# Biosynthesis of Citrinin and Synthesis of its Biogenetic Precursors

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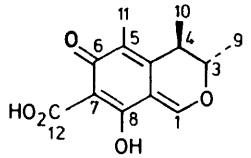
The biosynthetic pathway to citrinin has been elucidated by incorporation studies with advanced precursors. These specifically labelled compounds were obtained from labelled citrinin produced by cultures of *Penicillium citrinum* in the presence of [Me-$^{14}$C]methionine. The method for testing incorporation specificity is also described.

THE fungal metabolite citrinin (1) has been the subject of extensive chemical [1] and biochemical investigations. Incorporation studies [2] with $^{14}$C-labelled substrates have established that (1) is derived from a single pentaketide chain, composed of head-to-tail acetate units and three $C_1$ units. An investigation [3] of metabolites of the wild type of *P. citrinum* and of related mutants was carried out in an attempt to distinguish alternative pathways of biosynthesis. Recently Staunton and co-workers found that 6,8-dihydroxy-3,4,5,7-tetramethylisocoumarin [4] (2) and 4,6-dihydroxy-3,5-dimethyl-2-(1-methyl-2-oxo-propyl)benzaldehyde [5] (3) were specifically incorporated into citrinin by *P. citrinum* although only the latter is a true biosynthetic intermediate. In fact compound (2) is not an obligatory intermediate [6] because the proton at C-4 derives from the original polyketide and not from an external reducing agent. The most probable sequence of reactions involved in citrinin biosynthesis, resulting from incorporation of the potential advanced precursors (2)—(8), was described in a preliminary communication.[7]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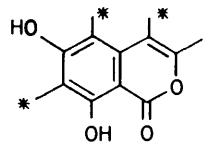
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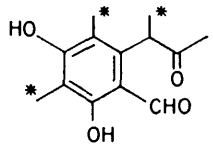
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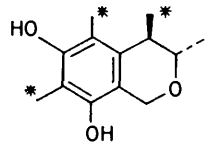
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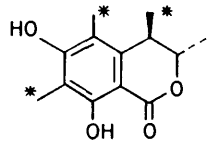
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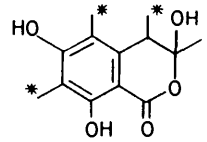
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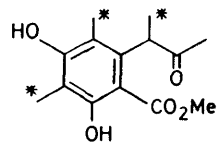
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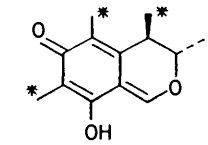

(8)

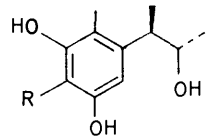
* = $^{14}$C

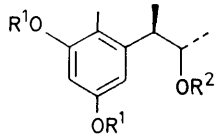
## RESULTS AND DISCUSSION

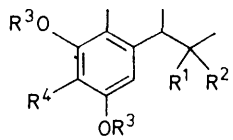
Various synthetic approaches were tried to synthesize the potential biogenetic intermediates of (1) in a convenient way to introduce labelling.

Citrinin was treated with alkali to obtain the alcohol (9) which was in turn carboxylated in glycerol with $CO_2$ and $NaHCO_3$ to yield the acid (10). Reduction of (10) with sodium bis-(2-methoxyethoxy)aluminium dihydride produced the alcohol (11). However, both the low overall yield and the high quantity of reagents ($CO_2$ and $NaHCO_3$) required for the carboxylation reaction, made the above mentioned scheme impracticable for the possible introduction of the labelling. We devised another way to introduce the labelling by means of a direct methylation of the aromatic nucleus with [$^{14}$C]methyl iodide. This method involved metallation of the carbon atom between the two hydroxy-groups which therefore had to be protected.

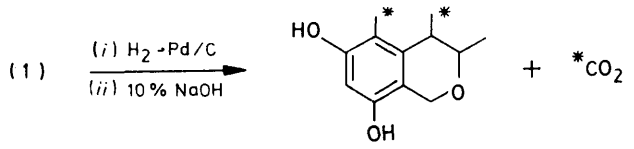
The choice of the protective groups was made taking


