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STABLE ISOTOPE STUDIES OF ANTHRAQUINONE INTERMEDIATES IN THE AFLATOXIN PATHWAY

CRAIG A. TOWNSEND† and SIEGFRIED B. CHRISTENSEN
Department of Chemistry, The Johns Hopkins University, Baltimore, MD 21218, U.S.A.

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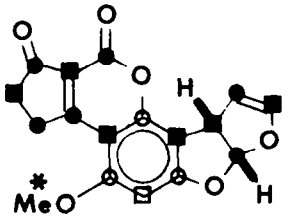
Abstract—Experiments with two specifically labeled samples of (±)-averufin in fermentations of *Aspergillus parasiticus* (ATCC 36537) show their intact incorporation into versicolorin A. The sites of labeling parallel those observed in earlier investigations with aflatoxin B_1 and versiconal acetate suggesting a common mechanistic path to the bisfuran. [1 – ^{13}C]-Hexanoic acid in cultures of *A. parasiticus* (ATCC 24551) unexpectedly gave intact incorporations into averufin, the pivotal C_{20}-anthraquinone intermediate of aflatoxin biosynthesis. On the basis of comparison to specimens obtained by total synthesis, the structure of nidurufin is revised and its intermediacy is suggested in a working hypothesis to account for bisfuran formation.

The extensive degradation studies of Biollaz, Büchi and Milne[1] to determine the location of radiolabel in aflatoxin B_1(**1**) derived from [$^{14}CH_3$]-methionine, [1-^{14}C]-and [2-^{14}C]-sodium acetate stand as an experimental *tour de force* in classical methodology and remain perhaps the single most important contribution toward understanding the biosynthesis of this potent mycotoxin. The origin of 12 of the 16 nuclear carbons was determined from acetate, with the OMe arising from methionine. The complementary but internally consistent levels of incorporation from the two labeled forms of acetate indicated derivation of the toxin from a single, albeit highly rearranged, polyketide chain.



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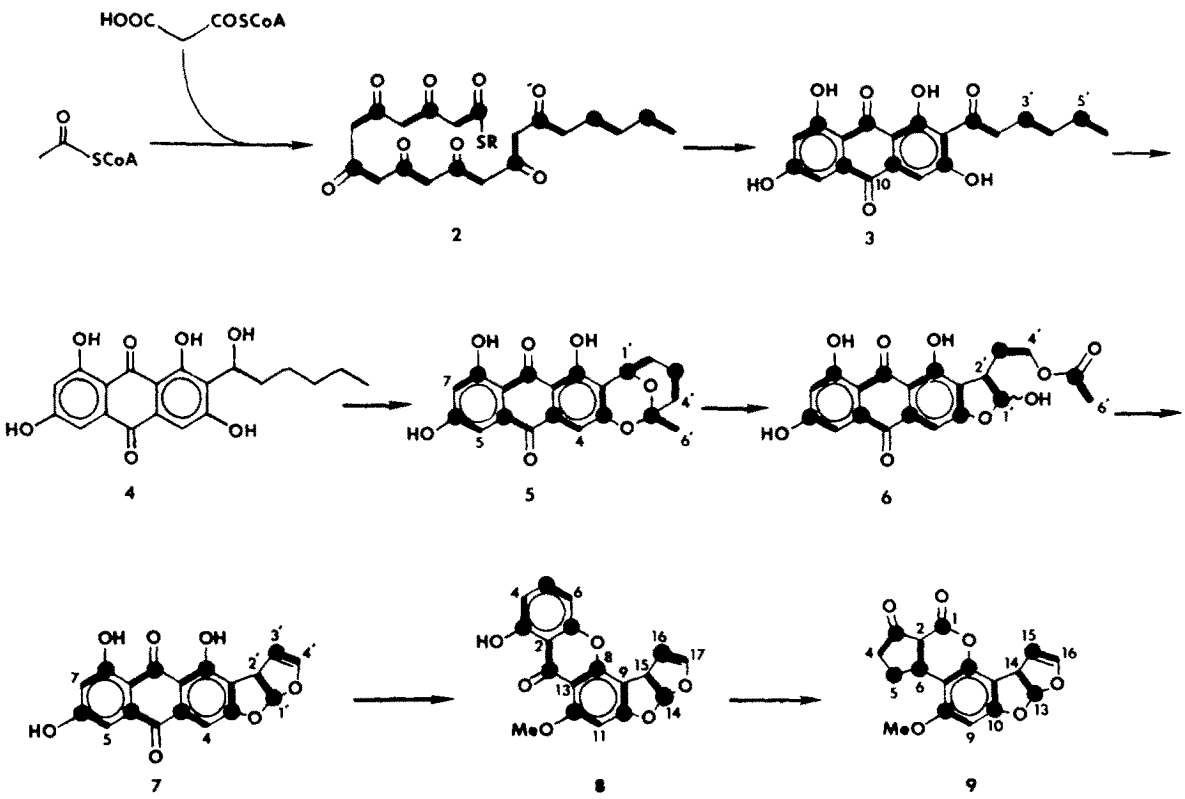
■ ● CH_3-COOH

