of the aromatic rings would then generate the anhydride moiety.



SCHEME 4

All of these biosynthetic ideas are interesting and merit further study. In this paper, we report the results of our own experiments to probe sclerin biosynthesis.[21] Initially, we decided to confirm the labelling pattern deduced by Kubota *et al.* using ^{13}C precursors,[22] which enable rapid detection of enriched sites within labelled molecules without recourse to chemical degradation. Subsequently, [1,2-$^{13}C_2$]acetate was used to differentiate between the alternative biosynthetic schemes because they all lead to different patterns of incorporation of intact acetate units into sclerin. The origins of the hydrogen atoms of sclerin were investigated using [$^{2}H_3$]acetate. Evidence, both in favour of a specific pathway and also concerning the timing of the introduction of the methyl substituents, was then established by classical, advanced precursor study with ^{14}C- and ^{2}H-labelled intermediates.

Experiments with Isotopically Labelled Acetates

Confirmation of Labelling Pattern.—Before any incorporation studies with expensive ^{13}C-precursors could be carried out, sclerin production was optimised. Initially, *Sclerotinia sclerotiorum* (strain IMI 148268) grew sluggishly and the yield of sclerin was unacceptably low. This problem was eventually traced to the type of yeast powder used in the culture medium. The isolated sclerin was found to be contaminated with lipid, but this could be removed by preferentially extracting sclerin into dilute sodium hydrogencarbonate solution. [1-^{14}C]-Acetate was then added to the culture medium in order to monitor incorporation levels; the isolated sclerin was found to be radioactive and the observed incorporation of 1.1% corresponded to a dilution per labelled site of 1 in 20. In equivalent feedings of ^{13}C precursors, this would give an excess of *ca.* 3% ^{13}C above the natural abundance at each labelled position.

The natural abundance proton-noise-decoupled (p.n.d.) ^{13}C n.m.r. spectrum of sclerin in deuteriochloroform is shown in Figure 1. It contains 13 signals of which 5 are more intense than the remainder and can therefore be assigned to proton-bearing carbons. The signal at δ_C 37.8 p.p.m. is a doublet in the proton-coupled spectrum and can thus be assigned to the methine position C-4. The remaining four up-field signals clearly belong to the methyl carbons C-11, C-12, C-13, and C-14; subsequent ^{13}C studies enabled their partial assignment. A partial assignment of the remaining eight signals was made using chemical-shift arguments. The two down-field signals at δ_C 165.2 and 167.5 p.p.m. belong to the two carbonyl groups. The six aromatic carbons were tentatively assigned by comparison of observed values with values calculated on the basis of standard data in which allowance is made for the influence of adjacent substituents.[23] In applying this approach to sclerin, it was assumed that the anhydride ring can be represented by an alkyl group at C-10 and a carboxy-group at C-9. Using this method, only C-5 and C-7 are too close together to allow distinction.

The ^{13}C n.m.r. spectrum of sclerin run in the presence of 0.1M chromium trisacetonylacetonate[24] showed uniform peak height. Spectra of ^{13}C-labelled sclerin samples were therefore run in the presence of this relax-

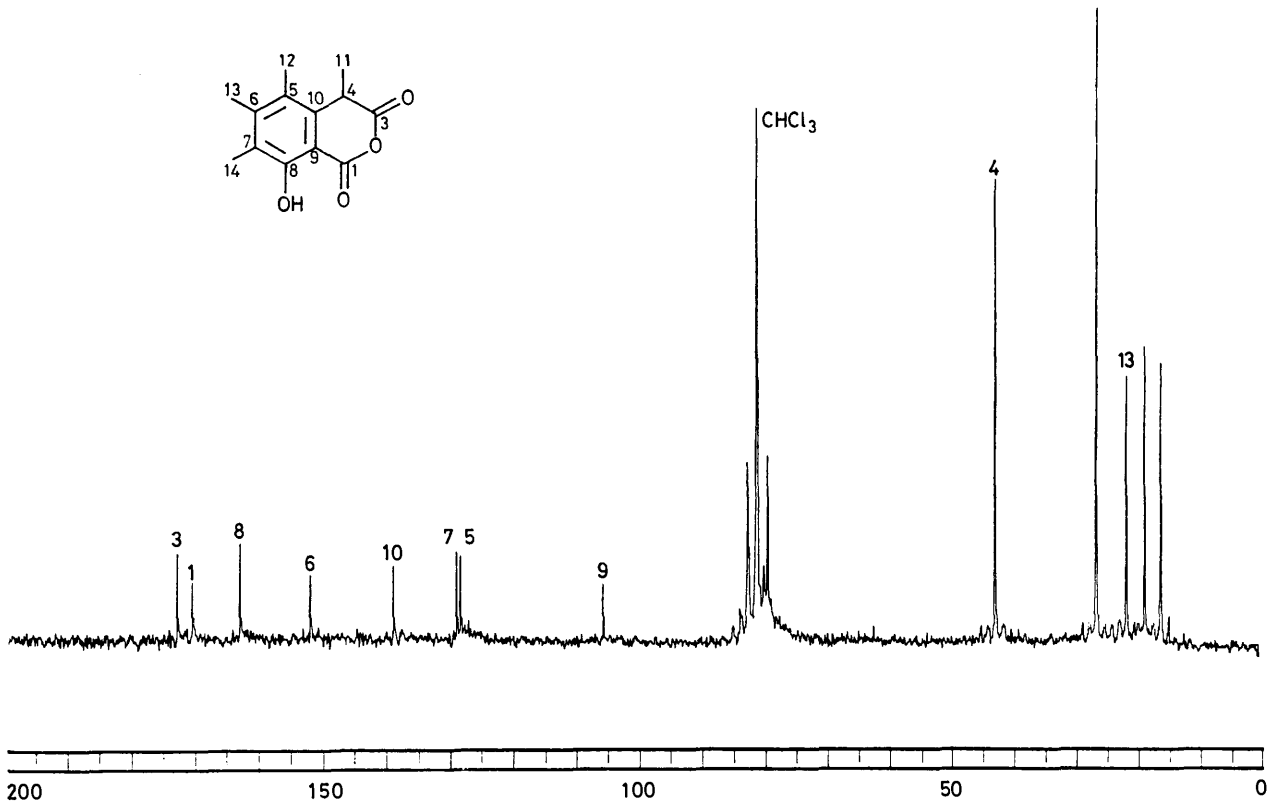


FIGURE 1 P.n.d. ^{13}C n.m.r. spectrum of sclerin (1)

