BIOSYNTHESIS OF AVERUFIN AND STERIGMATOCYSTIN IN ASPERGILLUS PARASITICUS AND A. VERSICOLOR:[1] APPLICATION OF MULTIPLE LABELLED ACETATE IN POLYKETIDE BIOSYNTHESIS

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Abstract-The biosynthesis of averufin and sterigmatocystin was studied by using [2-^{13}C,2-^{2}H$_3$]- and [1-^{13}C,^{18}O$_2$]-acetate as precursors. The patterns of ^{2}H and ^{18}O incorporation suggested new machanisms for the formation of dihydrobisfuran ring and xanthone skeleton.

Biosynthesis of aflatoxin(7) has been a subject of intense studies owing to its highly toxic property.[2] Particularly, successive rearrangement reactions starting from an anthraquinone with a linear C_6 side chain and leading to aflatoxin b_1(7) drew attention of many workers. The scheme of aflatoxin biosynthesis based on experimental data hitherto obtained is summarized in Chart 1. Most of the intermediates in the pathway have been evidenced by direct incorporation experiments with the labelled intermediates. Averufin(3) was first isolated from Aspergillus versicolor along with averantin(2), versicolorin A(5), sterigmatocystin(6) and other metabolites.[3] Later, a mutant strain of A. parasiticus, whose mother strain produced aflatoxin, was found to accumulate averufin(3) and following incorporation study with ^{14}C

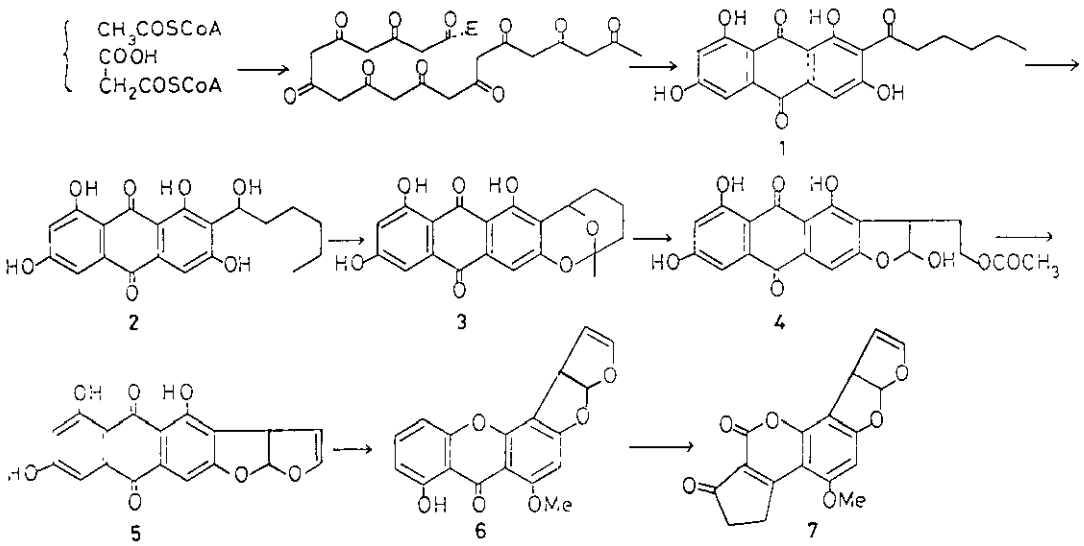


Chart 1 Biosynthetic scheme of averufin(3), sterigmatocystin(6) and aflatoxin B_1(7)

labelled averufin(3) has demonstrated that it is a potent precursor of aflatoxin B_1(7).[4] This was further confirmed by the incorporation of ^{13}C labelled averufin(3), prepared biosynthetically from ^{13}C labelled acetate, into aflatoxin B_1(7).[5] On the other hand, sterigmatocystin(6) which was isolated as the main metabolite of A. versicolor is the first natural product characterized to have dihydrobisfuran in its structure.[6] Holker and Underwood postulated sterigmatocystin(6) as an intermediate in aflatoxin biosynthesis,[7] and, later, this was evidenced by a direct incorporation experiment with ^{14}C labelled sterigmatocystin(6).[8] Our interest in the biosynthesis of averufin(3) and sterigmatocystin(6) was in the application of multiple labelled acetate in the studies of polyketide biosynthesis to obtain further informations concerning rearrangement mechanisms.[1]

