The Biosynthesis of Citrinin by *Penicillium citrinum*

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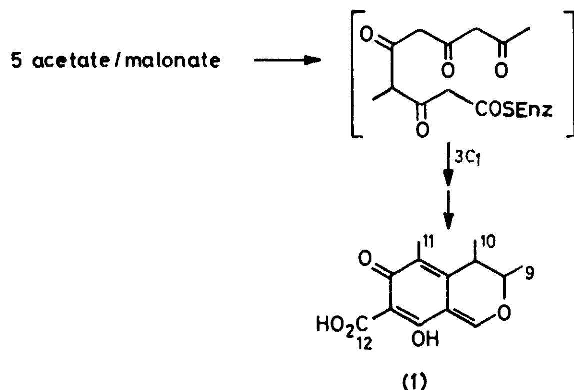
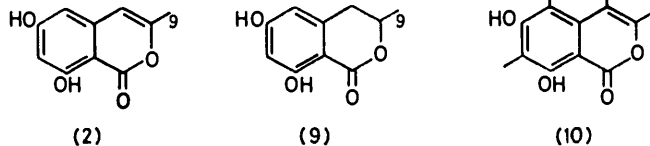
Incorporation studies have been carried out with [*Me*-^{13}C]methionine and aromatic compounds labelled with ^{14}C or ^{2}H. Evidence is presented that 4,6-dihydroxy-3,5-dimethyl-2-(1-methyl-2-oxopropyl)benzaldehyde (13) is the first enzyme-free aromatic intermediate in the biosynthesis of citrinin in *P. citrinum*, and that all three methylations take place on the polyketide synthetase complex.

CITRININ (1) is a highly crystalline, golden yellow metabolite of *Penicillium citrinum* and a number of Aspergillus species, which has attracted much interest because of its broad-band antibiotic activity.[1] It has been shown to be derived from five acetate/malonate and three C_1 units [2-4] and there is substantial evidence that the biosynthesis proceeds *via* an enzyme-bound poly-β-ketone chain (Scheme 1). This paper describes experiments to identify the first enzyme-free intermediate in the biosynthesis of citrinin.



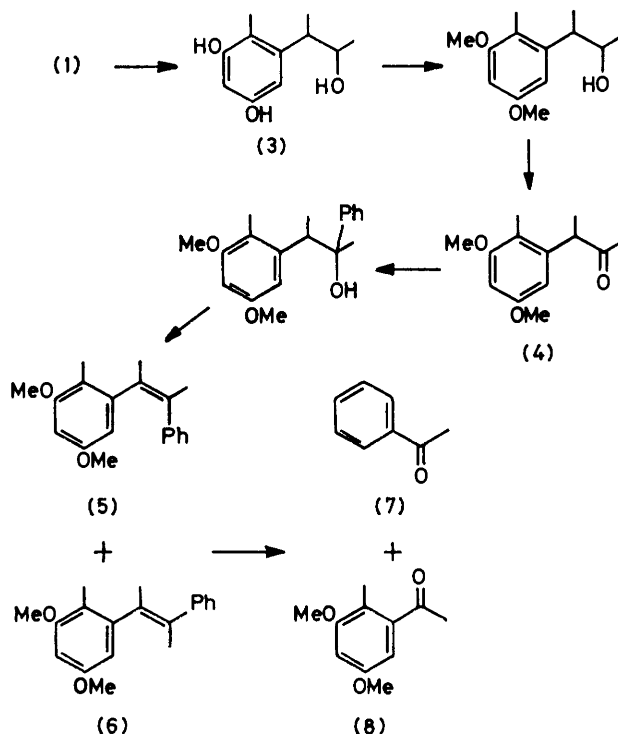
SCHEME 1

One of a number of strong candidates was the isocoumarin (2). This has the same carbon skeleton as the poly-β-ketone chain, and is at the same oxidation level. It was therefore decided to test its intermediacy by a



conventional advanced precursor feeding. The compound was prepared by a method described elsewhere [5] with a ^{14}C label in the methyl group C-9. *Penicillium citrinum* was grown from spores on a Czapex–Dox medium and the isocoumarin (2), in aqueous solution, was injected into the medium after 24 days of growth. Citrinin was isolated after a further week, and was found to contain 0.7% of the radioactivity present in the administered sample of (2) (Table 1).

