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THE BIOSYNTHESIS OF POLYKETIDE-DERIVED MYCOTOXINS

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Abstract - The biosynthesis of a few representative polyketide-derived mycotoxins is reviewed viz. patulin, citrinin, diplosporin, ochratoxin A, maltoryzine, xanthomegnin, cytochalasans, ergochromes, zearalenone, citreoviridin, and the aflatoxins. Particular attention is given to the conversion:acetate → averufin → versiconal acetate → versicolorin A → sterigmatocystin → aflatoxin B_1.

INTRODUCTION

The structural complexity and unique biological properties of mycotoxins have long excited the curiosity of natural product chemists. However, the dramatic discovery of the aflatoxins in 1960 (1) initiated the present renaissance of interest in the isolation, structure, synthesis, and biosynthesis of mycotoxins.

Mycotoxins are produced by a consecutive series of enzyme-catalysed reactions from a few biochemically simple intermediates of primary metabolism e.g. acetate, malonate, mevalonate, and amino acids. The important biosynthetic reactions involve condensation, oxidation/reduction, alkylation, and halogenation steps which lead to the diverse range of structures with their secondary metabolic origin as the only common factor. Administration of isotopically-labelled precursors to growing cultures of the fungi and analysis of the labelling patterns in the molecules established the "building units" of several mycotoxins, and the mechanisms involved in the biotransformations of intermediates. The application of stereospecifically-labelled precursors gave clear insight into the subtle stereochemical control which is typical of enzyme-mediated reactions; particular progress was made in this respect on the biosynthesis of ergot toxins and the trichothecanes.

The principal pathways involved in the formation of mycotoxins are : the polyketide route (e.g. aflatoxins), the terpene route (e.g. trichothecanes), the amino acid route (e.g. gliotoxin), and the tricarboxylic acid route (e.g. the rubratoxins). An increasing number of recently discovered mycotoxins (e.g. the cyclopiazonic acids) are formed from a combination of two or more of the principal pathways.

TABLE 1. Some Mycotoxins derived from the Acetate-Polymalonate Pathway

Number of C_2 units involved	Mycotoxin
Tetraketide	Patulin, Penicillic Acid
Pentaketide	Citrinin, Diplosporin, Ochratoxin A
Hexaketide	Maltoryzine
Heptaketide	Viomellein, Xanthomegnin
Octaketide	Ergochromes, Luteoskyrin
Nonaketide	Zearalenone, Cytochalasans, Citreoviridin, Aurovertin B
Decaketide	Norsolorinic Acid, Averufin, Versiconal Acetate, Versicolorins, Sterigmatocystin, Aflatoxins

This review is devoted solely to the biosynthesis of polyketide-derived mycotoxins. More fungal secondary metabolites, and also mycotoxins, are produced by this route than by any other route. Polyketides are formed by the linear condensation of the "starter unit" of acetyl coenzyme A with three or more units of malonyl coenzyme A, with concomitant decarboxylation, but without the obligatory reduction of the intermediary β-dicarbonyl system. The resulting poly-β-ketomethylene chain is made up of repeating two carbon units.

The first experimental proof of the polyketide rule was derived from the finding that radioactively-labelled acetate was incorporated in the anticipated manner into 6-methylsalicylic acid. The basic principles of polyketide metabolism have been adequately covered by Packter (2), Turner (3), Birch (4), and Money (5).

Polyketides originating from various numbers of acetate units (from C_8-C_{20}) are involved in the biogenesis of many mycotoxins (Table 1). The formed metabolites are named, tetraketides, pentaketides, hexaketides etc., according to the number of C_2 units which have contributed to the biosynthesis of the chain. A sharp cutout at the decaketide level (C_{20}) is evident.

TETRAKETIDE

Patulin

The biological reactions involved in the formation of patulin and penicillic acid are closely related. The biosynthesis of patulin as a representative tetraketide is discussed.

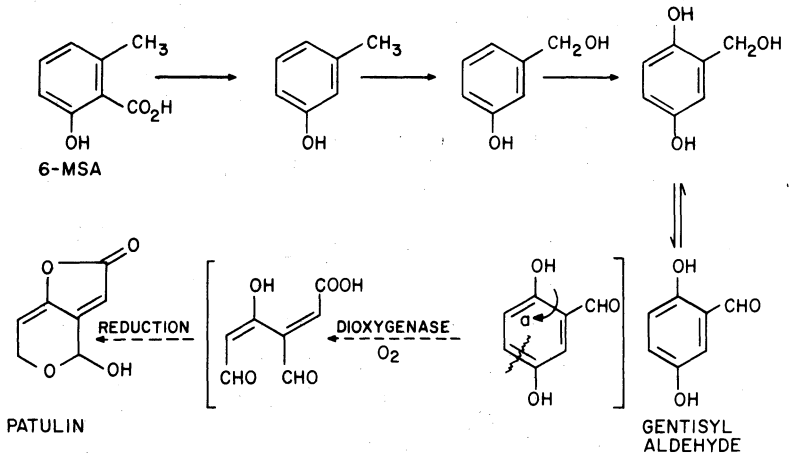

FIGURE 1 The biosynthesis of patulin

The biosynthesis of patulin attracted considerable attention since it involved the prototype of polyketide metabolism, 6-methylsalicylic acid (6-MSA) which forms the pivotal compound in the metabolic grid. Several eminent research groups, notably those of Scott (6) and Bu'Lock (7) studied the biogenesis of patulin. Labelling and kinetic pulse-labelling studies suggested a pathway from acetate via 6-MSA, m-hydroxybenzyl alcohol, m-hydroxybenzaldehyde, gentisyl alcohol, and gentisaldehyde (8). Experiments employing deuterated intermediates and analysis of the metabolites indicated the biosynthetic sequence (6) (Figure 1).

PENTAKETIDES

A. Citrinin

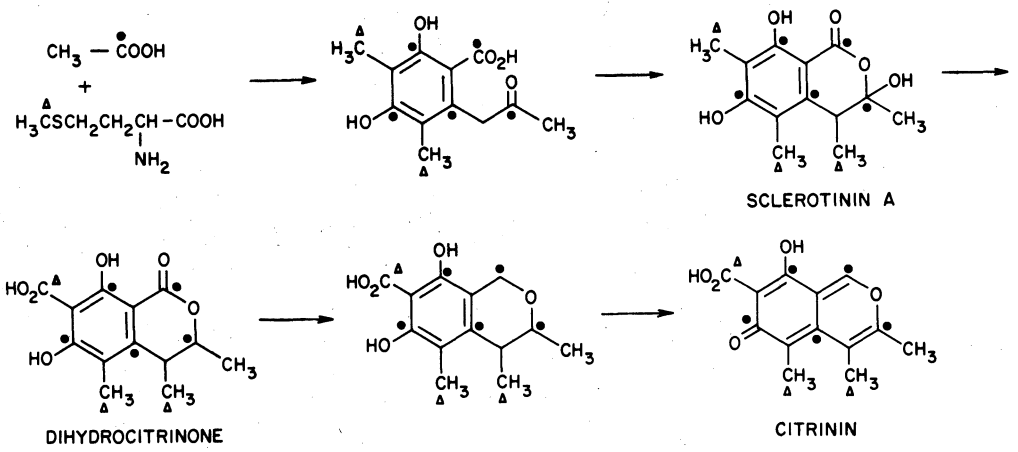

FIGURE 2 The biosynthesis of citrinin

Citrinin was first isolated from Penicillium citrinum, and is produced by several Penicillium and Aspergillus species. Citrinin is regarded as an important nephrotoxic mycotoxin. The biosynthesis of citrinin was studied by Birch et al. (9) in their classical investigations on the biosynthesis of polyketides. Administration of sodium [1-^{14}C]acetate or [methyl-^{14}C]methionine to cultures of Aspergillus candidus gave labelled citrinin. Chemical degradation of the citrinin established a labelling pattern in accordance with a pentaketide containing three methionine-derived C_1 units at C(4), C(5) and C(7). In an attempt to clarify the biosynthetic sequence, Curtis et al. (10) examined the metabolites from mutants of Penicillium citrinum. A biosynthetic sequence (Figure 2) although not experimentally proved, was proposed for the formation of citrinin. The study of citrinin exemplifies the rôle of both methionine and formate in the C-methylation of aromatic polyketides.

