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Biosynthetic studies on some polyene mycotoxins

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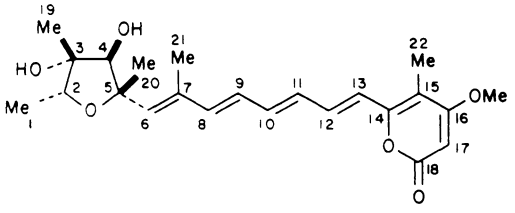
Abstract - Citreoviridin, asteltoxin and aurovertin B are members of a group of mycotoxins which act as inhibitors of oxidative phosphorylation in mitochondria. The biosynthetic origin of the carbon skeleton of these metabolites was studied by incorporation of ^{13}C-labelled precursors: [1-^{13}C]-, [2-^{13}C]-, [1,2-$^{13}C_2$]-, [1-^{13}C,2-$^{2}H_3$]-, and [2-^{13}C,2-$^{2}H_3$]acetate, (2S)-[methyl-^{13}C]methionine and [1-^{13}C]- and [3-^{13}C]propionate. The results show that aurovertin B and asteltoxin can be formed via two biosynthetic pathways. The first pathway involves the methylation of an eventual C_{20}-polyketide, followed by loss of the chain-initiating acetate unit. The second pathway involves a polyketide precursor formed from a propionate chain-initiating unit and eight malonate units. In addition the arrangement of intact acetate units in asteltoxin derived from [1,2-$^{13}C_2$]acetate proves that a 1,2-bond migration occurs during the formation of the 2,8-dioxabicyclo-[3.3.0]octane moiety. Citreoviridin is derived from a C_{18}-polyketide formed from an acetate chain-initiating unit and eight malonate units. Incorporation of [1-^{13}C,$^{18}O_2$]acetate and $^{18}O_2$ gas into citreoviridin, aurovertin B, and asteltoxin established the origin of the oxygen atoms and provided stereochemical and mechanistic information on the biosynthetic pathways of these metabolites. The incorporation of (2S)-[methyl-^{13}C]methionine and [1,2-$^{13}C_2$]acetate into the fusarins, metabolites of *Fusarium moniliforme*, points to a biosynthetic pathway involving the condensation of a C_{14}-polyketide and a C_4 intermediate, e.g. oxaloacetate, of the Krebs tricarboxylic acid cycle.

INTRODUCTION

Over the past ten years part of our research effort has been committed to a study of the biosynthesis of a number of polyketide-derived mycotoxins. The investigations to be reported here concern the study of three structurally-related members of a group of mycotoxins which act as inhibitors of ATP-synthesis and ATP-hydrolysis catalyzed by mitochondrial enzyme systems[1-3] viz. citreoviridin (1), aurovertin B (2), and asteltoxin (3), as well as a mutagen, fusarin C (4) isolated from cultures of *Fusarium moniliforme*.

CITREOVIRIDIN

Acute cardiac beri-beri, a mycotoxicosis prevalent for more than three centuries among man in rice-eating countries of East Asia, was first reported in Edo (the present Tokyo) in the late 17th century.[4] The disease is characterized by vomiting, convulsions, ascending paralysis, lowering of body temperature, and respiratory arrest as major symptoms.[4] Investigations of rice infected with *Penicillium citreoviride* resulted in the isolation of a toxic yellow compound, named citreoviridin,[5] which caused the typical symptoms of acute cardiac beri-beri in animals.[4]



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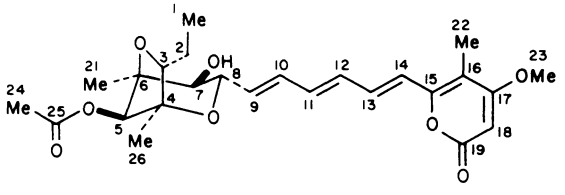

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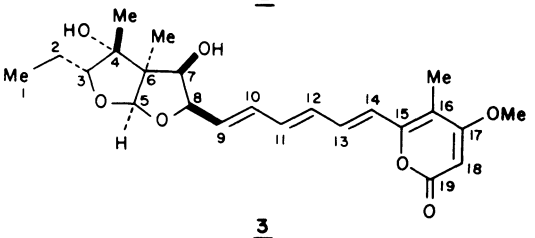

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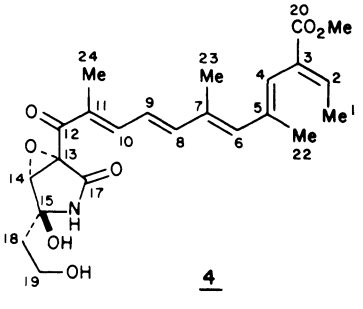

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The structure of citreoviridin was determined in 1964 by Sakabe et al.[6] by means of chemical degradation and ^{1}H n.m.r. spectroscopy. The structure and relative configuration, with the polyene having the all-trans configuration, were subsequently confirmed by X-ray crystallography.[7] Mulheirn[8] determined the absolute configuration at C-4 as R by comparison of the ^{1}H chemical shifts of the neighbouring groups in either the C-4 (S)-(+)- or (R)-(-)-α-phenylbutyrate ester with those of the acetate ester.[9] The synthesis of the substituted tetrahydrofuran moiety of citreoviridin starting from D-glucose, confirmed the proposed absolute configuration.[10]

The biosynthesis of citreoviridin by cultures of P. pulvillorum, CSIR 1406 was first investigated by Nagel et al.[11] It was postulated that the metabolite is formed from a C_{18}-polyketide, derived from acetyl-CoA as a starter unit and eight malonyl-CoA units, and five C_1 units derived from methionine.

This postulate was tested by incorporation of (2S)-[methyl-^{14}C]methionine (absolute incorporation 70%) and [2-^{14}C]acetate (absolute incorporation 5%) into citreoviridin. Some of the locations of the ^{14}C-label in the enriched citreoviridins could be determined by chemical degradation. Thus citreoviridin derived from [methyl-^{14}C]methionine was treated with hydroiodic acid in refluxing acetic anhydride and the methyl iodide formed was converted to the methylthiourea picrate. The activity of the methoxy group represented 20.4% of the total activity of the citreoviridin, indicating that four of the five C-methyl groups are derived from the C_1 pool. In the postulated biosynthesis C-1 would be derived from acetate. Kuhn-Roth oxidation of citreoviridin derived from

[methyl-^{14}C]methionine yielded acetic acid (characterized as the p-bromophenacyl derivative) containing 80.8% of the specific activity of the starting material. Schmidt decarboxylation of the acetic acid gave methyl amine, which was converted to 2,4-dinitro-N-methylaniline, and showed that all the activity was located in the methyl group of the acetic acid.

In the second experiment cultures of P. pulvillorum were incubated with [2-^{14}C]acetate. Kuhn-Roth oxidation of the labelled citreoviridin yielded acetic acid (characterized as the p-bromophenacyl derivative) which had a specific activity of 51.8% of that of the starting material (theory: 5/9 ≡ 55.5%, assuming a total of nine labelled positions). Schmidt decarboxylation of the acetic acid showed that the methyl group contained 11.1% of the activity of the starting material (theory: 1/9 ≡ 11.1%) and the carboxy group 31.4%.

The above results, consistent with the postulated biosynthetic pathway, also illustrate the difficulties in determining the location of ^{14}C-labels by chemical degradation. Nowadays, stable isotopes, such as ^{2}H, ^{13}C, ^{15}N, ^{17}O, and ^{18}O, in conjunction with n.m.r. methods of analysis[12,13] play an important role in biosynthetic studies and circumvent the problems associated with ^{14}C-labelling studies. A prerequisite of such studies using ^{13}C n.m.r spectroscopy for the location of ^{13}C-labels, is an unambiguous assignment of the resonances in the natural abundance ^{13}C n.m.r. spectrum of the metabolite.