(9) R = H
(10) R = $CO_2H$
(11) R = Me


(12) $R^1$ = MEM; $R^2$ = THP
(18) $R^1$ = MEM; $R^2$ = H


(13) $R^1$,$R^2$ = O; $R^3$ = H ; $R^4$ = H
(14) $R^1$,$R^2$ = $SCH_2CH_2S$; $R^3$ = H ; $R^4$ = H
(15) $R^1$,$R^2$ = $SCH_2CH_2S$; $R^3$ = Me ; $R^4$ = H
(17) $R^1$,$R^2$ = O; $R^3$ = H ; $R^4$ = Me

into account the feasibility of their removal. Upon treating alcohol (9) with β-methoxyethoxymethyl (MEM) chloride under phase-transfer conditions with tetrabutylammonium hydroxide, the two phenolic groups were converted into MEM ethers. The hydroxy-group of the side chain was transformed into its THP ether under usual conditions. The compound (12), treated with n-butyl-lithium in cyclohexane and then with methyl iodide, was not transformed into the desired product but into a complex reaction mixture. We therefore modified the protective groups to obtain an *O*-methylated product, in a manner similar to that successfully used in the case of ascochitine.[8] Alcohol (9) was then oxidized to ketone (13) by Jones reagent at low temperature (−40 °C) and this compound converted into its ethylene thioacetal (14). Finally methylation of the phenolic groups under phase-transfer conditions produced compound (15) on which metallation was ineffective. This result is probably due to the polarization induced by the methyl group *meta* to the metallation site. Labelling was then introduced by using citrinin produced in the presence of [Me-$^{14}$C]-methionine and synthesizing all compounds (2)—(8) from citrinin having equally labelled C-9, C-10, and C-12 carbon atoms. The specificity of incorporation was tested in a very simple way: reduction of (1) to dihydrocitrinin, and subsequent decarboxylation by alkaline treatment to compound (16) (Scheme 1). Acid was




SCHEME 1

added to the basic mixture; the liberated $CO_2$ was quantitatively absorbed by hyamine hydroxide and counted. Compound (16) was acetylated to facilitate isolation and counted. In the case of specific incorporation, the percentage recovery of label in $CO_2$ is half that in compound (16). The synthesis of labelled intermediates was performed as follows: citrinin, after hydrogenation and treatment with $CH_2N_2$, was reduced to (4) with sodium bis-(2-methoxyethoxy)aluminium dihydride; lactone (5) was obtained from (4) as previously described.[3] Treatment with aqueous alkali led to alcohol (11), which was oxidized to ketone (17) with Jones reagent. Conversion of (17) into aldehyde (3) was accomplished with triethyl orthoformate and $AlCl_3$ while (11) was converted into the quinone methide (8) with triethyl orthoformate and HCl. Lactol (6) was obtained by treating aldehyde (3) with $NaClO_2$ and $NH_2SO_3H$. Methyl ester (7) resulted from the treatment of lactol (6) with diazomethane. Dehydration of (6) with toluene-*p*-sulphonic acid in toluene gave the enol-lactone (2).

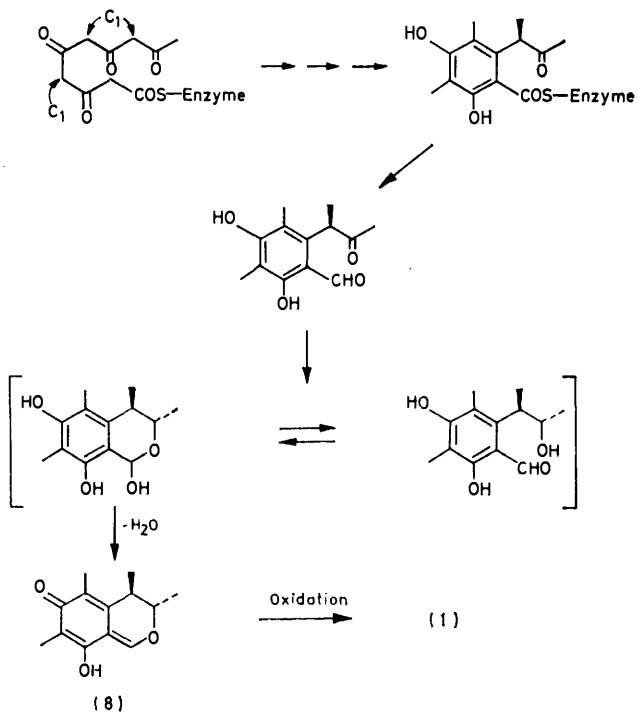
The labelled compounds (2)—(8) were added to intact surface cultures of *P. citrinum* (60 h culture broths). Four and a half days after the addition, compound (1) was isolated as previously described.[9] The contact between the mycelium and the precursor-containing medium was facilitated by mild magnetic stirring. Markedly lower incorporation percentage rates were observed when the above procedure was not followed. Percentage recovery data of label in $CO_2$ and in (16) (Table 1) indicate that some minor scrambling of the label occurred. Therefore a partial degradation of the intermediates to acetate prior to their incorporation cannot be excluded, as already shown by other authors.[5]