ation agent because of the ease of detection of enriched sites in the molecule. The p.n.d. spectrum of sclerin labelled with [1-^{13}C]acetate is shown in Figure 2. The five low-field signals, assigned to C-1, C-3, C-6, C-8, and C-10, are all enhanced by *ca.* 100% relative to the other carbons and are therefore derived from the carboxy-group of acetate, in agreement with the labelling pattern elucidated by Tokoroyama and Kubota. The remaining carbons are all derived from either C-2 of acetate or the one-carbon pool. A parallel study with [2-^{13}C]acetate was not considered necessary; because the carboxy-derived carbons had been identified, a [1,2-$^{13}C_2$]acetate experiment serves to identify methyl-derived carbons.

Pattern of Incorporation of Intact Acetate Units.—In the p.n.d. spectrum of sclerin derived from [1,2-$^{13}C_2$]acetate (Figure 3) ten carbons have satellite signals resulting

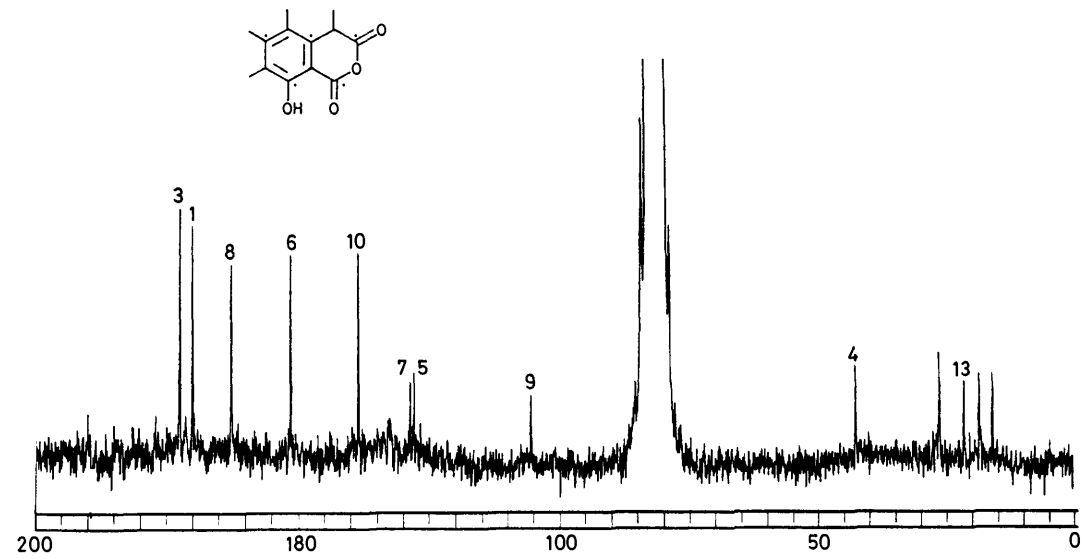


FIGURE 2 P.n.d. ^{13}C n.m.r. spectrum of sclerin (1) enriched with [1-^{13}C]acetate run in the presence of 0.05M Cr(acac)$_3$

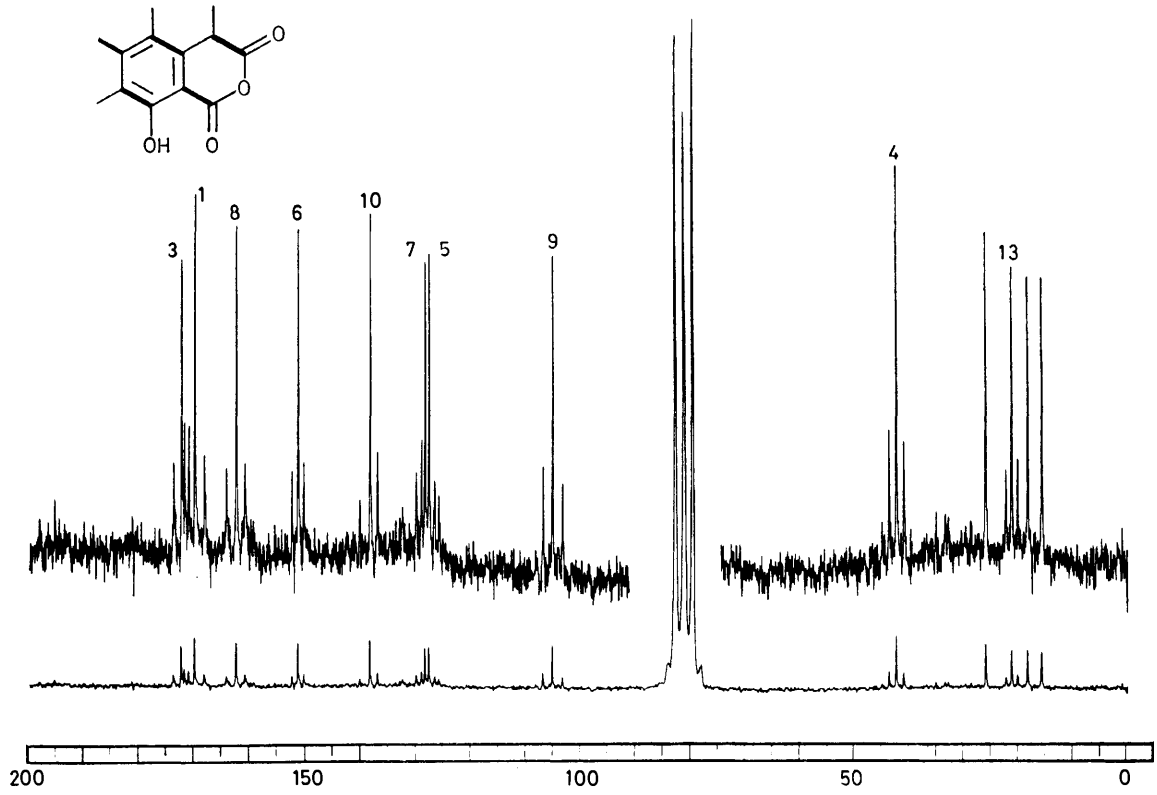


FIGURE 3 P.n.d. ^{13}C n.m.r. spectrum of sclerin (1) enriched with [1,2-$^{13}C_2$]acetate run in the presence of 0.05M $Cr(acac)_3$