B. Diplosporin

Diplosporin, a novel mycotoxin is produced by strains of Diplodia macrospora on both solid and liquid media. The γ-pyrone-containing structure of diplosporin was recently elucidated and its natural abundance ^{13}C n.m.r. spectrum assigned completely (11) (Figure 3a). The biosynthetic origin of diplosporin was not obvious from a cursory chemical inspection of its structure. Three plausible mechanisms can be presented (12). Two of the postulates involve the condensation of two preformed polyketide units; similar mechanisms have been proposed for mollisin (13). The third postulate involves a single polyketide chain and has the ethyl side-chain derived via successive methylations with methionine, as in the case of the phytosterols (14). The biosynthesis of diplosporin was subsequently elucidated by application of ^{13}C techniques. Pertinent reviews on the use of ^{13}C n.m.r. spectroscopy in biosynthesis appeared recently (15), (16).

Several advanced techniques were applied in the assignment of the ^{13}C resonances of diplosporin (11), ochratoxin A(17), aflatoxin B_1 (18) and its congeners (19) viz. (i) ^{13}C chemical shifts, (ii) ^{13}C, ^{1}H coupling constants, (iii) relaxation times, (iv) proton decoupling techniques : (a) off-resonance proton decoupling, (b) selective proton decoupling, (c) selective population inversion; (v) deuterium isotope shifts, (vi) lanthanide induced shifts, (vii) information from biosynthetic studies.

There are several advantages in the application of ^{13}C techniques in biosynthetic studies : (i) Analysis of labelling patterns by ^{13}C n.m.r. spectroscopy does not require any laborious chemical degradation of the product. The results are more reliable as rearrangements can occur during chemical degradations. The ^{13}C techniques furthermore give access to chemically inaccessible carbon atoms. (ii) Incorporation of sodium [1,2-^{13}C]acetate into metabolites is very informative, since all acetate-derived cabon atoms will be enhanced by this technique, and carbon atoms derived from an intact acetate unit will show (^{13}C, ^{13}C) coupling. The magnitude of the (^{13}C, ^{13}C) coupling (30-90 Hz) is related to the hybridization of the two carbon atoms involved and increases with increased 's' character. A major advantage is the elucidation of anomalous pathways, e.g. the cleavage of acetate units. Lower levels of incorporation can be detected in this way.

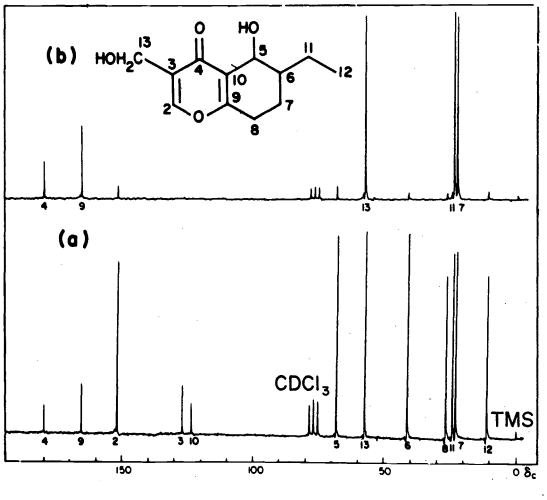

FIGURE 3 (a) Proton noise decoupled (p.n.d.) natural abundance ^{13}C n.m.r. spectrum of diplosporin
(b) P.n.d. ^{13}C n.m.r. spectrum of [1-^{13}C]acetate-derived diplosporin

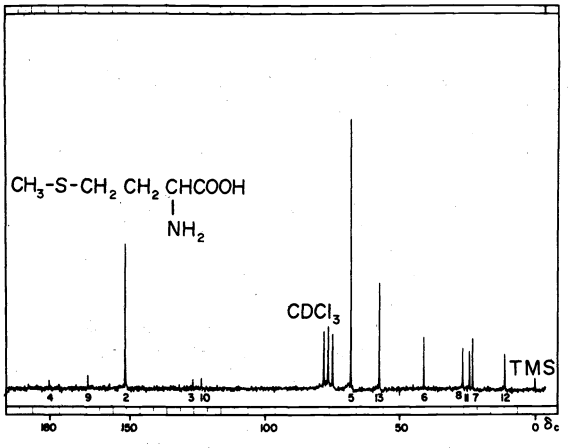

FIGURE 4 P.n.d. ^{13}C n.m.r. spectrum of [methyl-^{13}C]methionine-derived diplosporin
(Permission for reproduction of figures 3 and 4 from the Chemical Society, London. See reference (11)).

The biosynthesis of diplosporin was investigated by administration of [^{13}C]labelled precursors and analysis of the labelling pattern by ^{13}C n.m.r. spectroscopy (12). Sodium [1-^{14}C]acetate was employed only to determine the level of incorporation (2.5%) in diplosporin and thereby the amounts of [^{13}C]acetate necessary for feedings. Supplementing cultures of D. macrospora with [1-^{13}C]acetate gave diplosporin enriched at carbon atoms C(4), C(7), C(9), C(11), and C(13) (Figure 3b). In a complementary experiment the [2-^{13}C]-acetate origin of carbon atoms C(2), C(6), C(8), C(10), and C(12) was confirmed. In a subsequent feeding experiment, [1,2-^{13}C]acetate was administered to cultures of D. macrospora. The p.n.d. ^{13}C n.m.r. spectrum of the [1,2-^{13}C]acetate-derived diplosporin exhibited (^{13}C, ^{13}C) couplings for all the carbon atoms except for C(2) and C(5). The magnitude of the coupling constants defined the location of the five acetate units, viz. C(13)-C(3) [49.2 Hz],C(4)-C(10)[55.1 Hz],C(9)-C(8)[47.95 Hz],C(7)-C(6)[33.1 Hz], and C(11)-C(12)[34.95Hz]. These data confirmed the mode of folding and the alternating nature of the pentaketide chain.

The origin of the C(2) and C(5) carbon atoms from the C_1 pool was verified by supplementing cultures of D. macrospora with L-[methyl-^{13}C]methionine. The ^{13}C n.m.r. spectrum of the L-[methyl-^{13}C]methionine-derived diplosporin showed the enhancement of the signals of C(2) and C(5), thereby establishing their origin (Figure 4). The foregoing data enabled us to present a sound biosynthetic scheme for diplosporin (Figure 5).

