In vitro Production and Biosynthesis of Fomajorin D and S by *Fomes annosus* (Fr.) Cooke

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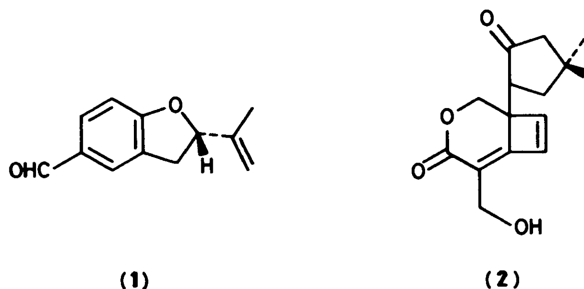
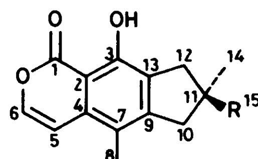
Incorporation of [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]-acetates and [2-^{13}C]-mevalonate into fomajorin D supports its biosynthetic derivation from a humulene-type precursor *via* cyclization of *trans, trans*-farnesyl pyrophosphate. [1,2-$^{13}C_2$]Acetate derived fomajorin D was not converted *in vitro* into fomajorin S. However, the stereochemistry of fomajorin S is proposed on the basis of its biosynthetic origin.

Fomannoxin (1)[1] and fomannosin (2)[2] have been implicated in the phytopathogenic activity of the economically important wood rot fungus *Fomes annosus*. In the course of our continuing search for biologically active metabolites produced by this fungus the unusual sesquiterpene isocoumarins fomajorin D (3) and fomajorin S (4) were isolated, the structures and initial biosynthesis of which formed the subject of preliminary communications.[3] In this paper we present the full evidence for the biosynthetic origin of these compounds.





(3) R = Me
(4) R = CO_2H
(5) R = CO_2Me

The initial vegetative growth of *F. annosus* is white, but after 15 days of surface culture in Raulins medium, deposition of crystalline material on the mycelial mat becomes apparent. Extraction of the mycelium with chloroform afforded fomajorin D (3) which was purified by column chromatography. Fomajorin S (4) has been isolated from the aged sporophores of this fungus.

The proposed structures of these unusual isocoumarins, established by chemical and spectroscopic methods,[3,4] were supported by the behaviour of the crystalline methyl ester (5) on electron impact. The fragmentation patterns follow two major pathways (Scheme 1).

The biosynthetic origin of the isolated isocoumarins were of interest as isocoumarins in nature are normally polyketide derived.[5]

However, biogenetic speculations for the derivation of (3) and (4) centred on a humulene precursor which might cyclize to a