TABLE 1

Incorporation of the advanced precursors (2)—(8) into (1)

| Experiment | Precursor | Incorporation into (1) (%) | Recovery of label in $CO_2$ (%) | Recovery of label in (16) (%) |
|---|---|---|---|---|
| 1 | $^{14}$Me-Methionine | 22.50 | 33 | 66 |
| 2 | (2) | 0.04 | 31 | 67 |
| 3 | (3) | 16.85 | 30 | 68 |
| 4 | (4) | 0.05 | 31 | 67 |
| 5 | (5) | 0.00 | | |
| 6 | (6) | 0.00 | | |
| 7 | (7) | 0.10 | 32 | 64 |
| 8 | (8) | 5.16 | 32 | 65 |

On the basis of our incorporation data and the results recently obtained by Staunton,[4-6] the most probable sequence of reactions and intermediates involved in citrinin biosynthesis is that shown in the Scheme 2.




SCHEME 2

## EXPERIMENTAL

*Culture Conditions.*—Previously reported [9] culture conditions were used. After 60-h fermentations, labelled compounds (2)—(8) were added to the cultures in acetone or dimethyl sulphoxide solution. Four and a half days after

the addition, (1) was isolated as previously described.[9] Table 2 reports the amounts and the radioactivity values of labelled compounds incubated and of citrinin obtained.

*Incorporation Specificity.*—A solution of citrinin (0.15 g) in tetrahydrofuran (30 ml) was treated with hydrogen, in the presence of 10% Pd–C (0.05 g), for 30 min at room temperature and pressure. The mixture was then filtered to give dihydrocitrinin. This product was treated with aqueous 10% NaOH solution (7.5 ml) and refluxed for 2 h. The cooled solution was then acidified dropwise with dilute HCl while a nitrogen stream was blown through: the $CO_2$ thus liberated was quantitatively absorbed by hyamine hydroxide [10] and counted. The acidified solution was extracted with ethyl acetate and the organic extracts were dried ($Na_2SO_4$) and evaporated. Reaction of the residue (0.105 g) with acetic anhydride (0.1 ml) in pyridine (0.5 ml) gave the *diacetyl*-(16) as an oil (0.093 g), which was purified by flash chromatography [11] [n-hexane–ethyl acetate (7 : 3)] to constant activity; δ ($CDCl_3$) 6.62 (1 H, s, Ar-H), 4.44 (2 H, s, ArC$H_2$), 3.78 (1 H, dq, CH–O, $J_1$ 8, $J_2$ 4 Hz), 2.55 (1 H, dq, ArC$H$, $J_1$ 8, $J_2$ 4 Hz), 2.16, 2.10, and 1.96 (3 H, s, COMe; 3 H, s, COMe, and 3 H, s, ArMe), 1.15 and 1.10 (3 H, d, *Me*CH, and 3 H, d, *Me*CH); *m/e* (%) 292 ($M^+$, 2), 250(4), 233(15), 208(37), 191(31), 190(27), and 164(100).

TABLE 2

| Compound | Quantity added (g) | Radioactivity (disint. $min^{-1}$ $mg^{-1}$) | Isolated (1)/g | Radioactivity of (1) (disint. $min^{-1}$ $mg^{-1}$) |
|---|---|---|---|---|
| $^{14}$Me-Methionine | 0.004 | 30,142,833 | 0.166 | 163 425 |
| (2) | 0.086 | 2,441,862 | 0.400 | 210 |
| (3) | 0.069 | 2,254,131 | 0.450 | 58 240 |
| (4) | 0.070 | 2,570,806 | 0.150 | 600 |
| (5) | 0.065 | 2,420,038 | 0.130 | |
| (6) | 0.075 | 2,262,309 | 0.270 | |
| (7) | 0.085 | 2,143,800 | 0.250 | 729 |
| (8) | 0.060 | 2,594,736 | 0.120 | 66 944 |

*Synthesis of Precursors.*—Mass spectra were recorded with a Varian MAT 112 spectrometer, i.r. spectra with a Perkin-Elmer 257 spectrophotometer, and n.m.r. spectra with a Varian XL-100 (100 MHz) instrument. Kieselgel 60 $F_{254}$ (Merck) was used for t.l.c.; 70—230 mesh silica gel (Merck) was used for column chromatography and 40—63 mesh silica gel (Merck) for flash chromatography.[11] Physical and spectroscopic data for compounds (2), (4), (5), (6), (9), (10), (11), and (13) have previously been reported.[3] The preparation of compound (11) from compound (9) is described in ref. 3, but we modified the reduction of the acid (10) to (11) by using sodium bis-(2-methoxyethoxy)-aluminium dihydride instead of lithium aluminium hydride. The synthesis of (5) from (4) is also reported in the above-mentioned reference.

