Biosynthesis of Palitantin, a Polyketide Metabolite of *Penicillium brefeldianum*: ^{13}C N.m.r. Assignment and Incorporation of ^{13}C-, ^{2}H-, and $^{18}O_2$-Labelled Acetates

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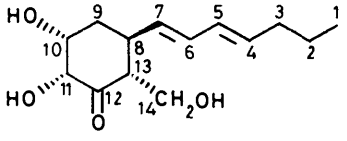
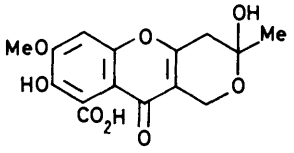
The biosynthesis of palitantin (**1**), a metabolite of *Penicillium brefeldianum*, has been studied using sodium [1,2-$^{13}C_2$]-, [2-$^{2}H_3$]-, [2-^{13}C,2-$^{2}H_3$]-, and [1-^{13}C,1-$^{18}O_2$]-acetate as simple precursors. Analysis of the labelled products by both n.m.r. and mass spectrometry established that no aromatic intermediates are involved in the generation of the six-membered carbocyclic ring. The assignment of the ^{1}H and ^{13}C n.m.r. spectra was made with the aid of two-dimensional homonuclear correlation experiments as well as more standard techniques. Incidentally, *O*-methyl fulvic acid (**2**) and *O*-methyl anhydrofulvic acid (**3**) were discovered as metabolites of *P. brefeldianum*.

Palitantin (**1**), a white crystalline heptaketide metabolite, was first isolated in 1936 by Birkinshaw and Raistrick [1] from culture filtrates of *Penicillium palitans* Westling; it is also produced by strains of *P. frequentans* [2] and *P. cyclopium*.[3] Experimental work by the discoverers, later extended by Birkinshaw,[4] revealed its principal reactions and determined some of its major features. However, it was not until 1959 that further investigation by Bowden *et al.*[5] established the structure and configuration shown in (**1**). The biosynthesis of palitantin was next investigated by Chaplen and Thomas (1960).[6] Since a polyketide pathway was visualised most readily, they started their experiments by administering [1-^{14}C]acetate to a palitantin-yielding strain of *Penicillium cyclopium*. Degradations of the isolated labelled metabolite supported a biosynthetic mechanism involving the head-to-tail condensation of seven acetate units (Scheme 1). We now report results of a further investigation using stable isotopes.[7]



(**1**)



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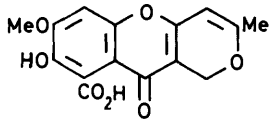

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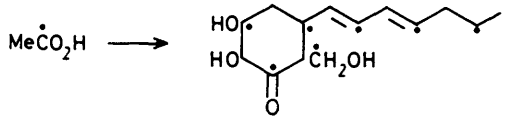


Scheme 1.