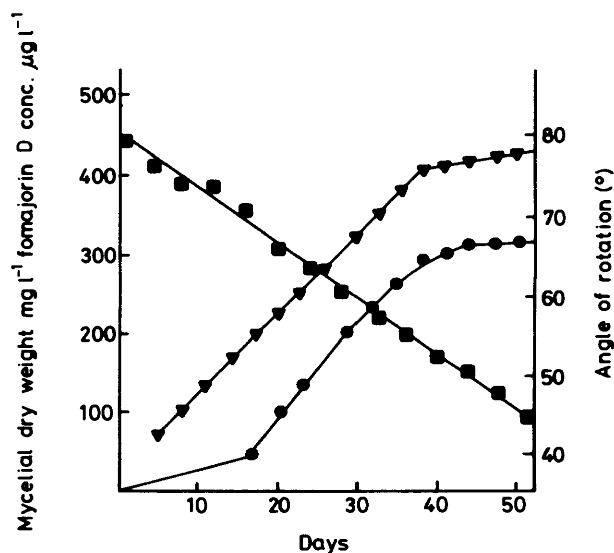


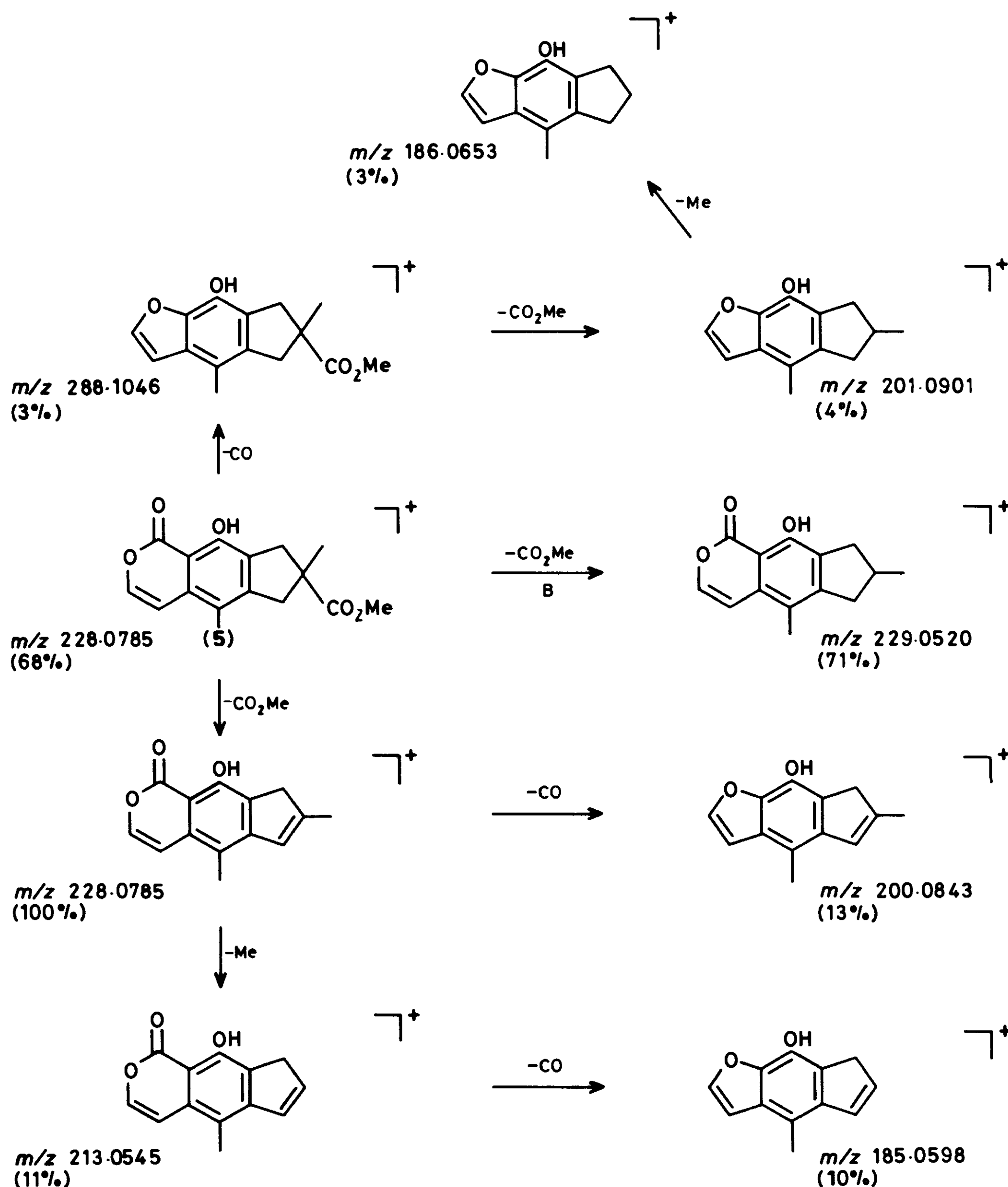
Figure 1. Time growth of *Fomes annosus*. ▼ Measurement of mycelial growth; ● weight of fomajorin D (3); ■ glucose utilization

tricyclic compound related to illudol followed by oxidative cleavage of the appropriate bond (Scheme 2) as observed in the biosynthesis of fomannosin (2)[6] and the biogenetically related illudins,[7] coriolins,[8] and hirsutic acid.[9]

Prior to incorporation of biosynthetic precursors a time growth study of *F. annosus* Fr. Cooke on a surface culture of Raulins medium was carried out to determine optimum feeding and harvesting conditions. Growth rate and production of compound (3) was measured over a period of 50 days (Figure 1). Production of (3) commenced after a 15 day lag phase and its concentration rose to a maximum of *ca.* 320 µg l^{-1} after 43 days of growth. Growth of *F. annosus* was accompanied by a decrease in glucose content of the medium and by a concomitant increase in the production of compound (3).