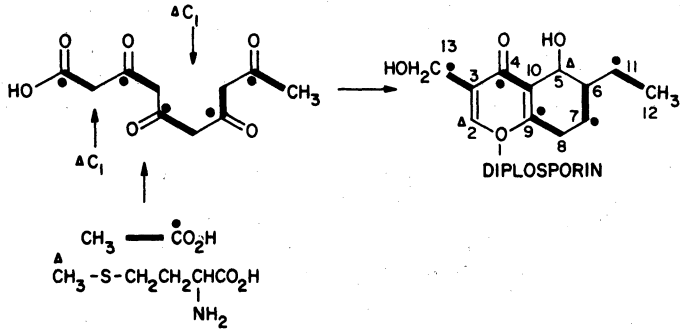

FIGURE 5 The proposed biosynthesis of diplosporin

The cofactor S-adenosyl methionine contributes N-methyl, O-methyl,and aromatic C-methyl groups during the biosynthesis of many secondary metabolites. The involvement of methionine in the formation of a carbocyclic ring has been demonstrated via a rearrangement of 3-methylorsellinic acid in the biogenesis of tropolones (20) and the formation of the cyclopropane ring in lactobacillic acid via C-methylation of cis-vaccenic acid (21). The incorporation of a methionine-derived carbon atom in the carbocyclic ring of diplosporin is, therefore, an unique biosynthetic process.

C. Ochratoxin A

The potent nephrotoxin, ochratoxin A contains a 3,4-dihydro-3-methylisocoumarin moiety which is linked over the carboxy-group at C(8) to L-β-phenylalanine (22). Cell-free extracts of A. ochraceus were capable of effecting this amide bond formation. L-β-Phenylalanine is derived from the shikimate pathway (23) and attention will thus be directed to the formation of the dihydroisocoumarin acid only.

Biosynthetic studies were carried out by Steyn et al. (24) using [^{14}C]labelled precursors. Hydrolysis of [1-^{14}C]acetate-derived ochratoxin A gave the isocoumarin acid which contained all the activity. Kuhn-Roth and Schmidt degradations of the acid indicated its pentaketide origin (Figure 6). This conclusion was unequivocally confirmed by administration of singly and doubly labelled acetate precursors to cultures and analysis of the labelling patterns in ochratoxin A (17). The p.n.d. ^{13}C n.m.r. spectrum of [1-^{13}C]acetate-derived ochratoxin A showed enhancement of C(1), C(3), C(5), C(7), and C(9). The p.n.d. ^{13}C n.m.r. spectrum of [1,2-^{13}C]acetate-derived ochratoxin A showed the presence of five intact acetate units as evidenced by the (^{13}C, ^{13}C) coupling constants C(11)-C(3)[40.0 Hz],C(4)-C(5)[42.3 Hz], C(6)-C(7)[67.4 Hz],C(8)-C(9)[68.9 Hz], and C(10)-C(1)[67.2 Hz].

The origin of the carboxy-group at C(8) was previously established by Schmidt degradation of the 9-O-methyl derivative of the dihydroisocoumarin acid derived from DL-[methyl-^{14}C]-methionine (24). Maebashi et al. (25) verified our findings by ^{13}C n.m.r. analysis of sodium [^{13}C]formate-derived ochratoxin A.

The skeleton of the dihydroisocoumarin moiety of ochratoxin A is therefore derived from the acetate-polymalonate pathway (pentaketide), whereas the carboxy-carbon atom C(12) is derived from the C_1 pool (Figure 6). It has not been determined at what point in the biosynthesis of ochratoxin A the chlorine atom is introduced at C(6).

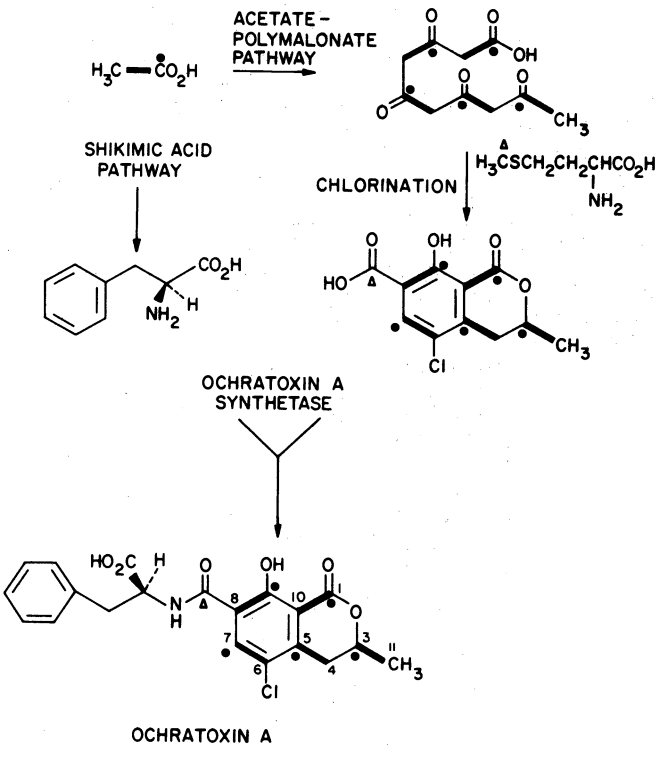

FIGURE 6 The biosynthesis of ochratoxin A

HEXAKETIDES

Maltoryzine

Maltoryzine is a mycotoxin from Aspergillus oryzae var. microsporus. Its hexaketide origin, although not experimentally verified, was recently proposed by Yamazaki (26).

HEPTAKETIDES

Xanthomegnin

Simpson assigned the ^{13}C n.m.r. spectrum of xanthomegnin and studied its biosynthesis by A. sulphureus, using singly and doubly ^{13}C-labelled acetate as precursors (27). The previously proposed angular structure of e.g. xanthomegnin was shown to be incorrect by Zeeck et al. (28). The linear structure was proposed (Figure 7). However, the change in structure has no influence on the conclusions of Simpson (27) on the biosynthesis of xanthomegnin or viomellein. The results indicated that xanthomegnin is derived from fourteen intact acetate units (two heptaketides) arising from foldings of a common heptaketide chain, followed by introduction of a C_1 unit and an oxidative coupling (dimerisation) (Figure 7).

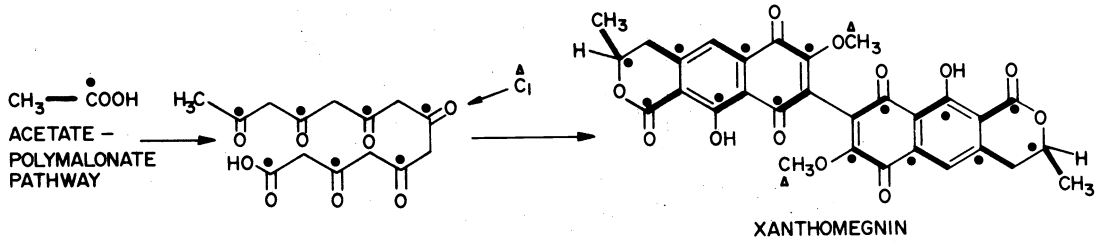

FIGURE 7 The biosynthesis of xanthomegnin

The isolation of the monomeric heptaketide semi-vioxanthin from a xanthomegnin- and viomellein-producing strain of Penicillium citreoviride clearly supports the pivotal rôle of this compound in the biogenesis of these pigments (28). Oxidation of demethyl-semi-vioxanthin with Fremy's salt gave after methylation semi-xanthomegnin. Semi-xanthomegnin could be transformed into xanthomegnin by a dimerisation reaction with potassium peroxydisulphate/sodium hydroxide. This facile conversion verified the biogenetic homogeneity of these pigments (28).

OCTAKETIDES

A. Cytochalasans (octaketides and nonaketides)

The cytochalasans contain a highly substituted perhydroisoindole moiety to which is linked a macrocyclic ring which can be either a carbocycle (e.g. cytochalasin H), lactone (e.g. cytochalasin F) or a cyclic carbonate (e.g. cytochalasin E).

