Biosynthesis of Ascochitine and Synthesis of its Biogenetic Precursors

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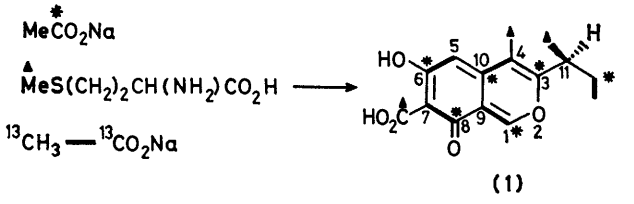
' Enzymic trap ' experiments show that the main biosynthetic pathway to ascochitine (1) involves the direct reduction of the enzyme-bound ester into aldehyde (4). Therefore the complete pathway has now been assessed. Various synthetic approaches have been tried to synthesize the precursors: the use of 1,3-dithians, of an organolithium equivalent to the Friedel–Crafts reagent, and of phase-transfer reactions allowed for a good introduction of the label.

THE polyketide origin of ascochitine [1] (1), a phytotoxic fungal metabolite from culture filtrates of *Ascochyta fabae* Speg.[2] and *Ascochyta pisi* Lib.,[3] was determined by a ^{13}C n.m.r. analysis [4] of [1-^{13}C]-, [1,2-$^{13}C_2$]-acetate-, and [Me-^{13}C]-methionine-derived (1). Ascochitine is derived from a single hexaketide chain, composed of head-to-tail acetate units, and three C_1 units introduced by *S*-adenosylmethionine (Scheme 1). The *ortho*-quinone

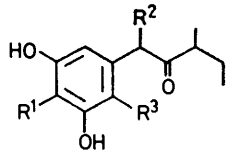


SCHEME 1

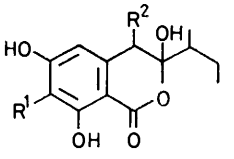
methide structure (1) was assigned to this phytotoxin, in chloroform solution, by the detailed analysis of the ^{13}C–^{1}H long-range coupling constants.[4] The most probable sequence of reactions involved in ascochitine biosynthesis was described in a preliminary communication,[5] resulting from incorporation of the potential advanced precursors (2)—(10).

RESULTS AND DISCUSSION

The labelled compounds (2)—(10) were added to cultures of *Ascochyta fabae* (three-day culture broths).

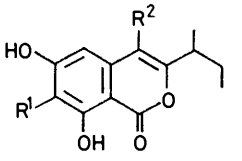


(2) R^1 = H, R^2 = Me*, R^3 = CHO
(3) R^1 = H, R^2 = Me*, R^3 = CO_2Me
(4) R^1 = Me*, R^2 = Me, R^3 = CHO
(5) R^1 = Me*, R^2 = Me, R^3 = CO_2Me

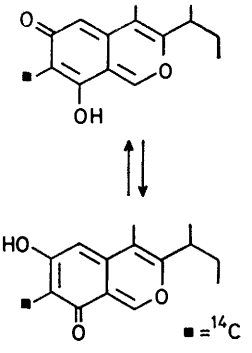


(6) R^1 = H, R^2 = Me*
(7) R^1 = Me*, R^2 = Me

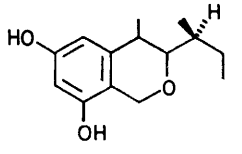
Nine days after the addition, (1) was isolated as previously described.[2] The specificity of incorporation was tested in a very simple way: reduction of (1) to tetrahydroascochitine, and subsequent decarboxylation to compound (11) by alkaline treatment. Therefore the percentage recovery of label in CO_2 is complementary to that in compound (11).



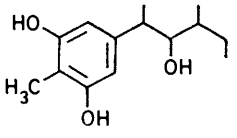
(8) R^1 = H, R^2 = Me*
(9) R^1 = Me*, R^2 = Me



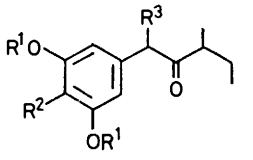
(10) * = ^{14}C



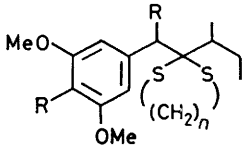
(11)



(13)