from the presence of ^{13}C at the adjacent position. These signals correspond to five matching pairs and therefore result from the incorporation of five intact units. The signal at δ_C 123.7 p.p.m. has a coupling constant of 67.4 Hz which matches the value (67.5 Hz) for the signal at δ_C 157.8 p.p.m. which had been unambiguously assigned to C-8; thus the resonance at δ_C 123.7 p.p.m. can be assigned to C-7. The remaining aromatic carbon at δ_C 122.9 p.p.m. must therefore be assigned to C-5; its coupling constant of 63.7 Hz matches that of C-10 (63.5 Hz). In a similar fashion, the two carbonyl groups were assigned; the signal for C-4 has the same J value as the lower field carbonyl group at δ_C 167.5 p.p.m. (54.0 Hz), therefore this signal is assigned to C-3. The signal at δ_C 165.2 p.p.m. for C-1 has, as expected, the same coupling constant (71.0 Hz) as C-9 (70.9 Hz). In the high-field region of the spectrum, only one carbon shows satellite signals; the J value is the same as that of C-6 (43.9 Hz) and thus this carbon is assigned to C-13. The remaining three high-field singlets C-11, C-12, and C-14 are not enriched, consistent with the derivation of these carbons from the one-carbon pool. A full assignment of the ^{13}C n.m.r. data for sclerin is presented in Table 1.

The interpretation of the [1,2-$^{13}C_2$]acetate spectrum was complicated by the fact that two of the five sets of couplings are not first order; the satellite signals for C-5/C-10 and C-7/C-8 are not symmetrically disposed about the natural abundance singlet. This perturbation arises when the coupling constant has the same order of magnitude as the chemical-shift difference between the two coupled carbons.*

TABLE 1

^{13}C N.m.r. data for sclerin (1) [$CDCl_3$; containing 0.1M $Cr(acac)_3$]

Carbon	Assigned chemical-shift value (p.p.m. relative to Me_4Si)	Calculated chemical-shift value (ref. 23)	$J(^{13}C-^{13}C)$ (Hz)
1	165.2 [a]		71.0
3	167.5 [a]		53.9
4	37.8		54.0
5	122.9	131.2	63.7
6	146.6 [a]	141.7	43.9
7	123.7	122.4	67.4
8	157.8 [a]	154.5	67.5
9	100.5	115.5	70.9
10	133.6 [a]	138.0	63.5
13	16.6		43.4
11, 12, 14	11.0, 13.6, 21.4		

[a] Enhanced in intensity after incorporation of [1-^{13}C]acetate.

This experiment shows that sclerin incorporates five intact acetate units, as shown in Scheme 5, in which heavy lines represent intact acetate units. This result rules out the involvement of naphthalenic precursors (Scheme 4) since the loss of a central carbon atom and a

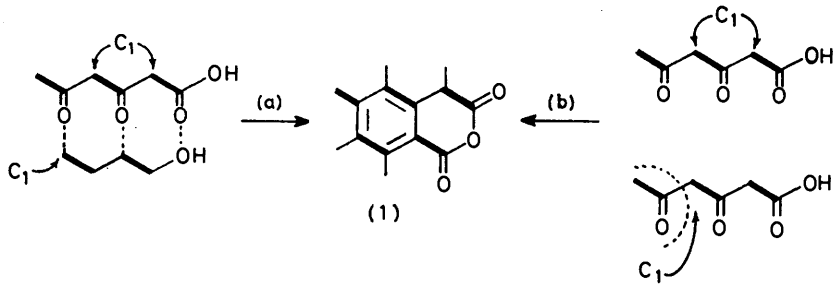
* It was possible to confirm that the couplings were genuine by use of the relationship $\delta_A - \delta_B = [(\nu_4 - \nu_1)(\nu_3 - \nu_2)]^{0.5}$ where δ_A and δ_B are the chemical-shift differences of the relevant carbon atoms and ν_1, ν_2, ν_3, and ν_4 the satellite frequencies (ref. 25).

terminal methyl group result in the retention of only four intact acetate units. The only pathway outlined in Schemes 1, 3, and 4 which is consistent with the labelling results is the two-chain hypothesis shown again in Scheme 5(a), in which a triketide condenses with a diketide

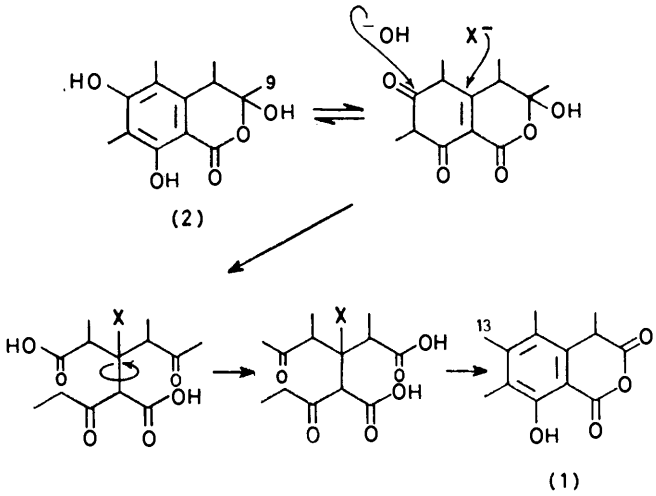
At this time, two other groups [26,27] independently published results of incorporation studies on sclerin with ^{13}C precursors. Holker and Cox [26] studied the incorporation of [*methyl*-^{13}C]methionine, which labelled C-11, C-12, and C-14; [^{14}C]propionate was incorporated randomly. Yamasaki *et al.*[27] assigned the methyl signals by comparison of their chemical-shift values with those of some sclerin analogues and found that [1-^{13}C]-formate labelled C-11, C-12, and C-14. Additionally, Yamasaki fed [2-^{13}C]malonate * in the presence of non-labelled acetate; he found that C-13 was labelled to a lower extent and C-7 to the same extent as the other methyl-derived carbon atoms. From this he concluded that only the two-carbon unit, C-6 + C-13, is a genuine starter unit and proposed a modified two-chain route [Scheme 5(b)] in which two triketides, which may or may not be methylated, condense with subsequent cleavage of a C-2 unit. This modified scheme has the advantage in that it avoids the exceptional features of the original two-chain route, namely cyclisation and methylation at a terminal methyl group. Although there is some biosynthetic precedent for methylation at a chain-starter unit, *cf.* barnol,[28] aureovertin,[29] and stellatin,[30] it is mechanistically more feasible for these processes to occur at a chain-building position.