The citrinin was then degraded so as to isolate C-9 (Scheme 2). Treatment with aqueous sodium hydroxide gave ' phenol A' (3) which was methylated with dimethyl sulphate, and then oxidised using chromium trioxide–pyridine to give the ketone (4), which was counted as its semicarbazone derivative. This compound appeared to contain about 8% less activity than (1) (Table 1) but in view of the difficulties associated with counting citrinin, a yellow compound with a low counting efficiency, it was reasonable to assume that the loss of activity had no



SCHEME 2

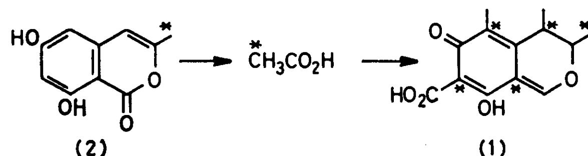
biosynthetic significance. The ketone (4) was then treated with phenylmagnesium bromide, and the product dehydrated to give a mixture of the two stilbenes (5) and (6). On ozonolysis this mixture gave rise to acetophenone (7), containing the carbon from C-9 and C-3 of citrinin [the dimethoxyacetophenone (8), containing the remainder of the carbon from phenol A was not isolated]. The acetophenone (7) was counted as its semicarbazone derivative, and its activity was found to be almost exactly 20% of that of (4) (Table 1). We conclude therefore that the incorporation of (2) is not specific, but that

TABLE 1

Results of incorporation of ^{14}C-labelled isocoumarin (2)

Compound	Quantity fed/ isolated (mg)	Specific activity (μCi mmol^{-1})
(2)	23	327
(1)	800	7.23×10^{-2}
(4)		6.73×10^{-2}
(7)		1.37×10^{-2}

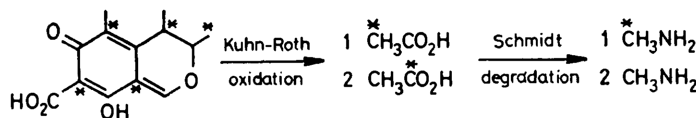
the molecule is degraded by the fungus to give [2-^{14}C]-acetate, which was incorporated into citrinin as shown in Scheme 3. Thus (2) is not a precursor on the biosynthetic pathway to citrinin.[6]



SCHEME 3

A second candidate, the lactone (9), was easily available by hydrogenation of (2)[7] and a similar feeding experiment was conducted with this compound. However, the citrinin isolated showed no radioactivity above background. It is unlikely that the permeability of the cell membrane to (9) and (2) would be very different so it is highly unlikely that (9) is a biosynthetic precursor to citrinin.

These results suggest that one or more of the methylation steps in the biosynthesis occur prior to aromatisation and so the next compound to be investigated was the trimethylated isocoumarin (10).[8] In this case, however, the specificity of labelling was checked by Kuhn–Roth oxidation followed by Schmidt degradation of the derived acetic acid[6] (Scheme 4). A control incorporation study with [2-^{14}C]acetate was therefore also carried out to check the method.



SCHEME 4

In the Kuhn–Roth degradation of [2-^{14}C]acetate labelled citrinin only three of the five ^{13}C-labelled positions were isolated, as 3 mol of acetic acid per mol of citrinin. As expected the % molar activity of the acetic acid (counted as its *p*-bromophenyl derivative) was 22.2%, approximately one fifth that of citrinin itself. Further confirmation of the labelling pattern was obtained by degradation of the acetic acid to methylamine (counted as its *p*-bromobenzoyl derivative). The activity dropped to one third that of the acetic acid indicating that, of the 3 molecules of acetic acid from each molecule of citrinin, one was labelled in the methyl group and two in the carboxy-group (Table 2).

The isocoumarin (10) was administered to $3\frac{1}{2}$-week-old cultures of *P. citrinum* and citrinin was isolated in the usual way. A low incorporation, 0.05%, was obtained but this was shown by degradation to be specific and therefore significant. The acetic acid isolated contained 28.1% of the activity of citrinin. This is lower than the 33% expected for a specific incorporation, but a high degree of specificity of labelling was established by the Schmidt degradation: the activity of methylamine was 97% that of the acetic acid. Hence all the radioactivity present in the acetic acid is present in the methyl group, and can only be derived from C-9 of citrinin.

TABLE 2

Results of incorporation of ^{14}C-labelled isocoumarin (10)

Feeding	Molar activities as % of activity of citrinin: Acetic acid	Methylamine
[2-^{14}C]acetate	22.2	7.3
[9-^{14}C]-(10)	28.1	28.0

The very low incorporation of (10) requires comment. It is possible that (10) is a true precursor to citrinin but that the cell membrane exhibits low permeability towards it. This appears to be the case in sclerin biosynthesis.[9] However, it is possible that (10) is not a true precursor but that it is capable of being converted in small amounts by non-specific enzymes in the cell into an intermediate on the biosynthetic pathway.

Experiments were then performed to determine whether or not (10) is a true precursor. These are described in detail elsewhere[10] and are presented here in outline only. When the fungus was grown on D_2O with unlabelled glucose as the carbon source, protium from the C–H bonds of glucose was incorporated to a significant extent at all the normally protonated carbon atoms of the metabolite. The presence of protium at C-4 proves that despite its specific incorporation the isocoumarin (10) cannot be on the normal biosynthetic pathway to citrinin.

