Pure & Appl. Chem., Vol. 58, No. 2, pp. 227–238, 1986.
Printed in Great Britain.

Progress toward a biosynthetic rationale of the aflatoxin pathway

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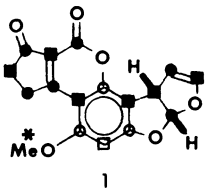
Abstract - Specifically-labeled samples of averufin have been synthesized and incorporated intact into aflatoxin B_1, versicolorin A and versiconal acetate as determined by NMR spectroscopy. [1-^{13}C]-Hexanoic acid has been shown to be utilized as the primer of the pathway while a group of alternative starters was found to be degraded to acetate prior to incorporation. The correct structure of nidurufin has been established and it and its 2'-epimer, pseudonidurufin, in labeled form have failed to be utilized in aflatoxin biosynthesis under two experimental protocols. [1'-^{18}O,5'-^{13}C]-Averufin, however, was observed to label versiconal acetate in such a way that the acetyl carbonyl bore 80% of the original ^{18}O-label. A biogenetic scheme is proposed to account for these findings that is supported in the cationic regime by preliminary model studies.

INTRODUCTION

Aflatoxin B_1 (1) is the major component of a family of secondary natural products produced by certain species of *Aspergillus*. It is perhaps the paradigm among mycotoxins both for the range and acuteness of its biochemical effects (ref. 1) and for its comparatively early discovery 25 years ago near the outset of a period of vigorous and diverse scientific activity in a field whose progress has been marked triennially by this series of Symposia.

From the perspective of biosynthesis, the structure of aflatoxin B_1 (1) presents a number of substantial problems. The dihydrobisfuran characteristic of this family of toxins is unique among polyketide-derived natural products and is generally held to be the principal site of activation in higher organisms and the chemical entity ultimately responsible for its severe *in vivo* effects (ref. 1). It is the natural origin of this structural feature and the question of starter units other than acetate in polyketide biosynthesis that will be the subject of the present lecture. Further questions are posed by 1 that are both of importance to our understanding of acetogenin biosynthesis in general and of 1 in particular where aryl cleavages, aromatic hydroxyl loss and deep-seated rearrangements, in large measure oxidative, are evident.



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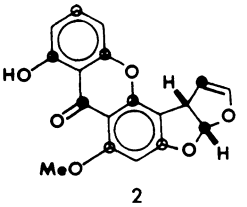

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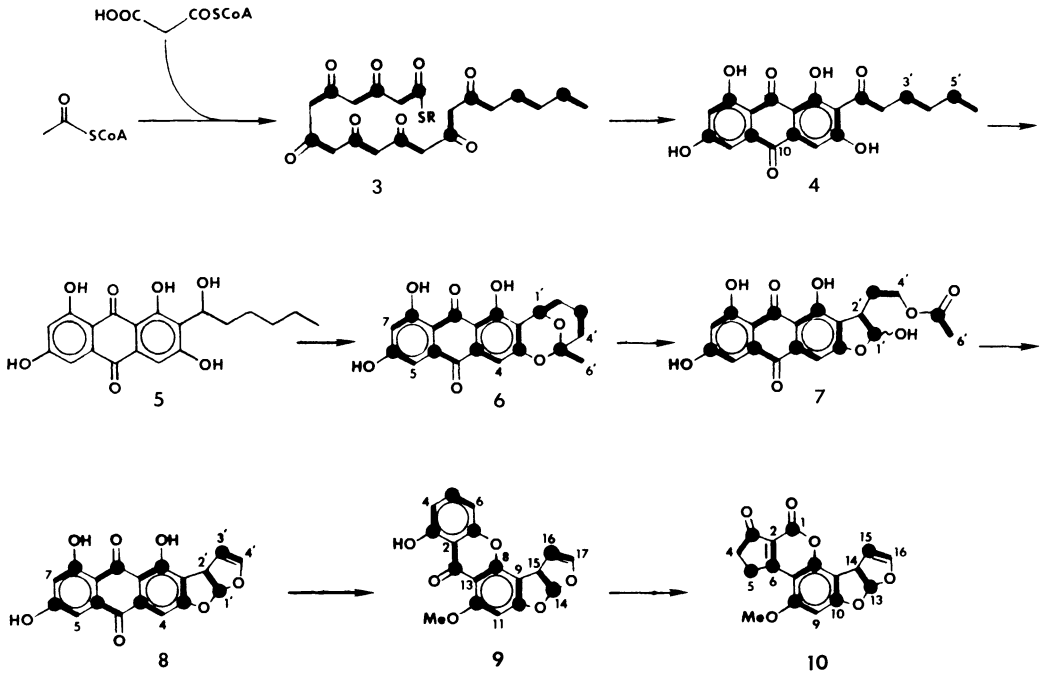
Büchi's group at MIT has figured prominently in the development of aflatoxin chemistry for contributions in structure proof (ref. 2), synthesis (ref. 3) and, in the late 1960's, for an exceptionally well-executed investigation of aflatoxin B_1 (1) biosynthesis by classical methods (ref. 4). In these benchmark studies careful degradations of 1 to determine the locations of radiolabel from incorporation of [^{14}C]-methionine, [1-^{14}C]- and [2-^{14}C]-acetate unambiguously established the origins of 12 of the 16 nuclear carbons from acetate (solid circles and squares for C-1 and C-2, respectively) and the methoxyl from methionine (*). For each of these acetate incorporation experiments in *A. flavus* a consistent level of radioactivity was observed throughout the molecule suggesting derivation of the toxin from a single, highly rearranged polyketide chain (ref. 4). Preliminary results of an analogous kind were published contemporaneously by Holker for the related *Aspergillus* metabolite

sterigmatocystin (2) from A. versicolor which gave a parallel pattern of acetate labeling in the bisfuran but notably at a level approximately 10% lower than the specific activity determined at nuclear sites (ref. 5). Several interpretations have been attached to this observation (ref. 6) but work at Hopkins has provided a new rationale and one grounded in experiment.

Progress with this problem in the 15 years since the publication of Büchi's experiments has been heavily dependent on the generation of A. parasiticus mutants by Donkersloot, and Bennett and Lee which accumulate norsolorinic acid (4) (ref. 7), averantin (5) (ref. 8), averufin (6) (ref. 9) and versicolorin A (8) (ref. 10) -- pigments which had been observed as metabolites in Aspergillus and other organisms (ref. 11). During this period it was also observed that an insecticide, dichlorovos (dimethyl-2,2-dichlorovinyl phosphate), at ppm concentrations reduced aflatoxin production in wild-type A. parasiticus to a fraction of its normal levels and resulted in the appearance of a new orange pigment identified as versiconal acetate (7) (ref. 12). As noted above, sterigmatocystin (9) is produced by the related species, A. versicolor (ref. 13). The first line of experimental evidence to support the sequence of intermediates shown in Scheme 1 was provided by the utilization of [^{14}C]-acetate in mutationally- or chemically-blocked fermentations to accumulate samples of radiolabeled potential intermediates. These were then observed to incorporate radioactivity into aflatoxin B_1 (10) with generally increasing levels of efficiency as the end of the pathway was approached. Corollary experiments demonstrated that labeled intermediates beyond a blockage point were converted to 10 while those before proceeded to the accumulated material but no further (ref. 1, 8, 14, 15). In sum these extensive experiments argue for the sequence of structures shown in Scheme 1. Kinetic pulse-labeling studies of Zamir (ref. 16) lend further support to this overall view.

