The Biosynthesis of Chlorine-containing Metabolites of *Periconia macrospinosa*

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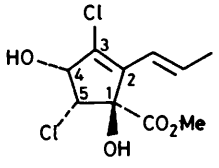
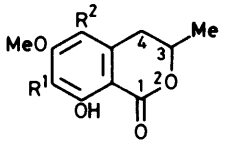
The biosynthesis of cryptosporiopsinol (methyl 2-prop-1-enyl-3,5-dichloro-1,4-dihydroxycyclopent-2-enoate (1) and 5-chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin (2), metabolites of the fungus *Periconia macrospinosa*, have been studied using dihydro[3-^{14}C]isocoumarins. 3,4-Dihydro-6,8-dihydroxy-3-methylisocoumarin (9), 5-chloro-3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin (10), 7-chloro-3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin (11), and 5,7-dichloro-3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin (12) were the most efficient precursors of cryptosporiopsinol, thus establishing that a ring contraction is involved in the biosynthetic route. The results of the incorporations of the dihydro[3-^{14}C]isocoumarins into (1) and (2) suggest that chlorination occurs early in the biosynthetic pathways of these metabolites.

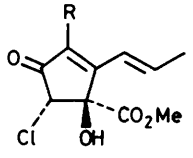
Cryptosporiopsinol (1) (methyl 2-prop-1-enyl-3,5-dichloro-1,4-dihydroxycyclopent-2-enoate) and 5-chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin (2) are the major polyketide metabolites of the fungus *Periconia macrospinosa*.[1] The ketone, cryptosporiopsin (3), was detected only as a minor metabolite in *P. macrospinosa*,[1] but it was found as a major metabolite in a *Cryptosporiopsis* sp.[2] and *Sporormia affinis*[3] where it is present with dechlorocryptosporiopsin (4) and the dihydroisocoumarins (5)—(7).[4] From the acetate-labelling pattern of cryptosporiopsinol (1) and its co-metabolite (2),[5] their origin from a common six-membered intermediate has been suggested (Scheme 1).[1,5] We have confirmed this hypothesis and investigated the order of the early stages of the biosynthesis by carrying out incorporation experiments with dihydroisocoumarins. The related metabolite terrein (8) has been shown to be formed from the dihydroisocoumarin (9) in *Aspergillus terreus*[6] (Scheme 2). In the case of terrein, carbon atoms derived from the carboxy-group of acetate are directly linked, but in cryptosporiopsinol (1) two carbon atoms from the methyl group of acetate are joined together, so although the two metabolites are superficially similar their biosynthetic pathways are different.



(1)