In the same experiment the ^{2}H : ^{1}H ratios at C-1 and C-3 were 1.2 : 1 and 1.9 : 1 respectively measured by comparison of the integrals of the signals in the ^{1}H n.m.r. spectrum with those of the hydroxy-groups which are fully protonated by exchange during work-up.

TABLE 3

^{1}H N.m.r. spectrum of citrinin (1) (in $CDCl_3$)

δ (p.p.m.)	Integral	J (Hz)	Position
1.23	3 H, d	7.3	10-H_3
1.35	3 H, d	6.8	9-H_3
2.02	3 H, s		11-H_3
2.99	1 H, q	7.3	4-H
4.78	1 H, q	6.8	3-H
8.24	1 H, s		1-H
15.09	1 H, s		8-OH
15.86	1 H, s		CO_2H

The hydrogen at both sites is delivered from a reducing coenzyme and the pool of transferable hydrogen carried by the coenzyme contains both protium and deuterium derived from the metabolic intermediates of the glycolytic pathway and of the citric acid cycle. If the aldehydo-alcohol (11) were the intermediate released by

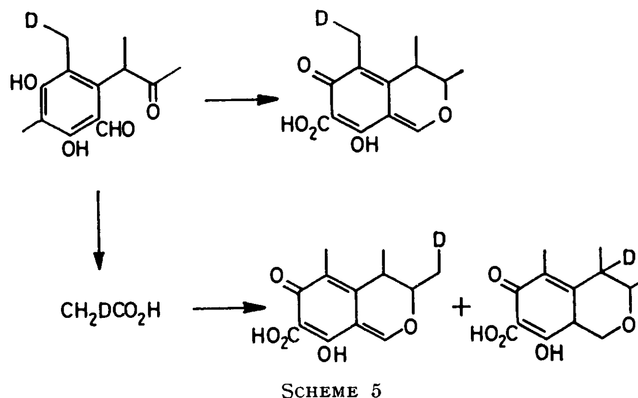
the polyketide synthetase both reductions would be carried out in rapid succession drawing on the same pool of coenzyme and the observed difference in isotopic ratios could only be explained in terms of different isotope effects operating on the rate of hydrogen transfer from the coezyme at the two sites. An alternative explanation which guided the next phase of the work is that separate enzymes are involved in the reduction steps possibly drawing on different pools of coenzyme in separate compartments of the cell with different isotopic enrichments in the pool of transferable hydrogen. In view of the non-intermediacy of the isocoumarin (10) it seemed likely that at least one of the reductions takes place prior to release of the intermediate from the polyketide synthetase. Hence, the choice of the first enzyme-free polyketide intermediate was narrowed to the lactone (12) in which reduction has taken place at C-3 but not at C-1, and the ketoaldehyde (13) in which C-1 but not C-3 is reduced. It is assumed in this analysis that all three methylation steps are carried out prior to release of the first aromatic intermediate; evidence to support this assumption is presented later.

The two compounds (12) and (13) were both synthesised [11] with a single deuterium label in the methyl group C-11, and administered to the fungus in separate experiments.[12] Deuterium rather than ^{14}C was chosen as the label for these experiments because ^{2}H n.m.r. had become routinely available since we carried out the experiments with (2), (9), and (10), described earlier; the ease with which specificity of incorporation can be detected without degradation makes this a more attractive tracer technique for advanced precursor studies.[13]

The citrinin resulting from the feeding of the lactone (12) contained essentially no deuterium above natural abundance. That derived from the feeding of the keto-aldehyde (13), by contrast, gave rise to a strong peak at δ 2.0 in the ^{2}H n.m.r. spectrum. By analogy with the chemical shifts in the ^{1}H n.m.r. spectrum of citrinin (Table 3) this can be shown to be due to the presence of deuterium in the C-11 methyl group of citrinin. Comparison of the intensity of this peak with that of natural abundance deuteriochloroform in the chloroform used as solvent showed that 6.5% of the administered keto-aldehyde was specificially incorporated into citrinin. This value is two orders of magnitude higher than the incorporation of the isocoumarin (10) and we are therefore confident in concluding that (13) is a true biosynthetic precursor to citrinin.

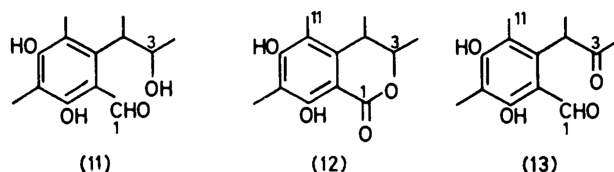
Flanking the major peak in the ^{2}H n.m.r. spectrum from this feeding are two smaller peaks whose positions correspond to the presence of deuterium at C-4 and at either C-9 or C-10. We attribute these peaks to the presence of a competing metabolic pathway in which (13) is degraded to acetate such that C-11 of (13) becomes C-2 of acetate, the acetate then being incorporated into citrinin in the usual way (Scheme 5). The ease with which this competing pathway was detected strikingly demonstrates the advantage of deuterium over other isotopes hitherto used routinely for advanced precursor studies.