Scheme 1



Since its introduction by Tanabe and Seto (ref. 17) and independently by McInnes and Wright (ref. 18), the application of doubly [^{13}C]-labeled acetate incorporation techniques has provided an important second experimental approach to investigation of the aflatoxin pathway. In the last dozen years several groups, most notably that of the organizer of this meeting, Pieter Steyn, and his coworkers at the CSIR in Pretoria, have carried out detailed singly- and doubly-labeled [^{13}C]-acetate incorporation experiments to map a common polyketide folding pattern in norsolorinic acid (4) (ref. 19), averufin (6), versiconal acetate (7), vesicolorin A (8), sterigmatocystin (9) and aflatoxin B_1 (10). These labeling patterns are shown in Scheme 1 in the conventional fashion by heavy lines, the dot signifying C-1 (ref.-20). Importantly, averufin enriched with carbon-13 as shown in 6 was shown in a reincorporation experiment to give the same labeling pattern in aflatoxin B_1 (10) as [1,2-$^{13}C_2$]-acetate itself (ref. 21). More recently developed spectroscopic methods have used carbon-13 as a reporter nucleus to monitor oxygen and hydrogen metabolism in natural product biosynthesis. Vederas (ref. 22, 23), Simpson (ref. 24, 25) and Sankawa (ref. 26) have carried out experi-

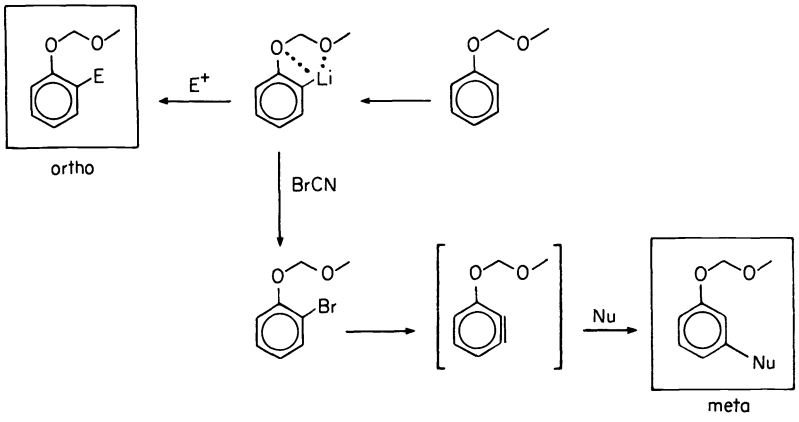
ments employing these techniques to examine some of the details of acetate utilization in several compounds noted in Scheme 1.

A possible biogenetic relation between sterigmatocystin (**9**) and aflatoxin B_1 (**10**) was recognized early on by Holker (ref. 27). Similarly, the potential intermediacy of anthraquinoid pigments in the pathway was proposed by Thomas (ref. 28) but it has been the complementary lines of experimental evidence gathered above that have identified and ordered the intermediates shown in Scheme 1. Nonetheless, the important fundamental issue of intact incorporation, while quite probable, was not strictly satisfied in these studies. Moreover, an understanding of the mechanisms that interconnect these remarkable structures has been lacking. These two objectives have guided our efforts at Hopkins.

INTERMEDIACY OF AVERUFIN AFLATOXIN BIOSYNTHESIS

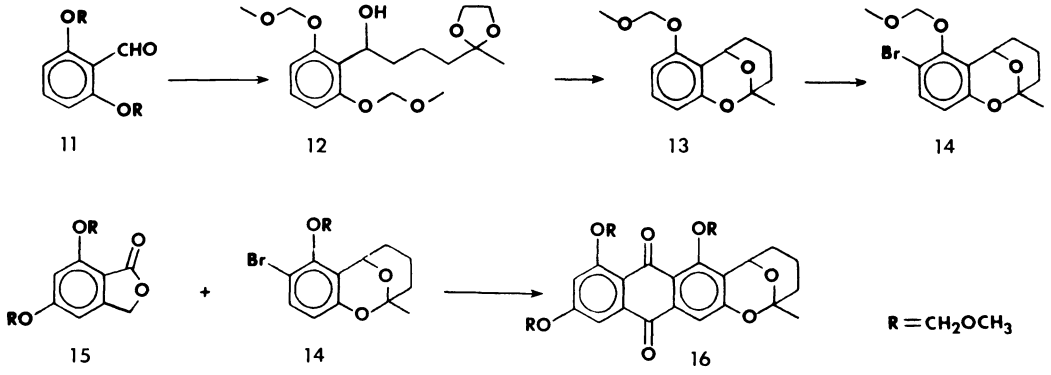
In considering Scheme 1, the origins of norsolorinic acid (**4**), averantin (**5**), and averufin (**6**) from a hypothetical intermediate as **3** are understandable, at least superficially, from the prevailing dogma of polyketide biosynthesis (ref. 11, 29). After averufin comes first the singular rearrangement and loss of two carbons to generate the bisfuran of versicolorin A (**8**) and second, cleavages and further reorganizations to lead to xanthone (**9**) and finally coumarin (**10**). Our first objective, therefore, was to prepare samples of averufin such that labels could be efficiently introduced to investigate bisfuran formation and to establish the intact utilization of averufin in aflatoxin formation. This was accomplished by extension of the principles shown in Scheme 2.

Scheme 2



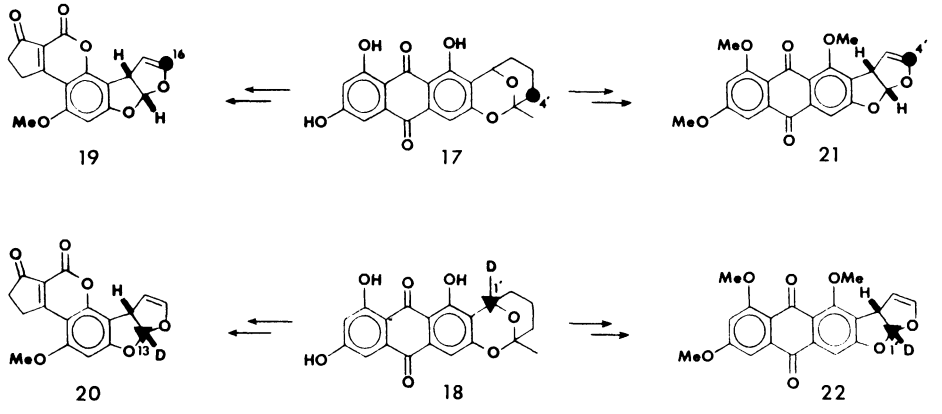
In recognition of the polyoxygenated nature of averufin, a decision was made at the outset to use phenolic hydroxyl groups to control the elaboration of simple benzenoid precursors to the substituted anthraquinone target. Methoxymethyl (MOM) protecting groups were chosen for their relative ease of removal under mildly acidic conditions, but also as directing groups of moderate strength to facilitate metallation specifically ortho. As illustrated in Scheme 2, this aryl anion could be reacted either directly with an electrophile to afford ortho-substituted products, or with an halonium donor, for example cyanogen bromide (ref. 30), followed by dehydrohalogenation with a hindered base, lithium tetramethylpiperidide (ref. 31) was found to be particularly effective, to afford in situ a benzyne, which in the presence of a nucleophile would lead to meta-substituted products. The latter regiospecificity was expected (ref. 32) based on the strong electron withdrawal of the adjacent methoxymethyl. Of the two possible modes of attack on the electron-deficient benzyne, meta-addition gives the more stable anionic intermediate. For our particular application shown below, this prediction was borne out in control experiments (ref. 33). Other synthetic applications along these lines have appeared subsequently (ref. 34).