Despite this modification, we still felt unconvinced by the two-chain approach. The biosynthesis of several fungal polyketides has been rationalised in terms of two chains, but for some of these, *cf.* mollisin [31] and citromycetin,[32] acceptable single-chain routes can also be proposed. At this stage, the relationship between sclerotinin A (2), citrinin (4), and sclerin (1) attracted our attention and we considered ways in which sclerotinin A could act as precursor to both citrinin and sclerin. The carbon skeleton required for sclerin can indeed be generated by a mechanistically plausible isomerisation of sclerotinin A (Scheme 6) which involves hydrolytic cleavage of a keto-tautomer of the aromatic ring, rotation about the central carbon–carbon bond, followed by ring closure. This leads to the pattern of incorporation of intact acetate units observed for sclerin; encouragingly, the malonate labelling results of Yamasaki were also consistent with this novel hypothesis.

Origin of Hydrogen Atoms in Sclerin.—If the pathway shown in Scheme 6 operates, then C-4, which is derived from C-5 of sclerotinin A, cannot retain any acetate-derived hydrogens. There has been much interest recently in the development of new methods for tracing the fate of hydrogen through biosynthetic pathways; [33] deuterium n.m.r. in particular has proved especially valuable.†



SCHEME 5



SCHEME 6 Isomerisation of sclerotinin A (2)

* Malonate is only required in the chain-building steps. Although the situation is complicated by cellular equilibration of acetate with malonate, malonate normally labels chain starter-units to a lower extent than the other positions.

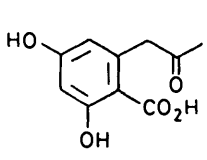
† Deuterium is a cheap isotope, readily available in a variety of chemical forms and, unlike tritium, does not require special handling. The availability of routine high-field Fourier-transform facilities overcomes the problem associated with the low sensitivity of detection and narrow spectral dispersion of deuterium n.m.r. spectra. Line broadening, which results from the quadropole nature of this nucleus, can be minimised and ^2H n.m.r. may be integrated because the short relaxation times, combined with the absence of an n.O.e., minimise the possibility of partial saturation. ^2H N.m.r. spectra are assigned by comparison with the corresponding ^1H n.m.r. spectra. The technique is effectively sixty times more sensitive than ^{13}C n.m.r. when applied to biosynthetic study using singly labelled precursors. Biosynthetic studies reported to date include penicillin G (ref. 34), griseofulvin (ref. 35), ovalicin (ref. 36), rosenonolactone (ref. 37), tetrahymanol (ref. 38), pterocarpans (ref. 39), camptothecin (ref. 40), dihydroborotriol (ref. 41), poriferasterol (ref. 42), formycin (ref. 43), and citrinin (ref. 44).

We therefore studied the incorporation of [$^2H_3$]acetate into sclerin.

A parallel study with [1-^{14}C]acetate resulted in a 0.8% incorporation of label into sclerin. The ^{2}H n.m.r. spectrum of the enriched sclerin clearly showed a singlet at δ 2.3 assigned to C-13 hydrogen atoms. No other signal was apparent; the lack of any signal in the methine region of the spectrum confirms that no acetate-derived hydrogen is retained at this position. Although this result provided good evidence in favour of Scheme 6, it did not exclude the two-chain hypothesis (Scheme 5) since there may be complete exchange of ^{2}H label from the activated position in the triketide precursor. It was unfortunate, therefore, that sclerin lacks other protonated centres through which to monitor the level of deuterium exchange.

Advanced Precursor Studies

The incorporation studies described above provided valuable pointers to the mechanism of sclerin biosynthesis, but did not allow complete definition of the



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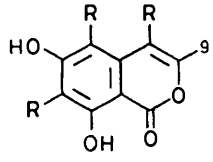
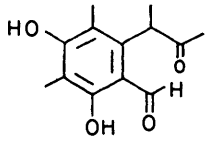


(9) R = H
(10) R = Me

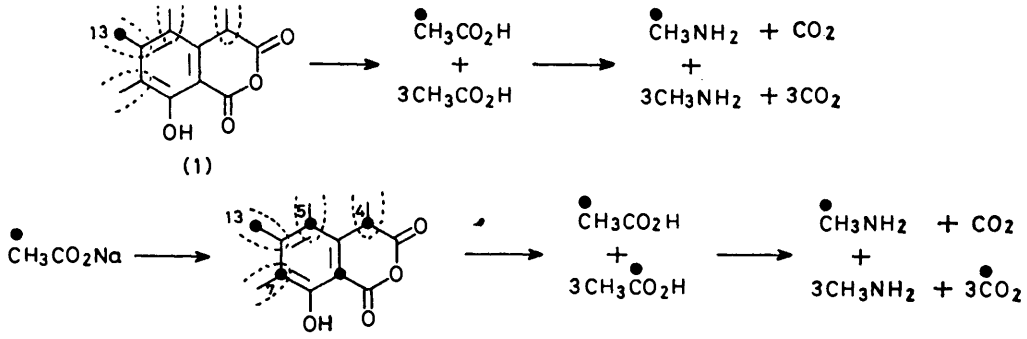


(11)

pathway involved. We therefore initiated a programme of advanced-precursor studies in order to test Scheme 6 (Scheme 5 is difficult to test by virtue of there being no free intermediates prior to sclerin). There are many possible sclerin precursors, depending on the timing of methylation and cyclisation. We chose to investigate, initially, the two extreme possibilities, acetylorsellinic acid (8) and sclerotinin A (2), following up with partially methylated intermediates if necessary. A complication is that the biosynthetic intermediate may exist either as a keto-acid or as a lactol, or even as the corresponding isocoumarin; these derivatives may or may not interconvert under the conditions of the biosynthetic study. It was decided to aim for the ring-closed forms (9) and (10) first, since these are amenable by synthesis,[45,46] and hope that they might equilibrate under physiological conditions.