These results strongly imply that (13) is the first enzyme-free intermediate in the biosynthesis of citrinin.



SCHEME 5

However, doubt remains concerning the timing of the three methylation steps. While it seems probable that all three methylations precede cyclisation it is possible that they are carried out by different enzymes in different parts of the cell which could lead to differential incorporations of labelled methionine into the carbon atoms C-10, C-11, and C-12. A precedent exists. In the biosynthesis of streptonigrin about the CMe groups which are inserted early in the biosynthesis each incorporate twice as much label from methionine as the OMe group which is added later.[14]



There are three studies in the literature of incorporation of [^{14}C]methionine into citrinin; two groups [2,3] report the presence of excess activity at C-12, while a third group [4] reports equal labelling. There are a number of difficulties associated with using a radiolabel in this context, in particular the unreliability mentioned earlier of counting activity in citrinin, and the necessity to degrade the metabolite chemically in order to test the activities of the three different carbon atoms. We decided therefore, to use ^{13}C as the label in a study of the incorporation of [^{13}C]methionine into the three C_1 units of citrinin.

First, it was necessary to establish conditions under which reproducible integrals in the ^{13}C n.m.r. spectrum of unenriched citrinin could be obtained. Using an acquisition time of 3.2 s in conjunction with a 25 s pulse delay the ratios of integrals for C-10, C-11, and C-12 in the expanded spectrum were found to be repeatable to within 10% (Table 4).

The methionine feeding was then performed using conditions similar to those used previously for acetate feedings.[10] *P. citrinum* was grown from spores on a

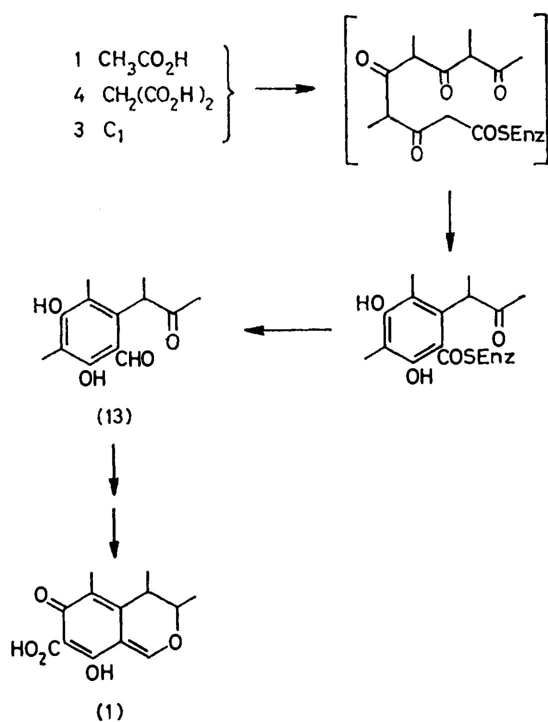
standard Czapek–Dox medium, which was replaced after eight days growth by fresh medium of lower glucose content. [*Me*-^{13}C]Methionine was administered in equal daily doses over the next eight days and citrinin was harvested after a further seven days. A ^{13}C n.m.r. spectrum showed that the enrichment of ^{13}C at C-10, C-11, and C-12 was around nine times natural abundance. When the spectrum was run under identical conditions and at similar concentration to the natural abundance sample, the ratios of the integrals of the enriched peaks due to C-10, C-11, and C-12 were found to be unchanged (Table 4).

TABLE 4

Ratios of signal intensities in the ^{13}C n.m.r. spectrum of citrinin

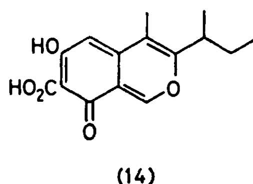
Sample	C-10	C-11	C-12
Nat. abundance	1.38	1.86	1.0
Nat. abundance	1.41	1.76	1.0
Nat. abundance	1.40	1.77	1.0
^{13}C-Enriched	1.39	1.82	1.0

We now conclude that the three methylation steps are carried out in rapid succession on the polyketide synthetase using material from the same pool of methionine.



SCHEME 6

The evidence is strong therefore that the ketoaldehyde (13) is the first enzyme-free aromatic intermediate in the biosynthesis of citrinin. Presumably it is released from the enzyme thioester, and the biosynthesis of citrinin proceeds as shown in Scheme 6. A similar mode of biosynthesis *via* a ketoaldehyde intermediate has been suggested for the hexaketide ascochitine (14).[15]



EXPERIMENTAL

Solutions were dried over magnesium sulphate or sodium sulphate (anhydrous). Analytical and preparative g.l.c. was carried out using a P & M 720 gas chromatograph with a hot wire detector. The column was ¼ in stainless steel, 5 ft 9 in long, packed with 15% silicone grease E301 (P & E) on Chromosorb P.