(2) R^1 = H, R^2 = Cl
(5) R^1 = R^2 = H
(6) R^1 = R^2 = Cl
(7) R^1 = Cl, R^2 = H

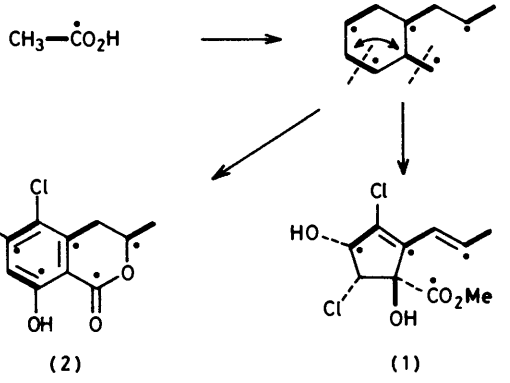


(3) R = Cl
(4) R = H

Results and Discussion

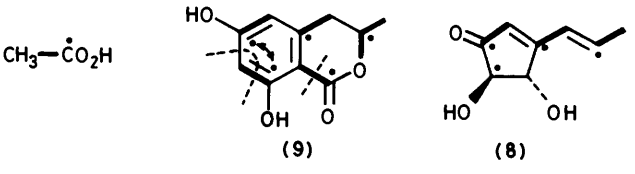
The naturally occurring 6-methoxydihydroisocoumarins (2) (5), (6), and (7) are thought to be likely candidates for intermediates in cryptosporiopsinol biosynthesis because of their co-occurrence with the cryptosporiopsin metabolites (1), (3), and (4). However the presence of the *O*-methyl group in these dihydroisocoumarins does not fit easily into the biosynthetic scheme as the methyl group must be subsequently lost. Therefore the dihydroxydihydroisocoumarins (9), (10), (11), and (12) were also selected for testing. These compounds were made by a route described earlier.[7] This route was adapted to produce dihydro[3-^{14}C]isocoumarins by treating the corresponding homophthalic anhydrides (13) with [1-^{14}C]acetic anhydride (Scheme 3). Better yields were obtained when R^2 of the homophthalic anhydride (13) was hydrogen rather than chlorine. Thus, dihydroisocoumarins with chlorine at this position (C-5) were prepared by chlorination of the C-5 unsubstituted dihydroisocoumarin [*e.g.* (11) to (12)] using sulphuryl chloride.[7]

The labelled dihydroisocoumarins were administered in aqueous solution as their sodium salts to 10-day-old cultures of *P. macrospinosa* (IMI 24411).[1] The incorporations into cryptosporiopsinol (1) and the dihydroisocoumarin (2) were determined and the extent of ^{14}C-labelling at C-2 of (1) and



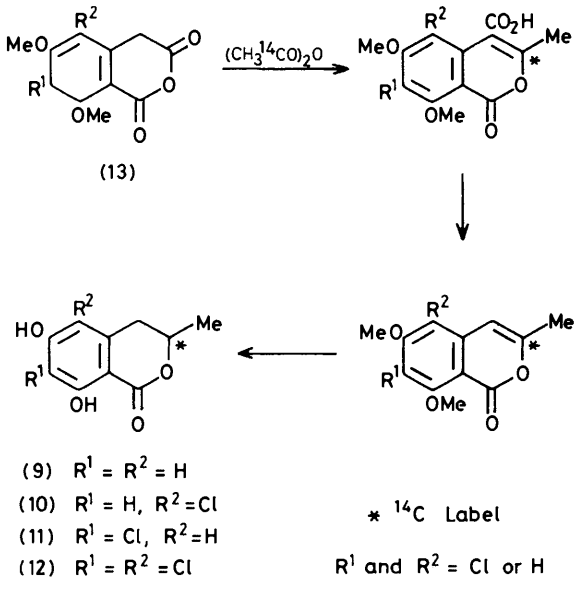
(2) (1)

Scheme 1.



(9) (8)

Scheme 2.



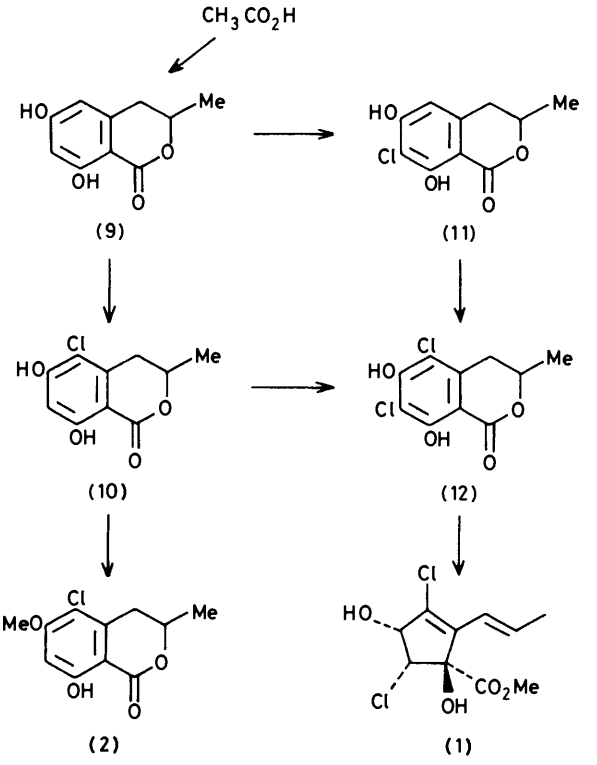
Scheme 3.