Bis(O-methoxymethyl)resorcinol was metallated with n-butyllithium and reacted with dimethylformamide to afford aldehyde **11** (ref. 33). Reaction with the appropriate C_5-Grignard reagent gave alcohol **12** which could be treated with dilute aqueous acid to give tricyclic ketal **13** in 60% or greater yield. Loss of the ethylene ketal was fast compared to the aryl MOM groups. The benzylic hydroxyl apparently assisted the hydrolysis of one of the latter at which point intramolecular cyclization to **13** was spontaneous. In the absence of the free hydroxyl group, loss of the second MOM group was slow and **13** accumulated in the reaction mixture. A second metallation and reaction with cyanogen bromide then gave aryl bromide **14**. Extending the chemistry of Hauser (ref. 35), Kraus (ref. 36) and Sammes (ref. 37), treatment of phthalide **15** (ref. 33) with excess lithium tetramethylpiperidide at -78°C., addition of **14** and warming to -40°C. generated the corresponding benzyne in the presence of the phthalide



anion. Reaction ensued to afford initially a dihydroanthraquinone product which readily air-oxidized to **16** (R=MOM) in moderate yield (ref. 33). Lastly, gentle acid hydrolysis gave racemic averufin (**16**, R=H).

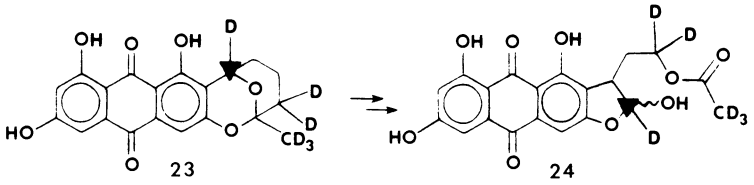
Development of this synthetic route and a related one (ref. 38) made possible the preparation of [4'-^{13}C]- and [1'-^{2}H,^{13}C]-averufin, (**17**) and (**18**), in a direct fashion (ref. 39). In mycelial suspensions of the wild-type A. parasiticus (ATCC 15517), **17** and **18** gave greater than 20% specific incorporations into aflatoxin B_1, (**19**) and (**20**) respectively. The sites of enrichment were established unambiguously by ^{13}C{^{1}H}-NMR spectroscopy. A necessary, but not sufficient, condition for the intermediacy of versicolorin A (**8**), the first bisfuran-containing pigment proposed in Scheme 1, would be the analogous pattern of enrichments from incorporation of **17** and **18**. Parallel experiments were, therefore, conducted with the appropriate blocked mutant (ATCC 36537) and the isolated versicolorins A were analyzed as their more soluble trimethyl ethers. Labels were found to be located as shown in **21** and **22**, in accord with expectation.



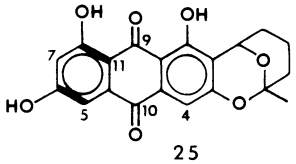
Several conclusions may be drawn. For the side chain carbons at least, specificity of labeling in both the first- and last-formed bisfuran-containing compound was established. Of the three intact acetate units evident in averufin (**6**, Scheme 1), two are retained in the bisfurans of versicolorin A (**8**) and aflatoxin B_1 (**10**). From the labeling patterns in **19**-**22** it is clear that the terminal unit of the former must be lost, the central unit becomes the outer of the bisfuran and the inner-most of averufin (**6**) becomes the central carbon-carbon bond of the bisfuran. Of pivotal importance to any mechanistic consideration of bisfuran formation, is that the oxidation state of the 1'-carbon in averufin, during the course of the chain branching process, is raised to that of an aldehyde but without loss of the attached hydrogen label, *cf.* **20** and **22**. This observation precludes a Favorski-like rearrangement (ref. 40) but other proposed mechanisms involving an epoxide (ref. 41) or open-chain pinacol rearrangement (ref. 42) are consistent with these labeling results.

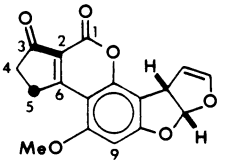
The second important consideration in bisfuran formation is the loss of the terminal pair of carbons. The structure of versiconal acetate (**7**, Scheme 1) suggests that these two carbons may be lost through a Baeyer-Villiger-like process as acetate from a methyl ketone derived from **6**. This is an appealing hypothesis for the adroit insertion of oxygen in a carbon chain to set the stage for generation of the second furan ring. However, given the uniform level of labeling of versiconal acetate by [1,2-$^{13}C_2$]-acetate, it is also fully possible that the O-acetyl function arises in this chemically-blocked fermentation by simple acylation by

endogeneous acetylCoA. To test for the intramolecularity implicit in a Baeyer-Villiger reaction, multiply-deuterated averufin (**23**) was prepared by exhaustive exchange of [1'-^{2}H,-^{13}C]-averufin (**18**). Upon incorporation into versiconal acetate and analysis by both mass spectrometry and deuterium NMR spectroscopy, the integrated ratios of the observed deuterium resonances clearly demonstrated the distribution of isotopic labels shown in **24**.



With regard to the side chain carbons of averufin, a one to one correlation was now secure for the right-hand portions of versiconal acetate (**24**), versicolorin A (**21**, **22**) and aflatoxin B_1 (**19**, **20**). To complete this correlation, a sample of A-ring-labeled averufin was prepared containing an equal mixture of (±)-[5,6-$^{13}C_2$]- and [8,11-$^{13}C_2$]-averufin **25** (ref. 43). In averufin (**6**, Scheme 1) C-5,6 are also labeled pairwise from [1,2-$^{13}C_2$]-acetate while C-8,11 are not. The patterns of ^{13}C-incorporation in the cyclopentenone ring of aflatoxin B_1 were determined to be as illustrated in **26**. By comparison of these enrichment sites to those observed from doubly-labeled acetate, *cf.* **10** in Scheme 1, it can be seen that the pairwise incorporation of label into C-2,3 of **26** must correlate with the unique C-8,11 site in **25**. It follows, therefore, that C-6, 8 and 11 in **25** map to C-5, 3 and 2, respectively, in aflatoxin (**26**). From the doubly-labeled acetate incorporation data (Scheme 1), it can be said that C-5 in averufin is lost and C-7 and 9 correlate to C-4 and C-6 in **26**. Specificity of labeling from the A-ring of averufin is maintained, therefore, from the anthraquinone level through to the fused cyclopentenone of **26** and in an overall manner consistent with published biogenetic schemes (ref. 1. 11, 20, 28, 44).