Radioactive samples were counted using a Packard Tricarb 3385 scintillation counter. Melting points were determined on a Kofler hot-stage apparatus. Infrared spectra were taken on a Perkin-Elmer 257 infrared spectrometer; all samples were in chloroform solution unless otherwise stated. Mass spectra were taken on an A.E.I. MS9, MS12, or MS30 mass spectrometer.

^{1}H N.m.r. spectra were run on a Varian HA 100 spectrometer, ^{2}H n.m.r. spectra on a Bruker 250 MHz spectrometer, ^{13}C n.m.r. spectra on a Varian XL 100 spectrometer.

Culture of Penicillium citrinum.—Cultures of *Penicillium citrinum* (IMI 24306) were obtained from the Commonwealth Mycological Institute, Kew. These were subcultured onto agar slopes made from one pellet of Czapex–Dox agar (Oxoid) and 4 cm^3 of distilled water, sterilised at 15 lbf in^{-2} for at least 15 min. After 14 days growth at 25 °C these stock cultures were stored at 4 °C.

Large-scale Growth of P. citrinum.—A liquid Czapex–Dox medium was made up as follows: glucose (300 g), sodium nitrate (12 g), potassium dihydrogen phosphate (6 g), potassium chloride (3 g), $MgSO_4 \cdot 7H_2O$ (3 g), $Fe_2SO_4 \cdot 7H_2O$ (0.06 g), $ZnSO_4 \cdot 7H_2O$ (0.06 g), and $CuSO_4 \cdot 5H_2O$ (0.03 g), made up to 6 l with distilled water, and sterilised.

P. citrinum was introduced as a vegetative innoculum from agar slopes and grown in stationary culture. Advanced precursors were introduced after 24 days in aqueous solution containing up to 20% dioxan or ethanol to aid solubility. Final concentrations of radioactive precursors were about 5 μCi l^{-1} of culture medium, of deuteriated precursors 100 mg l^{-1}. Citrinin was harvested 10 days after addition of precursors.

Harvesting and Isolation of Citrinin.—The mycelium was removed by gravity filtration of the medium through a glass-wool plug. The medium was reduced to *ca.* ¼ volume by evaporation then acidified to pH 2 with concentrated hydrochloric acid. Two alternative procedures were used from this point.

Method 1. The acidified mixture was left to stand at 4 °C for 15 h then filtered. The filtrate was discarded and the residue dried. The crude solid was extracted with benzene in a Soxhlet apparatus. The extract was evaporated and recrystallised.

Method 2.—The acidified mixture was extracted with ethyl acetate. The ethyl acetate solution was washed with water then re-extracted into saturated sodium hydrogencarbonate solution. Acidification (conc. HCl) and re-extraction into ethyl acetate gave a solution which on drying and evaporation gave citrinin, which was recrystallised.

Yields in both cases were 0.5 g per litre of culture medium; m.p. of citrinin 170—177 °C (decomp.), (lit.,[2] 169—171 °C). In feedings of radioactive precursors it was recrystallised twice from ethanol.

Degradation of Citrinin.—Method A. 3-(3,5-*Dihydroxy*-2-*methylphenyl*)*butan*-2-*ol* ('*Phenol A*'). Citrinin (1.0 g, 4 mmol) was heated with sodium hydroxide solution (10%; 4 ml) under reflux in an atmosphere of nitrogen for 4 h; an orange solution was formed. After cooling, a saturated solution of barium hydroxide was added until precipitation of barium carbonate was complete; it was then filtered. The residue contains the 7-carboxy-group of citrinin as barium carbonate. The pH of the filtrate was adjusted to 7.5—8.0 with hydrochloric acid and the solution was then extracted with ether (4 × 50 ml). Evaporation of the combined ether solutions gave a golden gum which crystallised on scratching to give 3-(3,5-dihydroxy-2-methylphenyl)butan-2-ol (phenol A) as a pale orange solid (0.77 g, 98%), which was recrystallised from chloroform as needles with 1 mol of chloroform of crystallisation, m.p. 128—129 °C [lit.,[3] 132—134 °C (from toluene)]; $\lambda_{max.}$ 224 (sh) and 280 nm; $\nu_{max.}$ (Nujol) 3 400—2 600, 1 610 (s), and 1 510 cm^{-1}; δ 7.82 (1 H, s), 6.25 (1 H, d, *J* 3 Hz), 6.16 (1 H, d, *J* 3 Hz), 3.85 (1 H, m), 3.05 (1 H, m), 2.06 (3 H, s), 1.12 (3 H, d, *J* 7 Hz), and 1.11 (3 H, d, *J* 5 Hz); *m/e* 196 (M^+), 152, 151 (100%), 137, and 123. The aqueous fraction was acidified to pH 1 with hydrochloric acid and steam-distilled. The distillate was titrated against 0.1M-sodium hydroxide solution with phenolphthaleïn as indicator. After evaporation, the residue contained C-1 of citrinin as sodium formate.