Cultures of P. pulvillorum, CSIR 1406 were grown in the dark at 23°C in stationary culture on an F14 medium.[14] Our studies on the course of fermentation indicated that citreoviridin production commenced on day 2 and that satisfactory yields of the metabolite (150 mg l^{-1}) could be obtained 12 days after the inoculation of the medium. In preliminary feeding experiments with [1-^{14}C]acetate as precursor we established conditions which would give a suitable ^{13}C enrichment at each individual, acetate-derived carbon atom of citreoviridin on feeding [^{13}C]acetate. A satisfactory dilution value for citreoviridin (19.5, assuming 9 labelled positions) and good incorporation (1.5%) was obtained by pulsing cultures of P. pulvillorum every 12 h from day 3 to day 11 with sodium acetate to a total amount 1.0 g l^{-1}.

The proton-decoupled ^{13}C n.m.r. spectrum of [1-^{13}C]acetate-derived citreoviridin showed nine enhanced signals attributed to C-2, C-4, C-6, C-8, C-10, C-12, C-14, C-16, and C-18 whereas that of citreoviridin derived from [2-^{13}C]acetate showed enhanced signals for C-1, C-3, C-5, C-7, C-9, C-11, C-13, C-15, and C-17. Satisfactory enrichment factors were obtained for the [1-^{13}C]- and [2-^{13}C]acetate-derived carbon atoms (average 5.3 and 4.0, respectively).[14] These results are in complete agreement with those reported by Franck.[15]

The arrangement of intact acetate units in citreoviridin was studied by addition of [1,2-$^{13}C_2$]acetate to cultures of P. pulvillorum.[14] All the signals in the proton-decoupled ^{13}C spectrum of citreoviridin enriched by [1,2-$^{13}C_2$]acetate, with the exception of those for C-19, C-20, C-21, C-22, and C-23, exhibited one-bond (C,C) coupling. The measured ^{1}J(CC) values proved the presence of nine intact acetate units arranged as shown in Fig. 1.

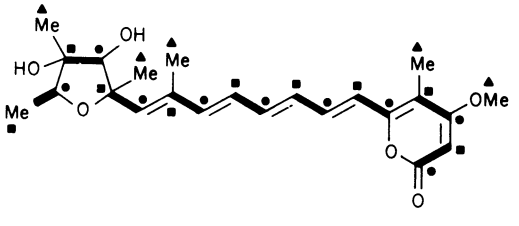
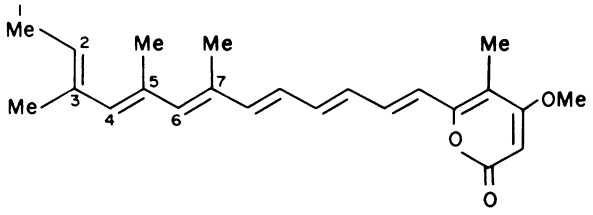


Fig. 1. ^{13}C-Labelling pattern for citreoviridin (1). ▲ ≡ (2S)-[methyl-^{13}C]-methionine; ■ ≡ C-2 of acetate; ● ≡ C-1 of acetate. Bold lines show the arrangement of intact acetate units.

The above results acount for the origin of 18 of the 23 carbon atoms in citreoviridin. The origin of the remaining five carbon atoms was determined by incorporation of (2S)-[methyl-^{13}C]methionine into citreoviridin. The proton-decoupled ^{13}C n.m.r. spectrum showed enhancement of the signals attributed to C-19, C-20, C-21, C-22, and C-23 (average enrichment factor 8.8).

The presence of an acetate starter unit in citreoviridin was demonstrated by a feeding experiment with [1-^{13}C,2-^{2}H$_3$]acetate. The incorporation of ^{2}H located β to a ^{13}C atom can be detected by the characteristic upfield β-isotope shift in the resonance position of the ^{13}C nucleus.[16,17] Experimental evidence reported in the literature shows that the methyl hydrogens of acetyl-CoA are incorporated in varying degrees into fatty acids: the predominant species (ca. 80%) at the terminal methyl group (i.e. the methyl group of the starter unit) is $^{13}C^{2}H_3$.[18,19] The retention of up to three ^{2}H atoms at C-1, evident from the β-isotope shift of -0.048 p.p.m. per ^{2}H atom for the C-2 resonance (δ_C 78.02 p.p.m.) in the proton-decoupled ^{13}C spectrum of citreoviridin derived from [1-^{13}C,2-^{2}H$_3$]-acetate confirmed the presence of an acetate starter unit in the metabolite. The presence of $C^{2}H_2{}^{1}H_1$ and $C^{2}H_1{}^{1}H_2$ at C-1 arises from loss of ^{2}H as a result of acetate-malonate interconversion by acetyl-CoA carboxylase.

The results obtained from the above feeding experiments with ^{13}C-labelled acetate and methionine prove that citreoviridin is derived from a C_{18}-polyketide formed from acetyl-CoA as a starter unit and eight malonyl-CoA units with methionine contributing the remaining five C_1 units. The stereochemical and mechanistic details of the intermediate transformations leading to the citreoviridin structure remained speculative. For instance, citreomontanin (5), a metabolite of P. pedemontanum,[20,21] has been proposed as a possible precursor for citreoviridin.[22]



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The use of ^{18}O labelled precursors in biosynthetic studies allows us to investigate the oxidation states of intermediates which can be involved in the biosynthesis and provides us with information on the reduction, deoxygenation and oxidation processes. The results of ^{18}O isotope incorporations, in conjunction with the known absolute configuration of citreoviridin, enabled us to propose a detailed mechanism for the formation of the tetrahydrofuran moiety.

[1-^{13}C,$^{18}O_2$]Acetate was added to cultures of P. pulvillorum, CSIR 1406 over days 3-11 before isolation and purification of the enriched citreoviridin. The incorporation of ^{18}O was detected by the presence of isotopically shifted resonances in the proton-decoupled ^{13}C n.m.r. spectrum.[23,24] The upfield isotope shifts observed for the C-14 (δ_C 154.90, Δδ -0.029 p.p.m.), C-16 (δ_C 171.22, Δδ -0.021 p.p.m.) and C-18 (δ_C 163.96, Δδ -0.047, -0.034, and -0.013 p.p.m.) resonances (Fig. 2) indicated that the corresponding carbon- oxygen bonds had remained intact throughout the biosynthetic pathway.[25] For the C-18 resonance, three isotopically shifted signals appeared due to species having ^{18}O in (a) the singly-bonded oxygen (Δδ -0.013 p.p.m.), (b) the doubly-bonded oxygen (Δδ -0.034 p.p.m.) and (c) both the singly- and doubly-bonded oxygens (Δδ -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. No ^{18}O was present at either C-2 or C-4 as the resonances at δ_C 78.02 and 86.27 p.p.m. 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.[25]

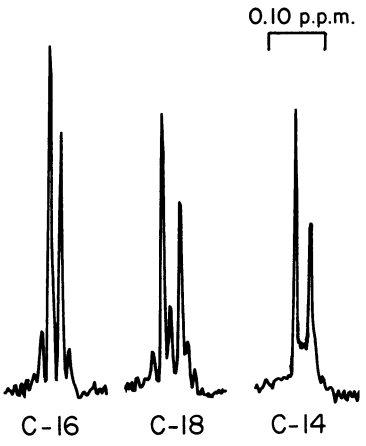


Fig. 2. ^{18}O Isotope shifts observed in the ^{13}C n.m.r. spectrum of citreoviridin derived from [1-^{13}C,$^{18}O_2$]-acetate.

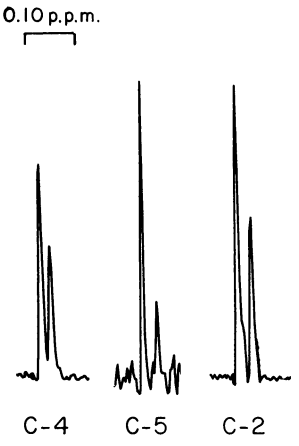


Fig. 3. ^{18}O Isotope shifts observed in the ^{13}C n.m.r. spectrum of citreoviridin derived from $^{18}O_2$ gas.

