# Biosynthesis of the Fungal Xanthone Ravenelin

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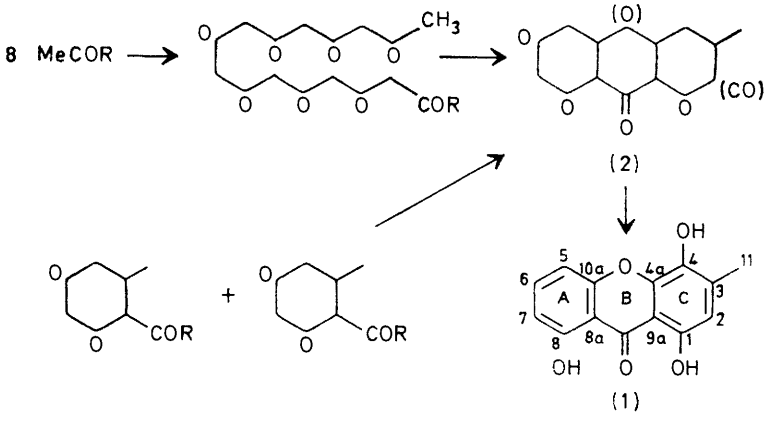
Carbon-13 and carbon-14 labelling experiments have shown that the fungal xanthone ravenelin (1) is acetate-derived. Enrichment studies with [1,2-$^{13}$C]acetate have demonstrated that an oxygenated benzophenone derivative is an intermediate in the biosynthetic pathway.

It has been suggested [1] that compounds with a benzophenone skeleton are biosynthesised by two types of route: wholly acetate–polyketide and shikimate–polyketide ($C_6C_1$ plus $3C_2$ and $C_6C_3$ plus $2C_2$). In the case of naturally-occurring xanthones, it has been shown in biosynthetic studies that the former route is probable in fungi and the latter in higher plants.[2-6]

the fungal xanthone, bikaverin is derived *via* the folding of a single polyketide chain without an anthraquinone intermediate.

Ravenelin (1), another naturally occurring xanthone, has been isolated [7] from the mycelium of two phytopathologically active members of the lower fungi, *Helminthosporium ravenelii* Curtis and *H. turcicum*




Scheme 1

The detailed mechanism of the biogenesis of xanthones has been studied in several cases. Labelling studies [3] have established that a benzophenone derivative is a precursor to gentisin, and that anthrone and/or anthraquinone intermediates most likely are involved as intermediates in the formation of several fungal xanthones.[4,5] In contrast, Vining and his co-workers [6] have shown that

Passerini. Its structure has been established by chemical studies [7] and two independent unambiguous syntheses.[8] The biosynthesis of ravenelin is of interest because, although inspection of the formula suggests a mainly polyketide origin, the position of the methyl substituent precludes derivation from one chain unless an intermediate containing an anthrone and/or an anthraquinone

[1] A. J. Birch and F. W. Donovan, *Austral. J. Chem.*, 1953, **6**, 1360.

[2] I. Carpenter, H. D. Locksley, and F. Scheinmann, *Phytochemistry*, 1969, **8**, 2013; O. R. Gottlieb, *ibid.*, 1968, **7**, 411; *Biochem. Systematics*, 1973, **1**, 111.

[3] P. Gupta and J. R. Lewis, *J. Chem. Soc.* (*C*), 1971, 629.

[4] J. S. E. Holker and L. J. Mulheim, *Chem. Comm.*, 1968, 1576; B. Franck, *Angew. Chem. Internat. Edn.*, 1969, **8**, 251; K. G. R. Pachler, P. S. Steyn, R. Vleggaar, and P. L. Wesels, *J.C.S. Chem. Comm.*, 1975, 355, and references cited therein.

[5] J. S. E. Holker, R. D. Lapper, and T. J. Simpson, *J.C.S. Perkin I*, 1974, 2135.

[6] A. G. McInnes, D. G. Smith, J. A. Walker, L. C. Vining, and J. L. C. Wright, *J.C.S. Chem. Comm.*, 1975, 66.

[7] H. Raistrick, R. Robinson, and D. E. White, *Biochem. J.*, 1936, **30**, 1303.

[8] R. P. Mull and F. F. Nord, *Arch. Biochem.*, 1944, **4**, 419; V. Kahluwalia and T. R. Seshadri, *Proc. Nat. Acad. Sci.*, India, 1956, **44A**, 1 (*Chem. Abs.*, 1957, **51**, 5059).

skeleton (2) is involved. Alternative routes involve a branched-chain polyketide such as that leading to citromycetin,[9] and the production of the carbocyclic rings in separate stages. In the latter case, a monocyclic precursor derived from a polyketide route could add either directly to another independently formed polyketide aromatic ring or to the malonate units required to produce such a ring (Scheme 1).

Incorporation of acetate into ravenelin was studied to distinguish between the alternative biosynthetic pathways. [$^{14}$C]Ravenelin, isolated from the mycelium of cultures of *H. ravenelii* fed with $^{14}$C-labelled sodium acetate or diethyl malonate, was degraded to establish labelling patterns.* These results prompted a thorough biosynthetic study with [1-$^{13}$C]- and [1,2-$^{13}$C]-acetate precursors.

