A Study of Biosynthesis of the Polyketide Polivione in *Penicillium frequentans* Using ^{13}C-, ^{2}H-, and ^{18}O-Labelled Precursors

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The biosynthesis of polivione (**5**), a metabolite of *Penicillium frequentans*, has been studied using ^{13}C-, ^{2}H-, and ^{18}O-labelled precursors. Analysis of the labelled products by both n.m.r. and mass spectrometry suggests that polivione [and probably citromycetin (**1**) and fulvic acid (**3**)] is formed *via* a naphthalene intermediate having the same carbon skeleton as fusarubin (**9**). Incorporation of [1,2-$^{13}C_2$]acetate resulted in compound (**5**) which contained seven intact acetate units arranged in a pattern consistent with that observed for citromycetin (**1**). Deuterium from CD_3CO_2Na was incorporated only in the starter methyl group and in the methine position of the aryl ring. The polivione produced after incorporation of [1-^{13}C,1-$^{18}O_2$]acetate showed isotopically shifted peaks in its ^{13}C n.m.r. spectrum at C-7, C-9, and C-11, showing that ^{18}O was retained at those sites. Significantly, there was no evidence that ^{18}O was retained at the other two carboxy-derived sites, C-2 or C-4. The oxygens at C-4, C-12, and C-14 were shown to be derived from $^{18}O_2$ gas by the presence of isotopically shifted peaks in the ^{13}C n.m.r. spectrum of polivione produced after incorporation of this precursor. Detailed pathways are proposed for the production of citromycetin (**1**), fulvic acid (**3**), and polivione (**5**) from a common polyketide precursor.

The biosynthesis of citromycetin (**1**), a polyketide metabolite of *Penicillium frequentans* has aroused much interest, since the unusual branched chain carbon skeleton (**2**) cannot arise in a standard way from a linear polyketide chain. Another metabolite possessing the same carbon skeleton is fulvic acid (**3**). The close resemblance between compounds (**1**) and (**3**), both in carbon skeleton and oxygenation pattern suggests that they are probably built up using a common biosynthetic strategy.

In order to explain the branched nature of the above metabolites, it was suggested that the carbon skeleton (**2**) could be derived by addition of a C_1 unit onto an acetate-derived chain (**4**).[1] Later, however, Birch[2] and his co-workers showed that seven C_2 units originating from [^{14}C]acetate were incorporated into citromycetin (**1**) by *Penicillium frequentans*, thus verifying that citromycetin (**1**) is derived solely from acetate. Since then, two fundamentally different biosynthetic strategies have been proposed to explain the structure of citromycetin and fulvic acid. According to the first,[3] the skeleton would be produced by combination of two separately formed chains, as exemplified by pathways (A) and (B) of Scheme 1. It has been observed by Turner[4] that known polyketides usually have structures in which the uncyclised residue from the methyl end of the polyketide chain is longer than the residue from the carboxy end of the chain; this would suggest that pathway (B) is less likely. The two-chain hypothesis was initially supported by Gatenbeck and Mosbach[5] and later by Birch *et al.*[6] who found evidence for two acetate starter units by chemical degradation of citromycetin labelled biosynthetically from diethyl [2-^{14}C]malonate. A ^{13}C n.m.r. study[7] of citromycetin (**1**) labelled from [1-^{13}C], [2-^{13}C], and [1,2-$^{13}C_2$]acetate showed a labelling pattern consistent with a two-chain origin but did not rule out routes involving a single-chain route. This latter possibility could occur by cleavage of a carbon–carbon bond in a polyketide skeleton produced in the conventional way by cyclisation of a single unbranched polyketide chain.[4,8,9] Two reasonable candidates, both with skeletons corresponding to naphthalene derivatives, are shown in pathways (C) and (D) of Scheme 1. Kurobane *et al.*[9] found evidence for the biosynthesis of fulvic acid (**3**) *via* oxidative ring-cleavage of a polyketide of the fusarubin type in accordance with pathway (C).