(12)
a; R^1 = Me, R^2 = R^3 = H
b; R^1 = Me, R^2 = H, R^3 = Me
c; R^1 = H, R^2 = H, R^3 = Me
d; R^1 = H, R^2 = CO_2H, R^3 = Me
e; R^1 = H, R^2 = Me, R^3 = Me



(14)
a; *n* = 2, R = Me
b; *n* = 3, R = H

These are the reasons why we have chosen to introduce the label into the methyl group which can become the carboxy-group of ascochitine. Compounds (4), (5), (7), (9), and (10) were obtained by alkylation with ^{14}MeI of the synthetic intermediate, using an organolithium equivalent to the Friedel–Crafts reagent. On

the other hand compounds (2), (3), (6), and (8) were labelled in the benzyl position by alkylation with ^{14}MeI of the respective ketone under phase-transfer conditions.

The incorporation data (Table 1) of compounds (2), (3), (6), and (8) compared with those of the analogous methylated compounds (4), (5), (7), (9), and (10) exclude, in agreement with other biosynthetic schemes,[6] methylation of the aromatic nucleus as a part of the biosynthetic pathway. The non-specific labelling of ascochitine derived from (7) indicates its degradation to acetate prior to incorporation (Table 1, experiment 5). The specific incorporation of the unnatural methyl ester (5) shows that the micro-organism can hydrolyse the ester with no formation of the lactol (7), and can apparently transform it into the enzyme-bound ester.

TABLE 1

Incorporation of the advanced precursors (2)—(10) into (1)

Experiment	Precursor	% Incorporation into (1)	% Recovery of label in (11)	% Recovery of label in CO_2
1	(2)	0.00		
2	(3)	0.00		
3	(6)	0.00		
4	(8)	0.00		
5	(7)	0.49	83	17
6	(5)	0.97	0	100
7	(9)	1.34	0	100
8	(4)	9.91	0	100
9	(10)	17.57	0	100

An ' enzymic trap ' experiment confirms that aldehyde (4) is a natural intermediate. Introduction of [Me-^{14}C]-methionine to the culture together with a large amount of unlabelled aldehyde (4), and interruption of the fermentation when only part of (4) had been transformed, allowed the recovery of label in (4) and in ascochitine (1) (Table 2). An exchange through the cell wall of the

TABLE 2

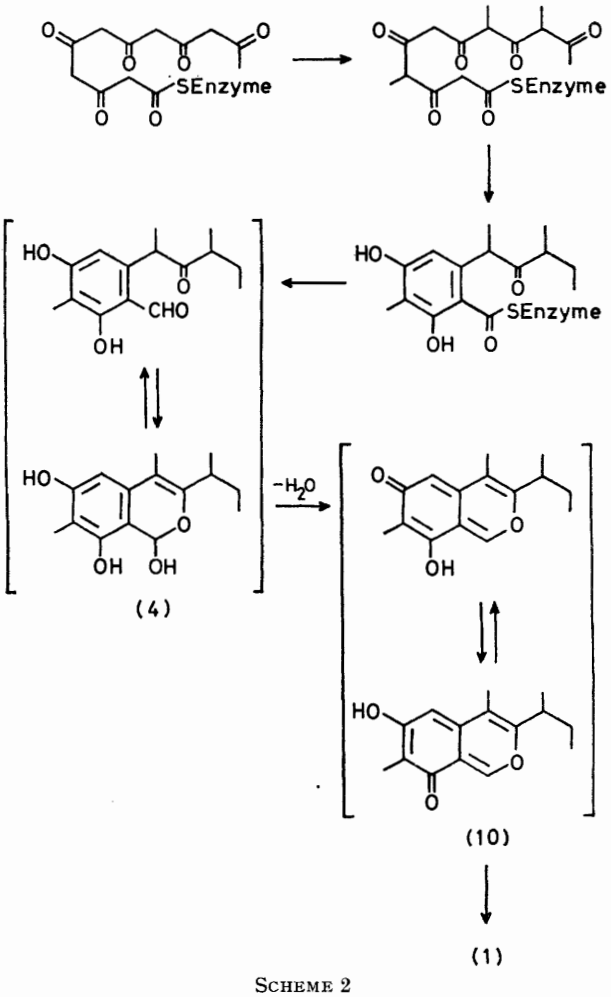
Incorporations of [Me-^{14}C]methionine into compounds (4), (9), and (1)

Fermentation time (h)	Total incorporation (%)	Compound
8	0.03	(4)
	0.80	(1)
8	0.00	(9)
	11.39	(1)

labelled (4), biosynthesized from [Me-^{14}C]methionine, with unlabelled (4), present in excess in solution, is thus operating.