$^{14}$C *Incorporation Studies.*—Acetate incorporation into ravenelin (1) by *H. ravenelii* was studied by degradation of [$^{14}$C]ravenelin, obtained from cultures grown with [1-$^{14}$C]acetate and with [2-$^{14}$C]acetate. The reactions are summarised in Scheme 2 and the results are collected in Table 1.

Sodium [1-$^{14}$C]acetate was incorporated into ravenelin (1) by cultures of *H. ravenelii* to the extent of 3.3%.

TABLE 1

Radioactivity of degradation products of [$^{14}$C]ravenelin trimethyl ether

| Compound | Labelled with [1-$^{14}$C]acetate (r.m.a. × $10^{-4}$) | Labelled with [2-$^{14}$C]acetate (r.m.a. × $10^{-4}$) |
|---|---|---|
| (4) | 152.3 | 90.60 |
| (6) | 129.3 | 88.83 |
| (11) | 21.6 | 0 |
| (12) | 0 | 12.90 |
| (9) | 64.07 | 38.40 |
| (10) | 65.28 | 52.23 |

Kuhn–Roth oxidation of the labelled xanthone (1) [11] gave carbon atoms 3 and 11 as acetic acid which was further degraded (Scheme 2) [12] to show that carbon atom 3 of the xanthone (1) possessed 1/7th of the total radioactivity of the parent molecule, and carbon atom 11 was devoid of isotopic carbon. The reaction of sodamide on [$^{14}$C]-ravenelin trimethyl ether (3) gave the diphenyl ether (5),[10] with concomitant loss of carbon atom 9 from (1) and 1/7th of the total radioactivity of the xanthone trimethyl ether (3).

These results support a complete polyketide origin for ravenelin (1). This xanthone cannot be derived from only one polyketide chain, however, unless it is produced by cleavage of an intermediate such as an anthrone and/or anthraquinone derivative.[4,5]

If ravenelin (1) is derived from a single polyketide chain then [1-$^{14}$C]acetate should be incorporated to an equal extent into rings A and C of the xanthone (1). Reduction of [$^{14}$C]ravenelin (1) with sodium and ethanol in liquid ammonia gave resorcinol monomethyl ether (7) and 2-methyl-1,4-benzoquinone (8), from rings A and C respectively of ravenelin (1). Radiochemical assay of purified derivatives of (7) and (8) showed equal incorporation of radioactivity into rings A and C, within the limits of accuracy of the experiment.

The results from the degradation of [$^{14}$C]ravenelin (1) derived from [2-$^{14}$C]acetate support the above observations, and also establish that the methyl group (carbon atom 11) of ravenelin (1) is acetate-derived and not an introduced $C_1$ unit. Carbon atom 11 of the labelled xanthone (1) is shown to contain 1/7th of the total radioactivity of the parent molecule, and C-3 and C-9 to possess no activity. Degradation of the metabolite to derivatives of rings A and C as described above demonstrates that the radioactive label is incorporated into rings A and C in the ratio 3 : 4, in support of the hypothesis that ravenelin (1) is derived from a single polyketide precursor.

Diethyl [1-$^{14}$C]malonate was incorporated into ravenelin (1) to the extent of 1.4% but there was no significant distinction of a ' starter unit ', suggesting that the long growth period allowed equilibration of acetate with the labelled malonate.

These degradations conveniently establish the mode of incorporation of [$^{14}$C]acetate into carbon atoms 3, 9, and 11 of ravenelin (1) and indicate the labelling pattern shown in (1) (Scheme 2). Acetate incorporation into the remaining carbon atoms in rings A and C and additional biosynthetic data were determined most conveniently by [$^{13}$C]acetate incorporation studies.

$^{13}$C *Incorporation Studies and Discussion.*—In order to establish the specificity of [1-$^{13}$C]- and [2-$^{13}$C]-acetate incorporations, by $^{13}$C n.m.r. spectroscopy, it was necessary to establish $^{13}$C chemical shift assignments for ravenelin (1). Signals for all fourteen carbon atoms were observed in the proton-decoupled natural-abundance $^{13}$C n.m.r. spectrum, and assigned chemical shifts are shown in Table 2. Partial analysis of the results for aromatic-ring carbon atoms was achieved by consideration of the shift data for suitable monosubstituted benzene derivatives.[13] Resonances associated with protonated aromatic carbon atoms were identified by off-resonance decoupling experiments. Of these signals that at lowest field (137.7 p.p.m.) was assigned to that carbon atom not subject to a shielding *ortho*-hydroxy-, or *ortho*-aryloxy-substituent effect, *i.e.* C-6.[13] This signal showed the largest residual proton-coupling on selective decoupling of the H-2 signal, consistent with its assignment to C-6, since the signal of H-6 is the furthest of the aromatic ring proton signals from the H-2 resonance. Of the above four carbon resonances only that at 111.1 p.p.m. does not show any residual proton coupling on

* Preliminary work [10] provided evidence of expected acetate incorporation, but a number of details were incomplete.