The fermentation of cultures of P. pulvillorum 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 (99.0 atom % ^{13}C) admixed with unlabelled acetate to the medium from day 4 to day 9 produced citreoviridin whose proton-decoupled ^{13}C n.m.r. spectrum (Fig. 3) exhibited ^{18}O-induced isotope shifts for the C-2 (δ_C 78.02, $\Delta\delta$ -0.028 p.p.m.), C-4 (δ_C 86.27, $\Delta\delta$ -0.018 p.p.m.) and C-5 (δ_C 84.47, $\Delta\delta$ -0.030 p.p.m.) resonances.[25] The C-4 and tetrahydrofuran ring-oxygen atoms in citreoviridin are thus derived from molecular oxygen by oxidative processes. The molecular ion cluster in the mass spectrum of citreoviridin derived from [1-^{13}C]acetate (99.0 atom %) and $^{18}O_2$ (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 established for the first time the origins of the oxygen atoms in citreoviridin. Analysis of the results afforded us considerable insight into the stereochemistry and mechanism of the events by which this metabolite is elaborated. For example, alkylation of a β-ketoacyl thioester by S-adenosylmethionine 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 reduction-elimination sequence analogous to that of fatty acid biosynthesis[26] to generate the 2Z, 4E, 6E, 8E, 10E, 12E polyene with the 3-s-cis, 5-s-cis conformation (6). 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 presence of deuterium only at C-1 in citreoviridin derived from [1-^{13}C,2-$^{2}H_3$]acetate indicates that significant losses of ^{2}H through exchange processes occur before reduction of the β-ketoacyl intermediates to the corresponding β-hydroxyacyl intermediates. If we assume that water elimination in the reduction-elimination sequence occurs in a syn fashion, as is the case in fatty acid biosynthesis, then E and Z double bonds can be formed only if the steric course of the reductions which generate the chiral secondary alcohols occur in the opposite sense. This

difference in stereospecificity is consistent with these reductions occurring at different active sites on distinct reductases. Alternatively, water could be eliminated either by a syn or an anti mechanism from the same chiral secondary hydroxy group.

The subsequent formation of the pyrone ring present in citreoviridin proceeds by nucleophilic attack of the C-14 enolic hydroxy group on the thioester carbonyl 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. Once released from its polyketide synthetase, the polyene is postulated to undergo epoxidation by a mono-oxygenase to give the (2R,3R,4S,5R)-bis-epoxide (7) (see Fig. 4). 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 water of the medium. A similar attack of the C-2 hydroxy group oxygen atom on an epoxide (as shown in Fig. 4) was used for the formation of the tetrahydrofuran moiety in the synthesis of citreoviridin from D-glucose.[10]

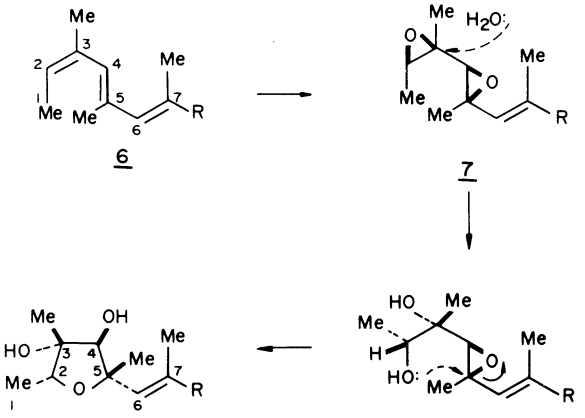
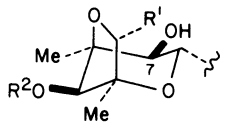


Fig. 4. Proposed mechanism for the formation of the tetrahydrofuran moiety of citreoviridin

Citreomontanin (5) differs from the putative polyene intermediate (6) in that it has the 2E configuration and exists in the solid state as the 3-s-cis conformer.[27,28] The epoxidation of citreomontanin (5) and subsequent ring formation as outlined above generates a tetrahydrofuran moiety with the wrong stereochemistry for the C-2 carbon atom. Citreomontanin is therefore not an intermediate in the biosynthesis of citreoviridin and this has been confirmed by feeding experiments.[28] The co-existence of citreomontanin and citreoviridin in cultures of P. pedemontanum, points to the presence of an early intermediate common to both biosynthetic pathways.[28]

THE AUROVERTINS

The aurovertins, a group of toxic metabolites isolated from cultures of the fungus Calcarisporium arbuscula (Preuss),[29,30] are potent inhibitors of ATP synthesis and ATPases.[2,31] The fungus produces a complex mixture of aurovertins[30] from which five components, named aurovertin A-E (Fig. 5) have been characterized.[30,32,33] Structural evidence for aurovertin B (2) was deduced mainly from its ^{1}H and ^{13}C n.m.r. spectra.[34] The relative configuration of the 2,6-dioxabicyclo[3.2.1]octane moiety is based on the observed proton-proton coupling constants and n.O.e experiments.[34] The structure and relative configuration have been confirmed by X-ray crystallography.[35] The absolute configuration followed when Mulheirn[32] established the C-7 chirality as S using the method of Helmchen.[9]



Aurovertin A : $R^1 = CH_2Me$; $R^2 = Ac$; 7-OAc
B : $R^1 = CH_2Me$; $R^2 = Ac$
C : $R^1 = Me$; $R^2 = Ac$
D : $R^1 = CH(OH)Me$; $R^2 = Ac$
E : $R^1 = CH_2Me$; $R^2 = H$

Fig. 5. Structures of the aurovertins A-E.

Several plausible mechanisms, some without firm precedent in fungal polyketide biosynthesis, can be formulated for the biosynthesis of aurovertin B and can be distinguished by the different origins of C-1—C-3. (a) Pathway 1 involves a C_{18}-polyketide precursor and requires the introduction of a methyl group from the C_1 pool at the methyl carbon atom of the acetate starter unit; C-1 is thus derived from methionine and C-2 from [2-C]acetate. A similar methylation has been postulated to explain the formation of the ethyl side-chain of barnol[36] and is indicated for the biosynthesis of stellatin.[37] (b) A more acceptable alternative (pathway 2) involves a C_{20}-polyketide which is methylated at C-18, followed by the loss of the acetate starter unit; once again C-1 in aurovertin B is derived from methionine and C-2 from [2-C]acetate (strictly from [2-C]-malonate). (c) Pathway 3 requires the loss of the methyl carbon atom of the acetate starter unit of a C_{20}-polyketide; C-1 of aurovertin B is thus derived from [1-C]acetate and C-2 from [2-C]acetate. (d) Incorporation of propionate[38] into aurovertin B can occur via pathway 4, in which propionate is used as a starter unit; C-1, C-2, and C-3 are thus derived from propionate. The participation of propionate in the biosynthesis of a polyketide derived metabolite viz. homo-orsellinic acid has been reported.[39]

^{13}C N.m.r. spectroscopy provides an efficient tool for distinguishing between the different postulated biosynthetic pathways and identifying a particular one. A study of the incorporation of different ^{13}C-labelled precursors into aurovertin B was therefore undertaken.