To define the biosynthetic origin of the carbon skeleton of (3) and (4) we administered in separate experiments sodium [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]-acetate, [2-^{13}C]mevalonate as well as [methyl-^{13}C]methionine to surface growing cultures of *F. annosus*. Backfeeding of fomajorin D (3) to a fomajorin S (4) producing strain was also carried out.

The ^{13}C n.m.r. spectrum of fomajorin D (3) was assigned using both broad band proton-decoupled and gated ^{1}H-decoupled spin-echo Fourier transform techniques. There was insufficient material available for two-dimensional INADEQUATE analysis which affords the carbon connectivity pattern by observation of natural abundance carbon-carbon coupling.



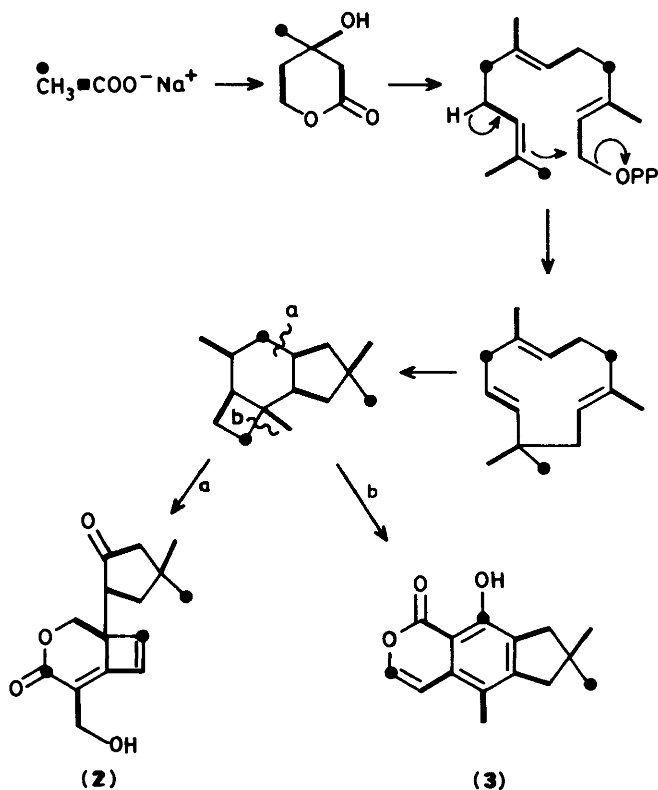
Scheme 1.

In feeding [1,2-$^{13}C_2$]acetate to *F. annosus* Fr. Cooke it was necessary to vary several experimental parameters before optimum feeding conditions were achieved. Optimum conditions for the incorporation of [1,2-$^{13}C_2$]acetate into fomajorin D (**3**) were obtained when the fungus was administered with 20 mg l^{-1} of sodium [1,2-$^{13}C_2$]acetate (90% enriched) diluted with 60 mg l^{-1} of unlabelled acetate and mixed with 12.69×10^6 d.p.m. of sodium ^{14}C acetate on day 27, and was harvested after a 6 day incubation period.

The ^{13}C n.m.r. spectrum of enriched fomajorin D (**3**) derived from [1,2-$^{13}C_2$]acetate feed exhibited six pairs of spin coupled doublets about the natural abundance signals, and three enhanced singlets (Table). As has been demonstrated previously,[6-10] when [1,2-^{13}C]acetate is used as a biosynthetic precursor of isoprenoid metabolites, all carbons derived from C-3 and C-3′ and C-4 and C-5 of mevalonate give rise to coupled doublets in the ^{13}C n.m.r. spectrum of ^{13}C-labelled metabolites provided the paired atoms remain connected. Adjacent carbon atoms derived from distinct molecules of acetate will not show spin-spin coupling as long as there is a significant pool of unlabelled acetate, either administered externally or endogenously generated.* Furthermore, since C-1 of mevalonate is lost as carbon dioxide in the formation of isopentenyl pyrophosphate, all carbons in any subsequent metabolite which are derived from C-2 of mevalonate give rise to enhanced singlets.