The isolation of polivione[10] from *Penicillium frequentans* renewed our interest in the biosynthesis of the above metabolites; polivione tautomers (**5**) and (**6**) have the carbon skeleton (**2**) with equivalent carbons at the same oxidation level as in compounds (**1**) and (**3**). It is likely, therefore, that all the metabolites are built up using a common biosynthetic pathway.

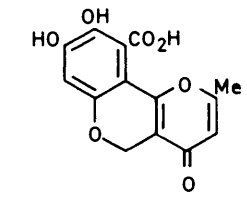
We elected[11] to study the biosynthesis of polivione (**5**), rather than citromycetin (**1**), because polivione is the major component in the crude ethyl acetate extract of a culture of *Penicillium frequentans*. Furthermore, polivione (**5**) is soluble in chloroform (in contrast to citromycetin itself) and hence very suitable for n.m.r. experiments. The ^{13}C n.m.r. spectrum of polivione was assigned during the course of its structure determination[10] using two-dimensional homonuclear and heteronuclear correlation experiments.[12]

Prior to incorporation studied using stable isotopes, optimum conditions for precursor administration were sought by performing a series of experiments using ^{14}C-labelled acetate. The isolated polivione was found to be radioactive in each of these experiments, in which the quantity of acetate and the time of feeding and isolation were varied. The highest incorporation was observed when [^{14}C]acetate was fed on the fifth day of fermentation and the metabolite harvested after an additional day of incubation. Under these conditions the observed incorporation was 2% of the labelled compound administered. This experiment established that the fungus can incorporate precursors at levels suitable for detailed ^{13}C feeding experiments.

With suitable conditions established, the origin of the carbon skeleton of polivione was first studied by administration of [1,2-$^{13}C_2$]acetate. In the ^{13}C n.m.r. spectrum of the resulting metabolite all 14 peaks were flanked by doublets due to ^{13}C–^{13}C coupling. The coupling constants could be paired unambiguously, thus demonstrating the existence of seven intact acetate units. Since both citromycetin[2,5,7] and fulvic acid[9] have been shown to incorporate acetate in a similar manner, this pattern is consistent with the proposed biosynthetic relationship.

However, the incorporation pattern of [1,2-$^{13}C_2$]acetate does not differentiate between the alternative biosynthetic schemes, because they can all lead to the same pattern of incorporation of intact acetate units into the branched structure (**2**).

The origins of the hydrogens in the C–H groups of polivione



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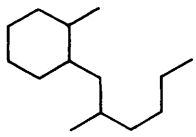

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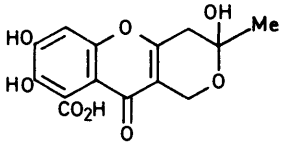

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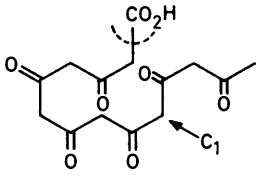

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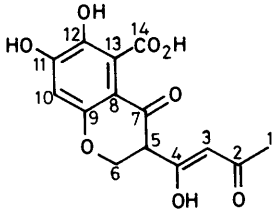

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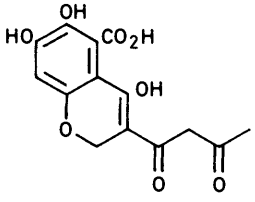

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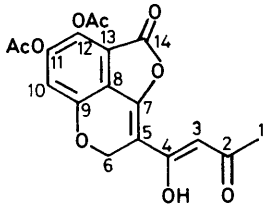

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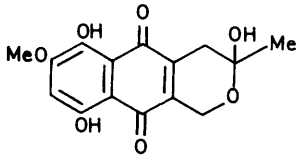