Results

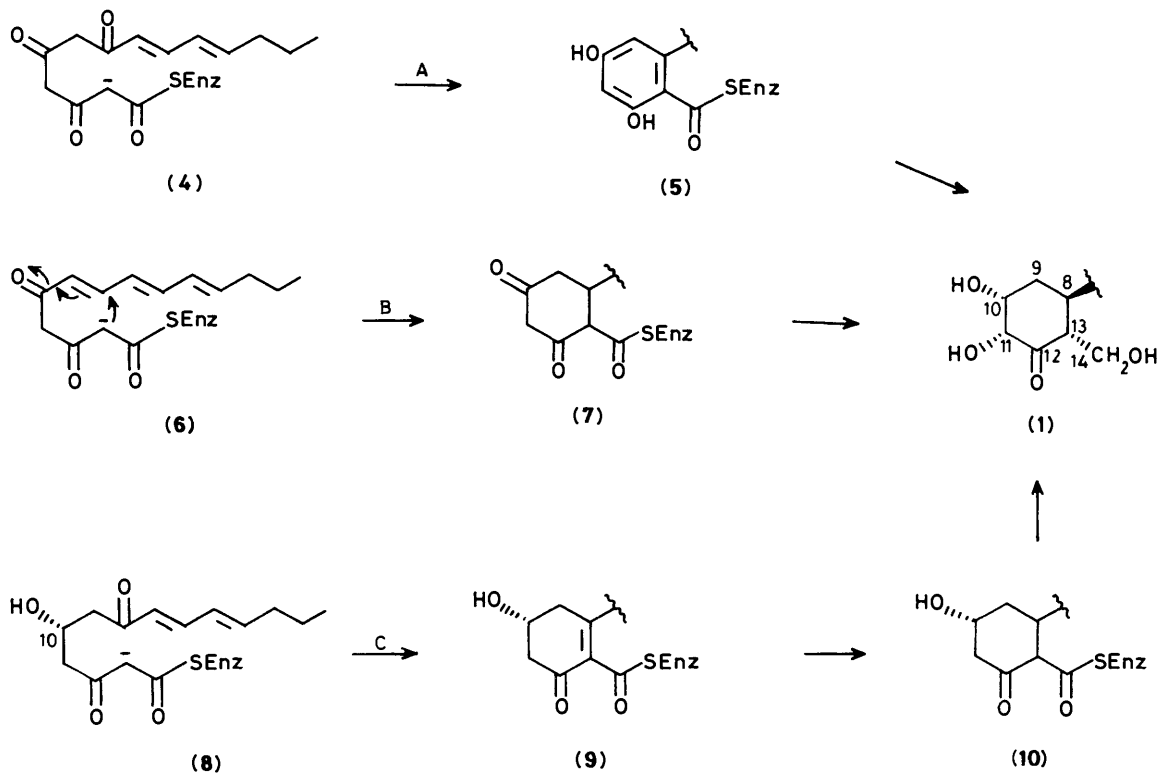
When a strain of *Penicillium brefeldianum* (C.M.I. 40592ii) was grown in a surface culture on Raulin-Thom medium (original method),[8,9] palitantin was only produced in trace amounts after the 22nd day of fermentation, the major product being a mixture (1:1) of two compounds whose spectra were consistent with their being the monomethyl ethers of fulvic acid (**2**) and anhydrofulvic acid (**3**). However, on Czapex-Dox medium, the organism was found to produce a reasonable quantity of palitantin (200—300 mg l^{-1}) with maximum peak yield occurring 10 days after inoculation.

The ^{1}H and ^{13}C n.m.r. spectra of palitantin were assigned as follows. The ^{1}H n.m.r. data in Table 1 are based on homonuclear decoupling experiments. Thus, irradiation of the triplet at δ 0.91 p.p.m. caused the multiplet at δ 1.41 p.p.m. due to 2-H to collapse to a triplet, the remaining coupling being to 3-H which was found to be at δ 2.06 p.p.m. by irradiation of 2-H. Irradiation of 3-H then simplifed the multiplet at δ 5.65 p.p.m. to a doublet, thus allowing its assignment to 4-H. Irradiation of 4-H simplified 3-H from a double triplet to a triplet and 5-H (δ 5.97 p.p.m.) from a multiplet to a doublet. Also 5-H was found to be coupled to a proton at δ 6.10 p.p.m. assigned to 6-H. A coupling was observed from 6-H to a one-proton multiplet at δ 5.49 p.p.m. and must be 7-H, which is also coupled to 8-H at δ 2.7 p.p.m. Similar arguments were used to assign the ^{1}H resonances around the carbocyclic ring. These assignments were checked by a ^{1}H–^{1}H two-dimensional correlation experiment.[10] The natural abundance p.n.d. ^{13}C n.m.r. spectrum of palitantin was then analysed. Of the 14 signals observed only C-1, C-2, C-12, and C-14 could be assigned on the grounds of chemical shift alone. The remaining carbons gave very complex signals in

Table 1. ^{1}H N.m.r. spectroscopic[a] data of palitantin (**1**)

Proton	Chemical shift (p.p.m.)
1	0.91 (t, *J* 7.39 Hz)
2	1.41 (m, *J* 7.39, 7.45 Hz)
3	2.06 (dt, *J* 7.45, 6.63 Hz)
4	5.65 (m)
5	5.97 (m)
6	6.10 (m)
7	5.49 (m)
8	2.70 (m)
9	1.93 (m)
10	4.29 (m)
11	4.28 (m)
13	2.40 (m)
14	3.65 (m)

[a] At 250 MHz in CD_3OD solution.