The ^{13}C n.m.r. spectrum of labelled (**3**) from the [1,2-$^{13}C_2$]acetate feed indicates that carbons 3 and 6 as well as one of the gem dimethyls are derived from the C-2 of acetate. All other paired carbon atoms of acetate remain intact resulting in the observed set of coupled signals. Our results are therefore consistent with the biogenetic speculations on the origin of fomajorin D (Scheme 2). The direct incorporation of [2-^{13}C]-

* Failure to dilute doubly labelled precursors with sufficient unlabelled material may lead to complications in interpretation of spectroscopic data as a result of excessive multiple labelling of products.



Scheme 2.

Figure 2.

Table. ^{13}C N.m.r. fomajorin D[a]

Carbon	δ_C/p.p.m.	J_{CC}/Hz[b]	Carbon	δ_C/p.p.m.	J_{CC}/Hz[b]
1	116.9 s[c]	69.2	8	14.2 q	46.3
2	105.9 s	*f*	9	154.1 s	41.8
3	155.9 s[d]	s	10	48.2 t	41.2
4	133.1 s	54.9	11	39.5 s	35.1
5	105.4 d	54.4	12	44.0 s	41.2
6	142.4 d[e]	s	13	129.2 s	41.2
7	119.1 s	46.0	14	29.1 q	35.1
			15	29.1 q[f]	s

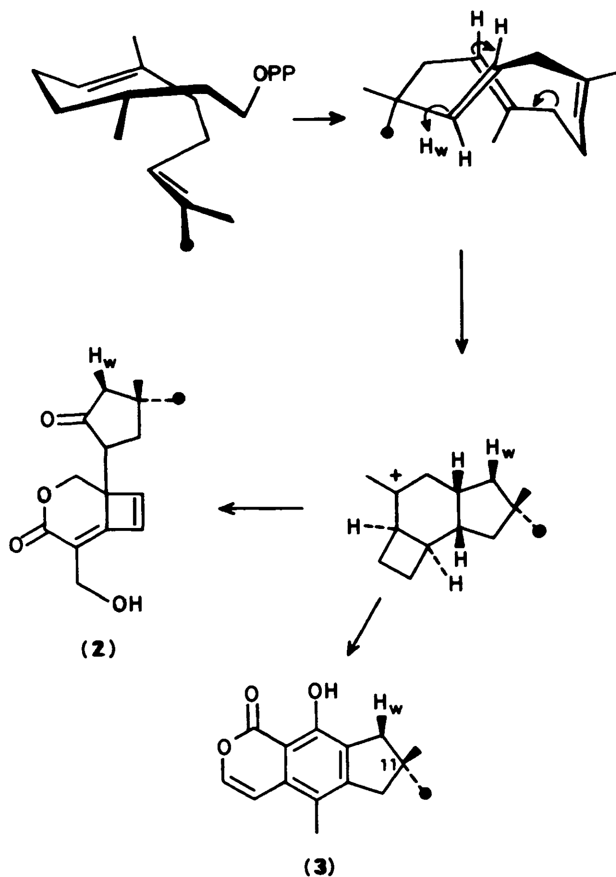
[a] Bruker WM 400 (100.62 MHz) determination in $CDCl_3$. [b] Observed coupling of satellite doublets for [1,2-$^{13}C_2$]acetate labelled fomajorin D. [c] Multiplicity in SFORD spectrum. s = singlet; d = doublet; t = triplet; q = quartet. [d] Enhanced intensity after incorporation of [2-^{13}C]mevalonate. [e] Could not be evaluated because of overlap with C-5.

mevalonate into carbons 3, 6, 14/15 of fomajorin D (3) (Table) is in accordance with the proposed pathway (Scheme 2).