Binder et al. (29) fed a number of labelled potential precursors to a growing culture of a Phoma sp. Chemical degradation of the cytochalasin B involved cleavage with periodate, Kuhn-Roth oxidation, and Schmidt degradation of the acids. It was established that the biosynthetic "building units" of cytochalasin B consist of phenylalanine (incorporated without any loss of the carboxy-group or any skeletal rearrangement), nine acetate units and two C_1 units (methionine) located as methyl groups at C(12) and C(16). Cytochalasin D was found to be an octaketide derived from the condensation of eight acetate units in the normal head-to-tail fashion to form an unbranched C_{16}-polyketide moiety (30) (Figure 8).

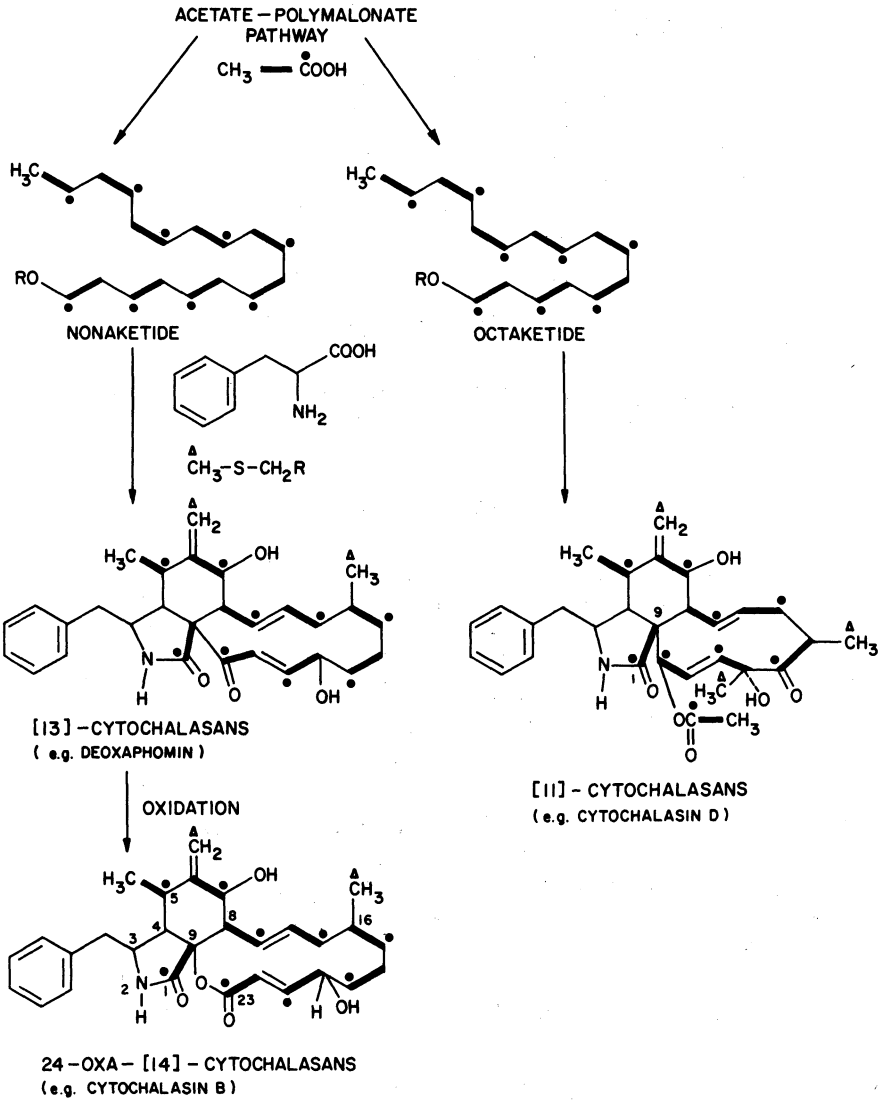

FIGURE 8 The biosynthesis of the cytochalasans

The ^{13}C n.m.r. studies of Graf et al.(31) in 1974 on cytochalasin B verified the previous proposals for the bio-origin of this metabolite. The ^{13}C data give clear insight into the "acetate" origin of C(1) and C(9) which form part of the lactam ring. C(1) and C(9) are the termini of the polyketide intermediate, occurring at the juncture of the amino acid and the polyketide biogenetic route.

The isolation of minor metabolites, e.g. deoxaphomin, suggested that such a carbocyclic intermediate could lead to macrolides, e.g. cytochalasin B, by an enzymatic Baeyer-Villiger type oxidation with the insertion of oxygen between C(9) and C(23). Robert and Tamm (32) demonstrated the precursor rôle of deoxaphomin in the biosynthesis of cytochalasin B.

B. Ergochromes

The ergochromes comprise a group of closely related bis(hexahydroxanthonyl) pigments elaborated by the parasitic fungus Claviceps purpurea (33) and several saprophytic fungi e.g. Penicillium oxalicum (34).

Ergot contains small amounts of the anthraquinonoidal carboxylic acids, endocrocin and clavorubin. The possible intermediacy of these anthraquinones in the biosynthesis of the ergochromes was recognized by Franck in 1964. Labelled ergochromes AA and BB were prepared by feeding sodium [1-^{14}C]-, [2-^{14}C]-, [2-^{3}H]-, and [1,2-^{13}C]acetate to growing cultures of the relevant fungi. In ^{14}C-labelled ergochromes, carbon atoms C(6) and C(11) were easily analysed by Kuhn-Roth oxidation.

Franck and Flasch (33) determined by incorporation studies the intermediacy of the anthraquinone, emodin in the ergochrome pathway. In very elegant competitive incorporation studies, two-component mixtures of [U-^{3}H]emodin and one of the four labelled anthraquinones viz. [U-^{14}C]emodin, [U-^{14}C]islandicin, [U-^{14}C]catenarin, and [U-^{14}C]chrysophanol were added to cultures of P. oxalicum, and the ^{3}H- and ^{14}C-activities of the isolated ergochrome EE determined (35). Chrysophanol was incorporated more effectively than emodin, whereas catenarin and islandicin were incorporated less efficiently. Emodin and chrysophanol therefore play an important rôle in ergochrome biosynthesis with the introduction of the additional hydroxy-group at a later stage.

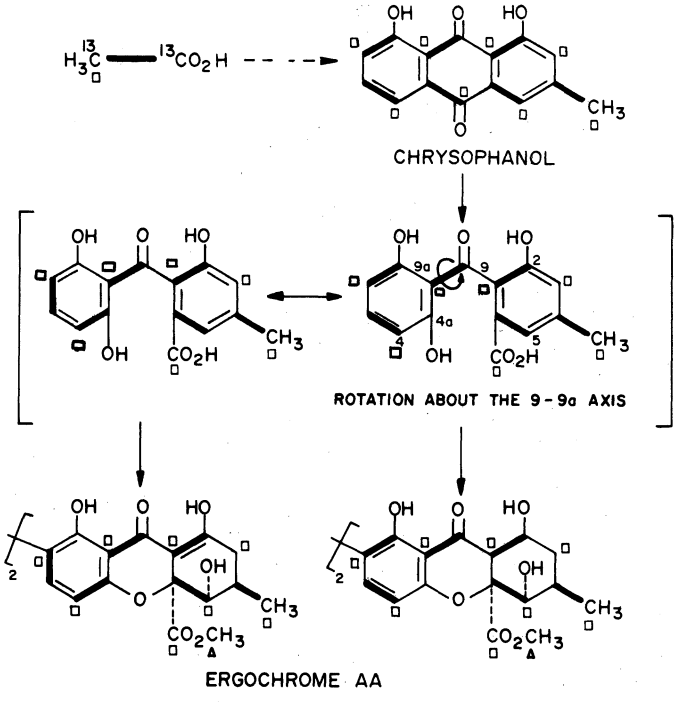

FIGURE 9 The biosynthesis of ergochrome AA

Kurobane et al. (36) administered [1,2-^{13}C]acetate to an ergochrome AA-producing mould, Pyrenochaeta terrestris and isolated ergochrome AA containing differently labelled xanthone units. The observed (^{13}C, ^{13}C) couplings indicated enrichment from rotation about the two-fold axis of symmetry in the trisubstituted ring of a benzophenone intermediate (Figure 9). This rotation can occur only if the intermediate is not bound to an enzyme surface; the oxidative cleavage therefore occurs at the monomeric anthraquinone prior to dimerisation.