Scheme 2.

the proton-coupled spectrum and were therefore more difficult to assign. However, having made the proton assignments, the carbon atoms to which they were bonded could be assigned using ^{1}H–^{13}C two-dimensional correlation spectroscopy.[11] At this stage, all the values were reasonably assigned except for those for C-3, C-9, C-10, and C-11. The final unambiguous assignment of these four resonances came from the ^{13}C–^{13}C couplings observed in the ^{13}C n.m.r. spectrum of a sample of palitantin enriched biosynthetically (see later) from sodium [1,2-$^{13}C_2$]acetate. Thus, the signal at δ 35.7 p.p.m. has a coupling constant of 43.3 Hz; this matches the value (43.3 Hz) for the signal at δ 134.95 p.p.m. which had been unambiguously assigned to C-4; thus the resonance at δ 35.7 p.p.m. can be assigned to C-3. Hence C-9 is at δ 37.9 p.p.m. In the same way the signals corresponding to the two hydroxylated carbons, C-10 and C-11, can be distinguished; the signal corresponding to the methylene carbon, C-9, has the same coupling constant (36.0 Hz) as the higher of the two hydroxy carbons at δ 73.84 p.p.m.(36.3 Hz), hence this signal is assigned to C-10 and that at δ 78.8 p.p.m. must therefore be due to C-11. As expected, the coupling constant of C-11 (37.8 Hz) matches that of C-12. The complete assignment is given in Table 2.

Three possible pathways for the biosynthesis of the ring in palitantin are shown in Scheme 2. According to pathway (A) the carbocyclic ring is formed by a standard cyclisation of a linear polyketide intermediate (**4**) (aldol condensation followed by dehydration) to form an aromatic intermediate such as compound (**5**). The aromatic ring would then be reduced in subsequent steps. In the cyclisation step of pathway (B), a carbanion is added to the enone to produce the carbocyclic ring

Table 2. Chemical shifts (δ) and ^{13}C couplings ($^{1}J_{CC}$) in the ^{13}C n.m.r. spectrum of palitantin (**1**)

Carbon	δ (p.p.m.)[a]	J ^{13}C–^{13}C (Hz)[b]
1	13.9	34.9
2	23.5	34.9
3	35.7	43.3
4	134.95	43.3
5	131.3	42.0
6	133.2	42.0
7	133.8	45.1
8	40.4	45.1
9	37.94	36.0
10	73.84	36.3
11	78.8	37.8
12	210.9	38.6
13	56.3	40.4
14	59.42	40.1

[a] At 100 MHz in CD_3OD solution (rel. to CD_3OD = 49.0 p.p.m.). [b] In palitantin derived from [1,2-$^{13}C_2$]acetate.

Table 3. Isotope shifts in ^{13}C n.m.r. spectra[a] of palitantin (**1**) following incorporation of ^{2}H- and ^{18}O-labelled acetates

Carbon	^{2}H-Shifts[b] (p.p.m.)	^{18}O-Shifts[c] (p.p.m.)
1	0.28, 0.58, 0.86	
3	0.4	
7	0.35	
9	0.37, 0.74	
10		0.02
11	0.38	
14		0.015

[a] At 100 MHz in CD_3OD solution. [b] δ-Shifts after incorporation of [2-^{13}C, 1-$^{18}O_2$]acetate.