The results of the singly labelled sodium acetate experiments are shown in Figure 2. A pulse feeding technique which momentarily floods the fermentation system with labelled substrate failed to reveal coupling at the C-10/C-11 positions. No carbon derived from labelled methionine was incorporated into the molecule.

It is proposed that the isocoumarin (3) is a biosynthetic precursor of the acid (4). Attempts to observe the oxidative conversion of (3) to (4) by backfeeding [1,2-^{13}C]acetate derived (3) to ageing cultures of *F. annosus* on synthetic growth medium failed. [1,2-$^{13}C_2$]-labelled fomajorin D, incubated for 100 days with a fomajorin S (4) producing strain of *F. annosus*, was recovered unaltered. It is most likely that this observation can be accounted for by failure of the large precursor molecule to permeate the fungal cell wall, with the substrate (3) not reaching the enzymic site of oxidative conversion.

The biogenetic origin of the carboxy group in fomajorin S can, however, be proposed on the basis of the biosynthetic study undertaken with fomajorin D in conjunction with a stereochemical analysis carried out by Cane & Nachbar on fomannosin and the biogenetically related illudin and marasmic acid.[6] In this work, Cane established that the *cis* methyl of fomannosin (2) is derived from the C-2 of mevalonate (Scheme 3). We propose that fomajorin S (4) is derived from mevalonate *via trans,trans*-farnesyl pyrophosphate and that the methyl at C-11 is derived from the C-2 of mevalonate and has the α-configuration (Scheme 3).



Scheme 3.

Experimental

M.p.s were determined on a Kofler hot-stage apparatus and are uncorrected. U.v. spectra were recorded on a Perkin-Elmer 124 Spectrometer. N.m.r. Spectra were recorded on a Bruker WM

400 instrument. Deuteriochloroform was used as solvent with $SiMe_4$ as internal standard. Mass spectra were determined at 70 eV on an AEI MS 30 instrument. Optical rotations were measured using a Perkin-Elmer 141 polarimeter. ^{14}C Was estimated by counting on an Intertechnique SL 30 scintillation counter. T.l.c.s were run on commercially prepared pre-coated plates (Merck, Kieselgel 60F 254). Merck Kieselgel PF 254 + 366 was used for p.l.c.

Culture.—The strain of *Fomes annosus* (Fr.) Cooke was no. 608 (CBS 169.28.) obtained from Centraal Bureau voor Schimmelcultures Baarn. Cultures were maintained on malt agar slopes and plates. Stocks of mycelial plugs were retained in sterile H_2O.

Time Course Experiment.—*Fomes annosus* (Fr.) Cooke (Strain 608) was grown in 14 Roux flasks containing 250 ml of Raulins medium. Mycelial plugs from developing malt agar plates were used as inoculum. The fungal cultures were incubated at 25 °C. At various time intervals, culture flasks were harvested in triplicate. The following determinations were made on the combined mycelia and filtrates on each occasion: (i) glucose concentration (determined approximately by measuring optical rotation of culture filtrate); (ii) mycelial weight (dry); (iii) fomajorin D concentration (determined by $CHCl_3$ extraction of macerated mycelium and growth medium). Extracts were pooled, dried ($MgSO_4$), and evaporated under reduced pressure. P.l.c. of the crude extract ($CHCl_3$) yielded fomajorin D, concentrations of which were measured by u.v. absorption (log ε fomajorin D = 6 100 at λ 340 nm).