[9] W. B. Whalley, ' Biogenesis of Natural Products,' 2nd edn. ed. P. Bernfield, Pergamon, Oxford, 1967, pp. 1058—1059.

[10] G. E. Blance, Ph.D. Thesis, Manchester University, 1960; S. F. Hussain, Ph.D. Thesis, Manchester University, 1964.

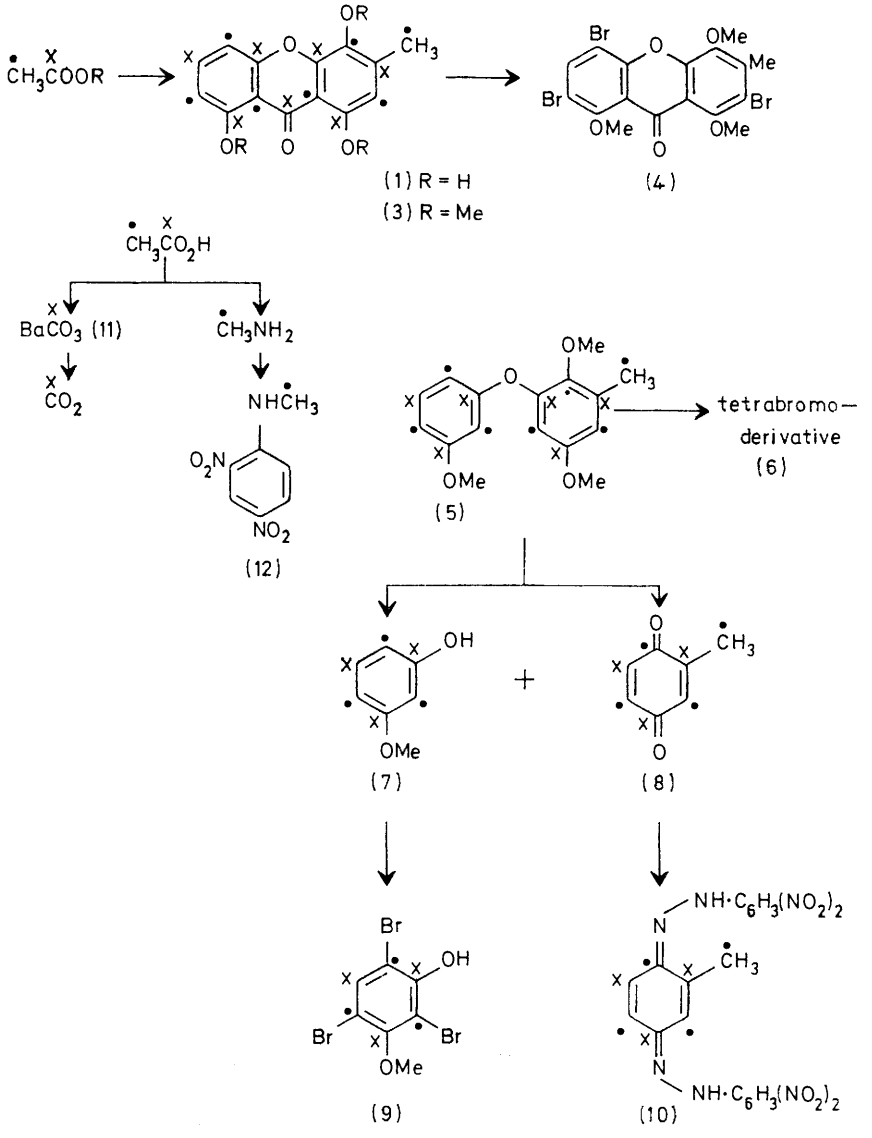
[11] R. Belcher and A. C. Godbert, ' Semimicro Quantitative Organic Analysis,' 2nd edn., Longman and Green, London, 1954, p. 160.

[12] E. F. Phares, *Arch. Biochem. Biophys.*, 1951, **33**, 173.

[13] J. B. Stothers, ' Carbon-13 NMR Spectroscopy,' Academic Press, New York, 1972, pp. 196—201.

decoupling of H-2; consequently this is assigned to carbon atom 2. In the proton-coupled $^{13}C$ n.m.r. spectrum the resonance at 107.4 p.p.m. appeared as a doublet of doublets [$^1J(^{13}C-H)$ 168; $^3J(^{13}C-C-CH)$ 7.4 Hz] and is assigned to carbon atom 5; the signal at 110.3 p.p.m. showed additional splitting probably arising from coupling with the proton of the C-8 hydroxy-group [14]

oxygen-substituted atoms at positions 1, 8, and 10a, not subject to an *ortho*-hydroxy- (aryloxy-) shielding effect, correspond to the three signals observed in the 151.7—160.4 p.p.m. range; the carbon atoms at positions 8a and 9a, subject to two shielding *ortho*-hydroxy- (aryloxy-) substituent effects and only a small deshielding *ipso*-carbonyl substituent effect, correspond to the two




SCHEME 2

[$^1J(^{13}C-H)$ 163.0; $^3J(^{13}C-C-CH)$ 7.4 Hz] and is assigned to carbon atom 7.