Cultures of C. arbuscula were grown in the dark at 23°C in stationary culture on an F14 medium at pH 6.2. Although the fungus is reported to produce at least nine aurovertins,[30] only aurovertins B and D were produced in our experiments. In fact, in a few cases when older culture material, obtained through repetitive subculturing, was used only aurovertin B was produced.[40]

Studies on the course of fermentation indicated that aurovertin production commenced on day 7 and reached a maximum 14 days after the inoculation of the medium. A satisfactory dilution value for aurovertin B (15.7) (assuming 10 labelled positions) and good incorporation (0.8%) was obtained by pulsing cultures of C. arbuscula every 12 h from day 7 to day 13 with sodium acetate to a total amount of 1.5 g l^{-1}.[40]

The proton-decoupled ^{13}C n.m.r. spectrum of [1-^{13}C]acetate-derived aurovertin B showed ten enhanced signals attributed to C-3, C-5, C-7, C-9, C-11, C-13, C-15, C-17, C-19 and C-25, whereas the spectrum of that derived from [2-^{13}C]acetate showed enhanced signals representative of C-2, C-4, C-6, C-8, C-10, C-12, C-14, C-16, C-18, and C-24.[40] High enrichment factors were obtained for both the [1-^{13}C]- and [2-^{13}C]acetate-derived carbon atoms (average 7.6 and 5.6, respectively). In some feeding experiments with [1-^{13}C]-acetate the derived aurovertin B exhibited a disparity in the enrichment of the carbonyl carbon atom of the O-acetate group, C-25 as this signal showed a three-fold enhancement

over those of the other [1-^{13}C]acetate-derived carbon atoms. A similar effect, but of lesser magnitude, was observed for the methyl carbon atom of the O-acetate group, C-27, in some [2-^{13}C]acetate-enriched aurovertin B samples.

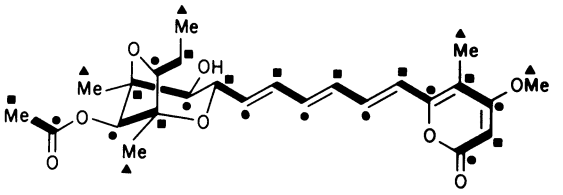


Fig. 6. ^{13}C-Labelling pattern for aurovertin B (2) formed via pathway 2. ▲ ≡ (2S)-[methyl-^{13}C]methionine; ■ ≡ C-2 of acetate: ● ≡ C-1 of acetate. Bold lines show the arrangement of intact acetate units.

The arrangement of intact acetate units in aurovertin B and thus in the original polyketide progenitor was studied by addition of [1,2-$^{13}C_2$]acetate to cultures of C. arbuscula. All the signals in the proton-decoupled ^{13}C n.m.r. spectrum of aurovertin B derived from [1,2-$^{13}C_2$]acetate with the exception of those for C-1, C-20, C-21, C-22, and C-23 exhibited one-bond (C,C) couplings. The measured ^1J(CC) values prove the presence of the following intact acetate units viz. C-2—C-3, C-4—C-5, C-6—C-7, C-8—C-9, C-10—C-11, C-12—C-13, C-14—C-15, C-16—C-17, C-18—C-19, and C-24—C-25 (Fig. 6). The origin of the C-1, C-20, C-21, C-22, and C-23 carbon atoms of aurovertin B was established by incorporation of (2S)-[methyl-^{13}C]methionine.

The results obtained from these feeding experiments with ^{13}C-labelled acetate and methionine exclude the formation of aurovertin B via pathway 3 which requires that C-1 originates from [1-^{13}C]acetate. The above evidence, however, points to an acetate-malonate origin for the metabolite and as such two pathways viz. 1 and 2, can be formulated. The two pathways differ in only one respect, in that a starter acetate unit is either present (pathway 2; C-2 and C-3) or absent (pathway 1) in the resulting metabolite.[40]

^{13}C-Labelled malonate has been used successfully to detect acetate-starter effects in herquinone,[41] sclerin,[42] and phomazarin.[43] A significant lower extent of labelling of the carbon atoms of the acetate starter unit allowed its identification in these polyketide-derived metabolites. Administration of [2-^{13}C]malonate admixed with unlabelled sodium acetate to cultures of C. arbuscula gave aurovertin B (and D) in good yield. The proton-decoupled ^{13}C n.m.r. spectrum of both metabolites showed high enhancement of the signals due to C-2, C-4, C-6, C-8, C-10, C-12, C-14, C-16 and C-18 and, more importantly, all were labelled to a similar extent.[40] Partial decarboxylation of the [2-^{13}C]malonate i.e. malonyl-CoA → acetyl-CoA interconversion, results in the enrichment of C-24, the methyl carbon atom of the O-acetate group, but only to one-eighth of the extent of the other skeletal carbon atoms.[40] As no significant lower extent of enrichment could be demonstrated it must be concluded that the starter unit of the original polyketide is lost in the biosynthesis of the aurovertins.

Additional evidence in favour of pathway 2 was obtained by feeding experiments with [2-^{13}C,2-$^2H_3$]- and [1-^{13}C,2-$^2H_3$]acetate. The incorporation of these precursors in aurovertin B by pathway 1 should result in the retention of two (or less) ^{2}H atoms at C-2 whereas no deuterium should be present at C-2 if pathway 2 is operative. The ^{13}C signals of ^{13}C-^{2}H species appear as triplets shifted to higher field (α isotope effect) compared with the ^{13}C signal due to the corresponding ^{13}C-^{1}H species in the proton-decoupled ^{13}C spectra. The latter signal exhibits a concomitant decrease in intensity. In a parallel experiment [2-^{13}C]- and [2-^{13}C,2-$^2H_3$]acetate were separately administered to cultures of C. arbuscula. A comparison of the proton-decoupled ^{13}C spectra of aurovertin B obtained

from each precursor showed no significant difference in the enhancement of the signals of the enriched carbon atoms except for the intensity of the C-24 methyl signal which was decreased by 85% in the spectrum of aurovertin B obtained from this doubly-labelled precursor. ^{2}H N.m.r. spectroscopy of this compound showed a doublet at δ 2.09 p.p.m. which is assigned to the ^{13}C-^{2}H species at C-24. The presence of ^{2}H only at C-24 in aurovertin B derived from [1-^{13}C,2-$^{2}H_3$]acetate was evident from the β-isotope shifts observed for the C-25 resonance in the proton-decoupled ^{13}C n.m.r. spectrum.

The evidence outlined above is consistent with the biosynthesis of aurovertin B via pathway 2: a C_{20}-polyketide formed from an acetate starter unit and nine malonate units is methylated at C-18, followed by the loss of the starter unit, through a retro-Claisen cleavage. The direct derivation of the O-acetate moiety from acetyl-CoA would explain the disparity in the enrichment of the carbon atoms compared with the other skeletal carbon atoms.[40]

An investigation of the one remaining biosynthetic hypothesis, as outlined in pathway 4, required a study of the incorporation of appropriately labelled propionate precursors.[40] In the proton-decoupled ^{13}C n.m.r. spectrum of aurovertin B derived from [1-^{13}C]propionate only the signal assigned to C-3 (δ_C 85.4 p.p.m.) was enhanced (enrichment factor 14.1). The incorporation of [3-^{13}C]propionate is more complex: although C-1 is preferentially labelled (enrichment factor of 11.4 for the signal at δ_C 11.7 p.p.m.) significant enrichment is also observed for the [2-^{13}C]acetate-derived carbons (average enrichment 1.5). The result is explained by the fact that the organism converts (ca. 12%) [3-^{13}C]propionate via [3-^{13}C]pyruvate into [2-^{13}C]acetate through the loss of C-1 of propionate. The process, however, does not proceed via a symmetrical intermediate (e.g. succinate) as the carbon atoms in aurovertin B derived from [1-^{13}C]acetate are not enriched. The results indicate that aurovertin B is formed from a propionate starter unit and eight malonate units (Fig. 7). The occurrence of a pathway involving propionate as a starter unit is unique amongst fungal metabolites. A single report[39] on this role of propionate in the biosynthesis of a fungal metabolite, homo-orsellinic acid, has appeared in the literature, but from the reported evidence it is apparent that propionyl-CoA is substituting for acetyl-CoA as the normal metabolic product is orsellinic acid.