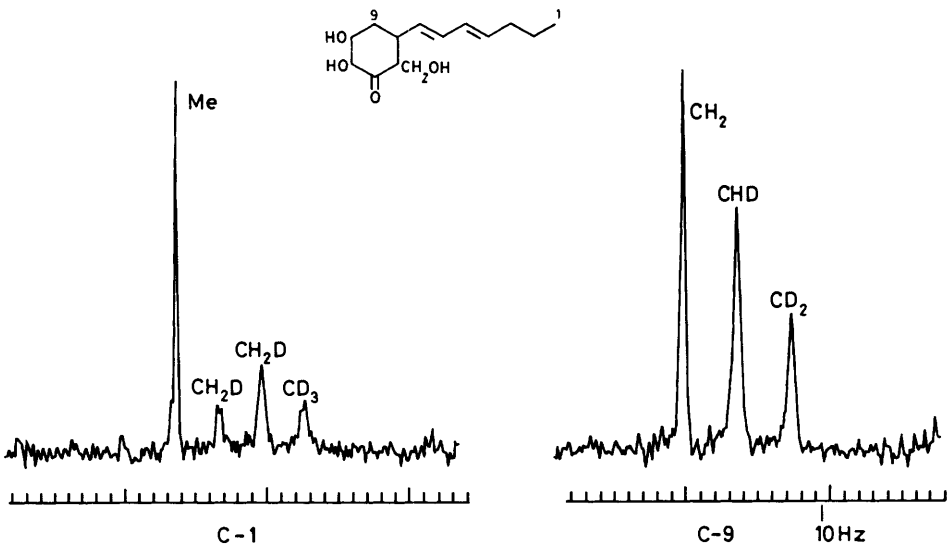


Figure 1. ^{2}H-Shifted resonances for C-1 and C-9 of palitantin (**1**) derived from [2-^{13}C,2-^{2}H$_3$]acetate

of (**7**) which is not prone to aromatise. Pathway (C) involves an aldol condensation followed by dehydration to produce the non-aromatic intermediate (**9**). This could aromatise but instead the enone residue is reduced to give compound (**10**), in which aromatisation is prevented. All three uncyclised intermediates (**4**), (**6**), and (**8**) have an array of functional groups consistent with their being generated on a polyketide synthase by standard biosynthetic reactions. The stereochemistry at C-10 of compound (**8**) is consistent with precedents set by the closely related fatty acid synthases.[12]

We elected to study the biosynthesis of this non-aromatic metabolite using stable isotopes and n.m.r. spectroscopy to determine the origin of the various key residues. In the first of these experiments sodium [1,2-^{13}C$_2$]acetate was administered to a 6 day old culture of *Penicillium brefeldianum* before isolation and purification of palitantin 2 days later. The optimum time for feeding the precursors was estimated from a growth curve which was obtained by isolating the metabolite after varying growth periods. The ^{13}C n.m.r. spectrum of the resulting metabolite showed doublets symmetrically disposed about every signal. The coupling constants could be paired up unambiguously (Table 2), thus demonstrating the existence of seven intact acetate units.

In a preliminary experiment to determine the origin of key hydrogens in palitantin, CD_3CO_2Na was fed to the organism. The ^{1}H n.m.r. spectrum of the resulting metabolite showed enriched signals for 1-H, 3-H, 5-H, 7-H, 9-H, and 11-H; of the methyl-derived sites, therefore, only 13-H failed to give evidence fof deuterium incorporation.

Next, [2-^{13}C,2-^{2}H$_3$]acetate was used as a precursor with the aim of determining the number of deuteriums retained at individual sites by observation of α-shifted peaks[13] in the ^{13}C n.m.r. spectrum of the derived metabolite. The ^{13}C n.m.r. spectrum, run under conditions of simultaneous proton and deuterium decoupling, contained a single isotopically shifted peak for C-3, C-7, and C-11 (Table 3), indicating that each of these carbons bore at the most one deuterium. The retention of only one deuterium at C-3 is consistent with the intermediacy of a compound with a carbon–carbon double bond at that site. In addition, C-1 showed an envelope of signals indicating the presence of molecules multiply labelled with deuterium at this position: the signal at δ 14.76 p.p.m., 0.86 p.p.m. upfield of the normal signal for protonated molecules, can be assigned to molecules triply substituted with deuterium. Further shifted resonances for C-1, 0.28 and 0.58 p.p.m. upfield, were observed corresponding to molecules labelled with CH_2D and CHD_2 residues, respectively (Figure 1). The detection of some molecules with three deuteriums at C-1 confirmed that this methyl is part of the chain starter unit.