The eight resonances due to the quaternary carbon atoms of the aromatic rings present the greatest assignment difficulties. The use of additivity of substituent-chemical-shift effects on aromatic rings requires caution in extrapolating from single ring to multi-ring aromatic systems, but does provide the following groupings, based on the *ipso*-hydroxy- (aryloxy-) deshielding and the *ortho*-hydroxy- (aryloxy-) shielding effects: the three resonances at 105.9 and 107.2 p.p.m.; and the carbon atoms at positions 3, 4, and 4a, subject to one shielding *ortho*-hydroxy- (aryloxy-) substituent effect and one deshielding *ipso*-hydroxy- (aryloxy-) or one deshielding *ipso*-alkyl substituent effect, correspond to the remaining three resonances in the range 135.0—143.7 p.p.m.

The multiplicities for this last group of signals, at 135.0, 136.9, and 143.7 p.p.m., in the proton-coupled carbon-13 n.m.r. spectrum, were a doublet of quartets [$^3J(^{13}C-C-$

[14] F. W. Wehrli, *J.C.S. Chem. Comm.*, 1975, 663.

CH) 9.0; $^3J$(C–C–$CH_3$) 4.0 Hz], a quartet [$^2J$($^{13}$C–$CH_3$) 6.7 Hz], and a singlet, and were assigned, on the basis of the known magnitudes of $^2J$($^{13}$C–$CH_3$) and $^3J$($^{13}$C–C–$CH_3$) in aromatic systems and the observation that $|^3J(^{13}C–C–CH)| > |^2J(^{13}C–CH)|$ in monosubstituted benzenes,[15] to carbon atoms 4, 3, and 4a, respectively. The first group of carbon resonances, at 151.7, 155.7, and 160.4 p.p.m., were a doublet [$^2J$ ($^{13}$C–CH) 2.3 Hz], a doublet of doublets {$^3J$[$^{13}$C–C–C(6)H] 11.5; $^2J$($^{13}$C–CH) 3.0 Hz}, and another doublet of doublets {$^3J$[$^{13}$C–C–C(6)H] 11.2; $^2J$($^{13}$C–CH) 2 Hz}, and were assigned to carbon atoms 1, 10a, and 8, respectively. Differentiation between the C-10a and C-8 signals was made on the basis of chemical shift data for a large number of substituted xanthone derivatives.[16]

The carbon signal at 105.9 p.p.m. is a doublet [$^3J$($^{13}$C–C–CH) 6.0 Hz] in the proton-coupled $^{13}$C n.m.r. spectrum and is assigned to carbon atom 9a; the signal at 107.2 appears as a triplet, coupled to protons 5 and 7, and is assigned to carbon atom 8a.

The following biosynthetic arguments are not altered if the doubtful assignments (C-5 and C-7) are reversed.

In order to obtain incorporations of $^{13}$C-labelled precursors sufficiently large to be readily observed in $^{13}$C n.m.r. spectra it is necessary to use much larger quantities of precursor than are usual in equivalent $^{14}$C studies for which radioactivity is used as an assay method. In the present investigation the amounts of [1-$^{13}$C]- and [1,2-$^{13}$C]-acetate necessary for the feedings were calculated by determining the overall dilution of $^{14}$C label in experiments with [1-$^{14}$C]acetate. Under our culture conditions the required enrichment was obtained by using 0.5 g of sodium acetate for every 50 g of sucrose in the medium.

The major disadvantage of loading the biological system with relatively large quantities of acetate precursor is the possible effect on the metabolism of the organism. Attempts to grow cultures of *H. ravenelii*, in either Czapek–Dox media supplemented with relatively large quantities of sodium acetate, or by replacing the Czapek–Dox medium with a solution of sodium acetate, sucrose, and peptone after 5 days of growth, gave mycelium, on further growth, much darker in colour than normal. Solvent extraction of these mycelia according to described procedures yielded little or no ravenelin. Optimum incorporation of sodium acetate was obtained by growing the mycelium in modified Czapek–Dox medium, removing two-fifths of the medium, and feeding the remainder with 1 ml samples of an aqueous solution of sodium acetate every 48 h.

Ravenelin was extracted from the mycelium according to described procedures and used without further purification for $^{13}$C n.m.r. spectral studies. An isotopic enrichment in ravenelin of approximately twofold above the natural $^{13}$C abundance, as established by mass spectrometry, was found when the medium was supplemented with sodium [1-$^{13}$C]acetate (57%). The proton-noise-decoupled $^{13}$C n.m.r. spectrum of ravenelin enriched with [1-$^{13}$C]acetate was recorded with spectrometer settings identical with those used for unenriched ravenelin, so that corresponding peak intensities in the two spectra were comparable. The $^{13}$C enrichments at individual positions in the $^{13}$C n.m.r. spectrum of the [1-$^{13}$C]acetate-derived sample of ravenelin were calculated as described in reference 5. The results thus calculated are summarised in the third column of Table 2 and