* [Me]-Methionine

In the years since 1970, further progress in this field has been dependent upon the generation of *Aspergillus parasiticus* mutants which accumulate various anthraquinone pigments as, for example, norsolorinic acid (**3**),[2] averantin (**4**),[3] averufin (**5**)[4] and versicolorin A (**7**)[5] – pigments which had been observed previously as metabolites of other *Aspergillus* species.[6] During this period it was found that an insectide, dichlorovos (dimethyl-2, 2-dichlorovinyl phosphate), markedly inhibited aflatoxin production by the wild-type strain and resulted in the appearance of a new orange pigment[7] whose structure was in time correctly identified as versiconal acetate (**6**).[8] The first line of evidence for a sequence of intermediates in aflatoxin biosynthesis utilized the mutational and chemical blocks to produce radiolabeled potential intermediates from labeled acetate which were then shown to be converted in the wild-type strain to aflatoxin B_1 (**1**) with generally increasing specific incorporations as the end of the pathway was approached. Corollary experiments demonstrated that radiolabeled intermediates in the sequence beyond a blockage point were converted in the respective modified strains or dichlorovos-inhibited fermentation to aflatoxin while those before it proceeded to the blocked conversion but no further. In sum, these data suggested the following series of intermediates in the pathway to aflatoxin B_1 (Scheme 1): hypothetical polyketide (**2**, *vide infra*)→norsolorinic acid (**3**)→averantin (**4**)→averufin (**5**)→versiconal acetate (**6**)→versicolorin A (**7**)→sterigmatocystin (**8**)→aflatoxin B_1 (**9**).[3,9-11] More recently, time course experiments with *A. parasiticus* largely support these conclusions.[12]

A second line of evidence has been developed over the past decade which has yielded complementary information in support of this proposed order of biosynthetic intermediates. Extensive singly- and doubly-labeled [^{13}C]-acetate incorporation studies have revealed a common polyketide folding pattern in norsolorinic acid (**3**),[13] averufin (**5**), versiconal acetate (**6**), veriscolorin A (**7**), sterigmatocystin (**8**) and aflatoxin B_1 (**9**) (so indicated in Scheme 1 by heavy lines, the dot signifying C-1).[14] In addition, a sample of averufin labeled as shown in **5** was accumulated by the fermentation of [1, 2-$^{13}C_2$]-acetate in the appropriate mutant (ATCC 24551) and upon reincorporation in the wild-type strain gave aflatoxin B_1 having the labeling pattern shown in **9**, i.e. identical to that obtained from doubly-labeled acetate itself.[15] Therefore, while the stable isotope studies afford important locations of label from acetate not provided by the earlier radiochemical investigations and

†Research Fellow of the Alfred P. Sloan Foundation (1982-84).



Scheme 1.

establish a common polyketide folding pattern, it is only this last incorporation experiment which addresses the fundamental issue of probable, but not strictly proven, specificity of incorporation of pathway intermediates into aflatoxin B_1. Demonstration of such specificity has been a goal of work at Hopkins.

We describe in this paper first the application of synthetic methods developed earlier in this laboratory[16] to prepare specifically labeled samples of averufin, whose polyketide origin appears clear (*vide infra*), and demonstrate their specific incorporation into versicolorin A paralleling recent findings in this laboratory for aflatoxin B_1 and versiconal acetate. The particular specimens of averufin synthesized were chosen not only to disclose which two of the three intact acetate units of the anthraquinone side chain become the two retained in the branched C_4-bisfuran but also to yield mechanistic details of the intriguing rearrangements themselves. The bisfuran characteristic of this family of metabolites is unique among polyketide-derived natural products and is widely held to be responsible for their toxic and carcinogenic properties.[17] Second, some unexpected observations pertaining to the formation of averufin from a presumed polyketide precursor **2** will be discussed, and, last, a tentative biogenetic scheme for bisfuran and subsequent aflatoxin formation will be advanced.

Incorporation of averufin into aflatoxin B_1, versicolorin A and versiconal acetate