Table. Incorporations of dihydro[3-^{14}C]isocoumarins into metabolites of *P. macrospinosa*

Compound fed	Incorp. into (1)/%	% Activity at C-2 of (1)	Incorp. into (2)/%	% Activity at C-3 of (2)
(5)	0.97	85	1.66	89
(2)	0.13			
(7)	1.75	96	<0.01	
(6)	2.3	87	<0.01	
(9)	4.6	89	17.6	83
(10)	5.0	81	16.2	85
(11)	8.0	84	<0.01	
(12)	17.6	81	<0.01	

C-3 of (2) (the expected positions of labelling) were determined by Kuhn–Roth degradation followed by Schmidt degradation of the acetic acid formed (Table).

Each of the eight labelled dihydroisocoumarins was incorporated to some extent into cryptosporiopsinol (1) and degradations of the metabolite showed that the compounds were being incorporated intact, that is without degradation to labelled acetate prior to incorporation. Degradation of the precursor to acetate would lead to 25% of the label at C-2 of cryptosporiopsinol (1). The results show that a small amount of degradation is taking place, but the major part of each dihydroisocoumarin is being incorporated intact. The incorporations of the 6-methoxy-dihydroisocoumarins (2), (5), (6), and (7) into cryptosporiopsinol (1) were found to be significantly lower than that of the corresponding 6-hydroxy-analogues (9), (10), (11), and (12); thus it seems unlikely that the methoxydihydroisocoumarins are on the direct pathway to cryptosporiopsinol. Furthermore, the dihydroisocoumarin (9) and its 5-chloro-derivative (10) are incorporated into compound (2) far more efficiently (17.6 and 16.2% respectively) than the 6-*O*-methyl derivative (5) (1.66%); thus it is probable that in this metabolite chlorination of the ring occurs before *O*-methylation. The methoxydihydroisocoumarins presumably can be converted into the corresponding hydroxydihydroisocoumarin by *O*-demethylation. Biological *O*-demethylation has been observed in the conversion of codeine into morphine[8] and also of codeine analogues into their corresponding unnatural morphine derivatives[9] by *Papaver somniferens* and the conversion of ricinine into *O*-demethylricinine[10] by *Ricinus communis*. Although the methoxydihydroisocoumarin (2) is a metabolite of *P. macrospinosa* and is incorporated into cryptosporiopsinol (1), it is unlikely to be on the direct biosynthetic pathway. We propose the biosynthetic pathway in Scheme 4.



Scheme 4.

When cultures of *P. macrospinosa* are grown on chloride-depleted media, no chlorinated metabolites are produced; instead there is a build up of the dihydroxydihydroisocoumarin (9) and its 6-*O*-methyl analogue (5). No ring-contracted metabolite was detected; thus it appears that chlorination is an early step in the biosynthetic scheme. The 6-*O*-methylation of the dihydroxydihydroisocoumarin may be a detoxification step by the fungus to prevent damage by build-up of its own metabolites. It is interesting to note that normal cultures of *P. macrospinosa* produce cryptosporiopsinol (1) and the chloroisocoumarin (2) in the medium, but the mycelium contains very little cryptosporiopsinol and large amounts of the chlorodihydroisocoumarin (2).

The existence of more than one pathway to cryptosporiopsinol was investigated by trapping experiments. We had so far established that the dihydroisocoumarins (9), (10), (11), and (12) could be converted by the fungus into cryptosporiopsinol (1), but this does not mean that they are intermediates in the normal biosynthesis. The isolation of the dihydroxydihydroisocoumarin (9) from chloride-depleted cultures and the presence of the natural metabolite (2) which implies the presence of the 5-chlorodihydroisocoumarin (10) means that these two compounds at least are made by the fungus. To test whether or not (11) and (12) are true intermediates, [1-^{14}C]acetate was fed to the fungus at the same time as large quantities of unlabelled (11) and (12) (in separate experiments). Thus, by increasing the metabolic pools of these isocoumarins, trapping of radiolabelled metabolites was made possible. In both experiments, the dihydroisocoumarins were found to be

labelled with acetate on isolation. Thus, the role of all the dihydroisocoumarins in the biosynthetic pathway has been established. The biological chlorination step appears to be relatively non-specific with regards to site of chlorination of the dihydroxydihydroisocoumarin (9).