Adjacent to the ^{13}C–^{1}H$_2$ signal for C-9, there were two sharp shifted resonances, 0.37 and 0.74 p.p.m. upfield, arising from molecules with one and two deuteriums, respectively, at that site (Figure 1). That two deuteriums can be retained at C-9 is very significant because this result is consistent with only one of the three postulated biosynthetic pathways shown in Scheme 2; in pathway (C) up to two acetate-derived deuteriums can be retained at C-9, whereas in pathways (A) and (B) only one acetate-derived deuterium can be retained. Confirmation of the assignment of the shifted resonances was obtained by running a [^{1}H$_2$,^{2}H]-decoupled quaternary-only DEPT spectrum[14] which identifies carbons without directly attached hydrogens. The spectrum obtained showed a single resonance at δ 37.31 p.p.m. due to C-9 which arises from molecules to which two deuteriums were attached. More evidence for the retention of two deuteriums at C-9 of compound (**1**) was obtained by re-running the spectrum of the [2-^{13}C,2-^{2}H$_3$]acetate-enriched palitantin with only proton decoupling. As only the protons, not the deuteriums have been noise decoupled during the experiment, the shifted C-9 carbon resonance consisted of a quintet pattern, of intensity 1:2:3:2:1, consistent with the presence of two deuteriums at that site.

In order to determine the source of the oxygens in palitantin (**1**), sodium [1-^{13}C,^{18}O$_2$]acetate[15] was next used as a precursor. The presence of [^{18}O]acetate-derived oxygens could be detected by isotope shifts in the ^{13}C n.m.r. spectrum of the resultant labelled metabolite at C-10, C-14 but not C-12 (Figure 2, Table 3). Since the oxygen attached to C-12 is almost certainly derived from acetate we decided to do a control experiment which showed that an oxygen label at that site would have been lost by exchange in the course of the biosynthetic experiment. Unlabelled palitantin was exposed to ^{18}O-labelled H_2O (50% enriched) containing a trace of toluene-*p*-sulphonic acid. Mass spectrometric analysis of the crude recovered material showed in addition to the normal M^+ peak, an $M + 2$ peak of equal intensity, consistent with exchange to equilibrium at one site, presumably C-12. Following purification by preparative thin

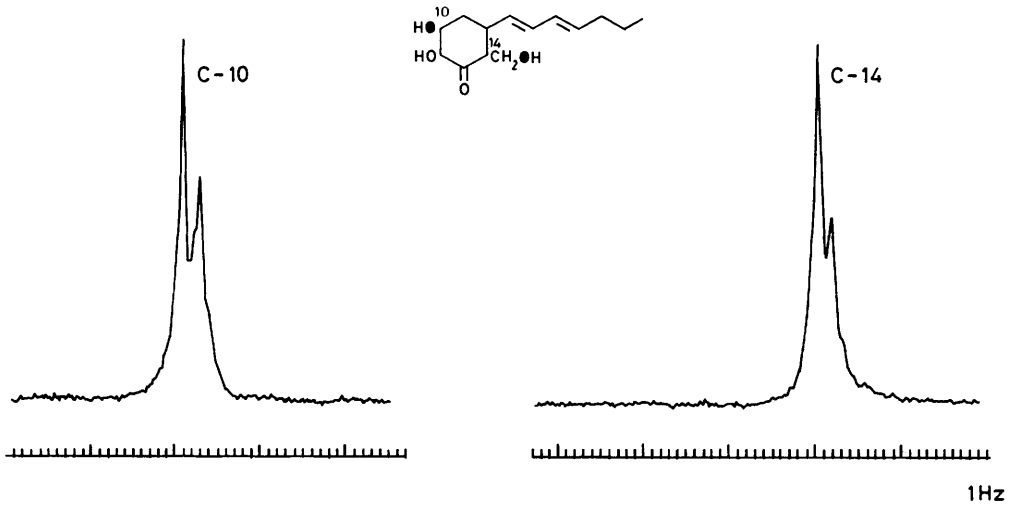
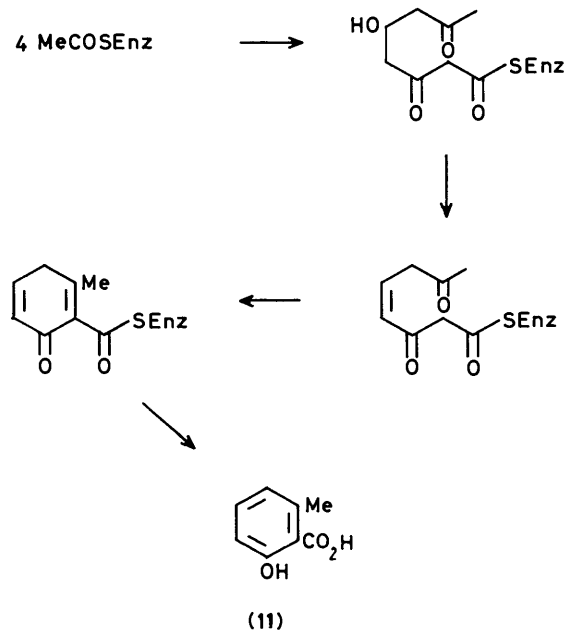