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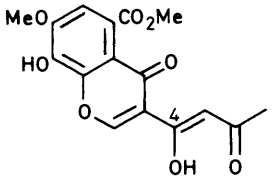
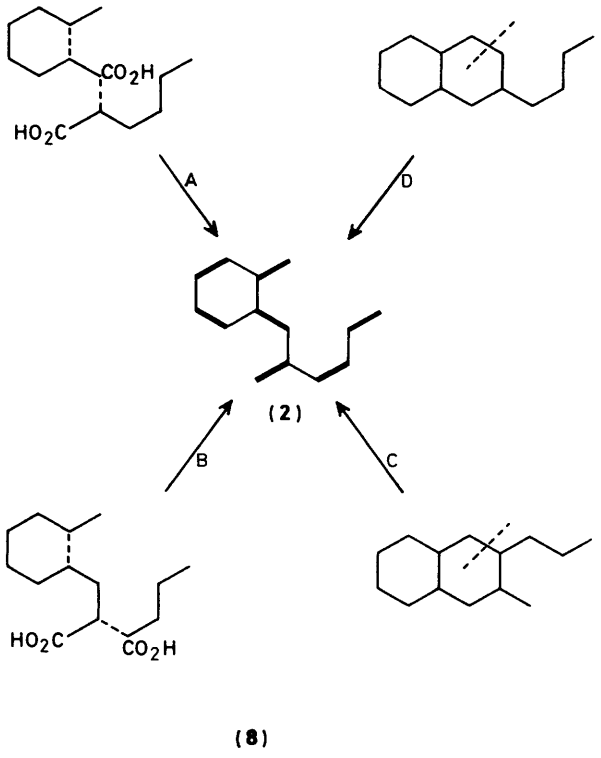


(10)

were investigated next, by administration of [2-$^2H_3$]acetate to the organism. The ^2H n.m.r. spectrum of the derived metabolite showed a strong signal from the methyl group at C-1, the presumed starter unit of the polyketide chain, and a weaker signal for 10-H (the aromatic proton). Disappointingly, no deuterium could be detected at the key position C-3 in either of the two principal tautomers of the metabolite. Presumably any label incorporated at that site is subsequently lost by exchange with the medium through equilibration of compound (**5**) with its tautomer (**6**).

The decisive experiments were those aimed at establishing origins of the oxygen atoms of polivione. Firstly, [1-^{13}C,1-$^{18}O_2$]acetate[13] was used as a precursor. The resulting polivione showed the incorporation of three ^{18}O atoms per molecule by mass spectral analysis but this technique did not allow exact determination of the positions of the ^{18}O labels. These were determined from the p.n.d. ^{13}C n.m.r. spectrum of compound (**5**) which revealed the presence of an enhanced amount of ^{18}O at C-7, C-9, and C-11 (Table). Each of these resonances appeared as an enhanced pair of signals corresponding to the $^{13}C^{16}O$ and $^{13}C^{18}O$ species, respectively. Interestingly, no ^{18}O was present at either C-2 or C-4, whose ^{13}C n.m.r. signals each appeared as enhanced singlets, as did those for the two non-oxygen-bearing carbons C-6 and C-13.



(8)

Scheme 1.

Next, the enriched polivione was converted into diacetylanhydropolivione[10] (**7**) by treatment with acetic anhydride and anhydrous sodium acetate. The 100 MHz ^{13}C n.m.r. spectrum of this compound displayed isotopically shifted resonances for the two oxygen-bearing carbons C-9 and C-11. The absence of an ^{18}O isotope shift at C-7 suggests that the lactone formation in diacetylanhydropolivione involves attack by the oxygen attached to C-14 on the carbonyl group at C-7, with subsequent loss of the labelled oxygen attached to C-7 of polivione.