We have very recently described the preparation of [4′-^{13}C]- and [1′-^{2}H,^{13}C]-averufin (**10** and **11**) and have shown their efficient (> 20%) and specific incorporation into aflatoxin B_1[18] using mycelial suspensions of the wild-type strain of *A parasiticus*. The sites of labeling were determined by $^{13}C\{^1H\}$-NMR spectroscopy to be cleanly as represented in structures **12** and **13**, respectively.[18] Some controversy from cell-free studies[19] surrounds the proposed intermediacy of veriscolorin A (**7**) in aflatoxin biosynthesis. While not addressing this problem directly, we sought to examine whether the labeling patterns observed in the bisfuran of aflatoxin B_1 from labeled averufins **10** and **11** would be paralleled earlier in the presumed pathway at the anthraquinone level in versicolorin A—a necessary but not sufficient requirement for intermediacy. To that end [4′-^{13}C]-averufin (**10**, 42 mg) and [1′-^{1}H, ^{13}C]-averufin (**11**, 42 mg) were separately administered to 14 500 mL Erlenmeyer flasks each containing 10 g of mutant *A. parasiticus* (ATCC 36537) pellets suspended in a replacement medium.[7b] After shaking 40 hr in the dark, the versicolorin A produced was isolated by extractive workup and converted to its trimethyl ether **14**.[5] $^{13}C\{^1H\}$-NMR analysis of the isolated pigment in deuteriochloroform gave an enhanced singlet at δ145.5 alone indicating a somewhat greater than 3% incorporation[20] of averufin (**10**) to label C-4′ exclusively. The corresponding sample of versicolorin A trimethyl ether (**15**), identically obtained from the incorporation of doubly-labeled averufin **11** (85% d_1[18]), gave a $^{13}C\{^1H\}$-NMR spectrum having a 1:1:1 triplet ($^1J_{CD}$ = 28 Hz) centered at δ111.9 superimposed on a weakly enhanced singlet at δ112.2. As was observed previously for the analogous aflatoxin

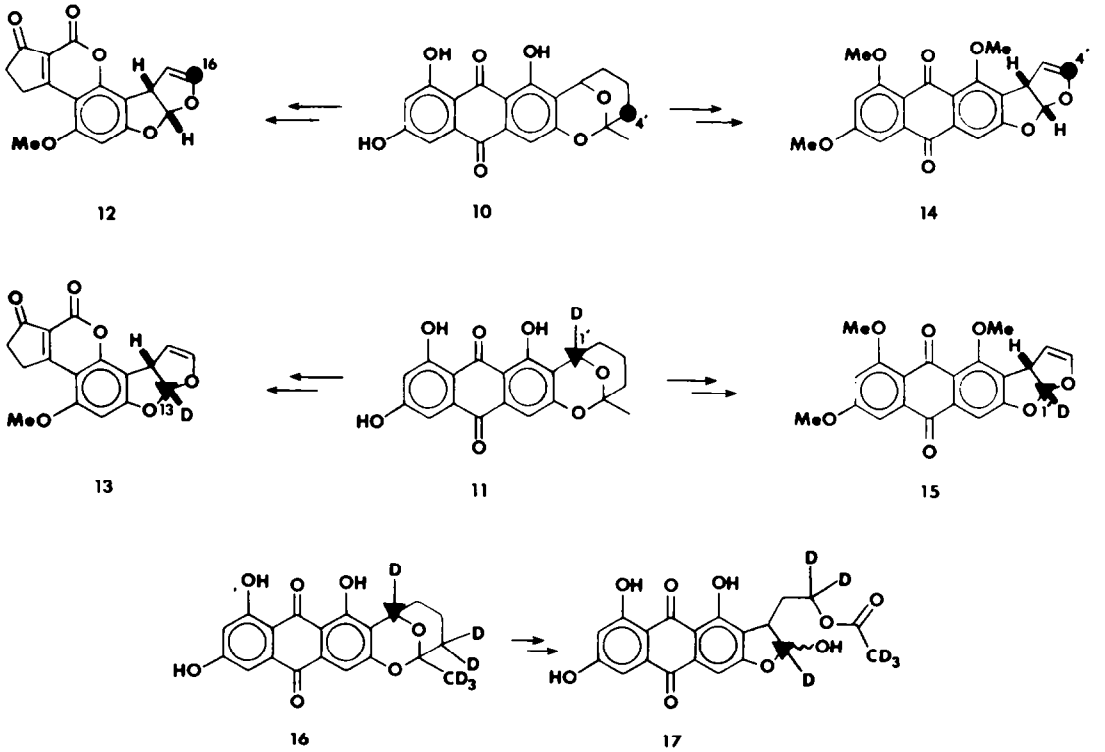
B_1 sample **13**,[18] the relative intensities of the three line manifold arising from the major $^{13}C/^{2}H$-species appeared disproportionately weak owing to the substantially reduced efficiency of ^{13}C-relaxation in the absence of a bound proton in a $^{13}C\{^1H\}$-experiment.[21] Taking this factor into account and the relative apparent levels of enrichment for $^{13}C/^{1}H$- and $^{13}C/^{2}H$-species present, it may be concluded, in keeping with the earlier experiment with aflatoxin B_1, that deuterium label at C-1′ in **11** was very largely if not completely retained during the rearrangement steps to bisfuran in versicolorin A (**15**) as it is in aflatoxin B_1 (**13**).