Using ^{14}C as the isotopic label, the choice of site of enrichment was determined by the availability of a good synthetic route and by the ease of degradation of sclerin to establish specificity of labelling. The C-9 methyl of (9) and (10) is a position in which a label can conveniently be introduced, but it cannot be isolated unambiguously in degradative work. However, sclerin specifically labelled at C-13 generates acetate labelled only in the methyl group in a Kuhn–Roth degradation (Scheme 7). Additionally, the Kuhn–Roth oxidation produces up to 4 mol equiv. of acetic acid; if these come equally from all methyl positions, the molar activity of the acetate should be 25% that of the degraded sclerin. In contrast, sclerin labelled by [2-^{14}C]acetate is labelled at carbons which emerge as C-2 of acetate in a Kuhn–Roth oxidation, as well as at C-13 which emerges as C-1 of acetate, and the molar activity of this acetate produced by oxidation will be only 20% that of sclerin, since four out of five labelled sites are isolated (C-4, C-5, C-7, and C-13). By degrading the acetic acid to methylamine and carbon dioxide in a Schmidt reaction to check the distribution of label between C-1 and C-2 it is possible to differentiate between the two patterns of incorporation, specific and general. The validity of the overall degradative sequence was tested using sclerin labelled with [2-^{14}C]acetate. As expected, the acetate from the Kuhn–Roth degradation had molar activity 20% that of sclerin and the distribution of label was shown by a Schmidt degradation to be close to 1 : 3 (Table 2, Expt. 1).

Radiolabelled synthesis of 6,8-dihydroxy-3-methylisocoumarin (9),[45] 6,8-dihydroxy-3,4,5,7-tetramethylisocoumarin (10), and 3,6,8-trihydroxy-3,4,5,7-tetramethyl-3,4-dihydroisocoumarin [46] (2) were carried out as described in the Experimental section. In each case the



SCHEME 7 Degradation of radiolabelled sclerin (1)

precursor was labelled at C-9 which, according to the biosynthetic pathway of Scheme 6, leads to sclerin specifically labelled at C-13. The results of incorporation studies with these compounds are given in Table 2. The isocoumarin (9) was incorporated efficiently (Expt. 2), but the labelling pattern was found to be non-specific and is consistent with degradation of this precursor to [2-^{14}C]acetate prior to incorporation. Similar results with the isocoumarin (9) have been obtained for terrein [47] and citrinin.[48]

In contrast, both the methylated precursors (2) and (10) were incorporated specifically. The incorporation was very low, but in experiments using intact organisms this can result from low permeability of the cell wall to the compound under test and hence a more reliable guide to the biosynthetic status of a proposed precursor is the specificity rather than the efficiency of incorporation. In carrying out the degradative sequence it was essential that the sclerin samples were not contaminated with either trace amounts of precursor or some structurally related product since these would interfere. Therefore, the radiochemical purity was in each case established by recrystallisation to constant activity from two different solvents (chloroform and benzene).

TABLE 2

Incorporation studies with ^{14}C precursors

Expt.	Precursor	Specific activity of precursor (μ Ci/mmol)	Amount fed (μ Ci)	Incorporation (%)	Kuhn–Roth oxidation [a] [% molar activity (acetate: sclerin)]	Schmidt degradation [b] [% molar activity (methylamine: acetate)]
1	[2-^{14}C]Acetate	58 × 10^3	100	1.34	18.6	27.1
2	[9-^{14}C]-(9)	519	7.5	0.69	19.1	26.1
3	[9-^{14}C]-(10)	9.8	1.7	0.045	25.6	99.2
4	[9-^{14}C]-(2)	15.9	2.2	0.011	25.9	

[a] Acetic acid (C_1 + C_2) counted as *para*-bromophenacyl derivative. [b] Methylamine (C_1) counted as *p*-bromo-*N*-methylbenzoylamide.

The intermediacy of compounds (9) and (10) in citrinin biosynthesis has also been studied;[48] again, a low, but significant, incorporation was observed. Subsequent work with [$^2H_3$]acetate revealed that the isocoumarin (10) cannot be an obligatory intermediate in the normal biosynthetic pathway to citrinin (4) since some ^{2}H label is retained at C-3 [49] and a keto-aldehyde (11), generated by reductive cleavage of an enzyme-bound thioester, was identified as the first true enzyme-free intermediate.[44,50] A related keto-aldehyde has also been identified in ascochitine biosynthesis.[51] We therefore decided to check the possible intermediacy of compound (11) in sclerin biosynthesis, even though the oxidation level of sclerin makes it unlikely that a keto-aldehyde would be a precursor. The compound [52] was produced *via* a route used in an earlier synthesis of sclerotinin A (2).[10] It was found convenient to introduce a ^{2}H label in the C-3 methyl group and use ^{2}H n.m.r. for detection of labelled sites in the enriched metabolite; sclerin labelled with [$^2H_3$]acetate was available for comparison. The ^{2}H n.m.r. spectrum of sclerin derived from compound (11) revealed no sign of label at the C-11 position. There was, however, a very low incorporation into the C-13 position and hence the keto-aldehyde can only be utilised as a carbon source by degradation to acetate [compare compound (9)]. Even in the case of citrinin, where a high, specific incorporation of compound (11) was observed, some incorporation of label *via* degradation to acetate as a competing pathway was also observed.

The specific incorporation of compounds (2) and (10), taken together with the result of the [$^2H_3$]acetate study, support the view that they are intermediates in sclerin biosynthesis, with at least one and probably all of the methylation steps preceding aromatisation. Subsequent novel, structural reorganisation then leads to sclerin which is in turn, possibly, converted into sclerolide (5). The origins of sclerone (6) and isosclerone (7) remain unclear. The biosynthesis of naphthalenes is not well understood and in many cases, the shikimate pathway may be involved rather than the acetate–polymalonate pathway.