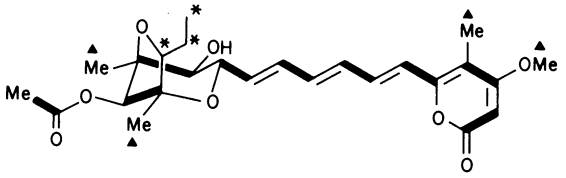
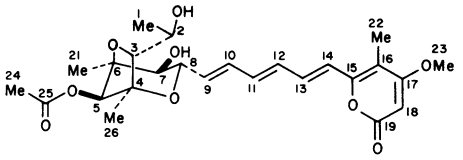


Fig. 7. ^{13}C-Labelling pattern for aurovertin B (2) formed via pathway 4. * ≡ carbon atoms derived from propionate; ▲ ≡ (2S)-[methyl-^{13}C]methionine. Bold lines show the arrangement of intact acetate units.

The formation of aurovertin B by two biosynthetic pathways raised the interesting question as to whether both pathways operate simultaneously. This aspect was investigated by addition of a mixture of (2S)-[methyl-^{13}C]methionine and [1-^{13}C]propionate to the culture medium.[40] The proton-decoupled ^{13}C n.m.r. spectrum of the enriched aurovertin B proved that C-3 (enrichment factor 9.3), derived from propionate, as well as C-1, C-20, C-21, C-22, and C-23 (enrichment factors 10.7, 13.5, 14.0, 14.4, and 13.8, respectively), derived from methionine, were enriched. The above results and especially the significantly lower enrichment of C-1 indicate that both biosynthetic pathways operate independently. An alternative explanation, for which there is no precedent involves the formation of propionyl-CoA by methylation of malonyl-CoA at C-2 and subsequent (or concomitant) decarboxylation.

The knowledge which we have on the origin of the carbon atoms of the aurovertins provides

no detail about the stereochemistry and mechanism of the intermediate transformations leading to the formation of the 2,6-dioxabicyclo[3.2.1]octane moiety. This information was obtained from incorporation of ^{18}O-labelled precursors into aurovertin B and D (8).[44] Thus [1-^{13}C,$^{18}O_2$]acetate was added to cultures of C. arbuscula. The ^{18}O-induced isotope shifts observed for the C-15 ($\Delta\delta$ -0.026 p.p.m.), C-17 ($\Delta\delta$ -0.021 p.p.m.) and C-19 ($\Delta\delta$ -0.032 p.p.m.) resonances indicated that the oxygen atoms of the α-pyrone moiety, as in the case of citreoviridin,[25] are derived from acetate. The upfield shift of 0.037 p.p.m. for the C-25 resonance shows that the sp^2 bonded oxygen of the acetoxy group, but not the sp^3 bonded oxygen, is derived from acetate.



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The oxygen atoms of the 2,6-dioxabicyclo[3.2.1]octane moiety must therefore be derived from atmospheric oxygen or water from the incubation medium. Fermentation of cultures of C. arbuscula in an atmosphere containing $^{18}O_2$ (50.0 atom %) and the simultaneous addition of [1-^{13}C]acetate produced ^{18}O-labelled aurovertin B.[44] The incorporation and location of ^{18}O was deduced from the ^{18}O-induced isotope shifts observed for a number of resonances in the proton-decoupled ^{13}C n.m.r. spectrum of aurovertin B: C-3 (δ_C 85.51; $\Delta\delta$ -0.026 p.p.m.), C-5 (δ_C 81.11; $\Delta\delta$ -0.032 p.p.m.), C-6 (δ_C 83.87; $\Delta\delta$ -0.029 p.p.m.), and C-7 (δ_C 76.86; $\Delta\delta$ -0.021 p.p.m.).

Similar ^{18}O shifts were observed for the C-3 ($\Delta\delta$ -0.021 p.p.m.), C-5 ($\Delta\delta$ -0.032 p.p.m.). C-6 ($\Delta\delta$ -0.032 p.p.m.), and C-7 ($\Delta\delta$ -0.021 p.p.m.) resonances of the enriched aurovertin D (8) (Fig. 8). The upfield shift of 0.026 p.p.m. observed for the C-2 (δ_C 65.48 p.p.m.) resonance confirms the origin of the C-2 oxygen atom from molecular oxygen. No ^{18}O isotope shift was observed for the resonances assigned to C-4 or C-8 in the aurovertins. The C-5, C-7 and C-6—O—C-3 oxygen atoms of the aurovertins are thus derived from molecular oxygen by oxidative processes and the C-4—O—C-8 oxygen atom from water from the medium.

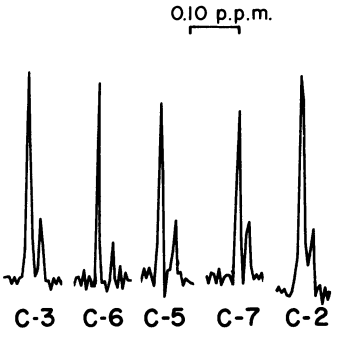


Fig. 8. ^{18}O Isotope shifts observed in the ^{13}C n.m.r. spectrum of aurovertin D (8) derived from $^{18}O_2$ gas.

The above results enabled us to make the following conclusions about the stereochemistry of the main events which occur during the biosynthesis of aurovertin B keeping in mind the known absolute configuration of the compound.

Alkylation of a β-ketoacyl thioester by S-adenosyl methionine introduces the eventual C-2, C-4, C-6, C-16 methyl groups and generates the C-17 O-methyl moiety. This methylation is

followed by the loss of the starter acetate unit through a retro-Claisen cleavage to give an intermediate which can also be formed from a propionate starter unit and malonyl-CoA units by methylation at the appropriate sites. The loss of oxygen from C-3, C-5, C-7, C-9, C-11, and C-13 presumably occurs by a reduction-elimination sequence similar to that postulated for citreoviridin (1) and analogous to that of fatty acid biosynthesis to generate the 3Z,5E,7E,9E,11E,13E polyene with the 4-s-cis, 6-s-cis conformation. The subsequent formation of the pyrone ring by nucleophilic attack of the C-15 hydroxy group on the thioester carbonyl generates the first enzyme-free intermediate. This polyene is postulated to undergo epoxidation by a mono-oxygenase to give the (3R,4R,5R,6R,7R,8S)-triepoxide (Fig. 9). Nucleophilic attack by water at C-4 initiates a cascade of ring closures to eventually generate the 2,6-dioxabicyclo[3.2.1]octane moiety of aurovertin B. This sequence, which accounts for the stereochemistry at C-3, C-4, C-5, C-6, C-7 and C-8 of aurovertin B (and D), is supported by the demonstrated derivation of the C-5, C-7 and C-3 (≡ C-6) oxygen atoms.

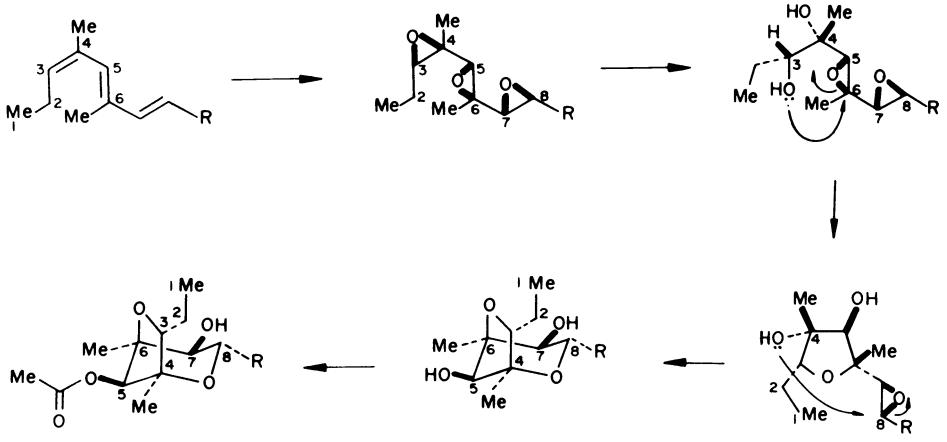


Fig. 9. Proposed mechanism for the formation of the 2,6-dioxabicyclo[3.2.1]-octane moiety of the aurovertins.