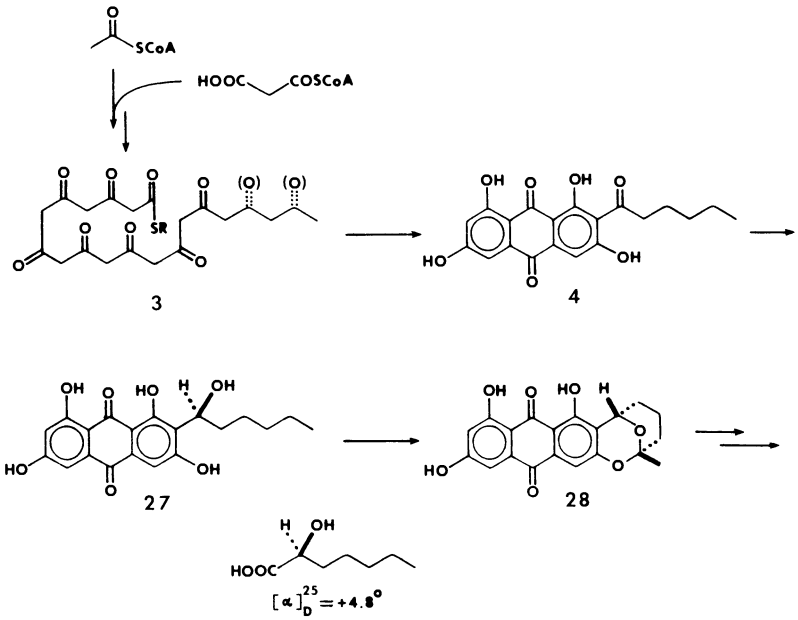


POLYKETIDE INITIATION BY HEXANOATE

It was noted near the outset of this discussion that unlike Büchi's acetate incorporation experiments with aflatoxin (**1**) where a consistent level of labeling was observed throughout the molecule (ref. 4), Holker in parallel investigations with sterigmatocystin (**9**) found experimentally significant (*ca.* 10%) lower incorporation rates at bisfuran sites relative to the xanthone nucleus (ref. 5). Similar observations have been made on occasion with other polyketide-derived natural products typically having saturated or nearly saturated hydrocarbon side chains where rates of acetate utilization have differed, usually lower by 5-10%, with respect to aryl nuclei to which they are bound (ref. 45, 46). Historically, however, attempts to demonstrate the intact incorporation of linear low molecular weight fatty acids C_4 or greater have failed owing to more rapid degradation by beta-oxidation and statistical incorporation of radiolabel as acetate throughout the molecule (ref. 46, 47). The point, therefore, has remained moot in the absence of experimental proof whether linear primers $\geq C_4$ can function in polyketide biosynthesis. By contrast benzenoid starters derived from shikimate are well known (ref. 11, 29) and in a few instances propionate (ref. 48) and the branched acids isobutyric (ref. 49, 50) and 2-methylbutyric (ref. 50) have been shown to function as initiators.

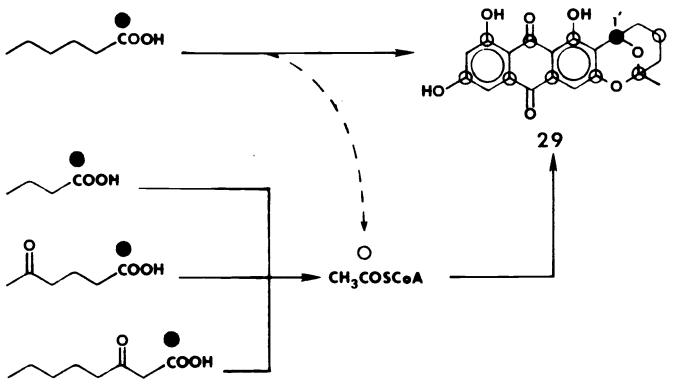
Returning for a moment to Scheme 1 and comparing it to Scheme 3 below, it is to be noted that after norsolorinic acid (**4**), the first-formed anthraquinone intermediate of the pathway, reduction gives averantin (**7**) and oxidation at C-5' followed by (spontaneous) ketalization gives averufin (**8**). This latter oxidation is surprising since this center is carbonyl-derived and it appears generally to be true that oxygens present at such sites in a number of natural products for which the relevant studies are available (ref. 51) have their origins in the progenitor polyketide, *eg.* **3**. If, however, hexanoate were the primer, this fact could account for Holker's apparently anomalous acetate incorporation results and provide a rationale for the seemingly redundant steps in averufin formation.

Scheme 3



To this end, therefore, [1-^{13}C]-hexanoic acid was administered to the averufin-accumulating A. parasiticus mutant (ATCC 24551) under two dissimilar feeding protocols. Averufin isolated from these experiments was found to show an enhancement of its C-1' resonance in the ^{13}C{1H}-NMR spectrum of about three times the natural abundance intensity. Superimposed on this intact incorporation (solid circle in **29**, Scheme 4) was a 0.5-1.0% statistical uptake of [1-^{13}C]-acetate throughout the molecule (open circles in **29**) from beta-oxidation and secondary incorporation. The significance of these observations was further refined by examining under identical conditions of incorporations of equimolar amounts of [1-^{13}C]-acetate, -butyrate, -5-oxohexanoate, and -3-oxo-octanoate. Notably, all of these latter substrates gave uniform incorporations of ^{13}C-label at the centers indicated in **29** by open circles at a level of 0.5-1.0% as was also seen for [1-^{13}C]-hexanoic acid. However, unlike hexanoate, no intact incorporation was evident (ref. 52).

Scheme 4

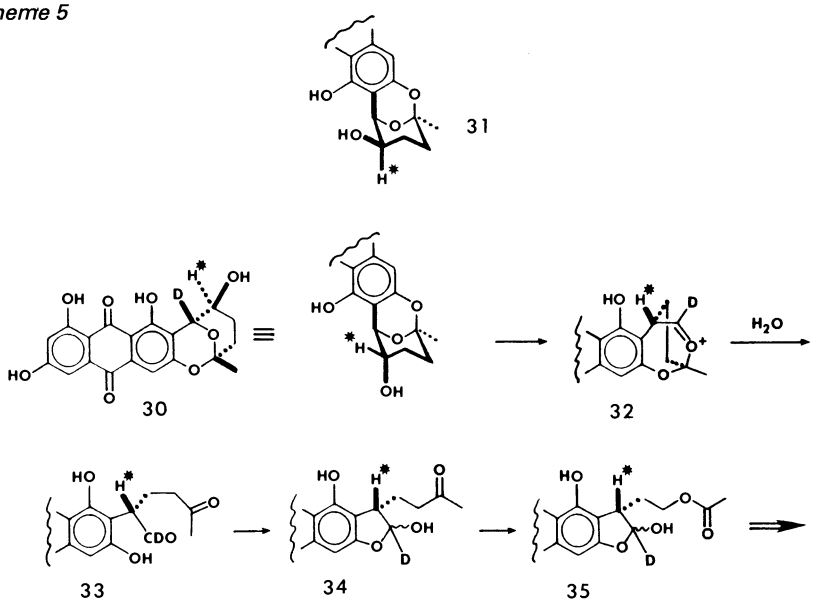


In sum, the data, by analogy to the other demonstrated primers of polyketide biosynthesis noted above, suggest a specific requirement for hexanoic acid with respect to chain length and oxidation state. It is possible that a specialized synthetase is present which produces hexanoylCoA or that it arises by degradation of larger fatty acids. It is at least partially exchangeable with labeled material derived from exogeneously supplied [1-^{13}C]-hexanoic acid. While doubtlessly prone to rapid catabolism, this material may be visualized to initiate polyketide synthesis by a second protein or complex that leads on to norsolorinic acid (**4**) after oxidation of the first-formed anthrone (ref. 22). Other interpretations of these experiments have been discussed previously (ref. 52).

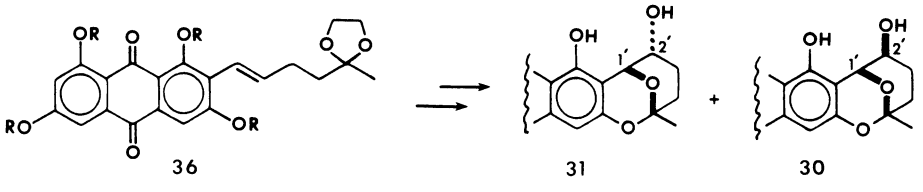
THE ROLE OF NIDURUFIN

The initiation of aflatoxin biosynthesis by hexanoic acid necessitates several seemingly superfluous redox steps to achieve the formation of averufin. The result of this labor, however, is the creation of a template whose latent chemistry is revealed in a series of oxidative rearrangement and cleavage reactions that yield the bisfuran of aflatoxin B_1. Retention of the 1'-deuterium label from averufin (**18**) and (**23**) into aflatoxin (**20**), versicolorin A (**22**) and versiconal acetate (**24**) at a carbon whose oxidation state changes from that of an alcohol to an aldehyde, was highly suggestive of a pinacol-like rearrangement. However, unlike the proposal of Kingston (ref. 42) proceeding through an open form of nidurufin **30** (relative and absolute configurations not defined), we have proposed (ref. 53) on stereoelectronic grounds that the closed form of nidurufin (**30**, Scheme 5) presents an antiperiplanar orientation of migrating and departing groups particularly favorable to rearrangement. Moreover, our synthetic experience with averufin had indicated that any equilibrium between the internal ketal and the open methyl ketone lay undetectably toward the latter.