Figure 2. ^{18}O-Shifted resonances for C-10 and C-14 of palitantin (**1**) derived from [1-^{13}C,1-$^{18}O_2$]acetate

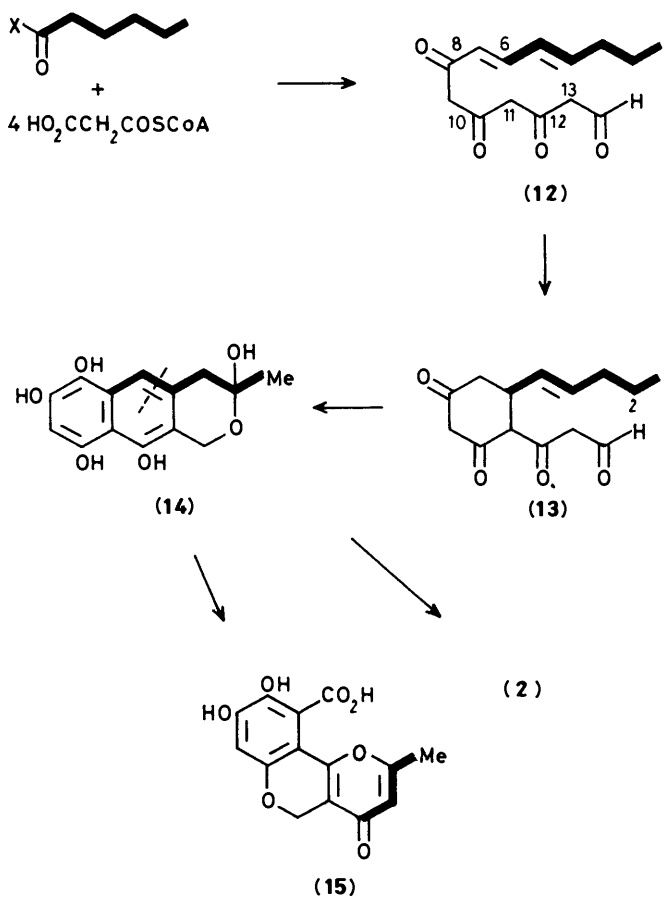
layer chromatography, this material failed to show an isotopically shifted peak in its ^{13}C n.m.r. spectrum. Mass spectrometric analysis of the sample recovered from the n.m.r. experiment confirmed that the ^{18}O label had been lost, presumably by exchange with water during the purification procedure.

The cyclisation mechanism shown for pathway (C) is consistent with all these results. It bears a remarkably close resemblance to that proposed[16] for the generation of the carbocyclic ring of the aromatic polyketide metabolite 6-methylsalicylic acid (**11**) (Scheme 3). All the corresponding carboxy-derived carbons are at the same oxidation level on both pathways; the key difference is that the hydroxy group at that site corresponding to C-10 or compound (**8**) is dehydrated prior to cyclisation in the proposed 6-methylsalicylic acid biosynthesis with the consequence that the six-membered ring of the intermediate corresponding to compound (**9**) can aromatise simply by enolisation of a ketone group. The carbocylic ring in compound (**9**) is much less prone to aromatise, and with the reduction of the enone residue the propensity to aromatise is removed. Subsequent steps in the proposed pathway involve the two-stage reduction of the thioester group to an alcohol, and the hydroxylation of the C-11 methylene, presumably at the expense of molecular oxygen.

Finally we propose in Scheme 4 a biosynthetic pathway



Scheme 3.