In a similar experiment involving enol-lactone (9) and [Me-^{14}C]methionine, unlabelled (9) together with labelled (1) was recovered. If compound (9) is able to cross the cell wall, the failure of the ' enzymic trap ' experiment indicates that the direct reduction of the enzyme-bound ester into aldehyde (4) is the main, if not the exclusive, biosynthetic route to (4) (Scheme 2). The specific incorporation of labelled (9) can be ascribed to some adaptability of the enzymic complex of *Ascochyta fabae*. The most probable sequence of reactions and intermediates involved in ascochitine biosynthesis is shown in Scheme 2.

The optical rotatory power of ascochitine derived from (4) and (10) is lower than the normal value. Thus the enzymatic systems of *Ascochyta fabae* can transform intermediates characterized by an unnatural *R*-configuration at C(11) into ascochitine. Thus, the optical purity of ascochitine derived from (4) and (10) and the molar % incorporation of these racemic intermediates are in good agreement. Various synthetic approaches were tried to synthesize the potential biogenetic intermediates of (1) in a convenient way to introduce labelling. The interaction of 3,5-dimethoxyphenylacetyl chloride with s-butylmagnesium bromide in the presence of copper(I)



SCHEME 2

bromide produced ketone (12a) which was methylated in the benzyl position with methyl iodide under phase transfer conditions, in order to provide (12b). Diphenol (12c), resulting from (12b) following demethylation by hydrobromic acid and tributylhexadecylphosphonium bromide,[7] was carboxylated in glycerol into the acid (12d). The reduction of (12d) with sodium bis-(2-methoxyethoxy)aluminium dihydride produced the alcohol (13). Finally the oxidation of this compound with Jones' reagent led to the formation of ketone (12e). However, both the low overall yield and the high quantity of reagents (CO_2 and $NaHCO_3$) required for the reaction of carboxylation, make the above mentioned

scheme impracticable for the possible introduction of the labelling. A more effective synthesis of (12e) was carried out: ketone (12b) was quant itatively converted into its corresponding ethylene thioacetal, and thereafter metallated selectively between the two methoxy groups [8] with n-butyl-lithium in cyclo hexane. The subsequent alkylation with [^{14}C]methyl iodide produced compound (14a) in a 65% yield. The conversion of (14a) into (12e), by removal of the th ioacetal residue with methyl fluorosulphonate, as well as the demethylation of the methoxy-groups by the above mentioned method, was accomplished in 80% overall yield. An alternative more direct route, that enables the attainment of ketone (12a), already protected as the dithian, involved the condensation of 3,5-dimethoxybenzyl bromide with 2-s-butyl-2-lithio-1,3-dithian to provide (14b) in 75% yield. The subsequent reactions to obtain (12e) were performed in the following sequence: metallation of (14b) and alkylation with methyl iodide, hydrolysis of the dithian with methyl fluorosulphonate, methylation of the benzylic position in phase-transfer conditions, and finally cleavage of the methyl ethers. The conversion of (12e) into aldehyde (4) was accomplished with triethyl orthoformate and hydrogen chloride, followed by a treatment with NaOH. The intermediate (10) was obtained treating aldehyde (4) in an ethanol solution with H_2SO_4 or P_2O_5. The oxidation of aldehyde (4), that results in the formation of (7), provides no satisfactory results with reagents such as $KMnO_4$, Collins' reagent, and Ag_2O in alkaline solution. On the other hand the process gives a satisfactory yield when $NaClO_2$ and NH_2SO_3H are used.[9]

The characteristics of (7), as already confirmed by other authors [10] in the case of analogous compounds, prove compatible with the lactol structure in balance with a minimum keto-acid percentage. The methyl ester (5) results from the treatment of lactol (7) with diazomethane. Dehydration of (7) with toluene-*p*-sulphonic acid in toluene gave enol-lactone (9). The intermediates (2), (3), (6), and (8) were synthesized starting from (12e) with a similar procedure.

EXPERIMENTAL

Cultural Conditions.—Previously reported [2] cultural conditions were used. After three days fermentation, the labelled compounds (2)—(10) were added to the cultures in acetone or dimethyl sulphoxide solution. Nine days after the addition, (1) was isolated as previously described.[2] Table 3 reports the amounts and the radioactivity values of labelled compounds incubated and of ascochitine obtained.