Scheme 5



Nidurufin is a known natural product isolated from A. nidulans (ref. 54). Its absolute configuration has not been determined but the relative configuration of the 2'-alcohol was assigned endo (structure **31** rather than exo as in **30**) from straightforward application of the Karplus relation to the observed vicinal coupling constant, $^3J_{1'2'}$=1.5 Hz. We prepared a sample of the endo-alcohol **31** by acid-catalyzed cyclization of the cis-diol derived from the trans-olefin **36** (R=MOM). The anthraquinone-olefin **36** was accessible by extension of the synthetic methods described earlier (ref. 33) and the cis-diol was readily generated by reaction with osmium tetraoxide in the presence of N-methylmorpholine N-oxide. The spectral and solubility properties of **31** were not in accord with those reported for nidurufin (ref.-54). In particular the vicinal coupling constant measured at H-1' was 4.7 Hz. The exo-diastereomer **30** could be prepared from the cis-olefin corresponding to **36** in an analogous fashion, but 15-20% of the trans-isomer was always present in the starting material yielding mixtures of **30** and **31**. In several trials various olefin ratios gave the corresponding proportions of cyclized products indicating no stereochemical crossover at the benzylic center. In an alternative strategy, the trans-olefin **36** could be epoxidized with m-chloroperbenzoic acid buffered with aqueous sodium bicarbonate and cyclized in acid to a ca. 5:1 mixture of **30** and **31** separable at an intermediate stage. Spectral data obtained for the exo-alcohol **30**, as expected, were identical to the reported characterizations of natural nidurufin. We have, therefore, named the endo-diasteromer **31** pseudonidurufin (ref. 53).



Discovery of the correct relative configuration of nidurufin led us to propose the biogenetic route that begins in Scheme 3 and continues on Scheme 5 (ref. 53). In Scheme 3, the absolute configuration of averantin (**27**) prefigures the stereochemical course of all subsequent transformations of the C_6-side chain. The steps proposed in Scheme 5 lead ultimately to the bisfuran of aflatoxin B_1 (**1**) having the correct absolute stereochemistry (ref. 55). At the time the absolute configurations of averantin (**27**), averufin (**28**) and nidurufin (**30**) were not known and were predicted to be as shown on the basis of the stereochemical requirements implicit in the biogenetic hypothesis set out in Schemes 3 and 5. Separate correlations have now established the validity of these assignments by unambiguous chemical means. From a large-scale fermentation of the A. parasiticus mutant AVR-1 a sample of **27** was obtained. Degradation of this material gave a specimen of (S)-2-hydroxyheptanoic acid which upon crystallization from pentane gave $[\alpha]_D^{25}=+4.8^\circ$, lit. (ref. 56) $[\alpha]_D^{25}=+6.9^\circ$, lit. (ref. 57) $[\alpha]_D^{26}=+5.5^\circ$.

Analogous attempts to determine the absolute configuration of averufin (**28**) by degradation failed. In collaboration with Professor M. Koreeda at The University of Michigan, a sample of the tricyclic ketal **13** was prepared in optically active form (ref. 58). The absolute configuration of this substance was secured non-empirically at an intermediate stage of the synthesis by application of the exiton chirality circular dichroism method (ref. 59). A sample of **13** of high enantiomeric purity was elaborated as before to afford a specimen of optically active averufin (**28**) to complete a stereochemical correlation with the natural product.

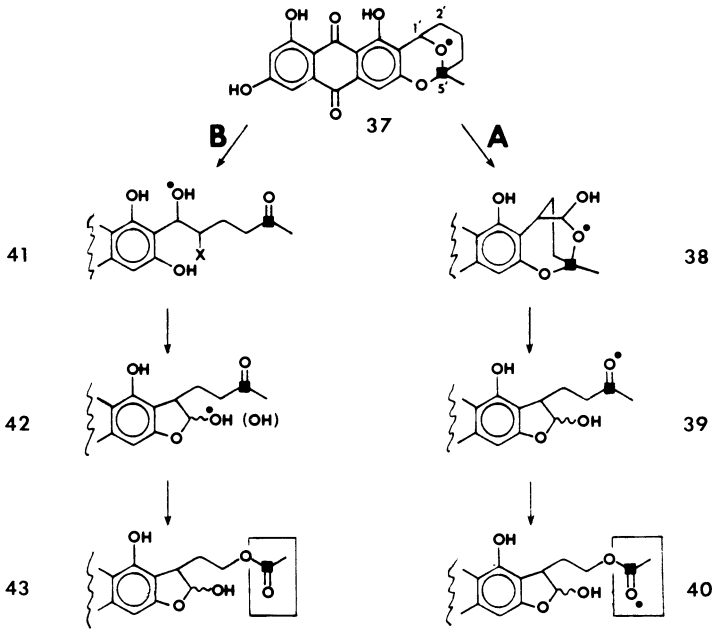
On the basis of comparisons of ORD/CD data (ref. 54), it may be inferred, but not strictly proved, that nidurufin (**30**) and averufin (**28**) are of the same stereochemical series. Nevertheless, having satisfied experimentally the predicted absolute configurational assignments of averantin (**27**) and averufin (**28**), the stereoelectronic significance of the exo-2'-hydroxyl in nidurufin and the possible role of the latter as an intermediate in bisfuran formation was to be tested. Racemic [1'-^{2}H]-nidurufin (**30**) and [1'-^{2}H]-pseudonidurufin (**31**) were prepared (both >98% d_1) via **36** from 2,6-bis(O-methoxymethyl)benzaldehyde (**11**) bearing a deuterated carboxaldehyde. These labeled compounds were administered to mycelial suspensions of A. parasiticus (ATCC 15517) under conditions where similarly labeled samples of averufin gave >20% incorporations into aflatoxin B_1 (ref. 39). To our acute disappointment, neither **30** nor **31** gave a detectable incorporation into the mycotoxin when analyzed by mass spectrometry. Impermeability seemed not to be the cause of this negative result as the mycelial pellets turned perceptibly from white to yellow with orange centers within three hours of exposure to the labeled anthraquinones. Unlike averufin, however, in an identical feeding protocol, **30** and **31** were excreted as polar, highly water-soluble conjugates turning the medium increasingly yellow after an additional three hours. In neither case could the water-soluble pigments be extracted into organic solvent, but on standing in aqueous solution for three weeks, the conjugates had largely decomposed to return the labeled anthraquinones (ref. 60).

This unfavorable outcome was further tested using FLUFF, a variant of A. parasiticus isolated by Bennett (ref. 61), which produces very little if any aflatoxin and appears to be blocked in the aflatoxin pathway before the formation of anthraquinones. In a parallel series of experiments averufin was found to support significantly enhanced aflatoxin production in this organism but both nidurufin and pseudonidurufin failed to do so.