Having established that three of the oxygen atoms of polivione are derived directly from the carboxylate oxygens of the carbon-skeleton precursor acetate, we investigated the origin of the remaining five oxygens, by growing a culture of *Penicillium frequentans* in the presence of ^{18}O-labelled molecular oxygen. A growing culture of the organism was exposed to $^{18}O_2$ gas for 24 h, at the time the rate of production of polivione reached its maximum (between 3—5 days after inoculation). The resulting metabolite showed ^{18}O-shifted peaks in its ^{13}C n.m.r. spectrum for C-4, C-12, and C-14 (two shifted peaks). The crucial finding was the presence of an ^{18}O-shifted peak at C-4. Since this peak was relatively weak (presumably due to loss of labelled oxygen by exchange with water), we decided to confirm that it was a true isotopically shifted peak by a control experiment in which unlabelled

Table. ^{18}O-Shifted peaks in the ^{13}C n.m.r. spectra[a] of ^{18}O-enriched samples of polivione (**5**)

Source of ^{18}O	^{18}O-Shifts (Hz)						
	C-2	C-4	C-7	C-9	C-11	C-12	C-14
[1-^{13}C,1-$^{18}O_2$]Acetate[b]			3.4	1.6	1.2		
$^{18}O_2$[b]		5.5				1.2	2.3, 4.6
$H_2{}^{18}O$[c]	2.6	5.6	3.4				

[a] At 100 MHz in CD_2Cl_2. [b] Biosynthetic experiment. [c] Exchange in vitro.

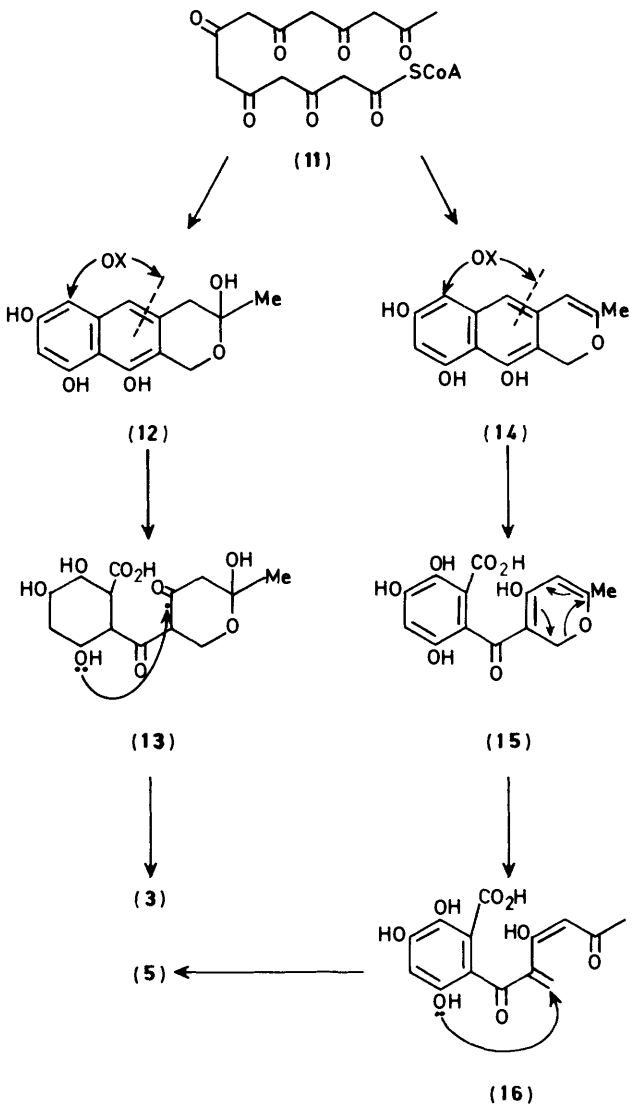
polivione was exposed to ^{18}O-enriched H_2O (50% enrichment) for 24 h. The p.n.d. ^{13}C n.m.r. spectrum of the recovered material showed isotopically shifted peaks for C-4 (at the expected position) and C-2 (Table), the shifted peaks being in each case approximately equal in intensity to the unshifted peak. In addition, an ^{18}O-shifted peak was observed for C-7; the size of the shift was exactly the same as that observed when [1-^{13}C,1-$^{18}O_2$]acetate was incorporated into the metabolite.