Scheme 4.

which relates the biosynthesis of palitantin (**1**) to that of its co-metabolite fulvic acid,[8] and, by implication, to those of related metabolites citromycetin (**15**),[17] lapidosin,[18] and polivione.[19] According to Scheme 4, the uncyclised polyketide intermediate released from the polyketide synthase is (**12**), which differs from compound (**8**), the corresponding palitantin intermediate, only in the oxidation level at C-10. The presence of a keto group at this site would facilitate an alternative cyclisation (between C-11 and C-6, rather than that between C-13 and C-8), leading to the formation of the intermediate (**13**). Further reasonable cyclisation and oxidation would lead to the naphthalene (**14**), which is the proposed aromatic precursor of the citromycetin family of metabolites.[20] The proposal[20] that a preformed C_6 starter acid may be involved in citromycetin biosynthesis can be satisfactorily accommodated by Scheme 4, as indicated by heavy lines in the structures.

A precedent for the required conversion of the C-2 methylene into a carbonyl group in going from (**13**) to (**14**) can be found in aflatoxin biosynthesis.[21] According to Scheme 4, this type of oxidative functionalisation would extend to residues involved in the generation of the aryl rings. In that sense it runs counter to accepted ideas of polyketide biosynthesis, which assume that a polyketone with the appropriate functionality is generated directly by the polyketide synthase. Given the paucity of experimental evidence, this modified mode of aryl ring generation deserves consideration, not just for the citromycetin group of metabolites, for which there is circumstantial evidence in the form of a non-aromatic co-metabolite, but also more generally for other classes of polyketide phenol.

Experimental

M.p.s were determined on a Reichert hot-stage apparatus and are uncorrected. I.r. spectra were recorded on a Perkin-Elmer 297 spectrometer for solutions in chloroform. U.v. spectra were recorded on a Pye-Unicam SP-80 or SP8-400 spectrometer. ^{1}H N.m.r. spectra were recorded on a Bruker WM-250 or WP-80 spectrometer. ^{1}H N.m.r. spectra were obtained either on the WM-250 running unlocked, or on the WH-400 using internal C_6F_6 and ^{19}F lock. ^{13}C N.m.r. spectra were obtained on a Bruker WM-250 or WH-400 spectrometer. Chemical shifts are given as p.p.m. downfield of tetramethylsilane. All spectra showing isotopic shifts measured on the WH-400 instrument. Mass spectra were obtained on an AEI MS 30 or MS 50 instrument.

Qualitative t.l.c. was carried out on commercially prepared plates coated with Merck Kieselgel 60 GF_{254}. Preparative t.l.c. was performed using plates coated with the same silica gel (20 × 20 × 0.1 cm). Solvents were distilled before use, organic solutions were dried over anhydrous sodium sulphate. Carbon-13 labelled precusors were obtained from Amersham International.

Growth of Penicillium brefeldianim *and Isolation of Palitantin* (**1**).—*Penicillium brefeldianum* (C.M.I. 40592ii) was grown at 27 °C in a static culture, each flask containing 500 ml of an aqueous solution[22] made up of glucose (50 g), sodium nitrate (2 g), potassium dihydrogen phosphate (1 g), potassium chloride (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), $CuSO_4 \cdot 7H_2O$ (0.005 g), $ZnSO_4 \cdot 7H_2O$ (0.01 g), and biotin (from Brewers yeast) (*ca.* 5 g) per litre of glass-distilled water. After 10 days of growth, the bright yellow medium was decanted off and filtered through a glass wool plug. It was then acidified with 50% H_2SO_4 to pH 1 and left at room temperature for about 1 h. The yellow flocculant material that formed was filtered off under suction through a bed of Celite, and the clear yellow solution was extracted with ethyl acetate (5 × 200 ml). The combined extracts were dried (Na_2SO_4) and evaporated under reduced pressure. The residue was purified by p.l.c. eluting with ethyl acetate–hexane (7:3) twice, to yield palitantin (**1**) (200—300 mg l^{-1}) as needles (from methanol), m.p. 159—161 °C (lit.,[1] 164—165 °C) (Found: C, 66.1; H, 8.67. $C_{14}H_{22}O_4$ requires C, 66.1; H, 8.72%); R_F [ethyl acetate–hexane (7:3)] 0.2; $\nu_{max.}$(Nujol) 3 350 (OH), 1 705 (ketone C=O) and 1 560 cm^{-1}; $\lambda_{max.}$(MeOH) 232 nm; $\delta_H(CD_3OD)$ 0.91 (3 H, t, *J* 7.39 Hz, Me), 1.41 (2 H, m, *J* 7.39 and 7.45 Hz, 2-H), 1.93 (2 H, m, 9-H), 2.06 (2 H, dt, *J* 7.45 and 6.63 Hz, 3-H), 2.4 (1 H, m, 13-H), 2.7 (1 H, m, 8-H), 3.69 (2 H, m, 14-H), 4.29 (2 H, m, 10- and 11-H), 5.49 (1 H, m, 7-H), 5.65 (1 H, m, 4-H), 5.97—6.14 (2 H, m, 5- and 6-H) (Found: M^+, 254.1505. $C_{14}H_{22}O_4$ requires *M*, 254.1518); *m/z* 254 (10%, M^+), 236 (20, $M - H_2O$), 224 (51, CH_2O), and 223 (100, CH_2OH).