It may be concluded from the experiments above that averufin is incorporated intact into versicolorin A and aflatoxin B_1. Of the three intact acetate units that constitute the averufin side chain (Scheme 1), the central and innermost of these are retained in the identical fashion in the bisfurans of versicolorin A and aflatoxin B_1; the outermost unit is lost. Of central importance to the rearrangement process, the migration of the tetrahydroxyanthraquinone nucleus from C-1′ to C-2′ of averufin must occur in such a way that deuterium is retained at the 1′-carbon to become C-1′ in versicolorin A and C-13 in aflatoxin B_1. Returning for a moment to Scheme 1, the structure of versiconal acetate (**6**) suggests that the terminal acetate unit of averufin (**5**) may be lost ultimately as acetate by way of a Baeyer Villger like oxidation[22] of a methyl ketone derived from the latter. From the data available,[23] however, it is equally possible that the O-acetyl group of **6** arises by trivial acylation by endogenous acetyl CoA. That the former *intra*-molecular rearrangement process holds was recently demonstrated[24] by incorporation of the multiply labeled averufin (**16**), synthesized from **11**, in a dichlorovos-inhibited culture of *A. parasiticus*. A sample of versiconal acetate (**17**) was obtained where deuterium label was retained in the acetyl methyl in the required ratio to labels at C-1′ and C-4′ as shown by $^{2}H\{^1H\}$-NMR and mass spectrometry. Whether the chain branching transformation(s) preceeds or succeeds this oxidative two-carbon cleavage cannot be determined at present.

Incorporation of hexanoate into averufin

As was noted at the outset of this discussion, experiments with the norsolorinic acid-accumulating mutant (ATCC 24690) suggest that norsolorinic acid (**3**) is the first anthraquinone intermediate formed in the aflatoxin pathway.[3,10] [^{14}C]-Labeled **3** was transformed into radiolabeled aflatoxin B_1 (**9**) in the wild-type strain of *A. parasiticus*, and aflatoxin production was restored in the mutant when later presumed intermediates in the pathway were supplied as, for example, averufin (**5**), versiconal acetate (**6**), versicolorin A (**7**) and sterigmatocystin (**8**).[10] Similarly [^{14}C]-averantin (**4**), derived from administration of labeled acetate to the *Aspergillus* mutant *ver-mu-39*, incorporated radioactivity into aflatoxin B_1 in the wild-type strain and into averufin (**5**) in the blocked mutant (ATCC 24551), but not into norsolorinic acid (**3**) in the corresponding mutant ATCC 24690.[3] However, [^{14}C]-norsolorinic acid was transformed into radiolabeled averantin in *ver-mu-39* which accumulates the latter.[3] In sum, these data indicate a linear pathway rather than a "metabolic grid"[25] proceeding from polyketide **2** (note reductions at the first acetate- and malonate-derived carbonyl carbons) to norsolorinic acid (**3**, presumably after oxidation at C-10 of the first-formed anthrone[26]) and then proceeding as in Scheme 1.

Further support for early reduction at the carbons



which become C-3′ and C-5′ in **3** may be inferred from the fate of [2-$^2H_3$, ^{13}C]- and [1-^{13}C, 2-$^2H_3$]-acetate in averufin biosynthesis.[27,28] Three deuteria appear at C-6′ in **5** as expected for the starter unit, but only one deuterium[29] was observed at C-2′ and C-4′. These findings are highly reminiscent of the well-studied sequence of reduction, dehydration, reduction steps characteristic of fatty acid biosynthesis.[31]

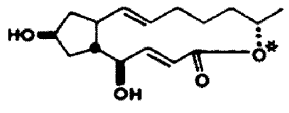
It struck us as particularly noteworthy that the biosynthetic pathway, therefore, appeared to proceed in such a way that the carbonyl of the starter unit would be completely reduced to a methylene only to be reintroduced at a later stage to generate averufin (**5**). Indeed for the (admittedly) limited number of [1-^{13}C, $^{18}O_2$]-acetate incorporation studies reported to date, it is generally true that oxygen bound to a carbonyl-derived carbon originates from the progenitor polyketide.[32] Even in the case of the heavily reduced brefeldin A (**18**), for example, the lactone oxygen (*) is derived from the acetate starter unit.[35]

One possible interpretation of these observations with respect to norsolorinic acid and averufin is that hexanoyl CoA serves as the starter unit for the hypothetical precursor **2**. That higher acids than acetic could serve as starters was suggested early on by Birch.[36] This possibility subsequently has been mooted by the observation in careful radiochemical analyses of a small but significant difference (usually lower) in the specific activity for saturated side chain carbons of particular polyketide metabolites with respect to nuclear carbons.[37] A particularly germane instance of this sort may be the report of Holker and Mulheirn[38] that the bisfuran carbons of sterigmatocystin (**8**, from *A. versicolor*) carried an approx. 10% lower specific radioactivity from [^{14}C]-acetate than nuclear carbons. On the other hand, the more extensive studies of Büchi's group on aflatoxin B_1 produced by *A. flavus* showed a consistent level of incorporation of acetate radiolabel throughout the molecule.[1] Nonetheless, despite the existence of several families of acetogenins that bear saturated hydrocarbon side chains,[39] few *bona fide* examples may be claimed where experimental evidence supports the intervention of starter units other than acetate or propionate.[40] In fact, when such experiments have been attempted, degradation to acetate almost without exception has been the observed result.[37b,41] For example, Tanenbaum examined the incorporation of [1-^{14}C]-hexanoate into pulvilloric acid (**19**) but found randomization of the label owing to secondary incorporation as acetate.[37b] However, aromatic acids, e.g. cinnamic from deamination of phenylamine, serve as starter units for a wide number of flavonoids, etc.[42] For a limited number of cases branched-chain fatty acids have been demonstrated to function similarly, e.g. isobutyric acid, derived apparently from valine, is incorporated intact into piloquinone[43] and isoleucine, evidently by way of 2-methylbutyric acid is said to be involved in avermectin biosynthesis.[44]