As a further check on the site of ^{18}O labelling the polivione was converted into diacetylanhydropolivione (**7**). The expected ^{18}O-shifted peaks were observed for C-12 and C-14 but not for C-4. The lack of isotope at the latter position implies that treatment of polivione with Ac_2O–NaOAc causes acetylation of the phenolic hydroxys and, in addition, the hydroxy at C-4, the latter being displaced by water in the work-up procedure.

On the basis of these results, we can propose a detailed sequence of steps by which polivione is formed. The finding that the oxygen at C-4 is derived from molecular oxygen points to an oxygenation reaction at that site at some stage of the biosynthesis. This would be expected for pathway (C) of Scheme 1, in which a naphthalene derivative with the carbon skeleton (**8**) undergoes oxidative cleavage of one of the carbon–carbon bonds to that carbon which subsequently becomes C-4 of polivione. In the other three postulated pathways (Scheme 1), the oxygen at C-4 would be expected to be derived from acetate (or from water by exchange).

A possible biosynthetic scheme to account for these results is presented in Scheme 2. A heptaketide chain (**11**) is cyclised and reduced at the chain terminus to produce compound (**12**) or (**14**) [quite likely *via* compound (**12**)], both of which are structurally related to fusarubin (**9**). Oxidative cleavage at the sites indicated would give intermediates (**13**) or (**15**), respectively. In the former case, cyclisation of the phenolic hydroxy as indicated would lead ultimately to fulvic acid (**3**). For compound (**15**) this potential cyclisation might be pre-empted by a facile Cope rearrangement leading to compound (**16**). The favoured cyclisation would now be the one leading to polivione (**5**). The absence of a detactable isotopically shifted peak for C-2 in the ^{13}C n.m.r. spectrum of polivione produced by incorporation of [1-^{13}C,1-$^{18}O_2$]acetate is in accord with this proposal, even though the attached oxygen is derived from acetate; the relevant oxygen is transferred to C-2 from a different acetate unit, and so, at the low level of isotopic enrichment in the metabolite, there is a very low probability that ^{18}O and ^{13}C labels will coincide at that site.

Recently, a similar scheme was proposed by Hutchinson to account for the biosynthesis of fulvic acid (**3**).[9] However, none of the evidence previously obtained discriminates between pathways (C) and (D) for this metabolite; the $^{18}O_2$ incorporation experiment which provided decisive evidence in our studies of polivione would probably not work with fulvic acid because the key oxygen at C-4 would always certainly be lost in the cyclisation and dehydration of compound (**12**) to form the pyrone ring of compound (**3**). Nevertheless, the close

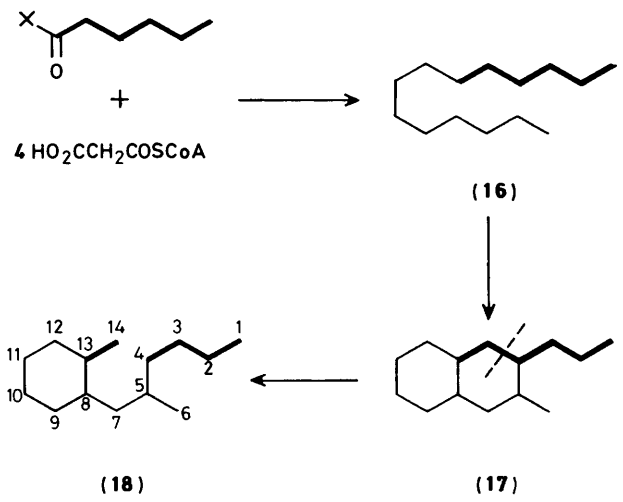


Scheme 2.

structural relationship which exists between compounds (**1**) and (**3**) strongly suggests a common biosynthesis. Another metabolite in this family of heptaketides for which an $^{18}O_2$ incorporation study might be useful is lapidosin (**10**),[14] which could retain an $^{18}O_2$-derived oxygen at C-4.