3-[3,5-*Bis*-(β-*methoxyethoxymethoxy*)-2-*methylphenyl*]-*butan*-2-*ol* (18).—A solution of (9) (0.4 g) in methylene chloride (16 ml) was treated with tetrabutylammonium hydroxide (40% in water, 3.2 g) and β-methoxyethoxymethyl chloride (3.05 g) at 0 °C. The mixture was then stirred at room temperature for 5 min. The organic phase was separated and washed with saturated brine while the aqueous phase was washed with methylene chloride. The two organic extracts were dried ($Na_2SO_4$) and evaporated. The crude product was chromatographed (methylene chloride) to give (18) as a colourless oil (0.302 g, 40%); δ ($CDCl_3$), 6.80—6.60 (2 H, m, Ar-H), 5.22 (2 H, br s, $OCH_2O$), 5.19 (2 H, br s, $OCH_2O$), 3.93—3.35 (8 H, m, $OCH_2CH_2O$), 3.02 (1 H, dq, C*H*OH, $J_1 = J_2 = 7$ Hz), 2.15 (4 H, m, ArCH and ArMe), 1.65 (1 H, m, OH), 1.23 (3 H, d, MeCH, $J$ 7 Hz), and 1.14 (3 H, d, MeCH, $J$ 7 Hz).

This product (0.025 g), treated with pyridinium chlorochromate and sodium acetate in methylene chloride, gave a product which, after preparative t.l.c., showed a band at 1 710 $cm^{-1}$ [ν(C=O)] in its i.r. spectrum.

2-[3,5-*Bis*-(β-*methoxyethoxymethoxy*)-2-*methylphenyl*]-1-*methylpropyl Tetrahydropyran*-2-*yl Ether* (12).—To a solution of the alcohol (18) (0.054 g) in dry dioxan (1 ml), toluene-*p*-sulphonic acid (0.002 g) and freshly distilled dihydropyran (0.042 ml) were added under nitrogen. The mixture was stirred for 1 h at room temperature and then treated with triethylamine (pH 7.5). The solution was evaporated, and the residue dissolved in methylene chloride, washed with aqueous $NaHCO_3$, and the organic phase dried ($Na_2SO_4$) and evaporated. The crude product was chromatographed (methylene chloride) to give (12) (0.037 g, 57%); *m/e* (%) 354(4), 281(5), and 71(100).

3-(3,5-*Dihydroxy*-2-*methylphenyl*)*butan*-2-*one* (13).—A solution of alcohol (9) (0.3 g) in acetone was treated at −20 °C with Jones reagent. After 5 min at −20 °C the excess of reagent was decomposed with isopropyl alcohol. The resulting mixture was worked up as usual and the residue was chromatographed [n-heptane–ethyl acetate (6 : 4)] to give (13) (0.11 g, 37%).

2-[1-(3,5-*Dihydroxy*-2-*methylphenyl*)*ethyl*]-2-*methyl*-1,3-*dithiolan* (14).—A solution of the ketone (13) (1.09 g) in acetic acid (15 ml) was treated with ethane-1,2-dithiol (2 ml) and $BF_3$–ether. After 30 min at reflux the solution was cooled and neutralized with 5% aqueous $NaHCO_3$. The mixture was extracted with methylene chloride and the organic phase dried ($Na_2SO_4$) and evaporated. The residue was purified by column chromatography [benzene–ethyl acetate (95 : 5)] to yield (14) (1.44 g, 95%); δ ($CDCl_3$) 6.75 (1 H, d, Ar-H, $J$ 2.5 Hz), 5.99 (1 H, d, Ar-H, $J$ 2.5 Hz), 3.63 (1 H, q, C*H*Me), 2.16 (3 H, s, ArMe), 1.75 (3 H, s, CMe), and 1.51 (3 H, d, CHMe); *m/e* (%) 270 ($M^+$, 9), 255(5), 151(7), and 119(100).