Detailed and elegant studies by the groups of Shibata and Sankawa defined the biosynthesis of anthraquinonoid mycotoxins from Penicillium islandicum Sopp (37). Their biosynthesis is, however, closely related to the biosynthesis of other anthraquinones discussed in this paper and is not included here.

NONAKETIDES

A. Zearalenone

Zearalenone is an anabolic uterotrophic toxin which is responsible for vulvovaginitis in farm animals fed mouldy grain. Cursory chemical inspection of the structure of zearalenone would suggest a biosynthetic origin from the condensation of nine acetate units via the acetate-polymalonate pathway. Biosynthetic studies on zearalenone were hampered by the inability to undertake these investigations on chemically defined media.

Steele and co-workers found that [1-^{14}C]acetate was readily incorporated into zearalenone (38). Chemical degradation of the [^{14}C]zearalenone yielded $^{14}CO_2$ which originated from the lactone carbonyl carbon atom. Results from degradation studies suggested labelling which is consistent with the acetate-polymalonate pathway (Figure 10). Zearalenone represents a relatively unmodified polyketide.

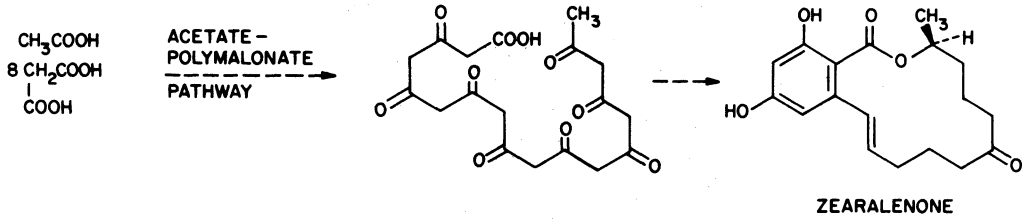

FIGURE 10 The proposed biosynthesis of zearalenone

B. Citreoviridin and related compounds

Citreoviridin, a potent neurotoxin was obtained from Penicillium citreoviride Biourge (39), one of the yellowed rice fungi and subsequently from a number of Penicillium sp. (40).

Labelled citreoviridin was obtained by administration of [methyl-^{14}C]methionine and [2-^{14}C]acetate to cultures of Penicillium pulvillorum (40). The labelling pattern was determined by Zeisel demethylation, Kuhn-Roth oxidation, Schmidt degradation and selective oxidation reactions. These data verified that citreoviridin was derived from one acetate unit and eight malonyl units to furnish the intermediary polyketide chain which is C-alkylated at four methylene positions by the methyl group of methionine. The methoxy-group also arises from methionine (Figure 13).

Citreoviridin is structurally related to aurovertin B (41) and to asteltoxin, a newly-discovered mycotoxin from Aspergillus stellatus (42) (Figure 12). Cursory chemical inspection of asteltoxin and aurovertin B indicates a polyketide origin. However, if the two compounds originated from a C_{18}-polyketide, C(1) must be derived from the introduction of the methyl group of methionine on the methyl carbon of the starter acetate unit. Alternatively a C_{20} acetate chain requires the loss of a methyl carbon from the chain initiating acetate unit. The incorporation of propionate into aurovertin was reported (43). Our recent ^{13}C n.m.r. analyses of aurovertin B derived from L-[methyl-^{13}C]methionine, [1-^{13}C]acetate, and [1,2-^{13}C]acetate established its nonaketide origin and that C(1) is derived from the methyl group of methionine (44). This particular involvement of methionine in fungal polyketide biosynthesis is without any clear analogy.

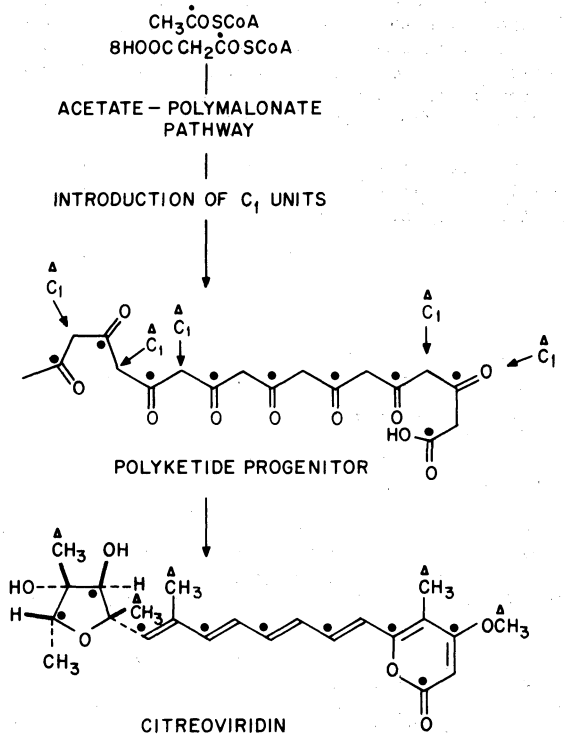

FIGURE 11 The biosynthesis of citreoviridin

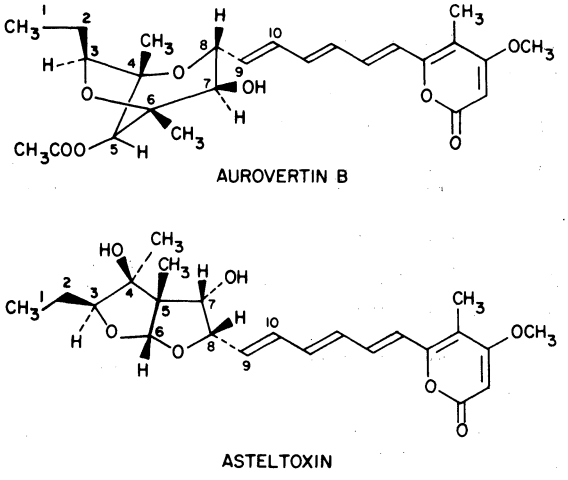

FIGURE 12 The structures of aurovertin B and asteltoxin

DECAKETIDES

A. Introduction

The conversion: acetate → averufin → versiconal acetate → versicolorin A → sterigmatocystin → aflatoxin B_1 is discussed in this section. Although versicolorin A, sterigmatocystin, and aflatoxin B_1 contain less than twenty skeletal carbon atoms they are classified as decaketides owing to their origin from averufin (C_{20}).

The aflatoxins contain a coumarin fused to a bisdihydrofurano-moiety and additionally either a cyclopentenone (B series) or a δ-lactone (G series). Special attention will be directed to the biosynthesis of the aflatoxins owing to their structural complexity and their established practical importance.

The structural similarity of aflatoxin B_1 and sterigmatocystin suggested that both compounds have a common biogenetic precursor or that sterigmatocystin is a precursor of aflatoxin B_1. Labelling studies of sterigmatocystin supported the acetate-polymalonate pathway of the xanthone moiety and indicated an apparent difference in the level of radioactivity of the side-chain and the xanthone nucleus (45) (Figure 13).