3-(3,5-*Dimethoxy*-2-*methylphenyl*)*butan*-2-*ol*. Phenol A (0.26 g, 1.3 mmol) was dissolved in dry acetone. Potassium carbonate (1.20 g) and dimethyl sulphate (0.47 ml, 0.64 g, 5 mmol) were added and the mixture refluxed for 3 h. It was then cooled and filtered; water (15 ml) was added and the reaction left to stand for 15 h. The mixture was then concentrated and extracted with ether (2 × 20 ml); the ether extracts were washed with sodium hydroxide solution (10%; 10 ml), dried, and evaporated to give a yellow oil which was purified by preparative t.l.c. using chloroform–ether (1 : 1 v/v) to give 3-(3,5-dimethoxy-2-methylphenyl)-butan-2-ol as an oil (0.257 g, 86%) which distilled at 110 °C and 0.5 Torr;[3] $\lambda_{max.}$ 223 (sh), 278 (sh), and 283 nm; $\nu_{max.}$ 1 600 cm^{-1}; δ 6.42 (1 H, *J* 2 Hz), 6.33 (1 H, d, *J* 2 Hz), 3.85 (1 H, m), 3.76 (6 H, s), 3.05 (1 H, m), 2.13 (3 H, s), 1.85 (1 H, broad), 1.24 (3 H, d, *J* 7 Hz), and 1.17 (3 H, d, *J* 7 Hz).

3-(3,5-*Dimethoxy*-2-*methylphenyl*)*butan*-2-*one*. Chromium trioxide (0.9 g, 9 mmol) was added to a solution of pyridine (1.5 ml, 1.5 g, 18.5 mmol) in dry dichloromethane (25 ml) and the mixture was stirred for 0.3 h at 20 °C. 3-(3,5-Dimethoxy-2-methylphenyl)butan-2-ol (0.26 g, 1.2 mmol) in dry dichloromethane (10 ml) was added and stirring continued for 0.3 h while a black tar formed. The solution was decanted and the residual tar washed with ether and water (×2). The combined organic solutions were washed with sodium hydroxide solution (5%; 3 × 40 ml), sulphuric acid (5%; 40 ml), and sodium hydrogencarbonate solution (10%; 40 ml), dried, and evaporated to give a pale yellow oil (0.22 g, 85%). Preparative t.l.c. using chloroform as eluant gave 3-(3,5-dimethoxy-2-methylphenyl)butan-2-one as a pale oil (0.2 g, 78%); $\lambda_{max.}$ 285 nm; $\nu_{max.}$ 1 710 and 1 610 cm^{-1}; δ 6.36 (1 H, d, *J* 2 Hz), 6.18 (1 H, d, *J* 2 Hz), 3.94 (1 H, q, *J* 6 Hz), 3.78 (3 H, s), 3.72 (3 H, s), 2.14 (3 H, s), 1.98 (3 H, s), and 1.32 (3 H, d, *J* 6 Hz). The semicarbazone of the product was prepared by adding the butanone (0.1 g, dissolved in the minimum quantity of methanol) to a saturated solution of semicarbazide (0.2 g) in water buffered with sodium acetate (0.3 g). The semicarbazone was recrystallised from ethyl acetate or methanol as needles, m.p. 204—208 °C, (lit.,[3] 198—198.5 °C) (Found: C, 60.1; H, 7.45; N, 14.9%. $C_{14}H_{21}N_3O_3$ requires C, 60.26; H, 7.58; N, 15.04%).

3-(3,5-*Dimethoxy*-2-*methylphenyl*)-2-*phenylbutan*-2-*ol*. Phenylmagnesium bromide was prepared as follows. Magnesium turnings (0.46 g, 0.02 mol) were placed in a flask with dry ether (20 ml) and stirred vigorously. A solution of bromobenzene (3.2 g, 0.02 mol) in dry ether (20 ml) was added at such a rate as to maintain gentle boiling. 3-(3,5-Dimethoxy-2-methylphenyl)butan-2-one (0.4 g, 1.8 mmol) was dissolved in dry ether (20 ml) and phenylmagnesium bromide solution was added to the stirred solution until present in excess. After stirring for 1.5 h, the reaction was poured into ammonium sulphate solution (10%; 50 ml); more ether was added and the phases separated. The ether solution was washed with sodium hydroxide solution (10%; 50 ml), dried, and evaporated to give a yellow oil which was purified by preparative t.l.c. (chloroform as eluant) to give 3-(3,5-*dimethoxy*-2-*methylphenyl*)-2-*phenylbutan*-2-*ol* as an oil, (0.042 g, 84%) which distilled at 170 °C and 0.1 Torr (Found: C, 75.75; H, 8.2%; *m/e*, 300.1723. $C_{19}H_{24}O_3$ requires C, 75.97; H, 8.05%; *m/e* 300.1725); $\lambda_{max.}$ 279—285 nm; $\nu_{max.}$ 3 600 (m), 3 500—3 200 (br), 1 600 (s), 1 460 (s), and 1 150 cm^{-1}; δ 7.6—7.2 (complex, 5 H), 6.60 (1 H, d, *J* 2 Hz), 6.40 (1 H, d, *J* 2 Hz), 3.81 (6 H, s), 3.76 (1 H, s), 3.59 (1 H, q, *J* 7 Hz), 2.24 (3 H, s), 1.92br (1 H, s, exchanges with D_2O), 1.36 (3 H, s), and 1.08 (3 H, d, *J* 7 Hz); *m/e* 300 (M^+), 238, 224, 180, 165, and 151.