Incorporation Specificity.—A solution of ascochitine (0.1 g) in ethanol (20 ml) was treated with hydrogen, in the presence of 10% Pd–C (0.06 g), for 90 min at room temperature and pressure. The mixture was then filtered and evaporated to give tetrahydroascochitine. This product was treated with aqueous 1% NaOH solution (20 ml) and heated at reflux for 2 h. The cooled solution was then acidified with dilute HCl while a nitrogen stream was blown through: the CO_2 thus liberated was quantitatively absorbed by hyamine hydroxide [11] and counted. The resulting solution was extracted with ethyl acetate and the organic extracts were passed down a column of Florisil. The eluted fractions were washed with water, dried (Na_2SO_4), and evaporated. The crude mixture was then chromatographed (n-hexane–ethyl acetate) to give the 3,4-*dihydroisochroman* (11) (0.045 g) which was crystallized from n-heptane–ethyl ether to constant radioactivity, m.p. 146—148 °C, δ(C_5D_5N) 6.80 (1 H, d, ArH, *J* 2 Hz), 6.71 (1 H, d, ArH, *J* 2 Hz), 5.42 and 4.94 (2 H, dd, ArCHH, *J* 15 Hz), 3.45—3.15 (1 H, m, ArC*H*), 3.05—2.60 (1 H, m, CHO), and 1.29 (3 H, d, ArCHCH_3), *m/e* 236 (M^+, 36%), 179 (42), 150 (62), and 121 (100) (Found: C, 79.5; H, 8.5. $C_{14}H_{20}O_3$ requires C, 79.7; H, 8.5%).

'Enzymic Trap' Experiments.—[Me-^{14}C]methionine (50 (μCi) was added to three flasks of culture after 36 h fermentation. After a further 24 h fermentation, unlabelled aldehyde (4) (100 mg) or enol-lactone (9) (100 mg) was added to the culture. After a further 8 h fermentation, the usual work-up gave a residue which was chromatographed over silica gel. Hexane–ethyl acetate (8 : 2) eluted aldehyde (4) (28 mg) which was crystallized from n-hexane–ethyl ether to constant radioactivity (1 190 disint min^{-1} mg^{-1}). With chloroform–ethyl acetate–acetic acid (55 : 45 : 1) as eluant, ascochitine (1) (4 mg) was obtained and crystallized from chloroform–carbon tetrachloride to constant radioactivity (2.22×10^5 disint. min^{-1} mg^{-1}).

In the other experiment, elution with hexane–ethyl acetate (8 : 2) gave enol-lactone (9) (75 mg) which was crystallized from n-hexane–ethyl ether to constant activity (0 disint. min^{-1} mg^{-1}). Further elution with chloroform–ethyl acetate–acetic acid (55 : 45 : 1) yielded ascochitine (1) (17 mg) which was crystallized to constant activity (7.45×10^5 disint. min^{-1} mg^{-1}).

TABLE 3

Compound	Quantity added (mg)	Radioactivity (disint. min^{-1} mg^{-1})	Isolated mg of (1)	Radioactivity of (1) (disint. min^{-1} mg^{-1})	$[\alpha]_D^{25}$ (°) of (1) (*c* 0.3, $CHCl_3$)[a]
(2)	53	127,989	102	0	−93.3
(3)	60	106,821	98	0	−92.9
(6)	60	101,220	107	0	−93.7
(8)	60	125.791	100	0	−93.6
(7)	60	127,739	80	471	−93.5
(5)	40	139,538	80	675	−92.8
(9)	28	154,432	118	491	−92.9
(4)	60	156,960	80	11 665	−83.1
(10)	60	121,453	80	16 005	−76.0

[a] $[\alpha]_D^{25}$ (*c* 0.3, $CHCl_3$) −93.7° is the observed value for (−)-(*S*)-ascochitine.