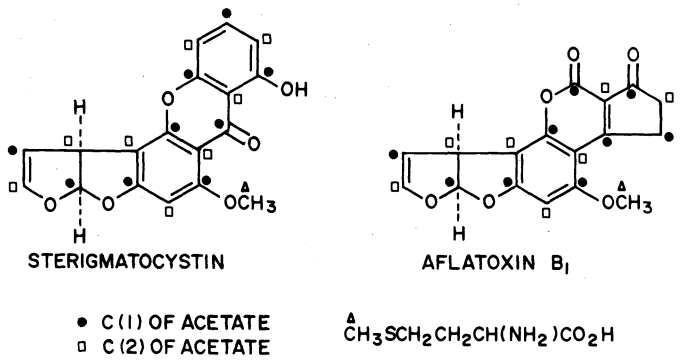

FIGURE 13 The labelling patterns of sterigmatocystin and aflatoxin B_1

Administration of [methyl-^{14}C]methionine, [1-^{14}C]acetate, and [2-^{14}C]acetate to cultures of A. flavus furnished labelled aflatoxin B_1 (46). Several ingenious degradation experiments established the specific incorporation of methionine and acetate (Figure 13). These data indicated an anomalous pathway as aflatoxin B_1 contained two unusual carbon-carbon linkages viz. C(5)-C(6) and C(11)-C(14), in which both carbon atoms are derived from the carboxy-and methyl-carbon atoms of acetate, respectively (46). Biollaz et al. (46) proposed a biosynthetic route consonant with these data. A C_{18} polyhydroxyanthraquinone could rearrange via a diradical or Zwitterion into versicolorin A. The postulate of Büchi was accepted until the publication of our definitive ^{13}C findings on aflatoxin B_1 (18), (47).

We subsequently undertook detailed biosynthetic studies of the precursors of aflatoxin B_1, and assigned the ^{13}C resonances of averufin, versiconal acetate, versicolorin A, and sterigmatocystin completely (Figure 14). The observed labelling patterns and modes of folding of the polyketides confirm their intermediacy in aflatoxin biosynthesis.

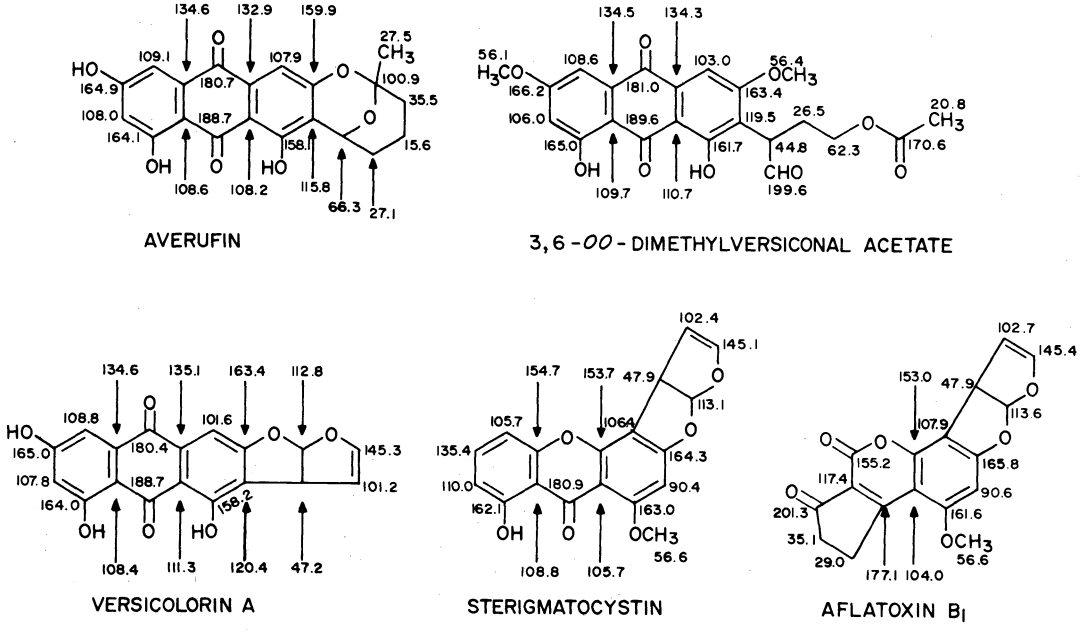

FIGURE 14 The chemical shift data (δ_C) of averufin, 3,6-O,O-dimethylversiconal acetate, versicolorin A, sterigmatocystin, and aflatoxin B_1. 3,5-O,O-Dimethylversiconal acetate was obtained from versiconal acetate.

The biochemical events prior to the elaboration of the aflatoxins were clarified by the use of mutants (derived from chemical treatment and U.V. illumination), enzyme inhibitors, information on the co-occurrence of these metabolites in fungal cultures, and conversion of advanced precursors by whole cells (48) and fungal protoplasts (49) into aflatoxin B_1. Labelled norsolorinic acid, versiconal acetate, versicolorin A, and sterigmatocystin are converted more efficiently than acetate into aflatoxin B_1, thereby indicating their direct conversion into aflatoxin without prior catabolism into "acetate" (18).

B. Averufin

Treatment of wild-type strains of A. parasiticus with N-methyl-N-nitroso-N-guanidine gave the mutant (W-49) which accumulates averufin in the mycelium (50) (Figure 15). [^{14}C]Averufin was efficiently converted into aflatoxin B_1 by incubation with wild-type A. parasiticus. However, addition of dichlorvos to this incubation medium inhibited the formation of aflatoxin B_1 and versiconal acetate accumulated during the incubation, indicating that versiconal acetate represents a step beyond averufin in aflatoxin biosynthesis (48).