cis- *and* trans-3,5-*Dimethoxy*-2,α,α'-*trimethylstilbene*. 3-(3,5-Dimethoxy-2-methylphenyl)-2-phenylbutan-2-ol (0.162 g, 0.57 mmol) was dissolved in trifluoroacetic acid (15 ml) and the solution stirred at 20 °C for 0.3 h. Evaporation gave a red oil which was purified by preparative t.l.c. using chloroform–benzene (1 : 1 v/v) as eluant to give a mixture (not separated) of cis- and trans-3,5-*dimethoxy*-2,α,α'-*trimethylstilbenes* (0.12 g, 79%) which distilled at 100 °C and 0.5 Torr (Found: C, 80.95; H, 8.1%; *m/e*, 282.1622. $C_{19}H_{22}O_2$ requires C, 80.81; H, 7.85%; *m/e*, 282.1619); $\lambda_{max.}$ 223, 278, and 284 nm; $\nu_{max.}$ 2 920, 2 830, 1 600, 1 460, 1 370, 1 140, and 1 070 cm^{-1}; δ (isomer 1) 6.98 (5 H, s), 6.16 (1 H, d, *J* 2 Hz), 6.03 (1 H, d, *J* 2 Hz), 3.68 (3 H, s), 3.60 (3 H, s), 2.14 (3 H, s), 2.04 (3 H, s), and 1.90 (3 H, s); δ (isomer 2) 7.20 (5 H, s), 6.40 (1 H, d, *J* 2 Hz), 6.32 (1 H, d, *J* 2 Hz), 3.82 (6 H, s), 2.08 (3 H, s), 1.79 (3 H, s), and 1.73 (3 H, s); *m/e* 282 (M^+, 100%), 268, 267, 253, 251, and 179.

Ozonolysis of the stilbenes; acetophenone isolation. A solution of 3,5-dimethoxy-2,α,α'-trimethylstilbene (*cis–trans*-mixture) (0.06 g, 0.27 mmol) in dry ethyl acetate (2 cm^3) was cooled to −70 °C (under a dry atmosphere) and ozonised until the solution became pale blue. The excess of ozone was flushed out with nitrogen, and dimethyl sulphide (1 cm^3, 0.85 g, 1.4 mmol) was added. The solution was allowed to warm to 20 °C; when it was no longer oxidising, it was concentrated to *ca.* 0.3 cm^3 under a stream of nitrogen

Acetophenone was separated from other components of the residue by preparative g.l.c.; its retention time was 2 min. The yield was *ca.* 0.04 g (0.036 g = 100%); no 3,5-dimethoxy-2-methylacetophenone could be detected.

Acetophenone was dissolved in methanol (0.3 cm^3) and a solution of 4-phenylsemicarbazide (0.15 g, 1 mmol) and sodium acetate (0.15 g) in water (saturated with 4-phenylsemicarbazone) was added. The mixture was allowed to stand at 20 °C for 15 h, then the white precipitate was filtered off, washed with water, dried, and recrystallised

from methanol to give *acetophenone 4-phenylsemicarbazone* as needles, m.p. 186—189 °C (Found: C, 70.8; H, 6.15; N, 16.65. $C_{15}H_{15}N_3O$ requires C, 71.12; H, 5.97; N, 16.59%).

Method B. Kuhn–Roth oxidation. Citrinin (80 mg) was heated at 100 °C with 5M-chromic acid (10 ml) and concentrated sulphuric acid (2 ml) for 1½ h. After cooling, the product was steam-distilled (*ca.* 500 ml collected) and the distillate boiled to remove carbon dioxide, cooled, and neutralised with 0.1M-sodium hydroxide using phenolphthalein as indicator. The solution was then evaporated to dryness.

p-*Bromophenacyl Acetate.* (*a*) 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 mixture refluxed for 2 h. The ethanol was then evaporated off and the residue dissolved in ether (10 ml) and water (10 ml). The aqueous layer was extracted into ether (2 × 10 ml) and the combined organic layers dried and evaporated to give a white crystalline solid which was purified by preparative t.l.c. on silica gel using ethyl acetate–benzene (1 : 9 v/v) 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.,[16] 86 °C).