The pathway from the dichlorodihydroisocoumarin (12) to cryptosporiopsinol (1) is being investigated.

Experimental

For general directions see ref. 7. Radioactive samples were counted in 10 ml of organic scintillator on a Philips liquid scintillation counter.

3,4-Dihydro-3-methyl[3-^{14}C]*isocoumarins* (2), (5), (6), (7), (8), (10), (11), *and* (12).—These compounds were prepared by the methods already described.[7] The label was introduced using [1-^{14}C]acetic anhydride on the corresponding homophthalic anhydride (13; R = H or Cl), by the following general method.

A solution of acetic anhydride (0.01 ml) in dry tetrahydrofuran (THF) (1 ml) was added to the anhydride (13) (400 mg) in dry THF (10 ml) and the mixture was stirred for 10 min. A solution of acetic anhydride (0.15 ml), and [1-^{14}C]acetic anhydride (20% w/w toluene solution, 4.8 μl, 30.3 mCi mmol^{-1}) in THF (15 ml) was added, followed by anhydrous pyridine (0.05 ml). The mixture was stirred at room temperature for 1 h, then a further quantity of acetic anhydride (0.15 ml) was added, and stirring was continued for a further 30 min. The solution was heated under reflux for 30 min and then left at room temperature for 16 h. The resulting orange solution was concentrated under reduced pressure and the residue dissolved in aqueous sodium hydroxide (10%, 25 ml). The basic solution was washed with ether (2 × 5 ml) and the aqueous solution heated under reflux for 1.5 h, cooled to 0 °C, then acidified with dilute hydrochloric acid (10%). The acidified solution was extracted with ethyl acetate (3 × 15 ml) and the organic extract was evaporated to give a crystalline residue. The residue was dissolved in methanol (15 ml) and an excess of sodium borohydride was added. The solution was stirred at room temperature for 6 h, then diluted with water (25 ml), and acidified with concentrated hydrochloric acid. The aqueous methanol solution was extracted with ether (3 × 25 ml), and the combined extracts were washed with aqueous sodium hydroxide (1%, 10 ml) and water (10 ml). The ether extracts were evaporated to give crystalline dihydroisocoumarin (299 mg, 70%; radiochemical yield, 10%).

Feeding Experiments to P. macrospinosa.—*P. macrospinosa* (IMI 24411) was grown as a surface culture in Roux bottles each containing Czapex-Dox medium (200 ml) according to the general directions of Giles and Turner.[1] Labelled compounds were dissolved in the minimum amounts of aqueous sodium hydroxide (0.1 M) and administered to 10-day-old cultures. After a further 11 days incubation (25 °C) the medium was extracted with ethyl acetate to give a brown gum. The mycelium was collected and dried (P_2O_5). The dried mycelium was powdered and continuously extracted in a Soxhlet apparatus (ethyl acetate; 24 h) and the ethyl acetate extract evaporated to give an oily residue. Preparative layer chromatography using silica gel (GF 254) and chloroform as eluant gave cryptosporiopsinol (1) and the dihydroisocoumarin (2) from the broth extract (recrystallised from methylene dichloride–hexane and ethyl acetate–hexane respectively). Preparative layer chromatography of the foregoing mycelium extract [chloroform–light petroleum (b.p. 40—60 °C)–diethyl ether then chloroform] gave further quantities of the dihydroisocoumarin (2).

Isolation of Metabolites from Chloride-depleted Cultures.—*P. macrospinosa* was grown as normal except that potassium chloride was not added to the medium. The medium and mycelium were extracted and chromatographed as described above. 3,4-Dihydro-6,8-dihydroxy-3-methylisocoumarin (9) and 3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin (5) were obtained (with properties agreeing with synthetic material [7]) in quantities which varied considerably from culture to culture. No inhibition of mycelium growth was observed under these conditions. The dried weight of chloride-depleted mycelium was on average equal to or greater than that of a normal culture.

Acknowledgements

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