Incorporation of Sodium [1,2-$^{13}C_2$]*Acetate into Fomajorin D.*—A modified Raulin's medium consisting of sucrose (46.6 g), D-tartaric acid (2.66 g), potassium carbonate (0.40 g), magnesium carbonate (0.26 g), calcium sulphate (0.16 g), zinc sulphate (0.06 g), ferrous sulphate (0.06 g), potassium dihydrogen orthophosphate (0.40 g), ammonium nitrate (2.70 g), and thiamine HCl (0.1 mg) in distilled water (1 l), pH adjusted to 4.50 with 0.1M NaOH, was divided between 1 l Roux flasks (250 ml both per bottle), fitted with Morton closures and autoclaved at 120 °C for 20 min. Each flask was inoculated with four mycelial plugs [cut from the inside edge of actively growing cultures with a sterile cork borer (10 mm)] of *F. annosus* (Fr.) Cooke. The surface cultures were incubated for 27 days at 25 °C in the dark. A solution of 20 mg of sodium [1,2-$^{13}C_2$]acetate (20 mg) diluted with unlabelled sodium acetate (180 mg) and mixed with sodium [2-^{14}C]-acetate (12.69×10^6 d.p.m.) in distilled water (19 ml) was administered on day 27 of growth *via* a millipore filter unit to 4 flasks of growing culture (5 ml aliquot per flask). Care was taken not to disturb the mycelial mat. After a total of 33 days of growth the cultures were harvested by filtration and the filtrate was extracted with chloroform (3 × 200 ml). Celite was used to break emulsions. The mycelium was air dried, macerated, and extracted with chloroform. The chloroform extracts were pooled, dried ($MgSO_4$), and the solvent was evaporated under reduced pressure to yield an oil which was purified by p.l.c. on silica gel (eluant $CHCl_3$) to yield fomajorin D (**3**) (22 mg, 22.317×10^5 d.p.m./mmol), ^{14}C (2.3%); m.p. 126—127 °C; $\nu_{max.}$(KBr) 3 240 and 1 675 cm^{-1}; δ ($CDCl_3$) 1.19 (6 H, s, 2 × Me), 2.78 (4 H, s, 2 × CH_2), 2.23 (3 H, s, ArMe), 6.63 (1 H, d, HCH, *J* 5.8 Hz), and 7.18 (1 H, d, CH, *J* 5.8 Hz).

General Procedures for the Administration of [1-^{13}C]-, [2-^{13}C]-*Sodium Acetate to* Fomes annosus *(Fr.) Cooke.*—To surface-growing cultures of *F. annosus* (Fr.) Cooke growing for 27 days on Raulins medium (4 × 250 ml broth) a mixture of labelled ^{13}C sodium acetate (30 mg), unlabelled sodium acetate (180 mg), [2-^{14}C]-sodium acetate (1 ml, 1.76×10^6 d.p.m. ^{14}C), and distilled water (19 ml) was administered *via* a millipore filter unit to each of four Roux flasks (5 ml aliquots). The flasks were incubated at 25 °C for a further 6 days. Extraction of the mycelium and growth medium yielded labelled fomajorin D.

A second sodium [2-^{13}C]acetate feed containing labelled acetate (60 mg), unlabelled acetate (180 mg), and distilled water (30 ml) was administered to surface cultures of *F. annosus* (4 × 250 ml Roux flasks, 7.5 ml aliquots). Extraction after six days incubation yielded fomajorin D, but no coupling between C-10 and C-11 was observed in ^{13}C n.m.r.

Incorporation of [2-^{13}C]*Mevalonate into Fomajorin D.*—A mixture of [2-^{13}C]mevalonolactone (60 mg), unlabelled mevalonolactone (200 mg), distilled water (1 ml), and EtOH (5 ml) was administered (5 ml aliquots) to 28-day old cultures of *F. annosus* (Fr.) on a surface culture of Raulins medium (4 × 250 ml broth). The fungus was harvested on day 33 of growth and fomajorin D was extracted as previously described. The ^{13}C n.m.r. spectrum indicated the incorporation of three enriched carbon atoms.

Backfeeding of [1,2-^{13}C]-*Labelled Fomajorin D to Fomajorin S-Producing Cultures.*—Fomajorin D (22 mg, sodium [1,2-$^{13}C_2$]-acetate labelled) dissolved in H_2O–EtOH (10 ml, 1:1) was administered to 48-day old surface cultures of Raulins medium (5 ml aliquot per 250 ml broth). The flasks were reincubated for a further 40 days. Labelled fomajorin D (20 mg), and unlabelled fomajorin S (4 mg) were recovered from this experiment.

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