Synthesis of Precursors.—Reactions based on the use of organolithium compounds were performed in purified argon. 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 A-60 (60 MHz) or 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. Physical and spectroscopic data for compounds (12a—c), (13), (12e), (14a and b), (4), (10), (7), (5), (9), (2), (6), (3), and (8) are deposited as Supplementary Publication No. SUP 22834 (6 pp.).*

Ketone (12a).—A solution of 3,5-dimethoxyphenylacetic acid (30 g) in dry benzene (90 ml) was treated with oxalyl

* For details see Notice to Authors No. 7 in *J.C.S. Perkin I*, 1979, Index Issue.

chloride (36 g). The mixture was stirred at room temperature for 2 h and then heated at reflux for 1 h. The solvent was distilled off under reduced pressure and the residue was dissolved in dry ether (120 ml). A Grignard reagent prepared from magnesium (8.58 g) and 2-bromobutane (49.2 g) in dry ether (270 ml) was added dropwise during 2 h to the above mentioned mixture, containing CuBr (43 g) at −50 °C. The temperature was slowly raised to 25° and the reaction was quenched in the normal manner. The crude mixture was chromatographed (n-heptane–EtOAc) and then distilled to yield ketone (12a) (25 g, 70%).

Compound (12b).—A solution of (12a) (6.8 g) in CH_2Cl_2 (32 ml) was treated with tetrabutylammonium hydroxide (40% in water, 22.6 g), water (32 ml), and CH_3I (2 ml). The mixture was stirred at 45 °C for 2 h. The organic phase was separated and treated again with tetrabutylammonium hydroxide (40% in water, 7.5 g) and CH_3I (0.65 ml) under the above mentioned conditions. After 1 h the organic phase was evaporated. The residue was taken up in ether, filtered from tetrabutylammonium iodide, dried (Na_2SO_4), and evaporated, giving (12b) (6.84 g, 95%).

Compound (12c).—Compound (12b) (1.15 g) was treated under nitrogen with aqueous 48% HBr (5 ml) and tributylhexadecylphosphonium bromide [7] (0.39 g). The stirred mixture was heated at reflux (120 °C) for 6 h. After cooling, the mixture was diluted with water and extracted with ether. The organic extracts were dried (Na_2SO_4) and evaporated. The crude product was chromatographed (n-hexane–ethyl ether) and then crystallized to give (12c) (0.87 g, 85%).

Alcohol (13).—Acid (12d) was prepared by carboxylation of (12c) as previously reported.[1] A solution of (12d) (2.97 g) in dry *p*-xylene (200 ml) was treated with sodium bis-(2-methoxyethoxy)aluminium dihydride [60% in toluene (41.2 ml)] at 80°. The mixture was stirred at 140° for 6 h. After cooling the excess 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 in turn with ether. The organic extracts were dried (Na_2SO_4) and evaporated. The crude mixture was chromatographed: elution with n-heptane–EtOAc gave the alcohol (13) (1.48 g, 55%). T.l.c. (n-heptane–EtOAc 6 : 4) showed the presence of three products. Each of these compounds, after isolation, was recognized, by spectroscopic data, as a diastereoisomer of (13).

Oxidation of Alcohol (13).—A solution of alcohol (13) (0.4 g) in acetone (100 ml) was treated at 0 °C with Jones' reagent [12] (0.53 ml). After 2 min at 0 °C the excess of the reagent was decomposed with isopropyl alcohol. The reaction mixture was worked up in the normal manner and the crude product chromatographed (n-heptane–EtOAc) to give (12e) (0.3 g, 76%).

Compound (14a).—A solution of ketone (12b) (3 g) in ethanedithiol (6 ml) was treated with BF_3–ether (18 ml). The mixture was stirred at room temperature for 3 h, then diluted with water, basified with NaOH, and extracted with ether.

The organic extracts were washed with aqueous 4% NaOH and thereafter with water, dried, and evaporated. The crude product was chromatographed (n-hexane–ether) to afford the protected ketone (3.35 g, 86%). A solution of this compound (3.9 g) in dry cyclohexane (300 ml) at 25 °C was treated with 1.5N-Bu^{n}Li in n-hexane (11.1 ml). The mixture was stirred at room temperature for 30 min and then heated at reflux (85 °C) for 1.5 h. After cooling, the dark red solution was treated with ^{14}MeI (4.8 g, 1 mCi) and heated again at reflux for 1 h. The work-up was accomplished by quenching with saturated aqueous NH_4Cl and extraction with ether. The extracts were dried and evaporated. The crude product was chromatographed (n-hexane–ether) to give (14a) (2.6 g, 65%; specific activity 1.61×10^5 disint. min^{-1} mg^{-1}).

