Oxygen-18 Labelling Studies on the Biosynthesis of Citreoviridin

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Incorporation of [1-^{13}C,^{18}O$_2$]acetate and ^{18}O$_2$ gas into citreoviridin by cultures of *Penicillium pulvillorum* established the origin of the oxygen atoms and provided stereochemical and mechanistic information on the biosynthetic pathway.

Citreoviridin (**1**) is a member of a group of mycotoxins which act as inhibitors of ATP-synthesis and ATP-hydrolysis catalysed by mitochondrial enzyme systems.[1] Our previous biosynthetic studies using ^{13}C-labelled precursors have shown that citreoviridin is derived from a C_{18}-polyketide formed from acetyl-CoA as a starter unit and eight malonyl-CoA units, and five C_1 units, derived from methionine. The stereochemical and mechanistic details of the subsequent intermediate transformations leading to the citreoviridin structure remained speculative. For instance, citreomontanin (**2**), a metabolite of *Penicillium pedemontanum*,[3—5] has been proposed as a possible precursor for citreoviridin.[6] In this paper we report results of ^{18}O isotope incorporations, which, in conjunction with the known absolute configuration of citreoviridin,[7] have enabled us to propose a detailed mechanism for the formation of the tetrahydrofuran moiety.

[1-^{13}C,^{18}O$_2$]Acetate (3.0 mmol) (53.2% ^{13}C^{18}O$_2$, 39.1% ^{13}C^{18}O, 7.7% ^{13}C^{16}O) was added to cultures of *P. pulvillorum*, CSIR 1406 (200 ml), over days 3—11 before isolation and purification of citreoviridin (**1**) (60 mg) on day 12 as previously described.[2] The incorporation of ^{18}O was detected by the presence of isotopically shifted resonances in the proton-decoupled ^{13}C n.m.r. spectrum[8] (see Figure 1 and Table 1). The C-14, C-16, and C-18 resonances exhibited upfield isotope shifts indicating that the corresponding carbon–oxygen bonds had remained intact throughout the biosynthetic pathway. For the C-18 resonance, three isotopically shifted signals appeared due to species having ^{18}O in (a) the singly bonded oxygen ($\Delta\delta$ −0.013 p.p.m.), (b) the doubly bonded oxygen ($\Delta\delta$ −0.034 p.p.m.), and (c) both the singly and doubly bonded oxygens ($\Delta\delta$ −0.047 p.p.m.). The presence of isotopomers with both ^{13}C and singly bonded ^{18}O at C-18 is the result of multiple labelling. Interestingly, no ^{18}O was present at either C-2 or C-4 as the resonances at δ_C 78.02 and 86.27 appeared as enhanced singlets. The lack of ^{18}O labelling at C-3 and C-5 is to be expected as these carbon atoms are derived from C-2 of acetate.[2]

The fermentation of cultures of *P. pulvillorum* (200 ml) in which the normal atmosphere was replaced by one containing ^{18}O$_2$ (50.0 atom %) and the simultaneous addition of [1-^{13}C]acetate (3.0 mmol, 99 atom % ^{13}C), admixed with unlabelled acetate (9.0 mmol), to the medium from day 4 to 9 produced citreoviridin (32 mg) whose proton-decoupled ^{13}C n.m.r. spectrum (see Figure 2) demonstrated the origin of the C-4 and tetrahydrofuran ring oxygens from oxidative processes. The molecular ion cluster in the mass spectrum of citreoviridin derived from [1-^{13}C]acetate (99.0 atom %) and

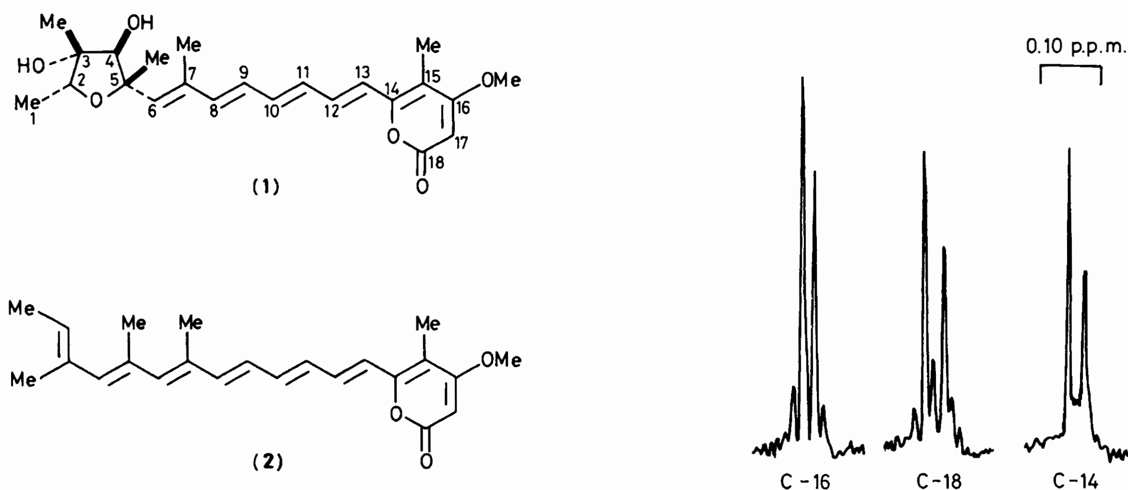


Figure 1. ^{18}O Isotope shifts observed in the ^{13}C n.m.r. spectrum† of citreoviridin (**1**) derived from [1-^{13}C,^{18}O$_2$]acetate.