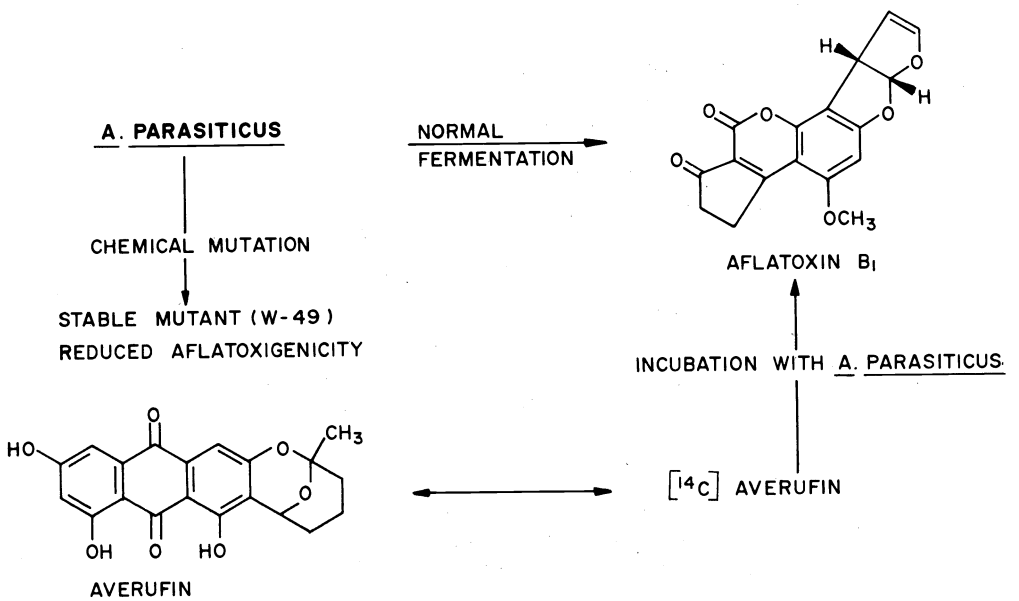

FIGURE 15 The intermediacy of averufin in the biosynthesis of aflatoxin B_1

Administration of [1-^{13}C]-, [2-^{13}C]- and [1,2-^{13}C]acetate to stationary cultures of the mutant gave labelled averufin (51). The p.n.d. spectrum of [1-^{13}C]acetate-derived averufin showed ten enhanced carbon atoms viz. C(1'), C(3'), C(5'), C(1), C(3), C(6), C(8), C(9), C(11), and C(14). A complementary feeding experiment with [2-^{13}C]acetate verified the origin of the remaining carbon atoms. In the p.n.d. ^{13}C n.m.r. spectrum of [1,2-^{13}C]acetate-derived averufin all the carbon signals showed (^{13}C, ^{13}C) couplings (51). The magnitude of the coupling constants established ten intact acetate units in averufin viz. C(6')-C(5'), (4')-C(3'), C(2')-C(1'), C(2)-C(3), C(4)-C(14), C(10)-C(11), C(5)-C(6), C(7)-C(8), C(12)-C(9) and C(13)-C(1), and the mode of folding of the polyketide progenitor (Figure 18).

The pivotal rôle of averufin in aflatoxin biosynthesis was furthermore shown by the conversion of [1-^{13}C]acetate- (52) and [1,2-^{13}C]acetate-derived averufin (53) by wild-type A. parasiticus into aflatoxin B_1.

C. Versiconal acetate

Treatment of cultures of A. parasiticus with the enzyme inhibitor, dichlorvos, resulted in reduced aflatoxigenicity and production of an orange-red pigment, versiconal acetate (54), minor quantities of versiconol acetate, and versiconol (19). The structure of versiconal acetate was elucidated (19) in order to clarify the intriguing rearrangement involved in the biotransformation of the straight C_6 side-chain of e.g. averufin into the branched C_4 bisdihydrofurano grouping present in the versicolorins, sterigmatocystins, and aflatoxins.

The physicochemical data of versiconal acetate indicated an equilibrium mixture of isomers in $(CD_3)_2SO$ (Figure 16). The mixture of isomers of versiconal acetate in solution seriously complicated the assignment of ^{13}C signals, particularly for the determination of enrichment sites in biosynthetic studies. Treatment of versiconal acetate with diazomethane gave

3,6-O,O-dimethylversiconal acetate. This product is present in $CDCl_3$ as the branched aldehyde as evidenced by the one-proton singlet at δ9.68. The lack of observable coupling from 2'-H indicates a dihedral relationship of close to 90°. However, in $(CD_3)_2SO$, the intramolecular hydrogen bonding is reduced and the compound is present as the angular hemiacetal.

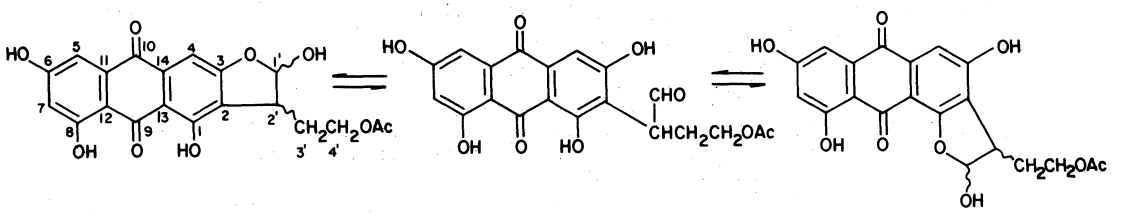

FIGURE 16 The isomers of versiconal acetate

The multiplicities observed in the n.O.e. enhanced single frequency spectrum of the dimethyl derivative of versiconal acetate in $CDCl_3$ were used in the assignment of the resonances (19). An important feature of the spectrum was the signal at δ199.6 [^1J(C,H) 178.6 Hz] which is characteristic of an aldehyde carbon atom and was assigned to C(1'). The only aliphatic methine carbon atom, viz. C(2') resonated at δ44.3 and showed a ^2J(C,H) value of 24 Hz which is typical of the coupling of an aliphatic aldehyde proton to an aliphatic carbon atom, thereby unambiguously proving the relationship between C(1') and C(2'). Feeding experiments using singly and doubly labelled acetate gave labelled versiconal acetate. Analysis of the labels by ^{13}C n.m.r. spectroscopy established an enrichment pattern and assembly of intact acetate units compatible with the intermediacy of versiconal acetate in aflatoxin biogenesis, and an epoxide rearrangement for the conversion of the straight C_6 side-chain of e.g. averufin into the branched C_4 benzylic side-chain of versiconal acetate (55).

Yao and Hsieh (54) reported the efficient conversion of [^{14}C]versiconal acetate (13.7%) into aflatoxin B_1 by a nitrogen-free medium of A. parasiticus.

D. Versicolorin A

The intermediacy of versicolorin A in aflatoxin biogenesis was established by (i) its accumulation in a mutant of A. parasiticus, (ii) conversion of [^{14}C]versicolorin A into aflatoxin B_1 by wild-type A. parasiticus (56), (iii) its ^{13}C labelling pattern (57), and (iv) the absolute configuration of the chiral centres at the cis-ring junction (57). The results are summarized (Figure 18).

E. Sterigmatocystin

In one of the pioneering applications of ^{13}C n.m.r. spectroscopy to biosynthetic problems, Tanabe et al. employed [1-^{13}C]- and [2-^{13}C]acetate as precursors in sterigmatocystin biosynthesis. The observed (^{13}C, ^{13}C) coupling between C(15) and C(9) in the p.n.d. spectrum of [2-^{13}C]acetate-derived sterigmatocystin confirms the unusual head-to-head linkage of the two acetate units. In subsequent labelling experiments, Seto et al. (59) employed [1,2-^{13}C]acetate but a wrong assignment of ^{13}C signals led to an erroneous location of intact acetate units in sterigmatocystin. A reassignment and reinterpretation of the ^{13}C n.m.r. data of sterigmatocystin by Pachler et al. (18) indicated only one possible arrangement of acetate units in sterigmatocystin. This information was consistent with its precursor rôle in aflatoxin biosynthesis (Figure 18). [^{14}C]Sterigmatocystin is efficiently converted into aflatoxin B_1 by cultures of A. parasiticus (60).

F. Aflatoxin B_1

The natural abundance ^{13}C n.m.r. spectrum of aflatoxin B_1 was completely assigned by Pachler et al. (18). P.n.d. spectra obtained from [1-^{13}C]acetate- and [2-^{13}C]acetate-derived aflatoxin B_1 showed nine and seven enhanced carbon atoms, respectively. The observed (^{13}C, ^{13}C) coupling constants of 34 Hz between C(5) and C(6) in [1-^{13}C]acetate-derived aflatoxin B_1 and 44 Hz between C(11) and C(4) in [2-^{13}C]acetate-derived aflatoxin B_1 are indicative of rearrangements involving bond fission during the biosynthetic process (18). The p.n.d. ^{13}C n.m.r. spectrum of [1,2-^{13}C]acetate-derived aflatoxin B_1 displayed ^1J(C,C) coupling constants which indicated seven intact acetate units viz. C(2)-C(6), C(3)-C(4), C(7)-C(12), C(8)-C(9), C(10)-C(11), C(13)-C(14), and C(15)-C(16). In this spectrum the C(1) and C(5) resonances were enhanced but showed no (^{13}C, ^{13}C) coupling, thus confirming that fission of acetate units occurred with the concomitant loss of methyl-derived acetate carbon atoms during the biosynthesis of aflatoxin B_1. The signal of the methoxy-carbon atom showed no enchancement of intensity.