Isolation of O-*Methylfulvic Acid* (**2**) *and* O-*Methylanhydrofulvic Acid* (**3**).—*Penicillium brefeldianum* (C.M.I. 40592ii) was grown in surface culture on Raulin-Thom medium[8] (500 ml per flask); after 18 days the mycelium and medium were separated. The medium was acidified with 50% sulphuric acid and extracted with ethyl acetate (4 × 300 ml). Saturated brine was used to break up the emulsions formed at this stage. The combined extracts were washed with water, dried (Na_2SO_4), and evaporated under reduced pressure to give a brown gum. Purification by p.l.c. on acidified silica gel[19] [ethyl acetate–hexane (1:1)] gave O-*methylfulvic acid* (**2**) (25 mg l^{-1}) as a yellow oil (Found: M^+, 322.0675. $C_{15}H_{14}O_8$ requires *M*, 322.0689); R_F [ethyl acetate–hexane (7:3)] 0.35; $\nu_{max.}$(Nujol) 3 500 (OH), 1 700 (ketone C=O), 1 660 (carboxy C=O), 1 600, and 1 580 cm^{-1}; $\lambda_{max.}$(MeOH) 225 and 315 nm; $\delta_H(CD_3OD)$ 1.46 (3 H, s, Me), 2.8 (2 H, q, J_{AB} 14.9 Hz, CH_2), 3.83 (3 H, s, OMe), 4.2 (2 H, br q, OCH_2), and 6.92 (1 H, s, ArH); *m/z* 322 (22%, M^+) and 290 (90, $M - CH_2O$). The second product isolated was O-*methylanhydrofulvic acid* (**3**) (20 mg l^{-1}) as an oil (Found: M^+, 304.0602. $C_{15}H_{12}O_7$ requires *M*, 304.0583); R_F [ethyl acetate–hexane (7:3)] 0.30; $\nu_{max.}$(Nujol) 2 900 (CH), 1 690 (ketone C=O), 1 656 (carboxy C=O), 1 594, and 1 570 cm^{-1}; $\lambda_{max.}$(MeOH) 212 and 286 nm; $\delta_H(CD_3OD)$, 1.96 (3 H, d, *J* 0.8 Hz, Me), 3.84 (3 H, s, OMe), 5.12 (2 H, s, OCH_2), 5.55 (1 H, d, *J* 0.8 Hz, vinyl-H), and 6.94 (1 H, s, ArH); *m/z* 304 (46%, M^+) and 294 (100, $M - CH_2O$). Both compounds, in alcohol, gave a deep green ferric reaction.

Techniques used in the Administration of Labelled Compounds.—Flasks were ready for the addition of precursors when the white mycelial mat was completely formed and the growing medium was yellow; this was usually 6 days after inoculation. The labelled precursors (150 mg) were dissolved in the minimum volume of water and were injected *via* a syringe through the mycelial mat into the medium. The flasks were swirled thoroughly, then incubated for two more days at 28 °C before work-up.

Acknowledgements

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