Against these largely negative precedents but in recognition of the seemingly elaborate formation of averufin, it was determined to attempt the incorporation of labeled hexanoate into the latter. To our very great surprise, utilization of this fatty acid under two rather different feeding regimens was remarkably efficient. In the first of these 12 g of 48 hr old mycelial pellets of the averufin-accumulating mutant ATCC 24551 were suspended in 100 mL of low-sugar replacement medium[45] in each of 12 500 mL Erlenmeyer flasks. [1-^{13}C]-Hexanoic acid (12 mg) was added under aseptic conditions to each flask and they were shaken for 24 hr at 28°. The averufin produced was purified by extractive workup and silica gel chromatography. In the second experiment a 100 mL, 48 hr standing culture of the same mutant grown on the low-salts medium[46] was treated with the labeled hexanoic acid (25 mg) and again at 72 and 96 hr. After 120 hr, the incubation was terminated and the averufin produced was isolated as above.

The $^{13}C\{^1H\}$-NMR spectrum of averufin at natural abundance is displayed as A in Fig. 1. The proton-decoupled spectra of the [^{13}C]-enriched averufin from the replacement culture and the low-salts medium, obtained under very nearly identical conditions of concentration, temperature and instrument parameters, are shown as B and C, respectively. When comparisons were made between B and C and the line intensities at natural abundance in A (normalized to C-5, an unenriched C derived from C-2 of acetate), it was found that the resonance in both spectrum B and C for C-1′ was enhanced to an extent 3–4 times natural abundance. Some degradation of the labeled hexanoate to [1-^{13}C]-acetate and secondary incorporation was observed in both experiments, the level of [^{13}C]-enrichment being about 0.5% in spectrum B and $1.0 \pm 0.1\%$ in spectrum C (acetate C-1 derived carbons are underlined in spectrum A). A possible starter unit effect may have been evident in the slightly higher level of [^{13}C]-enrichment at C-5′, 0.7% in B and 1.3% in C.

The intact incorporation of [1-^{13}C]-hexanoate may indicate a linear C_6-starter unit produced by a separate fatty acid synthetase. It is also possible that such an observation may arise from exchange of hexanoyl CoA onto a single polyketide synthase, itself capable of producing this reduced segment.[47] This process presumably would have to be quite efficient since the level of specific incorporation, 3–4%, is at least comparable to or greater than that observed with labeled acetate.[48] A less likely but conceivable course may be that acylation of a preformed tetrahydroxyanthraquinone takes place. 1,3,6,8-Tetrahydroxyanthraquinone has been observed in *A. versicolor*.[61] However, while this anthraquinone is symmetrical, its labeling pattern from [1,2-$^{13}C_2$]-acetate is



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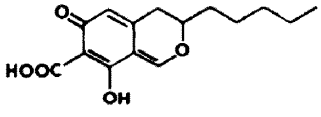