(4)

† Spectroscopic parameters were as follows: the spectrum of the citreoviridin was accumulated at 303 K in a 5 mm bore tube using a sweep-width of 21 739 Hz with a 64 K data block, 512 scans, pulse angle 90° and an acquisition time of 1.507 s. For resolution enhancement a line broadening factor of −2.0 was applied together with a Gaussian multiplier of 0.4 and the free induction decay was zero filled to 128 K prior to Fourier transformation, 0.33 Hz/data point.

Figure 2. ^{18}O Isotope shifts observed in the ^{13}C n.m.r. spectrum† of citreoviridin (**1**) derived from $^{18}O_2$ gas.

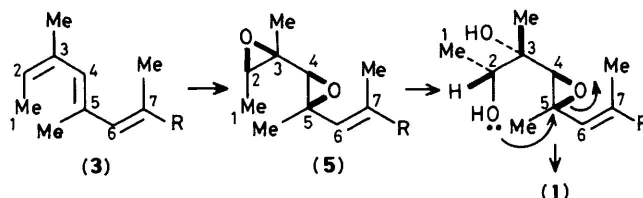
Table 1. ^{18}O Isotopically shifted resonances observed in the 125.76 MHz ^{13}C n.m.r. spectra of citreoviridin.[a]

Carbon	δ_C	$\Delta\delta$ (p.p.m. × 100)
C-2	78.02	2.8[b]
C-3	81.10	—[b,c]
C-4	86.27	1.8[b]
C-5	84.47	3.0[b]
C-14	154.90	2.9[c]
C-16	171.22	2.1[c]
C-18	163.96	4.7, 3.4, 1.3[c]

[a] Recorded on a Bruker WM-500 spectrometer; solvent CD_2Cl_2. [b] Enriched by $^{18}O_2$. [c] Enriched by [1-^{13}C,$^{18}O_2$]acetate.

$^{18}O_2$ (69.7 atom % ^{18}O, 48.6% $^{18}O_2$, 42.2% $^{18}O^{16}O$) indicated the presence of multiple labelled molecules *viz.* $^{12}C^{16}O_2$ 67.4% $^{13}C^{16}O_2$ 4.6%, $^{12}C^{18}O^{16}O$ 14.5%, $^{13}C^{18}O^{16}O$ 4.1%, $^{12}C^{18}O_2$ 6.8%, and $^{13}C^{18}O_2$ 2.6%. The ratio of isotopomers with a single ^{18}O atom compared to those with two ^{18}O atoms *i.e.* the ratio $(^{12}C^{18}O^{16}O + {}^{13}C^{18}O^{16}O)/(^{12}C^{18}O_2 + {}^{13}C^{18}O_2)$, equals 2.0, and suggests that oxygen is introduced by a mono-oxygenase enzyme. The introduction of the C-4 and tetrahydrofuran ring oxygen atoms from a single oxygen molecule by a dioxygenase enzyme requires a value of 0.9 for this ratio.

The above experiments establish for the first time the origins of the oxygen atoms in citreoviridin. Analysis of the results affords considerable insight into the stereochemistry and the mechanism of the events by which this metabolite is elaborated. For example, alkylation of a β-ketoacyl thioester by *S*-adenosyl methionine introduces the eventual C-3, C-5, C-7, and C-15 methyl groups, and generates the C-16 *O*-methyl moiety. The subsequent loss of oxygen from C-2, C-4, C-6, C-8, C-10, and C-12 presumably occurs by a



Scheme 1. Proposed mechanism for the formation of the tetrahydrofuran moiety of citreoviridin (**1**).

reduction–elimination sequence analogous to fatty acid biosynthesis[9] to generate the 2*Z*,4*E*,6*E*,8*E*,10*E*,12*E* polyene with the 3-s-*cis*,5-s-*cis*-conformation (**3**) (Scheme 1). The exact timing of the methylation step and the reduction–elimination sequence is not known but these reactions do not have to involve a C_{18} β-ketoacyl thioester and could occur at an earlier stage of the β-ketoacyl chain assembly. The subsequent formation of the pyrone ring proceeds by nucleophilic attack of the C-14 enolic hydroxy group on the thioester carbonyl [see (**4**)] and does not involve a free carboxylic acid intermediate as the ^{18}O enrichment at C-18 is the same as that at C-16 and C-14 (see Figure 1). Once released from its polyketide synthetase, the polyene is postulated to undergo epoxidation by a mono-oxygenase to give the (2*R*,3*R*,4*S*,5*R*)-bisepoxide (**5**). Nucleophilic attack by water at C-3 would initiate the formation of the tetrahydrofuran moiety of citreoviridin with the correct absolute configuration of the substituents. The oxygen of the C-3 hydroxy group is thus derived from the medium.

Citreomontanin (**2**) differs from the putative polyene intermediate (**3**) in that it has the 2*E* configuration and exists in the solid state as the 3-s-*cis* conformer.[4,10] The epoxidation of (**2**) and subsequent ring formation as outlined above generates a tetrahydrofuran moiety with the wrong stereochemistry for the C-2 carbon atom. Citreomontanin (**2**) is therefore not an intermediate in the biosynthesis of citreoviridin (**1**) and this has been confirmed by feeding experiments.[10] The co-existence of these two compounds in cultures of *P. pedemontanum* points to the presence of an intermediate common to both biosynthetic pathways.[10]

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