The weight of the evidence at this point, while of a negative kind, did not support a precursor role for **30** or **31** in aflatoxin biosynthesis. We were loath, however, to abandon certain appealing features of the mechanistic events proposed in Schemes 3 and 5. In particular, a discriminating test of the overall rationale could be visualized as illustrated in Scheme 6 where path A, invoking oxidation at C-2' of averufin (**37**) but not direct hydroxylation, would initiate migration of the anthraquinone nucleus and, either by hydration of an oxonium ion intermediate or perhaps by consumation of an hydroxylation reaction, generate **38**. Collapse of this intermediate would give the presently unknown methyl ketone **39**, which upon Baeyer-Villiger-like oxidation (ref. 62) would afford versiconal acetate (**40**). The interesting aspect of this proposal is that the averufin 1'-oxygen/5'-carbon bond remains intact to ketone **39** and, provided oxygen exchange in this hypothetical intermediate were slow relative to Baeyer-Villiger oxidation, both labels in principle could be intercepted in versiconal acetate (**40**) prior to their loss as acetate in bisfuran formation. In contrast, path B is representative of many mechanistic permutations which have as their common element, opening of the ketal side chain of averufin, hence cleaving the 1'-oxygen/5'-carbon bond. Rearrangement and Baeyer-Villiger reaction in any one of a number of possible ways or in reverse order would ultimately give versiconal acetate (**43**) whose acetyl carbonyl would bear ^{13}C-label but no ^{18}O.

[1'-^{18}O, 5'-^{13}C]-Averufin (**37**) was prepared from 2,6-bis(O-methoxymethyl)benzaldehyde (**11**) that had been exchanged with ^{18}O-water containing a trace of hydrochloric acid. Homologation of the appropriate ^{13}C-labeled side chain and completion of the synthesis as before gave **37**. Examination of the 100.7 ppm region of the ^{13}C{^{1}H}-NMR spectrum of this material at high

Scheme 6



digital resolution revealed two peaks separated by 0.02 ppm [^{18}O-induced upfield shift (ref. 63)] from whose intensities a 48/52 ^{16}O/^{18}O-ratio at C-5' was determined (spectrum A, Fig. 1). Administration of **37** to cultures of the wild-type A. parasiticus in the presence of dichlorovos led to the accumulation of versiconal acetate (**40**) whose analogous ^{13}C{^{1}H}-NMR spectrum showed two slightly broadened peaks at about 170.3 ppm separated by 0.04 ppm (spectrum B top, Fig. 1). However, recalling that versiconal acetate exists in dimethylsulfoxide solution as an equilibrating mixture of the hemiacetal shown in **40** at the anthraquinone 3-hydroxyl and that formed at the anthraquinone 1-hydroxyl, these two resonances could simply reflect the populations of these two ^{13}C-labeled species. That the upfield signal corresponded to the ^{18}O-labeled material was shown in a natural abundance spectrum of versiconal acetate (spectrum B bottom, Fig. 1) where only the downfield peak was observed. When the spectrum of [5'-^{13}C, ^{18}O]-versiconal acetate (**40**) was normalized for natural abundance contributions, it could be shown that approximately 80% of the ^{18}O-label originally present in averufin (**37**) survived in versiconal acetate (**40**) (ref. 60).

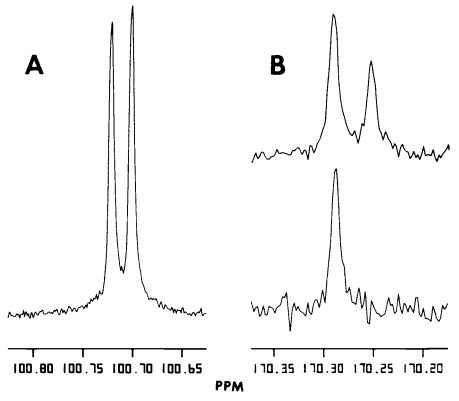
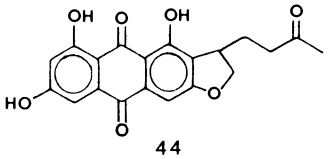


Fig. 1. 50.3 MHz ^{13}C{^{1}H}-NMR spectra of: (A) (±)[1'-^{18}O,5'-^{13}C]-averufin (**37**). (B) Top: [5'-^{13}C,^{18}O]-versiconal acetate (**40**); Bottom: versiconal acetate (natural abundance).

The requirement that the 1'-oxygen/5'-carbon bond remain intact is mechanistically a highly restrictive one. It is implicit in Scheme 5 that side chain rearrangement preceeds Baeyer-Villiger-like cleavage. Circumstantial evidence to support this order of events may be found in the structure of versicolorone (**44**) isolated form A. versicolor (ref. 64). As depicted in Schemes 5 and 6 path A, oxidative rearrangement of the six-carbon ketal side chain of averufin through its closed form satisfies the ^{18}O/^{13}C-incorporation data. However, abnormal openings of this ketal side chain may be visualized which would both meet the strictures of the labeling studies presented above and provide entry by alternative routes to bisfuran formation. These proposals will be evaluated elsewhere as experimental data become available to consider them critically.



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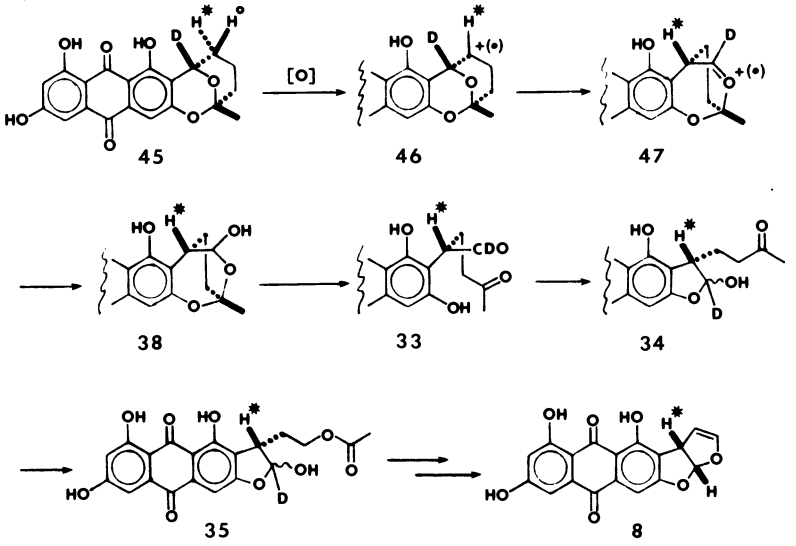
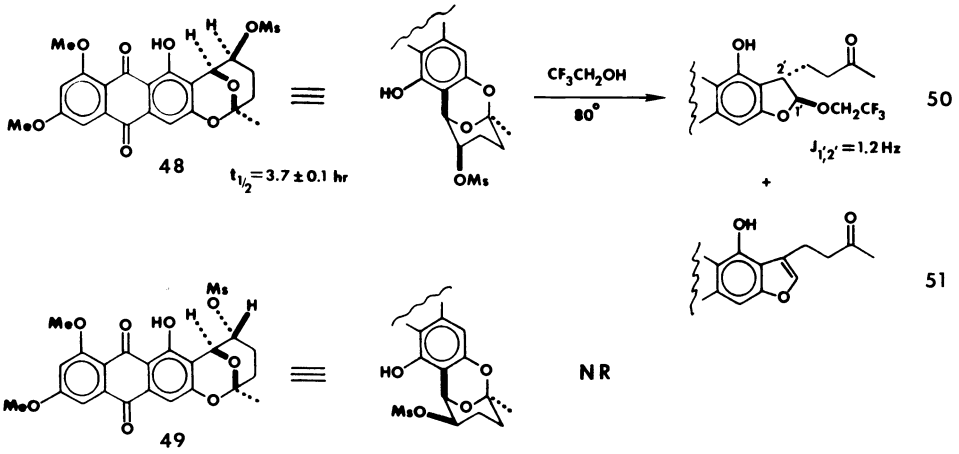
In what is plainly an oxidative conversion of the averufin side chain, the non-incorporation of nidurufin was at once disappointing and intriguing. If it is proposed as above that oxidation does indeed take place at C-2' initially to form presumably a radical or subsequently a cation by electron transfer, rearrangement of reactive intermediate **46** (Scheme 7) directly to **47** bypasses the intermediacy of nidurufin. Such a proposition finds analogy in investigations of gibberellin biosynthesis (ref. 65). Were nidurufin a precursor, ionization at C-2' could be reasonably suggested to initiate rearrangement through a cation.