2-[1-(3,5-*Dimethoxy*-2-*methylphenyl*)*ethyl*]-2-*methyl*-1,3-*dithiolan* (15).—A solution of (14) (1.4 g) in methylene chloride (25 ml) and water (25 ml) containing benzyltributylammonium chloride (0.320 g) was treated with dimethyl sulphate (2.2 ml). After stirring at room temperature for 12 h the organic phase was separated while the aqueous layer was extracted with methylene chloride. The organic extracts were collected, dried ($Na_2SO_4$), and evaporated. The residue was dissolved in water and extracted with ether. The ethereal phase was washed with 2N ammonia and saturated brine, dried ($Na_2SO_4$), and evaporated. The crude product was chromatographed [n-hexane–ethyl acetate (9 : 1)] to give (15) (1.166 g, 75%); *m/e* (%) 298 ($M^+$, 9), 179(8), and 119(100).

3,4-*Dihydro*-6,8-*dihydroxy*-3,4,5,7-*tetramethylisochroman*

(4).—Citrinin (1) (11 g) was treated with hydrogen as above. Crude dihydrocitrinin was then dissolved in ether (50 ml) and treated with $CH_2N_2$ at 0 °C. The resulting solution was evaporated and the residue was chromatographed (methylene chloride) to give a product (10.94 g), which was treated with sodium bis-(2-methoxyethoxy)aluminium dihydride [70% in benzene, 4.4 ml] in toluene at 80 °C. The mixture was stirred at 120 °C for 1 h. After cooling the excess of reducing agent was decomposed with aqueous 20% $H_2SO_4$. The organic phase was extracted with aqueous 10% NaOH and the aqueous extracts were acidified with HCl and extracted with ether. The organic phases were dried ($Na_2SO_4$) and evaporated. The crude mixture was chromatographed: elution with n-heptane–ethyl acetate (8 : 2) gave (4) (8.5 g, 87%).

3-(3,5-*Dihydroxy*-2,4-*dimethylphenyl*)*butan*-2-*ol* (11).—A suspension of lactone (5) (5.03 g) in 10% aqueous NaOH (70 ml) was refluxed for 2 h. The cooled solution was acidified (pH 3) with 1M HCl and extracted with methylene chloride. The organic phase was dried ($Na_2SO_4$) and evaporated. The residue was purified by flash chromatography [n-hexane–ethyl acetate (1 : 1)] to give (11) (4.76 g, 94%).

3,4-*Dihydro*-8-*hydroxy*-3,4,5,7-*tetramethyl*-6-*oxo*-6H-2-*benzopyran* (8).—A solution of (11) (0.150 g) in ether (3.5 ml) and triethyl orthoformate (0.7 ml) was treated with hydrogen chloride for 15 s. Ether (10 ml) was added to the mixture and the precipitate was collected, washed with ether and dried under vacuum to yield the quinone methide (8) (0.140 g, 95%); δ ($C_5D_5N$), 6.85 (1 H, s, ArC*H*O), 4.18 (1 H, m, OC*H*Me) 2.50 (1 H, m, C*H*Me), 2.23 and 2.03 (3 H, s, ArMe and 3 H, s, ArMe), 1.13 and 1.08 (3 H, d, MeCH and 3 H, d, MeCH); *m/e* 220 ($M^+$), 205, 202, and 191.

Compound (8) was an unstable yellow amorphous solid; when this product (50 mg) in ethanol (20 ml) was shaken with hydrogen and palladised charcoal for 5 h and worked up in the usual way, the isochroman (4) was obtained as a white crystalline solid, m.p. 176 °C (benzene).

3-(2,4-*Dimethyl*-3,5-*dihydroxyphenyl*)*butan*-2-*one* (17).—A solution of alcohol (11) (4.5 g) in acetone (600 ml) was treated at −40 °C with Jones reagent (5.58 ml). After 20 min at −45 °C the excess of reagent was decomposed with isopropyl alcohol. The reaction mixture was diluted with water and filtered through a Florisil cake. The solution was reduced by evaporation to a small volume and extracted with ethyl acetate. The organic phase was dried ($Na_2SO_4$) and evaporated, and the crude product was purified by flash chromatography [n-hexane–ethyl acetate (65 : 35)] to give unreacted (11) (1.04 g, 23%) and the *ketone* (17) (1.26 g, 28%), m.p. 128—130 °C (n-hexane–diethyl ether) (Found: C, 69.0; H, 7.9. $C_{12}H_{16}O_3$ requires C, 69.25; H, 7.76%); δ ($CDCl_3$) 6.75 (1 H, s, ArH), 4.03 (1 H, m, ArCH), 2.30, 2.22, 2.12 (3 H, s, ArMe; 3 H, s, ArMe; 3 H, s, MeCO), 1.37 (3 H, d, MeCH, *J* 7 Hz); *m/e* 208 ($M^+$), 179 and 165.