TABLE 2

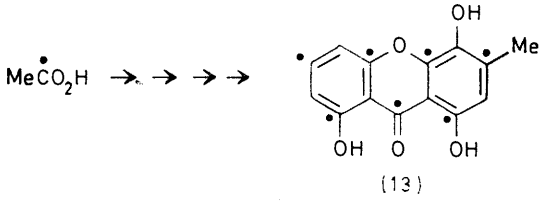
$^{13}$C Chemical shifts of ravenelin, excess $^{13}$C abundance at individual positions in [1-$^{13}$C]acetate-enriched ravenelin, and coupling constants [$^1J$($^{13}$C–$^{13}$C)/Hz] of [1,2-$^{13}$C]acetate-enriched ravenelin

| Carbon no. | Signal † | $E$ ‡ | $^1J$($^{13}$C–$^{13}$C) |
|---|---|---|---|
| 1 | 151.7 | 9.6 | 64.1 |
| 2 | 111.1d | 0.7 | 56.8 |
| 3 | 136.9 | 14.1 | 42.1 |
| 4 | 135.0 | 1.9 | 76.9 |
| 4a | 143.9 | 6.9 | 78.7 |
| 5 | 107.4 | 1.1 | 62 § |
| 6 | 137.7d | 4.4 | 56.8 |
| 7 | 110.3d | 0.6 | 62 § |
| 8 | 160.4d | 10.7 | 69.6 |
| 8a | 107.2 | 1.0 | 53.8 |
| 9 | 185.2 | 9.8 | 54.9 |
| 9a | 105.9 | 1.2 | 64.1 |
| 10a | 155.7 | 10.6 | 67.7 |
| 11 | 17.0q | 0.4 | 42.1 |

† Determined on 0.09M-solutions of unenriched ravenelin at ambient probe temperature (37 °C); in p.p.m. downfield from $Me_4Si$; measured from internal $Me_2SO$ and corrected by using the expression $\delta(Me_4Si) = \delta(Me_2SO) + 39.6$. Multiplicities are indicated from the proton off-resonance decoupled spectrum. ‡ Determined as described in reference 5. § Broad unresolved signals.

indicate that carbon atoms 1, 3, 4a, 10a, 6, 8, and 9 in ravenelin are derived from the carboxy-carbon atom of acetate. The range of values is wide owing to the limited number of instrumental plot data points; the individual differences probably have no biogenetic significance.

The observed labelling pattern in ravenelin enriched with [1-$^{13}$C]acetate is shown in structure (13).




(13)

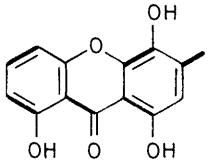
The proton noise-decoupled $^{13}$C n.m.r. spectrum of ravenelin enriched with [1,2-$^{13}$C]acetate showed intense satellite resonances due to $^{13}$C–$^{13}$C spin–spin couplings [C(11)–C(3), C(4)–C(4a), C(9)–C(8a), and C(1)–C(9a)] indicating their origin from four intact acetate units. The C(5) signal also showed intense satellites, indicating origin from an intact acetate unit. However these

[15] F. J. Weigert, J. Husar, and J. D. Roberts, *J. Org. Chem.*, 1973, **38**, 1313; F. J. Weigert and J. D. Roberts, *J. Amer. Chem. Soc.*, 1967, **89**, 2967.

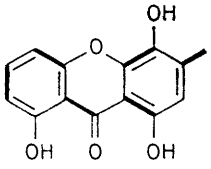
[16] P. W. Westerman, M. U. S. Sultanbawa, S. P. Gunasekera, and R. Kazlauskas, unpublished data.

satellites were very broad, due to couplings of C(5) to *both* C(6) and C(10a) to the same extent. Similarly C(7) is coupled to *both* C(6) and C(8).

These observations indicate that C(10a), C(5), C(6), C(7), and C(8) are derived from two intact acetate units,



(14)



(15)

distributed in equal amounts as shown in (14) and (15). In agreement with this, the relative intensities of the C(8) and C(10a) satellites are only half those of the remaining nuclei derived from intact acetate units (Table 3). The C(2) signal shows no intense satellites, indicating its origin from a cleaved acetate unit.

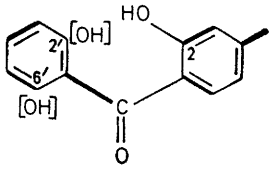
TABLE 3

Ratio of satellite intensities to natural-abundance peak intensities in [1,2-$^{13}$C]acetate-enriched ravenelin

| Carbon no. | Ratio | Carbon no. | Ratio |
|---|---|---|---|
| 1 | 1.2 | 7 | 1.5 |
| 2 | 0.6 | 8 | 0.7 |
| 3 | 1.5 | 8a | 1.4 |
| 4 | 1.6 | 9 | 1.4 |
| 4a | 1.4 | 9a | 1.5 |
| 5 | 1.4 | 10a | 0.8 |
| 6 | 1.5 | 11 | 2.5 |

The high value for the satellite intensities of C(11) in Table 3 arises because the satellite signals are not further split by $^{13}$C–$^{13}$C spin–spin coupling with adjacent intact acetate units. Such coupling is observed for most other signals because of the relatively high level of acetate incorporation. The weak satellites of the C(2) signal arise from spin–spin coupling of the C(2) nucleus with adjacent acetate units C(11)–C(3) and C(1)–C(9a), so a low value in Table 3 is anticipated.