Scheme 7



In preliminary model studies of such a process (ref. 66), we have examined the solvolysis of 6,8-di(O-methyl)nidurufin and -pseudonidurufin mesylates, **48** and **49**, in hot trifluoroethanol. The exo-mesylate **48** rearranged with clean first-order kinetics, albeit slowly (half-life=3.7±0.1hr, 80° C.) to give the mixed acetal **50** and fused furan **51** in a 5:1 ratio. In contrast, the endo-isomer **49** was completely unreactive under identical conditions. One can point, with ample precedent (ref. 67), to the strong inductive withdrawal of the 1'-oxygen as a major cause of rate retardation for this solvolytic rearrangement as well as to the comparative electron deficiency of the migrating anthraquinone nucleus (ref. 62). On inescapable thermodynamic grounds, therefore, Nature may chose to avoid the intermediacy of nidurufin owing to its inability to readily initiate cationic rearrangement of the linear C_6-side chain. More definitve resolution of these propositions must await the outcome of further experiments.



In closing it is my pleasure to acknowledge the exceptional efforts of Siegfried B. Christensen who carried out the incorporation experiments and much of the spectroscopy and synthesis I have described, and those of Steven G. Davis for developing the benzyne route to averufin, its extension to materials labeled in both the side chain and A-ring and for successfully

demonstrating the rearrangement of nidurufin mesylate to furanoid products. Important contributions to the development of this project were made by a small group of Hopkins undergraduates, John C. Link, Leslie M. Bloom and Katrin Trautwein, and by Dr. Charles P. Lewis. I am grateful to Professor Joan W. Bennett for providing laboratory space and experienced help without which the microbiological aspects of this problem would have been very much more difficult; and to Professor Masato Koreeda for his interest in the absolute configuration of averufin. Last, I wish to acknowledge the essential financial support of the National Institutes of Health (ES01670), the Alfred P. Sloan Foundation and the Camille and Henry Dreyfus Foundation.

REFERENCES

1. J. G. Heathcote and J. R. Hibbert, Aflatoxins: Chemical and Biological Aspects, Elsevier, Amsterdam (1978). For a review of aflatoxin biosynthesis see especially pp. 151-172.
2. T. Asao, G. Büchi, M. M. Abdel-Kader, S. B. Chang, E. L. Wick and G. N. Wogan, J. Amer. Chem. Soc., 85, 1706-1707 (1963); 87, 882-886 (1965).
3. G. Büchi, D. M. Foulkes, M. Kurono and G. F. Mitchell, J. Amer. Chem. Soc., 88, 4534-4536 (1966). G. Büchi, D. M. Foulkes, M. Kurono, G. F. Mitchell and R. S. Schneider, Ibid., 89, 6745-6753 (1967). G. Büchi and S. M. Weinreb, Ibid., 93, 746-752 (1971). G. Buechi, M. A. Francisco, J. M. Liesch and P. F. Schuda, Ibid., 103, 3497-3501 (1981).
4. M. Biollaz, G. Büchi and G. Milne, J. Amer. Chem. Soc., 90, 5017-5019, 5019-5020 (1968); 92, 1035-1043 (1970).
5. J. S. E. Holker and L. J. Mulheirn, J. Chem. Soc., Chem. Commun., 1576-1577 (1968).
6. See for example: J. G. Heathcote, M. F. Dutton and J. R. Hibbert, Chem. Ind., 1027-1030 (1973) and ref. 1, pp. 157-159, for a discussion.
7. L. S. Lee, J. W. Bennett, L. A. Goldblatt and R. E. Lundin, J. Amer. Oil Chem. Soc., 48, 93-94 (1971). R. W. Detroy, S. Freer and A. Ceigler, Can. J. Microbiol., 19, 1373-1378 (1973).
8. J. W. Bennett, L. S. Lee, S. M. Shoss and G. H. Boudreaux, Appl. Environ. Microbiol., 39, 835-839 (1980).
9. J. A. Donkersloot, R. I. Mateles and S. S. Yang, Biochem. Biophys. Res. Commun., 47, 1051-1055 (1972).
10. L. S. Lee, J. W. Bennett, A. F. Cucullu and J. B. Stanley, J. Agric. Food Chem., 23, 1132-1134 (1975).
11. W. B. Turner and W. B. Aldridge, Fungal Metabolites II, Academic Press, London (1983).
12. R. H. Cox, F. Churchill, R. J. Cole, and J. W. Dorner, J. Amer. Chem. Soc., 99, 3159-3161 (1977). D. L. Fitzell, R. Singh, D. P. H. Hsieh and E. L. Motell, J. Agric. Food Chem., 25, 1193-1197 (1977) and refs. cited.
13. E. Bullock, J. C. Roberts and J. G. Underwood, J. Chem. Soc., 4179-4183 (1962) and refs. cited.
14. R. Singh and D. P. H. Hsieh, Arch. Biochem. Biophys., 178, 285-292 (1977). D. P. H.-Hsieh, R. Singh, R. C. Yao and J. W. Bennett, Appl. Environ, Microbiol., 35, 980-982 (1978).
15. D. P. H. Hsieh, M. T. Lin, R. C. Yao and R. Singh, J. Agric. Food Chem., 24, 1170-1174 (1976).
16. L. O. Zamir and K. D. Hufford, Appl. Environ. Microbiol., 42, 168-173 (1981).
17. M. Tanabe, Biosynthesis, 4, 204-247 (1976).
18. A. G. McInnes and J. L. C. Wright, Accts. Chem. Res., 8, 313-320 (1975).
19. P. S. Steyn, R. Vleggaar and P. L. Wessels, S. Afr. J. Chem., 34, 12-17 (1981).
20. P. S. Steyn, R. Vleggaar and P. L. Wessels in The Biosynthesis of Mycotoxins: A Study in Secondary Metabolism, P. S. Steyn, ed., pp. 105-155, Academic Press, London (1980).
21. A. E. DeJesus, C. P. Gorst-Allman, P. S. Steyn, R. Vleggaar, P. L. Wessels, C. C. Wan and D. P. H. Hsieh, J. Chem. Soc., Chem. Commun., 389-390 (1980).
22. J. C. Vederas and T. T. Nakashima, J. Chem. Soc., Chem. Commun., 183-185 (1980).
23. T. T. Nakashima and J. C. Vederas, J. Chem. Soc., Chem. Commun., 206-208 (1982).
24. T. J. Simpson and D. J. Stenzel, J. Chem. Soc., Chem. Commun. 890-892 (1982). T. J.-Simpson, A. E. de Jesus, P. S. Steyn and R. Vleggaar, Ibid., 338-340 (1983).
25. T. J. Simpson, A. E. de Jesus, P. S. Steyn and R. Vleggaar, J. Chem. Soc., Chem.-Commun., 631-634 (1982).
26. U. Sankawa, H. Shimada, T. Kobayashi, Y. Ebizuka, Y. Yamamoto, H. Noguchi and H. Seto, Heterocycles, 19, 1053-1058 (1982).
27. J. S. E. Holker and J. G. Underwood, Chem. Ind., 1865-1866 (1964).
28. J. C. Bu'Lock in Comprehensive Organic Chemistry, Vol. 5, D. Barton and W. D. Ollis, eds., Pergamon, Oxford (1979). R. Thomas in Biogenesis of Antibiotic Substances, Z. Vanek and Z. Hostalek, eds., pp. 160-161, Academic Press, New York (1965).
29. U. Weiss and J. M. Edwards, The Biosynthesis of Aromatic Compounds, Wiley, New York (1980).
30. K.-H. Boltze, H.-D. Dell and H. Janzen, Ann., 709, 63-69 (1967).
31. R. A. Olofson and C. M. Dougherty, J. Amer. Chem. Soc., 95, 582-584 (1973).
32. R. W. Hoffman, Dehydrobenzene and Cycloalkynes, Academic Press, New York (1967).