Insertion of an oxygen atom, derived from molecular oxygen into a C-2 carbon-hydrogen bond of aurovertin B (2) or one of its precursors, leads to the formation of aurovertin D (8).

ASTELTOXIN

The investigation of toxic maize meal cultures of Aspergillus stellatus Curzi*, MRC 277 led to the isolation of a novel mycotoxin, asteltoxin (3).[45] The compound has an LD_{50} of 5.9 mg kg^{-1} when tested in mice and paralysis of the hind legs and respiratory impairment are the major symptoms observed in the experimental animals. Evidence in favour of structure (3) for asteltoxin was deduced mainly from its ^{13}C and ^{1}H n.m.r. spectra. A single crystal X-ray study confirmed the proposed structure and relative configuration.[45] The total synthesis of asteltoxin has been reported by Schreiber.[46]

Extensive incorporation studies with ^{13}C-labelled precursors have revealed the simultaneous operation of two apparently independent pathways in the biosynthesis of aurovertin B (2) (see above). These results prompted us to investigate the biosynthesis of asteltoxin (3) as, in addition to the same two pathways, a rearrangement of the polyketide chain must be invoked to explain the formation of the 2,8-dioxabicyclo-[3.3.0]octane moiety, a masked branched aldehyde.[17]

* Aspergillus stellatus Curzi is synonomous with Aspergillus variecolor (Berk. and Br.) Thom and Raper, the imperfect state of Emericella variecolor Berk. and Br.

Cultures of Emericella variecolor, strain NHL 2881 were grown on a malt extract medium (15%). Studies on the course of fermentation indicated that asteltoxin production commenced on day 8 and reached a level of 60-80 mg l^{-1} after 25 days. Preliminary feeding experiments with [1-^{14}C]acetate as precursor established that high but satisfactory dilution values (200.5, assuming nine labelled positions) were obtained by feeding cultures of E. variecolor every 24 h from day 6 to day 24 with sodium acetate to a total amount of 2.5 g l^{-1}.[47]

The proton-decoupled ^{13}C n.m.r. spectrum of asteltoxin derived from [1-^{13}C]acetate showed nine enhanced signals (average enrichment factor 1.5) attributed to C-3, C-5, C-7, C-9, C-11, C-13, C-15, C-17 and C-19[47] whereas the spectrum of asteltoxin derived from [2-^{13}C]-acetate showed enhanced signals representative of C-2, C-4, C-6, C-8, C-10, C-12, C-14, C-16 and C-18 (average enrichment factor 2.0). These results pointed to the involvement of nine acetate units in the formation of the metabolite. The arrangement of intact acetate units in asteltoxin was studied by addition of [1,2-$^{13}C_2$]acetate to cultures of E. variecolor. The proton-decoupled ^{13}C n.m.r. spectrum of the enriched asteltoxin exhibited as a result of multiple labelling, one-bond (C,C) coupling between carbon atoms derived from adjacent acetate units (interacetate coupling), in addition to the expected spin-spin coupling between carbon atoms derived from intact acetate units (intra-acetate coupling). The intra-acetate (C,C) couplings could be distinguished readily by their greater (5-fold) intensities. The measured ^{1}J(CC) values of these couplings prove the presence of eight intact acetate units arranged as shown in Fig. 10: C-2—C-3 (40.8 Hz), C-6—C-7 (37.0 Hz), C-8—C-9 (51.7 Hz), C-10—C-11 (56.3 Hz), C-12—C-13 (57.2 Hz), C-14—C-15 (69.5 Hz), C-16—C-17 (61.5 Hz), and C-18—C-19 (78.3 Hz).[47] The additional much lower intensity one-bond (C,C) couplings observed for the C-3 (38.0 Hz), C-7 (37.0 Hz), and C-5 (35.2 Hz) resonances are ascribed to interacetate coupling with C-4, C-8 and C-6, respectively. The results indicate that a 1,2-shift of the eventual C-4 carbon in asteltoxin from C-5 to C-6 occurs in the course of the biosynthesis. In this 1,2-bond migration an intact acetate unit is cleaved in a pinacol or epoxide rearrangement to generate a branched aldehyde which is subsequently utilised in the formation of the 2,8-dioxabicyclo[3.3.0]octane moiety.

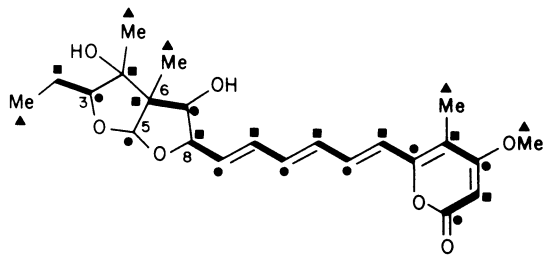


Fig. 10. ^{13}C-Labelling pattern for asteltoxin (3). ▲ ≡ (2S)-[methyl-^{13}C]-methionine; ■ ≡ C-2 of acetate; ● ≡ C-2 of acetate. Bold lines show the arrangement of intact acetate units.

The incorporation of [2-^{13}C]acetate into asteltoxin in initial experiments was subject to a too high dilution value as no reliable enhancement factors were obtained from the proton-decoupled ^{13}C n.m.r. spectrum of the enriched asteltoxin. However, a number of resonances exhibited low intensity satellite signals due to one-bond (C,C) coupling. Analysis of the one-bond (C,C) coupling constants indicated the presence of eight intact acetate units with an arrangement identical to that observed in [1,2-$^{13}C_2$]acetate enriched asteltoxin. In addition the spectrum revealed one-bond interacetate (C,C) couplings for C-4 (38.3 Hz), and C-5 (35.4 Hz) which probably arise from couplings in each case with C-6. It is evident that some [1,2-$^{13}C_2$]acetate is formed during the fermentation by the frequent recycling of [2-^{13}C]acetate in the Krebs citric acid cycle.

The above results obtained from feeding experiments using ^{13}C-labelled acetates account for the origin of 18 of the 23 carbon atoms of asteltoxin. The proton-decoupled ^{13}C n.m.r. spectrum of asteltoxin derived from (2S)-[methyl-^{13}C]methionine showed enhancement of the signals attributed to C-1, C-20, C-21, C-22, and C-23.

The origin of C-1—C-3, as in the case of aurovertin B (2), from acetate and methionine, indicates that asteltoxin is formed by pathway 2 (see earlier). The possible involvement of propionate in the biosynthesis of asteltoxin (pathway 4) was investigated by administration of [1-^{14}C]propionate (specific activity 11.29 μCi mmol^{-1}) to growing cultures of *E. variecolor*, to give asteltoxin (specific activity 0.76 μCi mmol^{-1}). The low dilution value of 14.9 (assuming one labelled position) indicates that high enrichment can be obtained in studies with ^{13}C-labelled propionate. In the proton-decoupled ^{13}C n.m.r. spectrum of asteltoxin derived from [1-^{13}C]propionate (93.2 atom % ^{13}C) only the signal assigned to C-3 (δ_C 90.10 p.p.m.) was enhanced (enrichment factor 5.4). The results indicate that asteltoxin can be formed via two biosynthetic pathways, as described earlier for aurovertin B, which are distinguishable by the different origins of C-1—C-3.[47]

In order to investigate the mechanism of the 1,2-shift which occurs in the course of the biosynthesis and the stereochemistry of the intermediate transformations leading to the asteltoxin structure, it was necessary to determine the origin of the oxygen atoms in the metabolite.[48]

Sodium [1-^{13}C,$^{18}O_2$]acetate admixed with unlabelled sodium acetate was added to cultures of *E. variecolor*, strain NHL 2881 over days 6-17 before isolation and purification of asteltoxin.[48] The proton-decoupled ^{13}C n.m.r. spectrum of the enriched asteltoxin exhibited ^{18}O-isotope shifts for the C-15 ($\Delta\delta$ -0.032 p.p.m.), C-17 ($\Delta\delta$ -0.021 p.p.m.) and C-19 ($\Delta\delta$ -0.032 p.p.m.) resonances indicating that the corresponding carbon-oxygen bonds had remained intact throughout the biosynthetic pathway. No ^{18}O was present at either C-3, C-5, or C-7 as the resonances at δ_C 90.10, 112.98 and 80.19 p.p.m., respectively, appeared as enhanced singlets. The lack of ^{18}O labelling at C-4 and C-8 is to be expected as these carbon atoms are derived from C-2 of acetate.