The above results indicate that an oxygenated benzophenone derivative (16), of polyketide origin, is an



(16)

intermediate in the biogenesis of ravenelin. The observed labelling distribution results from the equal probability of cyclisation between positions 2 and 2′, or 2 and 6′, to give the xanthone skeleton. There are several *in vitro* precedents for such a cyclisation.[17] It has been established from tritium incorporation experiments in the plant *Gentiana lutea*, that 2,3′,4,6-tetrahydroxybenzophenone is a precursor of the xanthone gentisin.[3] The coexistence of a benzophenone with related xanthones has also been observed in the plants *Symphoria globulifera* [2] and *Chlorophora tinctoria* [18] and the fungus *Penicillium patulum*.[19]

The biosynthetic origin of naturally occurring benzophenones is usually discussed in terms of an acetate–malonate or a polyketide–shikimate pathway; our results show that the former occurs for ravenelin. Acetate-derived benzophenone (16) may be formed from two distinct units each separately derived from acetate and malonate, or through an anthrone and/or anthraquinone intermediate derived from a single $C_{16}$ polyketide chain. The latter process has been established for biosynthesis of the benzophenone sulochrin.[20] The even distribution of the $^{14}$C label and $^{13}$C label (Table 3) favours a similar biosynthetic pathway in the formation of the intermediate (16). If an anthraquinone derivative is an intermediate our results rule out a concerted oxidation mechanism for the replacement of the carbonyl group by an oxygen atom.

## EXPERIMENTAL

$^{14}$C *Radioactivity Assays.*—$^{14}$C-Labelled organic compounds were recrystallised to constant radioactivity, measured {1 mg sample in a mixture of toluene (1 ml) and the scintillator solution [0.5% 2,5-diphenyloxazole in toluene (9 ml)]} on a Beckman LS-150 liquid scintillation system, calibrated by the external standard channel-ratio method. The product of counts min$^{-1}$ mg$^{-1}$ and the molecular weight gave the relative molar activity (r.m.a.). Barium [$^{14}$C]carbonate was decomposed as described later [21] and a sample (1 ml) of the ethanolamine absorbent was used in place of the toluene solvent (1 ml) in the counting solution. [$^{14}$C]Ravenelin trimethyl ether (3) and [$^{14}$C]-2,3′,5-trimethoxy-3-methyldiphenyl ether (5) were best purified as their respective tribromo- and tetrabromo-derivatives, (4) and (6). Radioactivity assay of the labelled bromo-compound (4) necessitated recounting of samples with an added $^{14}$C standard to correct for scintillator quenching by the bromo-derivative. A convenient standard was a sample (1.0 ml) of a solution of [$^{14}$C]-L-tryptophan benzyl ester in 20% ethanolic toluene of activity 3 700 counts min$^{-1}$ ml$^{-1}$. Merck adsorbents were used for column chromatography.

$^{13}$C *N.m.r. Determinations.*—The $^{13}$C n.m.r. spectra were obtained from samples in hexadeuteriodimethyl sulphoxide ($\leqslant$0.09M depending on sample availability). Chemical shifts were measured from the centre peak of the $Me_2SO$ signal, corrected by use of the expression $\delta(Me_4Si)$ =

[17] D. H. R. Barton and A. I. Scott, *J. Chem. Soc.*, 1958, 1767; W. J. McMaster, A. I. Scott, and S. Trippett, *ibid.*, 1960, 4628; J. R. Lewis, *Proc. Chem. Soc.*, 1963, 373; F. M. Dean, 'Naturally Occurring Oxygen Ring Compounds,' Butterworths, London, 1963, p. 269; P. D. McDonald and G. A. Hamilton, *J. Amer. Chem. Soc.*, 1973, **95**, 7752; P. K. Grover, G. D. Shah, and R. C. Shah, *J. Chem. Soc.*, 1955, 3982.

[18] A. Jefferson and F. Scheinmann, *Nature*, 1965, **207**, 1193.

[19] A. Rhodes, B. Boothroyd, M. P. McGonagle, and G. A. Somerfield, *Biochem. J.*, 1961, **81**, 28.

[20] R. F. Curtis, C. H. Hassall, and D. R. Parry, *J.C.S. Perkin I*, 1972, 240.

[21] L. Cattel, J. F. Grove, and D. Shaw, *J.C.S. Perkin I*, 1973, 2626.

$\delta(Me_2SO) + 39.6$; and reported as $\delta$ values (p.p.m. downfield from $Me_4Si$). $^{13}C$ N.m.r. spectra of natural-abundance samples of ravenelin and samples enriched with [1-$^{13}$C]-acetate were recorded in 5 mm tubes at ambient probe temperature with a Bruker HX-90 FT spectrometer operating at 22.6 MHz. Sweep widths of 6 024 Hz with 4 096 plot data points were used throughout to give chemical shift values accurate to within $\pm 1.5$ Hz, *i.e.* $\pm 0.06$ p.p.m. A pulse width of 3—5 μs was used throughout and the computer data memory size (8 192 addresses) limited the data acquisition time to 0.68 s. Pulse delays of 0.1—2.5 s were used. Proton gated-decoupled, noise-decoupled, and single-frequency off-resonance decoupled spectra were obtained to assist in signal assignment. For the sake of comparison of signal intensities the proton-noise-decoupled spectrum was recorded with spectrometer settings identical with those used to obtain the spectrum of ravenelin enriched with [1-$^{13}$C]acetate.