33. C. A. Townsend, S. G. Davis, S. B. Christensen, J. C. Link and C. P. Lewis, J. Amer. Chem. Soc., 103, 6885-6888 (1981).
34. A. I. Meyers and W. Rieker, Tetrahedron Lett., 2091-2094 (1982). A. I. Meyers and P. D. Pansegrau, Ibid., 4935-4938 (1983). R. D. Clark and J. M. Caroon, J. Org. Chem., 47, 2804-2806 (1982). K. Shankaran and V. Snieckus, Tetrahedron Lett., 2827-2830 (1984).
35. F. M. Hauser and R. P. Rhee, J. Org. Chem., 43, 178-180 (1978); J. Amer. Chem. Soc., 101, 1628-1629 (1979).
36. G. A. Kraus and H. Sugimoto, Tetrahedron Lett., 2263-2266 (1978). G. A. Kraus and J. O. Pezzanite, J. Org. Chem., 44, 2480-2482 (1979).
37. N. J. P. Broom and P. G. Sammes, J. Chem. Soc., Chem. Commun., 162-164 (1978). P. G.-Sammes and D. J. Dodsworth, Ibid., 33-34 (1979). D. J. Dodsworth, M.-P. Calcagno, E. U.-Ehrmann, B. Devadas and P. G. Sammes, J. Chem. Soc., Perkin I, 2120-2124 (1981).
38. C. A. Townsend and S. B. Christensen, unpublished results.
39. C. A. Townsend, S. B. Christensen and S. G. Davis, J. Amer. Chem. Soc., 104, 6152-6153 (1982).
40. T. J. Simpson and J. S. E. Holker, Tetrahedron Lett., 4693-4696 (1975). M. Tanabe, M. Uramoto, T. Hamasaki and L. Cary, Heterocycles, 5, 355-365 (1976).
41. C. P. Gorst-Allman, K. G. R. Pachler, P. S. Steyn, P. L. Wessels and D.-B. Scott, J. Chem. Soc., Perkin I, 2181-2188 (1977).
42. D. G. I. Kingston, P. N. Chen and J. R. Vercellotti, Phytochem., 15, 1037-1039 (1976).
43. C. A. Townsend and S. G. Davis, J. Chem. Soc., Chem. Commun., 1420-1422 (1983).
44. J. W. Bennett and S. B. Christensen, Adv. Appl. Microbiol., 29, 53-92 (1983).
45. See for example: J. R. Hadfield, J. S. E. Holker and D. N. Stanway, J. Chem. Soc (C), 751-755 (1967). J. L. Gellerman, W. H. Anderson and H. Schlenk, Lipids, 9, 722-725 (1974).
46. S. W. Tannenbaum and S. Nakajima, Biochemistry, 8, 4626-4631 (1969).
47. A. J. Birch, Proc. Chem. Soc., 3-13 (1962). J. F. Grove, J. Chem. Soc. (C), 1860-1865 (1970). B. E. Cross and P. Hendley, J. Chem. Soc., Chem. Commun., 124-125 (1975). J. C.-Vederas, W. Graf, L. David and Ch. Tamm, Helv. Chim. Acta, 58, 1886-1898 (1975). However, see: P. G. Gordon, A. Penttila and H. M. Fales, J. Amer. Chem. Soc., 90, 1376-1377 (1968).
48. See for example: W. D. Ollis, I. O. Sutherland, R. C. Codner, J. J. Gordon and G. A.-Miller, Proc. Chem. Soc., 347-348 (1960). K. Mosbach, Acta Chem Scand., 18, 1591-1595 (1964). R. C. Paulick, M. L. Casey and H. W. Whitlock, J. Amer. Chem. Soc., 98, 3370-3371 (1976).
49. J. Zylber, E. Zissman, J. Polonsky, E. Lederer and M. A. Merrien, Eur. J. Biochem., 10, 278-283 (1969).
50. F. Drawert and J. Beier, Phytochem., 15, 1695-1696 (1976).
51. For a recent review see: T. J. Simpson, Nat. Prod. Repts., 1, 281-297 (1985).
52. C. A. Townsend, S. B. Christensen and K. Trautwein, J. Amer. Chem. Soc., 106, 3868-3869 (1984).
53. C. A. Townsend, S. B. Christensen and S. G. Davis, Tetrahedron, 39, 3575-3582 (1983).
54. P. J. Aucamp and C. W. Holzapfel, J. S. Afr. Chem. Inst., 23, 40-56 (1970).
55. S. Brechbuehler, G. Büchi and G. Milne, J. Org. Chem., 32, 2641-2640 (1967).
56. D. H. Nugteren, D. A. Van Dorp, S. Bergstrom, M. Hamberg and B. Samuelsson, Nature, 212, 38-39 (1966).
57. F. M. Hauser, M. L. Coleman, R. C. Huffman and F. I. Carroll, J. Org. Chem., 39, 3426-3427 (1974).
58. M. Koreeda, B. Hulin, M. Yoshihara, C. A. Townsend and S. B. Christensen, unpublished results.
59. N. Harada and K. Nakanishi, Accts. Chem. Res., 5, 257-263 (1972).
60. C. A. Townsend and S. B. Christensen, J. Amer. Chem. Soc., 107, 270-271 (1985).
61. J. W. Bennett, R. B. Silverstein and S. J. Kruger, J. Amer. Oil Chem. Soc., 58, 952A-955A (1981).
62. C. A. Townsend, S. B. Christensen and S. G. Davis, J. Amer. Chem. Soc., 104, 6154-6155 (1982).
63. J. C. Vederas, Can. J. Chem., 60, 1637-1642 (1982).
64. Y. Berger, Phytochem., 19, 2779-2780 (1980).
65. R. Evans, J. R. Hanson and A. F. White, J. Chem. Soc. (C), 2601-2603 (1970). J. R.Hanson, J. Hawker and A. F. White, J. Chem. Soc., Perkin I, 1892-1895 (1972). J. E. Graebe, P. Hedden and J. MacMillan, J. Chem. Soc., Chem. Commun., 161-162 (1975).
66. C. A. Townsend, S. G. Davis, M. Koreeda and B. Hulin, unpublished results.
67. See for example: J. C. Martin and P. D. Bartlett, J. Amer. Chem. Soc., 79, 2533-2541. L. A. Paquette and I. R. Dunkin, Ibid., 95, 3067-3068 (1972).