In conclusion, our study of sclerin biosynthesis neatly illustrates the advantages and disadvantages of different isotopic labelling techniques used in biosynthetic studies. Although the exploratory studies with [1,2-$^{13}C_2$]acetate supplied valuable information, the decisive incorporation results came from advanced-precursor studies. The experiments with ^{14}C-labelled precursors gave useful results, but the work involved in synthesis and degradation was laborious when compared with that when ^{2}H was used as the tracer isotope. With the increasing availability of ^{2}H n.m.r., there is much scope for the use of ^{2}H in advanced-precursor work.

EXPERIMENTAL

Solutions were dried over magnesium sulphate or sodium sulphate (anhydrous). Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. I.r. spectra were recorded with a Perkin-Elmer 257 spectrophotometer for solutions in chloroform, unless otherwise stated. Mass spectra were taken on an A.E.I. MS 12 or MS 30 mass spectrometer. ^{1}H N.m.r. spectra were run on either a Varian HA 100 or a Perkin-Elmer R12 B instrument for solutions in deuteriochloroform, unless otherwise stated, using Me_4Si as internal standard. ^{13}C N.m.r. spectra were recorded on a Varian CFT 20 n.m.r. spectrometer for solutions in deuteriochloroform. ^{2}H N.m.r. spectra were recorded as solutions in chloroform on a Bruker WH-300 MA 7 spectrometer locked onto deuteriochloroform. Radio-

active samples were counted in organic or aqueous scintillators (7 ml) on a Packard Tri-Carb 3385 instrument and standardised using radio labelled n-hexadecane as internal standard. Column chromatography was carried out on silica gel (Merck; 500 mesh) and preparative t.l.c. on glass plates coated with Merck Kieselgel GF_{254}.

Isolation of Sclerin.—*Sclerotinia sclerotiorum* (I.M.I. 148268) was maintained on Czapex–Dox agar slopes at 0 °C. Culture medium (6 l) containing sucrose (300 g), sodium nitrate (12 g), potassium dihydrogenphosphate (6 g), potassium chloride (3 g), magnesium sulphate heptahydrate (3 g), ferrous sulphate heptahydrate (0.06 g), and yeast powder (Oxoid, 30 g) was divided among 12 culture pans and these were then autoclaved at 120 °C/15 lb in^{-2} for 30 min. The vessels were inoculated from a spore suspension made from a single slope and incubated at 28 °C for 14—18 d. The medium was then filtered off through a Celite column and extracted with ether (4 × 2 l). The organic fractions were evaporated under reduced pressure to give a brown semi-solid (*ca.* 1.8 g) which was dissolved in ether–cyclohexane (1 : 1, 30 ml), partitioned with sodium hydrogencarbonate solution (5%, 30 ml), and left overnight. The mixture was then evaporated to remove the organic solvents and the residual liquid extracted into ether (3 × 20 ml) to remove fatty-acid impurities. The hydrogencarbonate layer was acidified and re-extracted into ether (3 × 30 ml). The combined ether fractions were dried and evaporated to give a pale brown solid (1.3 g) which could be recrystallised from chloroform–ether (1 : 1, 10 ml) to give sclerin (1) as white needles, m.p. 122.5—123 °C (lit.,[1] 123 °C); $\lambda_{max.}$ (EtOH) 263 and 320 nm; $\lambda_{max.}$ (KOH–EtOH) 242s and 313 nm; $\nu_{max.}$ 3 260br, 1 800, 1 690, 1 610, 1 580, 1 370, and 1 345 cm^{-1}; δ 10.5 (1 H, br, OH), 4.14 (1 H, q, *J* 7 Hz), 2.28, 2.22, 2.17 (each 3 H, s), and 1.53 (3 H, d, *J* 7 Hz); *m/e* 264 (M^+).

Techniques used in the Administration of Labelled Compounds.—Flasks were ready for the addition of precursors when the white mycelial mat was completely formed and the silvery black conidia were darkening in colour; this was usually one week after inoculation. ^{14}C-Labelled precursors were dissolved in water (10 ml) which contained up to 10% dioxan if required to ensure solubility. The solution was divided equally amongst 2 culture pans; samples (5 ml) were injected *via* a syringe through the mycelial mat into the medium. The flasks were swirled thoroughly, then incubated for 1 week at 28 °C before work-up. The procedure involved in ^{13}C incorporation studies differed; one week after inoculation, the culture medium was removed and the mycelial mat refloated onto fresh medium to which the labelled precursor (1 g l^{-1}) had been added.

Degradation of Sclerin.—Kuhn–Roth oxidation. Sclerin (80 mg) was heated at 100 °C with chromic acid (5M, 10 ml) and concentrated sulphuric acid (2 ml) for 1.5 h. The solution was cooled, water (500 ml) was added, and the solution steam-distilled. The distillate (500 ml) was boiled to remove carbon dioxide, cooled, and neutralised with 0.1M sodium hydroxide solution using phenolphthalein as indicator. The solution was then evaporated to dryness.

p-*Bromophenacyl acetate.* The residue from the Kuhn–Roth oxidation was dissolved in water (1 ml) and ethanol (9 ml). *p*-Bromophenacyl bromide (50 mg) was added and the solution was refluxed for 2 h. After removal of the solvent under reduced pressure, the residue was partitioned between ether (10 ml) and water (10 ml). The aqueous layer was further extracted into ether (2 × 10 ml). The combined organic layers were dried and evaporated to give a white crystalline solid which was purified by preparative t.l.c. using ethyl acetate–benzene (1 : 9) as eluant. The product, R_F 0.5, was recrystallised from light petroleum (b.p. 40—60 °C) as white needles, m.p. 85—86 °C (lit.,[53] 86 °C).