Fermentation of cultures of *E. variecolor* in a closed system in which the normal atmosphere was replaced by one containing $^{18}O_2$ (50.0 atom % ^{18}O) and the simultaneous addition of [1-^{13}C]acetate (99.0 atom % ^{13}C) admixed with unlabelled acetate to the medium from day 6 to day 17 produced labelled asteltoxin whose proton-decoupled ^{13}C n.m.r. spectrum demonstrated the origin of the C-7 and the 2,8-dioxabicyclo[3.3.0]octane ring oxygens from oxidative processes.[48] ^{18}O Isotope shifts were observed for the C-3 ($\Delta\delta$ -0.032 p.p.m.), C-7 ($\Delta\delta$ -0.021 p.p.m.) and C-5 resonances (Fig. 11). For the C-5 resonance, two isotopically shifted signals appeared due to species having ^{18}O in (a) both tetrahydrofuran rings ($\Delta\delta$ -0.042 p.p.m.) and (b) in either of the two tetrahydrofuran rings ($\Delta\delta$ -0.021 p.p.m. in each case). The presence of the two isotopomers mentioned under (b) was deduced from the intensity of the ^{18}O shifted signal ($\Delta\delta$ -0.021 p.p.m.). The ratio of $^{13}C^{16}O$:$^{13}C^{18}O$ for this signal is twice the ratio of that of the C-3 and C-7 resonances (Fig. 11). The low intensity of the C-8 resonance precluded the observation of an ^{18}O isotope shift.

The above results allow us to propose a mechanism for the formation of the 2,8-dioxabicyclo[3.3.0]octane moiety of asteltoxin. The initial steps in the biosynthesis leading to the 3Z, 5E, 7Z, 9E, 11E, 13E polyene with the 4-s-cis, 6-s-trans conformation (9) occurs by a process analogous to that described for aurovertin B (see earlier). Epoxidation of this polyene by a mono-oxygenase gives the (3R,4R,5R,6R,7S,8S)-triepoxide or its enantiomer (see Fig. 12). Nucleophilic attack by water at C-4 would initiate the formation of the 2,8-dioxabicyclo[3.3.0]octane system. The 1,2-shift occurs with retention of configuration at C-4 and inversion at C-6 to generate the branched aldehyde. Formation of the lactol by nucleophilic attack of the C-3 hydroxy group on the aldehyde

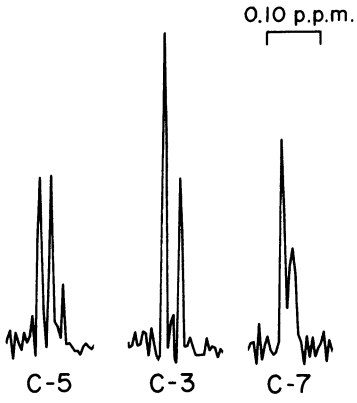


Fig. 11. ^{18}O Isotope shifts observed in the ^{13}C n.m.r. spectrum of asteltoxin (3) derived from $^{18}O_2$ gas.

and subsequent ring closure generates the bicyclic ring system. The conversion of the triepoxide to the 2,8-dioxabicyclo[3.3.0]octane moiety must be a concerted process as no apparent loss of ^{18}O present in the aldehyde moiety occurs through exchange with water of the medium.

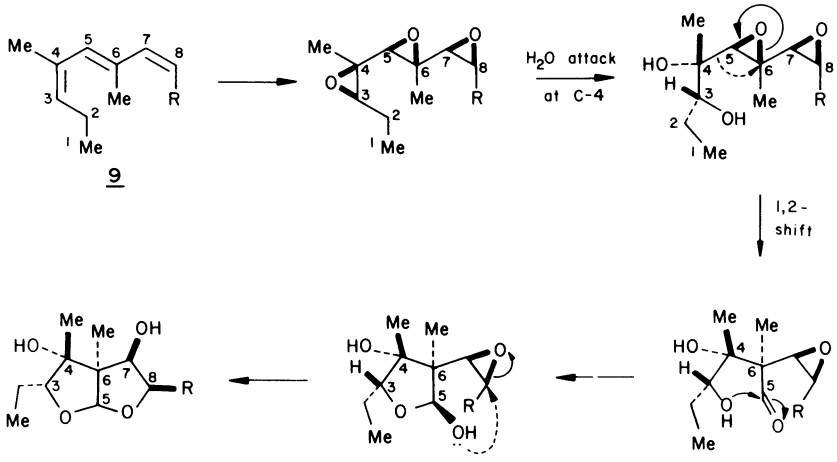


Fig. 12. Proposed mechanism for the formation of the 2,8-dioxabicyclo[3.3.0]-octane moiety of asteltoxin.

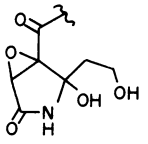
In summary, our results on the biosynthesis of citreoviridin, aurovertin B (and D) and asteltoxin, allow us to define the stereochemistry and conformation of the putative polyene precursors of these metabolites. Thus the same polyene intermediate, but having either the 7E or 7Z stereochemistry and the 6-s-cis or 6-s-trans conformation (see Figs. 9 and 12, respectively) is involved in the biosynthesis of the aurovertins and asteltoxin. Furthermore, the initial nucleophilic attack of water on an epoxide to initiate the concerted formation of the hetereocyclic rings is strikingly similar in each case.

THE FUSARINS

Fusarium moniliforme Sheldon occurs world-wide on a great variety of plant hosts and is one of the most prevalent fungi on maize (Zea mays L.).[49] An isolate of F. moniliforme, strain MRC 826, obtained from maize in an area of the Transkei, Southern Africa, with a

high oesophageal cancer rate[50] was found to be not only highly toxic and able to induce leukoencephalomalacia in horses,[51] but also mutagenic.[52]

High-resolution mass spectrometric analysis of the molecular ion m/z 431.1970 of fusarin C (4) gave the molecular formula as $C_{23}H_{20}NO_7$. The assignment of structure (4) to fusarin C is based on a detailed study of its ^1H and ^{13}C n.m.r. spectra.[53] Selective population inversion (SPI)[54] experiments established the two- and three-bond (C,H) connectivity pattern for the metabolite but did not allow us to differentiate between the substituted 2-pyrrolidone in (4) and that shown in (10). The structure elucidation of two related nonmutagenic metabolites, fusarin A (11) ($C_{23}H_{29}NO_6$) and D (12) ($C_{23}H_{29}NO_7$), based on a similar detailed study of their high-field ^1H and ^{13}C n.m.r. spectra, confirmed the presence of the 2-pyrrolidone (4) moiety in fusarin C. In the event additional evidence for the structure (4) was provided by X-ray crystallography of an isomer of fusarin C.