(*b*) The residue from the Kuhn–Roth oxidation was dried thoroughly, then stirred with *p*-bromophenacyl bromide (50 mg) in dry dimethylformamide (15 ml) at 90 °C for 2 h. The solvent was then removed, the residue dried at high vacuum, and then extracted into ether. The ether-soluble portion was purified by preparative t.l.c. as above.

The first method, although less reliable in that yields of product were low (it was necessary to ensure that the pH was *ca.* 8—9 throughout, adding more *p*-bromophenacyl bromide as required), gave purer product than the second method.

Radiochemical Synthesis of 3,4,5,6-*Tetramethyl*-6,8-*dihydroxyisocoumarin.—Ethyl 2-methyltriphenylphosphorylidene acetate.* Ethyl triphosphorylidene acetate (69.5 mg, 2 mmol) in dry ethyl acetate (10 ml) was heated to reflux with stirring. A solution of methyl iodide (1 ml) in ethyl acetate (10 ml) was prepared; 0.1 ml of this solution was added to the reaction mixture. [^{14}C]Methyl iodide (500 μCi; 59.7 mCi mmol^{-1}) was vacuum-transferred onto a solution of methyl iodide (0.1 ml) in ethyl acetate (1 ml) and the resulting mixture allowed to equilibrate by allowing it to warm to room temperature then refreezing twice. The chilled solution was then injected into the reaction mixture and the flask residues rinsed in with a further 1 ml of ethyl acetate. After refluxing for 1 h, the reaction was completed by addition of methyl iodide solution (0.5 ml), then stirred at reflux for a further 1 h; a gummy solid separated. The solvent was removed *in vacuo* and the residual gum (1.02 g) dried at high vacuum, then dissolved in water (10 ml) containing tetrahydrofuran (1 ml) and treated with dilute sodium hydroxide solution (10%) until an alkaline reaction to phenolphthalein was obtained. The ylide which precipitated was extracted into ethyl acetate (4 × 15 ml), washed with saturated brine (10 ml), dried, and evaporated to give a yellow crystalline solid (637 mg, 88%) which was recrystallised from ethyl acetate–light petroleum (b.p. 40—60 °C) as a yellow powder, m.p. 155—157 °C (lit.,[17] 156—157 °C); $\lambda_{max.}$ 225 and 268 nm; $\nu_{max.}$ 1 605 (s) and 1 109 cm^{-1}; δ 7.95—7.93 (15 H, m, ArH), 3.93 (2 H, q, *J* 7 Hz, CO_2CH_2-CH_3), 1.98 (3 H, d, *J* 15 Hz, $PCCH_3$), and 1.34 (3 H, t, *J* 7 Hz, $CO_2CH_2CH_3$); *m/e* 362.

Ethyl 2-methylcrotonate. Ethyl 2-methyltriphenylphosphorylidene acetate (390 mg) was dissolved in dry dichloromethane (30 ml) and cooled to 0° C. Freshly distilled acetaldehyde (0.2 ml, 0.16 g) was added dropwise with stirring, then the reaction mixture was stirred for a further 18 h. The solvent was removed at atmospheric pressure and the residual solid extracted with light petroleum (b.p. 40—60 °C). Triphenylphosphine was removed by filtration, and evaporation of the solvent as before gave crude ethyl 2-methylcrotonate as a yellow oil (110 mg, 78%) which was purified by distillation at 60 °C and 16 Torr (lit.,[18] 156 °C at 752 torr, 55 °C at 11 Torr); $\nu_{max.}$ 1 705 cm^{-1}; δ 3.02 (1 H, m, olefinic H), 5.82 (2 H, q, $CO_2CH_2CH_3$), 8.18br (3 H, s, 2-Me), 8.24 (3 H, d, *J* 5 Hz, 3-Me), and 1.30 (3 H, t, *J* 7 Hz, $CO_2CH_2CH_3$); *m/e* 128.

2-*Methylcrotonic acid* (*tiglic acid*). Crude ethyl 2-methylcrotonate (110 mg) was refluxed with sodium hydroxide solution (10%; 10 ml) for 3—4 h until there were no oily droplets left on the surface of the solution. The solution was then cooled and extracted with ether (3 × 15 ml). The aqueous layers were acidified with dilute hydrochloric acid and re-extracted into ether (3 × 10 ml). The combined organic extracts were dried and evaporated to give a semi-solid (76 mg, 75%) which was recrystallised from water as needles, m.p. 63—64 °C (lit.,[19] 62—64 °C). 2-Methylcrotonyl chloride was prepared from 2-methylcrotonic acid by treatment with an excess of thionyl chloride; the crude acid chloride was used in a Friedel–Crafts reaction with an excess 1,3-dimethyl-2,4-dimethoxybenzene to give 2,3,4,6-tetramethylindan-1-one. This compound was then used for the rest of the radiochemical synthesis as described in reference 8.

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