A proton-noise-decoupled spectrum of a sample enriched with [1,2-$^{13}$C]acetate was recorded with a Bruker HX-270 FT spectrometer operating at 67.89 MHz. A sweep width of 15 000 Hz with 16 192 plot data points was used to give chemical shift values accurate to within $\pm 0.9$ Hz, *i.e.* $\pm 0.01$ p.p.m.

*Incorporation of Sodium* [1-$^{14}$C]- and [2-$^{14}$C]-*Acetate*.—Four penicillin flasks, each containing 500 ml of Czapek–Dox medium and mycological peptone (1 g) were inoculated with cultures of *Helminthosporium ravenelii* Curtis obtained from Centraalbureau voor Schimmelcultures, Baarn, Holland. After growth for 4 days at 25 °C, the mycelium had begun to darken and form a mat over the surface and either (i) 300 mCi of sodium [1-$^{14}$C]acetate or (ii) 300 mCi of sodium [2-$^{14}$C]acetate was distributed equally under sterile conditions among the four flasks. The cultures were harvested after a further 10 days growth and the [$^{14}$C]-ravenelin [(i) 420 mg, 3.3% incorporation; (ii) 480 mg] was extracted and purified as described previously.[7] For degradation the [$^{14}$C]xanthone (100 mg) was diluted with unlabelled material (900 mg).

Incorporation of diethyl [1-$^{14}$C]malonate into ravenelin occurred to the extent of 1.4%.

[$^{14}$C]*Ravenelin Trimethyl Ether* (3).—Dimethyl sulphate (5 ml) was added to a solution of [$^{14}$C]ravenelin (1.25 g) in 10% sodium hydroxide (10 ml). The mixture was warmed with stirring and treated alternately with alkali and dimethyl sulphate until it remained alkaline and none of the initial dark green colour was evident. The alkaline suspension was cooled and the precipitated [$^{14}$C]ravenelin trimethyl ether was filtered off and dried. The product (0.92 g), m.p. 176—178° (lit.,[7] 178—179°), was purified by chromatography on alumina (chloroform–benzene mixtures) and recrystallised from aqueous ethanol. This material could not be rigorously purified to constant radioactivity, and the tribromo-derivative (4) was prepared for assay of radioactivity.

[$^{14}$C]-2,5,7-*Tribromoravenelin Trimethyl Ether* (4).—A stirred solution of crude [$^{14}$C]ravenelin trimethyl ether (80 mg) in glacial acetic acid (7 ml) was treated with an excess of bromine. An excess of bromine was maintained until t.l.c. (alumina; benzene) showed complete conversion into a single product. The mixture was diluted with benzene and ethyl acetate, and washed with water, saturated sodium hydrogen sulphite solution, 5% sodium hydrogen carbonate solution, and saturated sodium chloride solution, dried ($MgSO_4$), and evaporated to leave a crystalline residue (120 mg). Percolation through alumina in 50% light petroleum (b.p. 60—80°)–benzene and recrystallisation from ethanol–chloroform gave the pure *product* (100 mg), m.p. 240—241° (Found: C, 38.1; H, 2.5; Br, 44.3. $C_{17}H_{13}Br_3O_5$ requires C, 38.0; H, 2.4; Br, 44.6%).

[$^{14}$C]-2,3′,5-*Trimethoxy*-3-*methyldiphenyl Ether* (5) *and its Tetrabromo-derivative*.—[$^{14}$C]Ravenelin trimethyl ether (120 mg) was added to a suspension of sodamide (1 g) in dry toluene (100 ml) and the mixture was refluxed under nitrogen for 5 h. Ice was added to the cooled mixture and the organic layer was separated, dried, and evaporated under reduced pressure. The dark oily residue (113 mg) was purified by column chromatography on alumina [light petroleum (b.p. 60—80°)–benzene mixtures] to yield the pure [$^{14}$C]diphenyl ether (72 mg) as a colourless oil, which was characterised as the tetrabromo-derivative (6). The [$^{14}$C]diphenyl ether (50 mg) was heated on a steam-bath in glacial acetic acid solution (1 ml) with an excess of bromine for 0.5 h. The cooled mixture was diluted with ethyl acetate and washed with water, dilute sodium hydrogen sulphite solution, 5% sodium hydrogen carbonate solution, and saturated sodium chloride solution, dried, and evaporated to leave a crystalline residue (120 mg), which was recrystallised from methanol to yield the [$^{14}$C]tetrabromo-derivative (59 mg), m.p. 156—157° (lit.,[7] 152°).