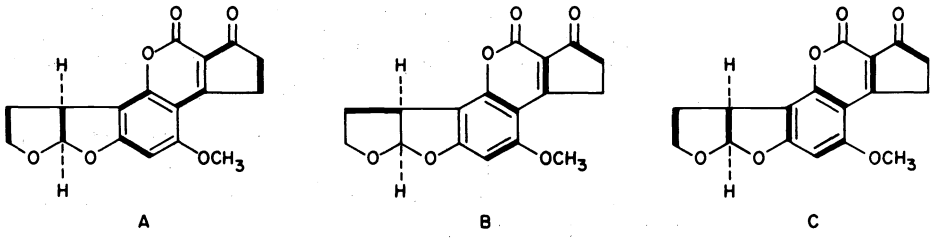

FIGURE 17 The distribution of intact acetate units in aflatoxin B_1, according to the Biollaz proposal (A,B), and according to ^{13}C results (C)

A salient feature of these ^{13}C data is the presence of three intact acetate units in the central ring of aflatoxin B_1 (Figure 17). This finding requires that the central ring of aflatoxin B_1 be derived from the outer ring of an anthraquinonoid precursor e.g. averufin and norsolorinic acid, thereby refuting the postulate of Biollaz et al. (46). A polyhydroxynaphthacene intermediate could lead to two possible arrangements of acetate units in aflatoxin B_1; however, in each case the central ring of aflatoxin B_1 contains only two acetate units (Figure 17).

The foregoing data from the ^{14}C and ^{13}C labelling studies with primitive and advanced precursors, mutant studies and co-occurance of metabolites are consonant with a general biosynthetic scheme (Figure 18).

Several mechanisms have been advanced for the conversion of the straight C_6 side-chain of averufin into the branched side-chain of versicolorin A. A possible mechanism was proposed by Gorst-Allman et al. (51), which involved ring opening and hydration of averufin, followed by dehydration and epoxidation. Rearrangement of the epoxide intermediate can lead to a branched benzylic aldehyde containing the anticipated labelling pattern. The terminal acetyl group can be removed by an enzymatic Baeyer-Villiger oxidation which involves insertion of an oxygen atom between C(4') and C(5'). Versiconal acetate is a compound of this nature. Oxidative cleavage of the C(9)-C(11) bond of versicolorin A with concomitant loss of the acetate-derived methyl carbon C(9) can lead, after rotation, to the angular xanthone, sterigmatocystin (Figure 18).

The foregoing labelling experiments established the primitive and advanced biogenetic precursors of the aflatoxins. However, some challenging problems in aflatoxin biosynthesis require further attention, e.g. the nature of the highly complex polyketide synthetase; the exact mechanism by which primary metabolites or even secondary metabolites induce the enzymes of aflatoxin biosynthesis; and the loss of the C(6) phenolic hydroxy-group during the conversion of the polyhydroxyanthraquinones (e.g. versicolorin A) into xanthones (e.g. sterigmatocystin) - such a loss is without clear analogy in fungal polyketide biogenesis.

The discovery of the aflatoxins led to an international awareness of the serious economic and health problems which are associated with the contamination of foodstuffs and feedstuffs by toxigenic fungi, to a better comprehension of the mechanism of chemical carcinogenesis, and a renewed interest in natural product chemistry and fungal secondary metabolism, particularly polyketide biogenesis.

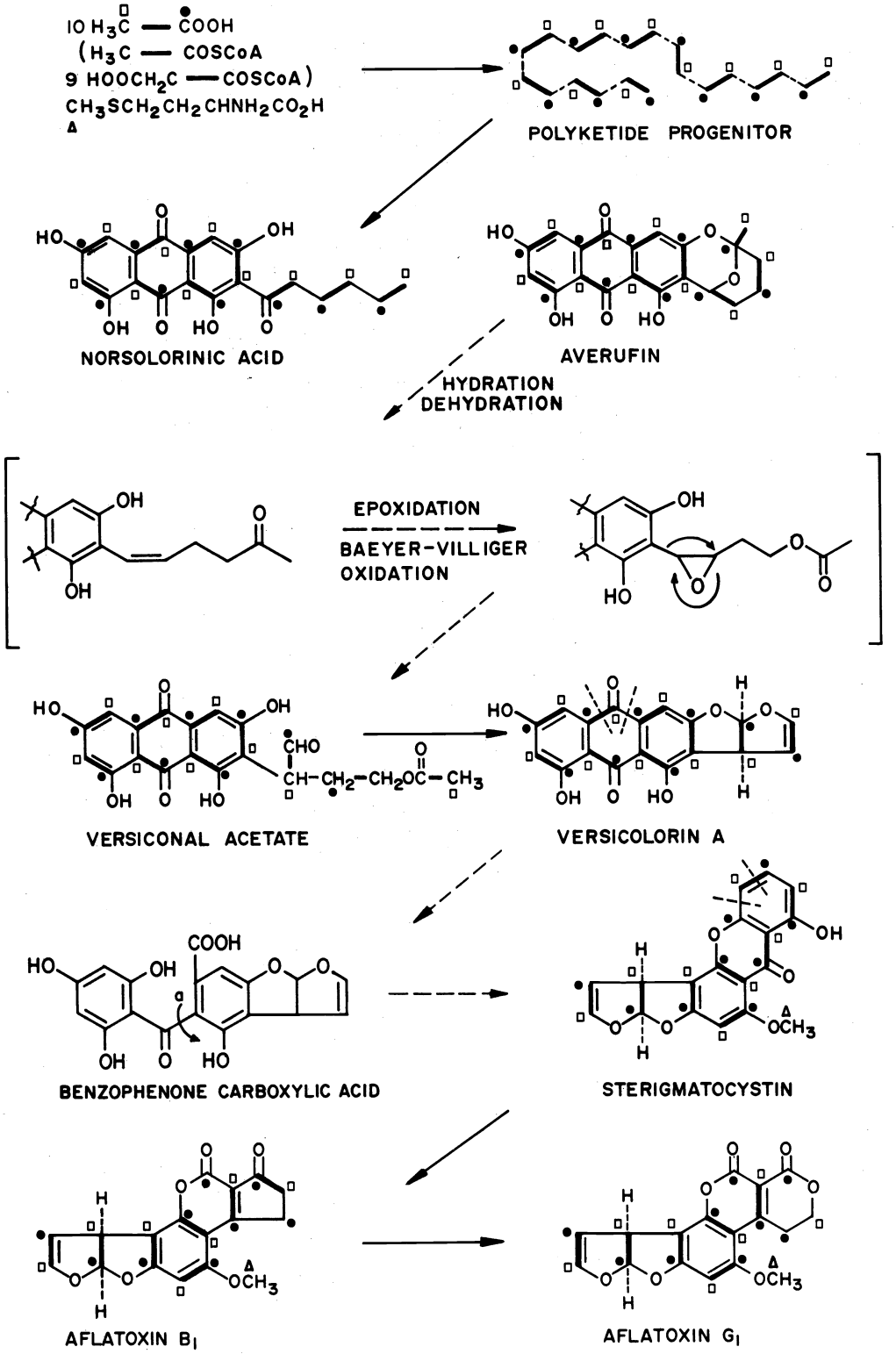

FIGURE 18 The proposed biosynthesis of norcolorinic acid, averufin, versiconal acetate, versicolorin A, sterigmatocystin, and the aflatoxins

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