Schmidt degradation. To sodium acetate (15 mg) was added, with cooling, chloroform (AnalaR, 3 ml) and sulphuric acid (AnalaR, 0.5 ml). The solution was heated to 50 °C and sodium azide (70 mg) added quickly. The reaction was kept at this temperature for 90 min, after which it was neutralised with potassium hydroxide (40%, *ca.* 3 ml) and distilled to dryness; the distillate was collected in hydrochloric acid (5M, 15 ml). The acidic solution was then evaporated to give methylamine hydrochloride, which was dissolved in dilute potassium hydroxide (1%, 5 ml); *p*-bromobenzoyl chloride (100 mg) was then added and the mixture was shaken for 15 min. The solution was maintained at pH 10—11 throughout. The reaction mixture was then extracted with ether (3 × 10 ml) and the combined organic layers dried and evaporated to give *N*-methyl-*p*-bromobenzamide, which was purified as a white powder by preparative t.l.c. on silica gel with ethyl acetate–benzene (1 : 3) as eluant and then recrystallised from ethyl acetate–cyclohexane as a white powder, m.p. 166.5—167 °C (lit.,[53] 166.5—167 °C).

Preparation of Radio-labelled Compounds for Advanced Precursor Study

6,8-*Dihydroxy*-3-[^{14}C]*methylisocoumarin* (9).—6,8-Dimethoxy-3-[^{14}C]methylisocoumarin [45] (32 mg, *ca.* 200 μCi) was combined with 6,8-dimethoxy-3-methylisocoumarin (45 mg) and recrystallised (71 mg). Dry dichloromethane (10 ml) was added and the solution was cooled to −78 °C. Boron tribromide (0.5 ml) was added, with stirring, and the solution was allowed to warm to room temperature, with stirring, overnight. Ether (20 ml) was added cautiously, followed by water (15 ml), and the two layers separated. The organic layer was extracted twice more with water (20 ml) and the combined, aqueous fractions were re-extracted with ether (2 × 50 ml). The combined organic layers were dried and evaporated to give a buff powder which was purified by sublimation at 180 °C/0.05 Torr to give a white powder (51 mg, 138 μCi, 70%), m.p. 250 °C (lit.,[54] 250—253 °C); $\lambda_{max.}$ 238sh, 245, 258sh, 278, 289, and 327 nm; $\nu_{max.}$ 3 200br, 1 670, and 1 620 cm^{-1}; δ[$(CD_3)_2CO$], 6.36 (3 H, s, Ar-H and ArC*H*=) and 2.22 (3 H, s, Me); *m/e* 192 (M^+).

Radiochemical Synthesis of 6,8-*Dihydroxy*-3-[^{14}C]*methyl*-4,5,7-*trimethylisocoumarin* (10) *and* 3,6,8-*Trihydroxy*-3-[^{14}C]*methyl*-4,5,7-*trimethyl*-3,4-*dihydroisocoumarin* (*Sclerotinin A*) (2).—The synthetic approach used is described in detail in ref. 46.

Ethyl 2-*triphenylphosphorylidene*[3-^{14}C]*propionate.* Ethyl triphenylphosphorylideneacetate (695 mg, 2 mmol) was dissolved in dry ethyl acetate (10 ml). The mixture was stirred at reflux while methyl iodide [0.1 ml of a solution which contained methyl iodide (1 ml) in ethyl acetate (10 ml)] was injected *via* a side arm. [^{14}C]Methyl iodide (500 μCi, 59.7 mCi $mmol^{-1}$) was vacuum-transferred onto a solution containing methyl iodide [0.1 ml in ethyl acetate (1 ml)]. The mixture was warmed and cooled twice to equilibrate it, then added as a chilled solution to the reaction mixture. The flask contents were rinsed in with ethyl acetate (1 ml). After the mixture had been stirred

at reflux for 1 h, the reaction was completed by the addition of methyl iodide (0.05 ml) in ethyl acetate (0.5 ml) and further stirring at reflux for an hour. The solvent was removed under reduced pressure to leave an oily residue (1.02 g) which crystallised on removal of the residual solvent at high vacuum. Distilled water (9 ml) and tetrahydrofuran (1 ml) were added and the solution was treated with dilute sodium hydroxide (10%) until alkaline (phenolphthalein). The solution was then extracted into ethyl acetate (4 × 10 ml), the combined organic layers were washed with saturated brine (10 ml), dried, and evaporated to give ethyl 2-triphenylphosphorylidene[3-^{14}C]propionate as a yellow, crystalline solid (781 mg, 94%; 152 μCi, 30.4% radiochemical yield), m.p. 155—157 °C (lit.,[55] 156—157 °C) from ethyl acetate–light petroleum (b.p. 40—60 °C); $\lambda_{max.}$ 225 and 268 nm; $\nu_{max.}$ 1 605 and 1 109 cm^{-1}; δ 7.93—7.95 (15 H, m, Ar-H), 3.93 (2 H, q, *J* 7 Hz, CO_2CH_2Me), 1.98 (3 H, d, *J* 15 Hz, PC*Me*), and 1.24 (3 H, t, *J* 7 Hz, CO_2CH_2*Me*); *m/e* 362.

2-[^{14}C]*Methylcrotonic acid*. Ethyl 2-triphenylphosphorylidene [3-^{14}C]propionate (781 mg) was dissolved in dry dichloromethane (10 ml) at 0 °C. Freshly distilled acetaldehyde (0.2 ml) was added as drops and the reaction mixture was stirred at room temperature under nitrogen for 18 h. The solvent was then removed (water-bath) and the gummy residue extracted with light petroleum (b.p. 40—60 °C) and filtered off to remove triphenylphosphine; the solvent was then distilled off. Sodium hydroxide [0.5 g in water (5 ml)] was added and the solution stirred at reflux for 2.5 h, cooled, and extracted with ether (2 × 5 ml). The aqueous solution was acidified by addition of drops of concentrated HCl and extracted into ethyl acetate (4 × 5 ml). The combined organic layers were evaporated and dried to give 2-[^{14}C]methylcrotonic acid (136 mg, 69%) as a semi-solid, m.p. 63—64 °C from water (lit.,[56] 62—64 °C).