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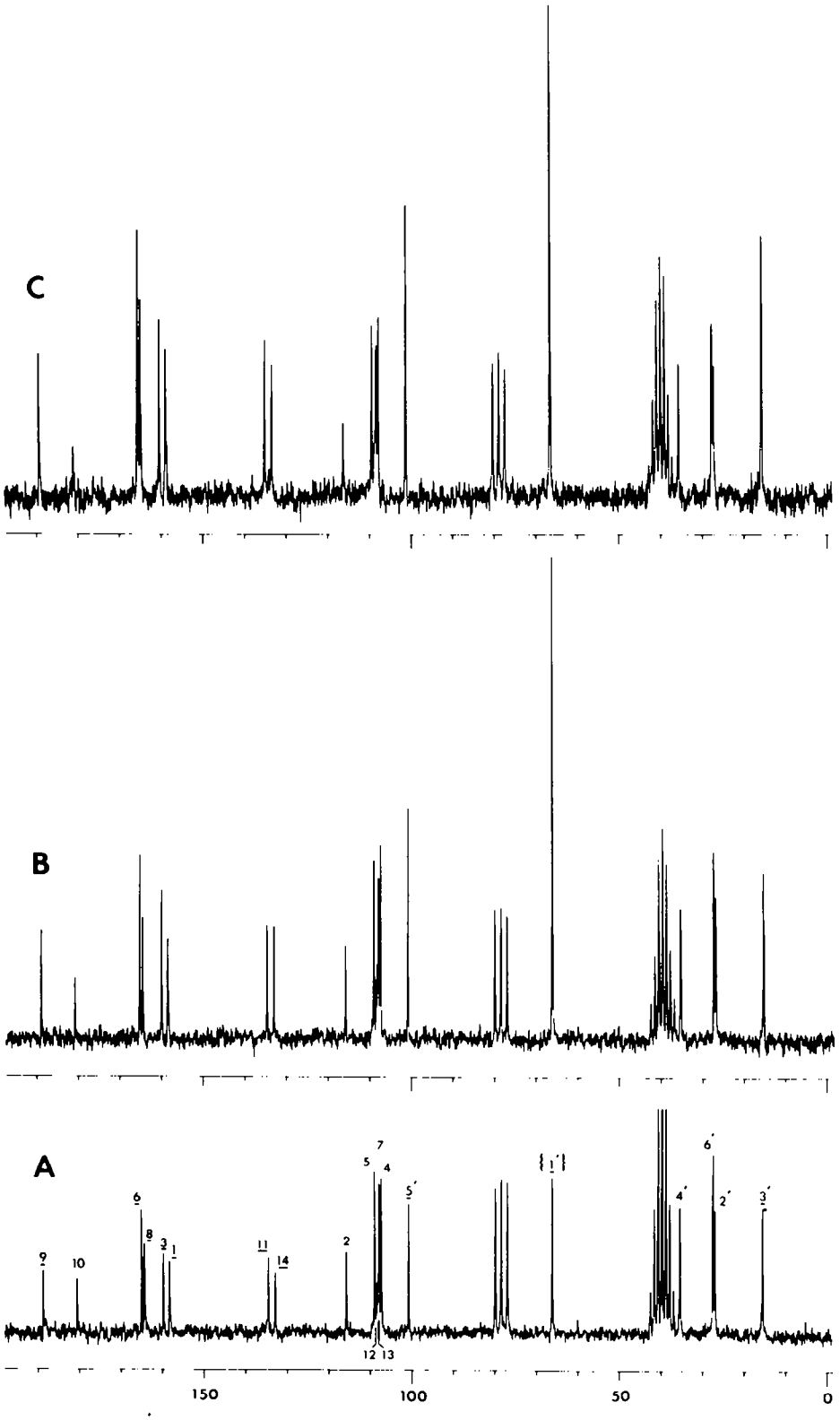


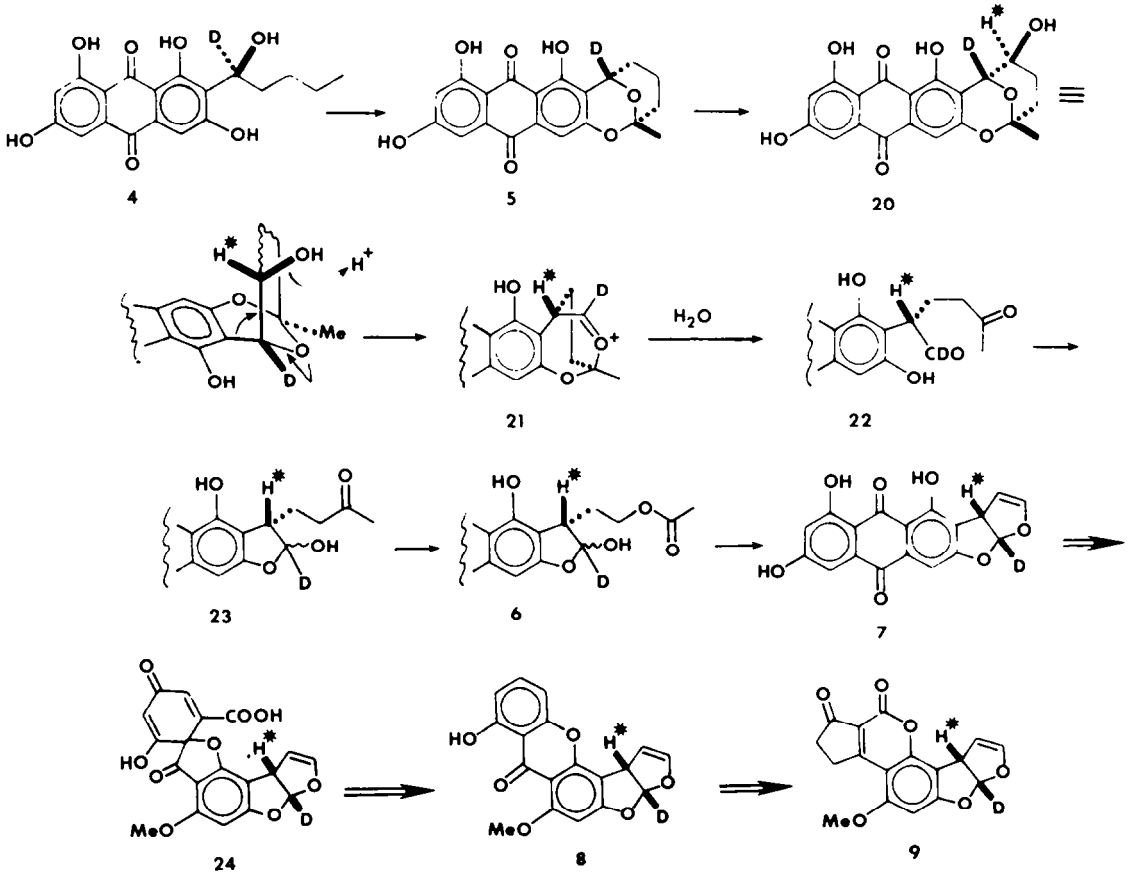
Fig. 1. $^{13}C\{^{1}H\}$-NMR spectra of averufin (all samples *ca* 20 mg in 0.5 mL of 1:1 d_6-DMSO—$CDCl_3$) obtained under the following conditions: Jeol FX-90Q, 22.5 MHz; spectral width 4500 Hz, 8K points, acquisition time 0.5 sec, pulse decay 0.5 sec. (a) natural abundance (19,500 transients). (b) derived from [1-^{13}C]-hexanoic acid in replacement medium[45] (13,300 transients). (c) derived from [1-^{13}C]-hexanoic acid in low salts medium[46] (12,800 transients).