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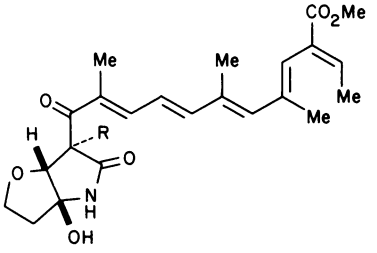


11 R=H
12 R=OH

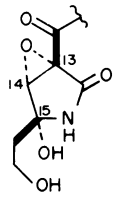
Prolonged exposure of fusarin C (4) to u.v. light as well as high temperatures results in extensive decomposition.[52] Irradiation of a dichloromethane solution of fusarin C at 366 nm for 5 min, however, results in the formation of a mixture of four compounds, which includes starting material. H.p.l.c. of the mixture on silica gel using dichloromethane-methanol (95:5 v/v) as eluant resulted in the isolation of three isomers of fusarin C which on the basis of their ^1H n.m.r. chemical shifts and (^1H,^1H) coupling constants, are assigned the 8Z, the 6Z, and the 10Z stereochemistries, respectively (see Table 1).[53]

TABLE 1 ^1H Chemical shifts and coupling constants for fusarin C and its isomers

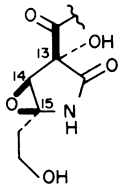
Proton	Fusarin C		8Z-isomer		6Z-isomer		10Z-isomer	
	δ_H	J(HH)/Hz	δ_H	J(HH)/Hz	δ_H	J(HH)/Hz	δ_H	J(HH)/Hz
8	6.790 d	15.0	6.400 d	11.0	7.465 d	15.0	6.567 d	14.9
9	6.670 dd	15.0,11.0	6.469 dd	11.0,11.0	6.716 dd	15.0,11.2	7.157 dd	14.9,11.7
10	7.492 d	11.0	7.675 d	10.9	7.473 d	11.2	6.560 d	11.7

The structure of the 8Z isomer of fusarin C as well as the relative configuration were determined by X-ray crystallography.[53] The conformation of the polyene chromophore deserves particular attention. The torsional angle, Ø for the constituent atoms of this moiety indicates that a high degree of distortion i.e. deviation from planarity exists about the C-3—C-4 (Ø 67.5°), C-5—C-6 (Ø 55.8°) and C-7—C-8 (Ø 51.3°) single bonds. It is evident that in the crystal the 2E, 4E, 6E, 8Z, 10E polyene chromophore exists as the distorted 3-s-cis, 5-s-cis, 7-s-trans, 9-s-trans, 11-s-trans conformer. This conformation also leads to intramolecular hydrogen bonding between the N-16 proton and the oxygen of the methoxycarbonyl group.

In solution and in the dark a slow equilibrium is established between fusarin C, the C-15 epimer (13) and the isomeric epoxide (14). The two equilibrium products could be isolated in the dark at 4°C by column chromatography on Sephadex LH-20 using dichloromethane as eluant.



13



14

Structural analysis strongly suggests a polyketide origin for fusarin C but the origin of the substituted 2-pyrrolidone moiety is obscure. Possible biosynthetic pathways include: (a) the condensation of two preformed polyketide chains; or (b) a 1,2-bond migration of a single polyketide chain; or (c) the oxidative cleavage of a suitable oxygenated phenyl ring; or (d) the cleavage of the terminal acetate unit of a C_{18}-polyketide chain. This diversity in possible biosynthetic routes prompted us to investigate the biosynthesis of fusarin C in order to identify the pathway.[55]

In our biosynthetic studies we used an isolate of F. moniliforme, strain MRC 826, which consistently produced mainly fusarin A (11). Cultures of F. moniliforme were grown in the dark at 23°C on cakes of yellow maize meal containing 50% water. Studies on the course of fermentation indicated the initial appearance of fusarin A on day 7 which reached a level of 8 mg per 100 g of maize on day 14.

On feeding (2S)-[methyl-^{13}C]methionine (90 atom % ^{13}C, 500 mg per 375 g of maize), admixed with (2S)-[methyl-^{14}C]methionine (50 μCi) as a tracer, to cultures of F. moniliforme, fusarin A with a specific activity of 4.87 μCi mmol^{-1} was obtained. This result corresponds to a dilution value of 15.4 (assuming the presence of 5 labelled positions). The proton-decoupled ^{13}C n.m.r. spectrum of the enriched metabolite showed enhancement of the signals attributed to C-20, C-21, C-22, C-23, and C-24.[55]

The arrangement of intact acetate units in fusarin A was studied by addition of [1,2-$^{13}C_2$]acetate (91.0 atom % ^{13}C, 2.0 g per 375 g of maize) to cultures of F. moniliforme. All the resonances in the proton-decoupled ^{13}C n.m.r. spectrum of the enriched fusarin A, with the exception of those for C-20—C-24, exhibited low intensity one-bond (C,C) coupling. The measured ^{1}J(CC) values prove the presence of nine intact acetate units arranged as shown in Fig. 13. The enrichment at each labelled site, deduced from the intensities of the satellite signals arising from one-bond (C,C) coupling relative to that of the central signal,[56] provided additional information on the biosynthesis of fusarin A. Both C-1 and C-2 showed somewhat higher enrichment (0.28%) than the average (0.22%) observed for the twelve carbon atoms C-3—C-13 and C-17. In contrast the average enrichment for C-14, C-15, C-18 and C-19 (0.12%) is distinctly lower. This difference in enrichment levels points to a biosynthetic pathway for fusarin A involving a C_{14}-polyketide derived from an acetyl-CoA starter unit (C-1 and C-2) and six malonyl-CoA units, and a C_4 unit derived most probably from a product of the Krebs tricarboxylic acid cycle, oxaloacetate. A similar biosynthetic pathway involving a polyketide chain and oxaloacetate, or a related intermediate of the tricarboxylic acid cycle has been demonstrated for the tetronic acids, carolic and carlosic acid, metabolites of Penicillium charlesii,[57] and for the marticins, metabolites of Fusarium martii.[58]

The involvement of oxaloacetate in the biosynthesis of fusarin A implies that C-15 is derived from C-2 of oxaloacetate and furthermore that the two contiguous carbon atoms, C-15 and C-18 are derived from C-2 of acetate. Incorporation of either [1-^{13}C]- or [2-^{13}C]acetate into fusarin A would test these conclusions. However, the low enrichment obtained on incorporation of [1,2-$^{13}C_2$]acetate precludes the detection of enrichment at these carbon atoms using singly ^{13}C-labelled acetate. This was indeed the case. The

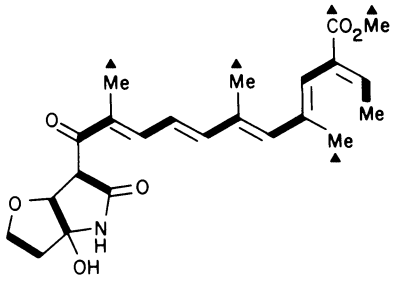


Fig. 13. ^{13}C-Labelling pattern for fusarin A (11). ▲ ≡ (2S)-[methyl-^{13}C]-methionine. Bold lines show the arrangement of intact acetate units.

incorporation of [1-^{13}C]acetate (99.0 atom % ^{13}C, 2.5 g per 375 g of maize) was subject to a too high dilution and no reliable enhancement factors were obtained from the proton-decoupled ^{13}C n.m.r. spectrum of the enriched fusarin A.[55]

The nature of polyketide biosynthesis indicates that C-1—C-2 constitutes the starter acetate unit which is extended by six malonyl-CoA units to form the proposed C_{14}-polyketide chain. Thus C-2, C-4, C-6, C-8, C-10, C-12, and C-17 are derived from C-1 of acetate as the subsequent methylations of the C_{14}-polyketide occur at positions derived from C-2 of acetate.

In conclusion, I hope that the work presented here illustrates the important role that stable isotopes, in conjunction with n.m.r. methods of analysis, can play in biosynthetic studies.

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