5,7-*Dimethoxy*-2-[^{14}C]*methyl*-3,4,6-*trimethylindan*-1-*one*. 2-[^{14}C]Methylcrotonic acid (136 mg) was diluted with 2-methylcrotonic acid (300 mg) and recrystallised (375 mg, 100 μCi). The dried product was treated with thionyl chloride (0.3 ml) at room temperature. The mixture was then distilled (kugelrohr), firstly at atmospheric pressure to remove the excess of thionyl chloride and then at water-pump pressure using dry ice–acetone to cool the receiver bulb. The oil which was obtained was dissolved in carbon disulphide (AnalaR, 5 ml) and added to a mixture of dimethylresorcinol (0.8 g) and stannic chloride (1.5 ml) in carbon disulphide (AnalaR, 30 ml). The reaction mixture was stirred for 2—3 h, then evaporated off and extracted between ice–concentrated hydrochloric acid and ether. The organic layers were dried and evaporated to give a brown oil (1.3 g) which was purified by column chromatography on silica gel using dichloromethane to give 1-(2,4-dimethoxy-3,5-dimethylphenyl)-2-[^{14}C]methylbut-2-en-1-one (659 mg) and 5,7-dimethoxy-2-[^{14}C]methyl-3,4,6-trimethylindan-1-one (66 mg). The acrylophenone was dissolved in AnalaR chloroform (passed down an alumina column, 40 ml) and cooled to 0 °C. Fluorosulphonic acid (0.2 ml) was added and the mixture was stirred for 7 d until t.l.c. showed the reaction to be complete. The solvent was removed under reduced pressure and the residue extracted between ice–water–ether (3 × 30 ml). The ether layers were dried and extracted to give the indan-1-one as a brown oil (350 mg, 50%), $\lambda_{max.}$ 260 and 302 nm; $\nu_{max.}$ 2 840, 1 695, 1 585, 1 325, and 1 120 cm^{-1}; δ 3.96 (3 H, s, OMe), 3.80 (3 H, s, OMe), 4.00—3.00 (1 H, m, COC*H*Me), 2.7—3.0 (1 H, m, Ar-C*H*Me), 2.30 (3 H, s, Ar-Me), 2.24 (3 H, s, Ar-Me), and 1.4—1.1 (6 H, m, *J* 7, 8.5 Hz, Me); *m/e* 248.

3-*Hydroxy*-6,8-*dimethoxy*-3-[^{14}C]*methyl*-4,5,7-*trimethyl*-3,4-*dihydroisocoumarin*. A freshly prepared sample of trifluoroacetic anhydride (10 ml) was vacuum-transferred from phosphorus pentoxide onto potassium carbonate and then onto 5,7-dimethoxy-2-[^{14}C]methyl-3,4,6-trimethylindan-1-one (250 mg, 28 μCi). The mixture was warmed to room temperature and then stirred for 4 h under nitrogen. After removal of the solvent, the darkish oil was dissolved in dry ethyl acetate (25 ml), cooled to −78 °C, and flushed out with nitrogen. Ozone was passed through until a pale blue colour developed, followed by nitrogen to remove the excess of ozone. Dimethyl sulphide (0.2 ml) was added and the solution warmed to room temperature overnight. The pale yellow solution was evaporated and dried to give an oil which was dissolved in ether (15 ml) and stirred with sodium hydrogencarbonate solution (10 ml) for 5 h. The basic layer was removed, acidified, then re-extracted to give 3-hydroxy-6,8-dimethoxy-3-[^{14}C]methyl-4,5,7-trimethyl-3,4-dihydroisocoumarin as a brown oil (180 mg); $\lambda_{max.}$ 255 and 301 nm, $\nu_{max.}$ 1 715 cm^{-1}; δ 3.78, 3.72 (each 3 H, s, OMe), 2.5—3.0 (1 H, m, Ar-C*H*Me), 2.21 (6 H, s, Ar-Me), 1.65 (3 H, s, *Me*C-OH), 1.25 (3 H, d, *J* 7 Hz, Ar-CH*Me*), and 4.4 (1 H, s, OH); *m/e* 280 weak), 262, and 248.

6,8-*Dihydroxy*-3-[^{14}C]*methyl*-4,5,7-*trimethylisocoumarin* (10) *and* 3,6,8-*trihydroxy*-3-[^{14}C]*methyl*-4,5,7-*trimethyl*-3,4-*dihydroisocoumarin* (2). The 3-hydroxy-3,4-dihydroisocoumarin (100 mg), described above, was dissolved in dry dichloromethane (10 ml) and cooled to −78 °C. Boron tribromide (0.5 ml) was added and the solution brought to room temperature overnight. Ether (10 ml) was added, followed by water (10 ml). The organic layer was extracted with water (2 × 10 ml) and the combined aqueous fractions were re-extracted into ether (3 × 15 ml). The combined organic layers were dried and evaporated to give a yellow powder (90 mg) which was purified by preparative t.l.c. with dichloromethane to give 3,6,8-trihydroxy-3-[^{14}C]-methyl-4,5,7-trimethyl-3,4-dihydroisocoumarin (2) (37 mg, 2.34 μCi, 15.9 μCi mmol^{-1}), R_F 0.1, m.p. 195—198 °C (lit.,[9] 205—208 °C); $\lambda_{max.}$ 222, 275, and 313 nm; $\nu_{max.}$ 1 651 and 1 620 cm^{-1}; δ 3.48 (1 H, q, *J* 7 Hz, Ar-C*H*Me), 2.30, 2.32 (each 3 H, s, Ar-Me), and 1.83 (3 H, d, *J* 7 Hz, Ar-CH*Me*); *m/e* 252 and 6,8-dihydroxy-3-[^{14}C]methyl-4,5,7-trimethyl-isocoumarin (10) (42 mg, 1.76 μCi, 9.8 μCi mmol^{-1}), R_F 0.8, m.p. 223—224 °C from methanol (lit.,[9] 224 °C); $\lambda_{max.}$ 247, 267, 285, 293, and 345 nm; $\nu_{max.}$ 3 200br, 1 728, 1 662, and 1 618 cm^{-1}; δ 2.44 (3 H, s, Ar-CH*Me*), 2.32, 2.28 (each 3 H, s, Ar-Me), 2.18 (3 H, s, Ar-C*Me*), and 1.29 (2 H, br, OH); *m/e* 234.

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