not, which would require such acylation to occur in an enzyme bound state.

DISCUSSION

The specificities of labeling obtained in aflatoxin B_1 (**12** and **13**) and versicolorin A (**14** and **15**) from incorporation of labeled averufins (**10** and **11**), respectively, demonstrate the intact incorporations of the latter into the former.[49] Of the three intact acetate units of the averufin side chain evident from earlier [1, 2-$^{13}C_2$]-acetate incorporation experiments, the inner and central of these become correspondingly the central and outer units in the bisfurans of both aflatoxin B_1 and versicolorin A. The terminal unit is lost by way of an apparent Baeyer-Villiger oxidation (intramolecular) as revealed by the conversion of multiply labeled averufin (**16**) to versiconal acetate (**17**). In the critical chain branching process deuterium label at C-1′ of averufin (**11**) is retained in versiconal acetate (**17**), versicolorin A (**15**) and aflatoxin B_1 (**13**). In the migration of the anthaquinone nucleus from C-1′ to C-2′, therefore, a Favorski-like rearrangement[22,50] is excluded but other proposed mechanisms involving an epoxide[48b] or an open-chain pinacol rearrangement[51] are consistent with the results of available labeling experiments. However, based on extensive experience with the chemistry of averufin,[16] we prefer a scheme wherein the ketal side chain of the latter remains closed prior to the rearrangement itself. This working hypothesis is outlined below.

The polyketide precursor (**2**, Scheme 1), apparently derived from a hexanoate starter unit, forms norsolorinic acid (**3**) as the first anthraquinone intermediate, following oxidation at C-10 as discussed above. Ketone reduction then yields averantin (**4**, Scheme 2) which on oxidation at C-5′ affords, upon thermodynamically favorable *intra*molecular ketalization,[16] averufin (**5**). It is tentatively suggested that the next intermediate is nidurufin (**20**), a known natural product that has been isolated from *A. nidulans*.[52] The absolute configurations of **5** and **20** are at present not known[53] but have been designated here solely on the basis of the established absolute configurations[38,54] of the bisfurans that must ultimately be formed. In the original structure proof of nidurufin, the relative configuration at C-2′ was quite reasonably assigned *endo* on the basis of the vicinal coupling constant readily measured at H-1′.[52] We have obtained the *endo*-alcohol by unambiguous total synthesis[55] but have found its spectral and physical properties to be similar but not identical to those reported for natural nidurufin. Synthesis of the epimeric *exo*-alcohol (**20**), however, gave a set of such data completely in accord with those reported.[52] Whereas the C–O bond in the former alcohol is orthogonal to that of the migrating anthraquinone C-1′ bond, in the latter, regardless of whether the E-ring is in a chair or boat conformation, these bonds are very nearly antiperiplanar and hence ideally disposed stereoelectronically to rearrange in the sense indicated in Scheme 2. Participation of 1′-O, there-



Scheme 2.

fore, leads directly to oxonium species **21** which upon hydration would collapse to aldehyde **22** and close to hemiacetal **23**. Baeyer–Villiger oxidation of the methyl ketone **23** would generate versiconal acetate **6**. Hydrolysis/oxidation[56] or oxidation/hydrolysis[19] of **6** then would yield versicolorin A (**7**).