*Fission and Reduction of* [$^{14}$C]-2,3′,5-*Trimethoxy*-3-*methyldiphenyl Ether* (5).—The [$^{14}$C]diphenyl ether (5) (0.4 g) in ether (50 ml) was added to liquid ammonia (380 ml). Sodium (0.1 g) was added and then, after stirring for 1 h, ethanol (8 ml), followed by more sodium (1 g). After disappearance of the metal, the ammonia was evaporated off and water (30 ml) was added. The alkaline solution was extracted with ether and the combined extracts were washed with water, dried, and evaporated. The residual oil was treated with Brady's reagent to give a yellow 2,4-dinitrophenylhydrazone which was purified by chromatography on Bentonite–Kieselguhr (3 : 1) with 30% ethanol–chloroform as eluant. Recrystallisation from ethanol–chloroform gave [$^{14}$C]-2-methylcyclohexa-2,5-diene-1,4-dione bis-2,4-dinitrophenylhydrazone (0.15 g), m.p. and mixed m.p. 235—236° (Found: C, 46.5; H, 3.75. Calc. for $C_{19}H_{18}N_8O_8$: C, 46.9; H, 3.75%).

The alkaline solution from the above reduction of the diphenyl ether was acidified and extracted with ether. The extract was suspended in 0.1M-hydrochloric acid (10 ml) and bromine was added until a colour persisted. The gum that precipitated on addition of sodium hydrogen sulphite solution was purified by chromatography on silica [elution with 5% ether–light petroleum (b.p. 60—80°)]. Recrystallisation from light petroleum gave colourless crystals of [$^{14}$C]-2,4,6-tribromo-3-methoxyphenol (89 mg), m.p. 106—107°, identical with an authentic sample.

*Kuhn–Roth Oxidation of* [$^{14}$C]*Ravenelin Trimethyl Ether and Schmidt Degradation of the Resulting* [$^{14}$C]*Acetic Acid*.—[$^{14}$C]Ravenelin trimethyl ether (72 mg) was oxidised under standard conditions [11] to acetic acid (12 mg), which was subjected to Schmidt degradation.[12] The [$^{14}$C]carbon dioxide was trapped as barium [$^{14}$C]carbonate (23.8 mg), and the [$^{14}$C]methylamine was assayed as [$^{14}$C]-*N*-methyl-2,4-dinitroaniline (21.8 mg). An accurately weighed sample of the barium [$^{14}$C]carbonate was decomposed in concentrated sulphuric acid in a slow stream of nitrogen and the [$^{14}$C]-carbon dioxide liberated was passed into ethanolamine (5 ml). After 0.5 h a sample (1 ml) of the ethanolamine solution was removed for radioactivity assay.

*Incorporation of Sodium* [1-$^{13}$C]- *and* [1,2-$^{13}$C]-*Acetate*.—The optimum yield of $^{13}$C-enriched ravenelin was obtained under the following conditions. A penicillin flask containing Czapek–Dox medium (500 ml) and mycological peptone (1 g) was inoculated with a macerate of *H. ravenelii* grown on 3 plates over 9 days. After 5 days incubation, 200 ml of the medium was removed and the remainder fed with a solution of sodium [1-$^{13}$C]acetate (0.1 g; 57% enriched) or sodium [1,2-$^{13}$C]acetate (0.1 g; 91.6 and 93.1% enriched, respectively) in water (1.0 ml). Similar feedings were repeated on alternate days until a total of 500 mg of $^{13}$C-enriched sodium acetate had been added. Three days after the last feeding, the mycelium was harvested by filtration, washed with water, and dried in a current of air. It was further dried *in vacuo* over phosphorus pentaoxide. Powdered mycelium was extracted (Soxhlet) with light petroleum (500 ml; b.p. 60—80%) for 24 h. Concentration of the solution to 50 ml and cooling to 0 °C gave a crystalline precipitate (0.051 g with sodium [1-$^{13}$C]acetate and 0.036 g with sodium [1,2-$^{13}$C]acetate) which was removed by filtration. The $^{13}$C-enriched samples showed no signals arising from impurities in either the $^{1}$H or the $^{13}$C n.m.r. spectrum.

The powdered mycelium was further extracted with chloroform (500 ml) to yield a crop of less pure ravenelin on concentration of the extract to 15 ml (0.076 g with [1-$^{13}$C]-acetate and 0.014 g with [1,2-$^{13}$C]acetate). Samples of all the above products on recrystallisation from acetone–chloroform gave intensely yellow crystals, m.p. 268—269°, alone or mixed with authentic ravenelin.

Isotope incorporation levels on $^{13}$C-enriched samples were determined mass spectrometrically by analysis of the molecular ion region. Ravenelin enriched with singly and doubly labelled acetate showed enrichment factors of 2.2 and 3.7, respectively.

$^{1}$H *N.m.r. Spectrum of Ravenelin*.—Ravenelin showed $\delta$[$(CD_3)_2SO$] 2.30 ($CH_3$, s), 3.30 (4-OH and 1- or 8-OH, s, exchangeable), 6.54 (2-H, s), 6.76 (5- or 7-H, d, $J_{5,6}$ or $J_{7,6}$ 8.2 Hz), 7.02 (7- or 5-H, d, $J_{5,6}$ or $J_{7,6}$ 8.2 Hz), 7.72 (6-H, dd, $J_{5,6} = J_{7,6} = 8.2$ Hz), and 9.04 (1- or 8-OH, s, exchangeable).

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