Conversion of (14a) *into* (12e).—A solution of (14a) (2.2 g) in CH_2Cl_2 (70 ml) was treated under nitrogen at 0 °C with CH_3OSO_2F (0.786 ml). The mixture was stirred at 0 °C for 10 min and at room temperature for 3 h. The work-up was accomplished by quenching with aqueous 3% $CuSO_4$ and extraction with CH_2Cl_2. The extracts were dried and evaporated. The crude product was chromatographed (n-hexane–ether) to give the related ketone (1.57 g, 92%). This compound (1 g) was demethylated with HBr and tributylhexadecylphosphonium bromide [7] (under the conditions described above) to give (12e) (0.77 g, 87%).

Compound (14b)—A solution of 2-s-butyl-1,3-dithian (7.14 g) in tetrahydrofuran (120 ml) at −40 °C was treated with 1.5N-Bu^{n}Li (19.8 ml). The mixture was stirred at −15 °C for 2 h. To the resulting solution, cooled at −78 °C, was added 3,5-dimethoxybenzyl bromide (6.25 g) in tetrahydrofuran (120 ml). After 1 h at −78 °C, the mixture was treated with saturated brine. The aqueous phase was extracted with ether and the organic extracts were dried (K_2CO_3) and evaporated. The crude mixture was chromatographed (n-hexane–ether) to give (14b) (6.6 g, 75%).

Conversion of (14b) *into* (12e).—Metallation of (14b) and alkylation with MeI (66%), hydrolysis of the dithian with CH_3OSO_2F (85%), methylation of the benzylic position (85%), and cleavage of the methyl ethers (86%) were accomplished under the above-mentioned conditions. Following this route (14b) was converted into (12e) in a 41% overall yield.

Aldehyde (4).—A solution of (12e) (0.86 g) in triethyl orthoformate (17 ml) was treated with hydrogen chloride for 20 min at 0 °C and the precipitate was collected. The yellow oxonium chloride [13] was washed with ether and dried under vacuum. The pyrylium salt was dissolved in aqueous 5% NaOH. The cooled mixture was then acidified with aqueous 12% HCl. The precipitate was collected, washed, and dried to yield the aldehyde (4) (0.88 g, 91%).

Quinone Methide (10).—A solution of aldehyde (4) (0.2 g) in ethanol (2 ml) was treated with concentrated H_2SO_4 (1 ml). The mixture was warmed to 60 °C for 3 min, and then cooled to −20 °C. The yellow precipitate was collected, washed with ethanol, and dried to give the quinone methide (10) (0.177 g, 95%).

Lactol (7).—A mixture of aldehyde (4) (0.5 g) in water (100 ml) and acetone (15 ml) was treated with NH_2SO_3H (0.266 g) and $NaClO_2$ (0.292 g). After 5 h at room temperature the resulting solution was extracted with ethyl acetate and the organic extracts were dried and evaporated. The crude product was chromatographed (n-heptane–ethyl acetate) to give the lactol (7) (0.48 g, 92%).

Methyl Ester (5).—A solution of lactol (7) (0.1 g) in ether (10 ml) was treated with CH_2N_2 at 0 °C. After 1 h the mixture was evaporated under vacuum. The crude product was crystallized to give the methyl ester (5) (0.063 g, 60%).

Enol-lactone (9).—A solution of lactol (7) (0.35 g) in toluene (70 ml) was treated with toluene-*p*-sulphonic acid (0.088 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 and extracted in turn with ether. The organic extracts were dried (Na_2SO_4) and evaporated to yield enol-lactone (9) which was crystallized from n-hexane–ether (0.3 g, 90%).

Aldehyde (2), *Lactol* (6), *Methyl Ester* (3), *and Enol-lactone* (8).—Labelled (12c) (1.58×10^5 disint. min^{-1} mg^{-1}) was obtained by alkylation with $^{14}CH_3I$ (1 mCi) of ketone (12b) under the above mentioned phase-transfer conditions. Aldehyde (2) was prepared by formylation of (12c) in 69% yield. Oxidation of (2) with $NaClO_2$ and NH_2SO_3H gave lactol (6) in 86% yield. Methylation of lactol (6) with CH_2N_2 gave methyl ester (3) in 95% yield, and dehydration of (6) with toluene-*p*-sulphonic acid in toluene gave enol-lactone (8) in 92% yield.

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