The presumed conversion of veriscolorin A (**7**) to sterigmatocystin (**8**) has been rationalized in a number of ways in recent years.[27,57] At present we favor cleavage of the anthraquinone nucleus and oxidative coupling to a dienone intermediate **24**[58] very much along the lines of a recent proposal by Sankawa.[27] To account for the loss of the 6-OH of **7** upon xanthone formation, reduction, dehydration, decarboxylation and rearrangement then would lead to sterigmatocystin (**8**). Further oxidative steps may be proposed[58,59] to account finally for aflatoxin B_1 (**9**) formation. Much experimental work remains to be done to test this biogenetic scheme and to reveal the details of the diverse transformations which characterize this pathway.

EXPERIMENTAL

General procedures. TLC was carried out with Analtech glass plates coated (0.25 mm) with silica gel (GHLF Uniplate) and preparative layer chromatography with 20 × 20 cm plates, 2.0 mm thickenss, of E. Merck silica gel F-254. All broths and slants were sterilized at 125°/20 psi for 20–30 min prior to inoculation. Liquid cultures were grown in cotton-stoppered Erlenmeyer flasks at 28° and shaken at the indicated revolutions in a New Brunswick Scientific model G-25K gyrotory incubator shaker. ^{13}C-NMR spectra were obtained using a Jeol FX-900 spectrometer operating at 22.5 MHz for solns in the indicated solvents (Fig. 1) using the central line of $CDCl_3$ (δ 76.9) or in $CDCl_3/d_6$-DMSO mixtures the central line of the latter (δ 39.5) as internal reference.

Incorporation of labeled averufin into versicolorin A by A. parasiticus (ATCC 36537)

Six 500 mL portions of minimum mineral medium[60] contained in 2 L Erlenmeyer flasks were inoculated with *ca* 10^6 conidia of the versicolorin A-producing mutant. After incubation for 52 hr at 150 rpm at 28°, the mycelial pellets were collected on cheesecloth and washed with a replacement medium[7b] containing 5.4 g/L glucose. 10 g (wet weight) of pellets were resuspended in 100 mL of the replacement medium contained in each of 14 500 mL Erlenmeyer flasks; 3 mg of the appropriate averufin **10** or **11** in 2 mL acetone were added to each flask and incubation was continued at 180 rpm at 28° for 40 hr. The pellets were collected on cheesecloth, washed with water and exhaustively extracted with acetone. The acetone extract was evaporated to *ca* 300 mL, poured into water (1.2 L) and extracted with hexane until the hexane extract was colorless and then with ether. The ether extract was washed with water, brine, dried over Na_2SO_4 and evaporated. The hexane extract was washed with brine (causing a ppt to form) until the hexane layer was colorless. The ppt was collected, spotted on two 2 mm preparative layer chromatography plates and developed in 7:6:4 hexane–acetone–ether. The versicolorin A band was collected, eluted with 9:1 $CHCl_3$–MeOH, added to the crude ether extract and evaporated. Anhydrous potassium K_2CO_3 (5 g), 1.25 mL Me_2SO_4 and 30 mL dry acetone were added and the mixture was heated to reflux.[5] After 6 hr, the mixture was cooled, filtered and evaporated to dryness. Silica gel chromatography of the residue (1:1:1 pentane–CH_2Cl_2–ether) yielded pure versicolorin A trimethyl ether: from [4′-^{13}C]-averufin (**10**), 5 mg of **14** were obtained from [1′-^{2}H, ^{13}C]-averufin (**11**), 12.5 mg of **15** were obtained.

Incorporation of [1-^{13}C]-*hexanoic acid into averufin by* A. parasiticus (ATCC 24551)

(a) *From replacement cultures.* Six 500 mL portions of minimum mineral medium[60] contained in 2 L Erlenmeyer flasks were inoculated with approx. 10^6 conidia of the averufin-producing mutant. After incubation at 150 rpm at 28° for 48 hr, the mycelial pellets were collected on cheesecloth and washed with a replacement medium[45] containing 1.62 g/L-glucose. 12 g (wet weight) of pellets were resuspended in 100 mL of the replacement medium contained in each of twelve 500 mL Erlenmeyer flasks; a suspension of 12 mg of [1-^{13}C]-hexanoic acid in 2 mL of the replacement medium was added to each flask and incubation was continued at 175 rpm at 28° for 24 hr. The mycelial pellets were collected on cheesecloth, washed with water and exhaustively extracted with acetone. The crude acetone extract was evaporated to near dryness, poured into water and extracted with ether. The ether extract was washed with water, brine, dried over Na_2SO_4 and evaporated. The averufin fraction from silica gel chromatography (98:2 $CHCl_3$:MeOH) was triturated with $CHCl_3$, filtered and washed with $CHCl_3$ to yield averufin (77 mg) as a bright orange solid.

(b) *From standing culture.* Conidia of the averufin-producing mutant were inoculated into 100 mL of low-salts medium[46] contained in a 500 mL Erlenmeyer flask and incubated as a static culture at 28°. [1-^{13}C]-Hexanoic acid (25 mg) in 2 mL water was added after 48 hr of incubation and at two 24 hr intervals thereafter. After an additional 24 hr, isolation and purification as above yielded 22 mg of purified averufin.

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