4,6-*Dihydroxy*-3,5-*dimethyl*-2-(1-*methyl*-2-*oxopropyl*)*benzaldehyde* (3).—A solution of (17) (1.3 g) in benzene (50 ml) was treated at 0 °C with triethyl orthoformate (28 ml) and $AlCl_3$ (8.7 g). The mixture was stirred at 0 °C for 2 h and quenched with water (80 ml). The organic phase was separated and the aqueous layer was extracted with ethyl acetate. The organic extracts were collected, dried ($Na_2SO_4$), and evaporated. The crude mixture was purified by flash chromatography [benzene–ethyl acetate (86 : 14)] to give (3) as a white amorphous solid (1.03 g, 70%); $\nu_{max.}$ ($CHCl_3$) 3 600 and 3 400 (OH), and 1 710 (CO) $cm^{-1}$; δ ($CD_3COCD_3$) 11 (1 H, s, OH), 9.47 (1 H, s, CHO), 4.68 (1 H, q, MeCH), 2.03 and 2.06 (3 H, s, ArMe and 3 H, s, ArMe), 1.93 (3 H, s, MeCO), and 1.37 (3 H, d, MeCH); *m/e* (%) 236 ($M^+$, 10), 218 (15), 193(19), 165(23), 105(54), and 64(100).

3,6,8-*Trihydroxy*-3,4,5,7-*tetramethyl*-3,4-*dihydroisocoumarin* (6).—A mixture of aldehyde (3) (0.85 g) in water (85 ml) and acetone (60 ml) was treated with $NH_2SO_3H$ (0.6 g) and $NaClO_2$ (0.65 g). After 30 min with stirring at room temperature the resulting solution was extracted with ethyl acetate and the organic extracts were dried ($Na_2SO_4$) and evaporated. The crude product was chromatographed [n-hexane–ethyl acetate (85 : 15)] to give the lactol (6) (0.79 g, 86%).

*Methyl* 4,6-*Dihydroxy*-3,5-*dimethyl*-2-(1-*methyl*-2-*oxopropyl*)*benzoate* (7).—A solution of lactol (6) (0.25 g) in ether (30 ml) was treated with $CH_2N_2$ at 0 °C. The mixture was then evaporated under vacuum and the residue was purified by column chromatography [n-hexane–ethyl acetate (90 : 10)] and crystallised from ether–n-hexane to give (7) (0.158 g, 60%) as *needles*, m.p. 142—144 °C; $\nu_{max.}$ ($CHCl_3$) 3 600 and 3 250 (OH), 1 710 and 1 665 (CO) $cm^{-1}$; δ ($CDCl_3$) 11.5 (1 H, s, OH), 4.10 (1 H, q, C*H*Me), 3.82 (3 H, s, $CO_2Me$), 2.20 (6 H, s, 2 ArMe), 1.98 (3 H, s, MeCO), and 1.37 (3 H, d, MeCH); *m/e* (%) 266 ($M^+$, 23), 235(16), 234(56), 223(26), 192(95), and 191(100) (Found: C, 63.1; H, 6.7. $C_{14}H_{18}O_5$ requires C, 63.16; H, 6.77%).

6,8-*Dihydroxy*-3,4,5,7-*tetramethylisocoumarin* (2).—A solution of lactol (6) (0.25 g) in toluene (60 ml) was treated with toluene-*p*-sulphonic acid (0.075 g). The mixture was stirred at reflux for 15 min and then extracted with aqueous 5% NaOH. The aqueous phase was acidified with dilute HCl (pH 3) and extracted in turn with ether. The organic extracts were dried ($Na_2SO_4$) and evaporated to yield the *enol-lactone* (2) (0.195 g, 85%), m.p. 212—214 °C (benzene) (Found: C, 66.8; H, 6.0. $C_{13}H_{14}O_4$ requires C, 66.7; H, 6.0%); δ ($C_5D_5N$) 10.73 (1 H, s, ArOH), 2.48 and 2.55 (3 H, s, ArMe; 3 H, s, ArMe), 2.05 and 2.12 (3 H, s, MeC=C; 3 H, s, MeC=C); *m/e* 234 ($M^+$), 219, 192, and 191.

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