In conclusion, we note, as did Hutchinson[9] earlier, that a metabolite formed in the conventional way by pathway (C) would be expected to incorporate only one C_2 starter unit. Therefore this pathway is not obviously compatible with the earlier evidence from three groups[5,6,7] that citromycetin (and by implication polivione) incorporates two C_2 starter units. This apparent conflict can be reconciled in terms of a modified biosynthesis of the linear heptaketide precursor (**16**) as shown in Scheme 3. Here the chain is started by a preformed C_6 unit (indicated by heavy lines) which condensed successively with four malonate units in the usual way to generate a linear C_{14} intermediate (**16**). Cyclisations convert compound (**16**) into the bicyclic system of (**17**) which undergoes carbon–carbon bond cleavage to generate the carbon skeleton of (**18**), corresponding to the four natural products (**1**), (**3**), (**5**), and (**10**).

On the basis of Scheme 3, three acetate-derived units (those of the C_6 starter acid) could differ in activity from the remaining four. Two of these, C-1 and C-2, and C-14 and C-13, were shown



Scheme 3.

to differ in the earlier experiments with malonate incorporation. The activity at the third was not measured because of the lack of a suitable degradation for citromycetin. This crucial piece of evidence, which would have discriminated between our new proposal and the earlier two-chain hypotheses is therefore missing.

The modified pathway presented in Scheme 3 will be elaborated in the following paper in the light of an investigation of palitantin biosynthesis. For the moment we note that a precedent for the incorporation of a preformed C_6 starter acid has been found recently in the biosynthesis of the aflatoxins.[15]

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 SP8-100 or SP8-400 spectrometer. ^{1}H N.m.r. spectra were recorded on a Bruker WM-250 or WP-80 SY spectrometer. ^{1}H N.m.r. spectra were obtained on the WM-250 running unlocked. ^{13}C N.m.r. spectra were obtained on a Bruker WM-250 or WH-400 spectrometer. All ^{18}O-shifts were measured on the WH-400 instrument. Mass spectra were obtained on an AEI MS30 or MS50 instrument. Solvents were distilled before use, organic solutions were dried over anhydrous sodium sulphate. Radioactive samples were counted in an organic scintillator solution (7 ml), on a Packard Tri-Carb 3385 instrument, and standardised internally with radioactive hexadecane. Carbon-13 labelled precursors were obtained from Amersham International.

Isolation of Metabolites.—*Penicillium frequentans* (C.M.I. 91914ii) was grown in surface culture of Czapex-Dox medium[7] (500 ml per flask); after 10 days of growth at 28 °C the cultures were acidified with 50% H_2SO_4 and filtered. The filtrate was extraced with ethyl acetate to give a brown gum (*ca.* 2.5 g l^{-1}) which was further purified by flash chromatography on acidified silica gel[10] [ethyl acetate–hexane (1:1)] followed by preparative t.l.c. [ethyl acetate–hexane (7:3)]. Polivione (**5**) and (**6**) and citromycetin (**1**) were isolated in order of decreasing R_F value. A full characterisation of the metabolites is given in a preceding paper.

Incorporation of Labelled Acetates.—In a feeding experiment involving labelled acetate the precursor (150 mg), dissolved in the minimum volume of water (2 ml), was administered to a flask containing a 5 day old surface culture of *Penicillium frequentans*. The labelled solutions were injected through the mycelial mat into the growing medium using a long-needled syringe. The resulting metabolite was isolated on day 6.

Administration of $^{18}O_2$ Gas.—A 48 h old culture of *Penicillium frequentans* was connected to a closed system utilising a 1-l gas burette ($^{18}O_2$-reservoir), a diaphragm pump with a bypass valve to circulate the $^{18}O_2$ gas directly through the fermentation medium, and a round-bottomed flask with concentrated potassium hydroxide, used to trap expired carbon dioxide. The gas burette was filled with oxygen (1 l) containing 21.4 atom % oxygen-18, and the flow-rate was set at approximately 1 bubble per second. After 24 h air (200 ml) was introduced into the gas burette and incubation continued for an additional day to ensure complete uptake of ^{18}O, after which the labelled poivione had reached its maximum concentration, was isolated and purified as described previously.

Acknowledgements

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