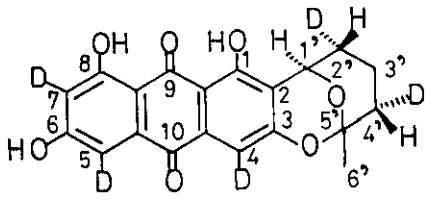
[2-^{13}C] and [2-^{13}C,2-$^{2}H_3$]-Acetate were separately administered to stationary cultures of a mutant strain of A. parasiticus (ATCC 24551) grown on synthetic low salt medium.[9] The incorporation of ^{2}H into C-4, -5, -7, -2', and -6' was readily shown by the comparison of the intensities of corresponding ^{13}C signals in the ^{13}C-NMR spectra of two kinds of labelled averufin(3). The retention ratios of ^{2}H versus ^{13}C were calculated from the decrease of signal heights in the ^{13}C-NMR spectrum of ^{2}H labelled averufin(3a), though they are not accurate figures(Table 1). The use of [2-^{13}C,2-$^{2}H_3$]-acetate is the only method to provide information concerning the numbers of ^{2}H atom bonded to particular carbons. If averufin(3) is an immediate intermediate released from the enzyme template of polyketide biosynthesis, C-4' methylene should be labelled with two ^{2}H. The ^{2}H decoupled ^{13}C-NMR spectrum of averufin(3a) labelled with ^{2}H showed complex signals arising from C-2', -4' and -6', which were impossible to be interpreted. To obtain a better separation for the signals, the labelled averufin(3a) was converted into a tribenzoate. Signals observed at 10 - 40 ppm were assigned as shown in Table 2. The results clearly demonstrate the presence of one ^{2}H at C-2' and -4' and are well in accord with the observations that ^{13}C labelled norsolorinic acid(1) and averantin(2) were efficiently converted into aflatoxin B_1(7) in A. parasiticus.[10,11] Incorporation study with [2-^{13}C,2-$^{2}H_3$]-acetate gave further support to the biosynthetic scheme that averufin(3) is formed from norsolorinic

Carbon	ppm	Retention%
C-4	108.3	42
C-5	108.9	68
C-7	108.7	65
C-2'	27.9	66
C-6'	27.8	88

Table 1. ^{2}H-Retention % in averufin(3a) labeled with [2-^{13}C,2-$^{2}H_3$]-acetate.

Carbon	ppm	Species	Multiplicity	J_{C-H}(Hz) obs.	lit.
C-3'	15.9	$^{13}C^{1}H_2$	t	133	130
C-6'	26.9	$^{13}C^{2}H_3$	s	-	-
	27.2	$^{13}C^{2}H_2{}^{1}H$	d	129	128
C-2'	27.7	$^{13}C^{2}H^{1}H$	d	129	128
	28.3	$^{13}C^{1}H_2$	t	130	128
C-4'	35.2	$^{13}C^{2}H^{1}H$	d	130	127
	35.4	$^{13}C^{2}H_2$	t	126	127

Table 2. Assignment of ^{13}C-^{2}H signals of averufin(3a) labelled with [2-^{13}C,2-$^{2}H_3$]-acetate.



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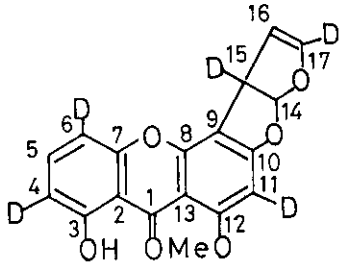

6a

acid(1) via averantin(2). It is of interest that the oxo-group forming ketal is formed by oxidation of a methylene group originally derived from the carboxyl of acetate. The stereochemical course of fatty acid biosynthesis has been rigorously established[12] and there are no reason to doubt the stereospecific incorporation of ^{2}H into C-2' and -4' of averufin(3a).

Next, [2-^{13}C] and [2-^{13}C,2-$^{2}H_3$]-acetate were separately administered to cultures of A. versicolor grown on synthetic low salt medium to obtain enriched sterigmatocystin(6). The ^{2}H-decoupled ^{13}C-NMR spectra of two kinds of labelled sterigmatocystin(6) readily showed that C-4, -6, -11, -15 and -17 were labelled with ^{2}H. The retention ratios of ^{2}H versus ^{13}C are shown in Table 3. Signals arising from ^{13}C labelled with ^{2}H were directly observed in the ^{2}H-decoupled ^{13}C-NMR spectrum as shown in Fig. 1*. The retention ratio of ^{2}H at C-11 of sterigmatocystin(6a) is markedly lower compared to the other carbons bearing ^{2}H. Since retention of ^{2}H at the corresponding carbon of averufin(3a), C-4, was more than 40%, the loss of ^{2}H should occur during its conversion into sterigmatocystin(6a). The ^{2}H labelled carbons in the dihydrobisfuran ring of sterigmatocystin(6a) are derived from C-2' and -4' of averufin(3a), which have been shown to be labelled with one ^{2}H atom. Therefore the retention of ^{2}H at C-15 and -17 of sterigmatocystin(6a) was the results of stereospecific rearrangement reactions.

The transformation mechanism of the C_6 linear side chain of averufin(3) into the C_4 dihydrobisfuran ring has been extensively discussed by many workers.[2] The key intermediate of the conversion reactions is versiconal acetate(4), which accumulated in culture of A. parasiticus under the presence of an insectcide, dichlorovos.[13] A feeding experiment followed has revealed that it is a potent precursor of aflatoxin B_1(7).[14] The structure of versiconal acetate(4)

Carbon	ppm	Retention%
C-15	47.9	49
C-11	90.3	7
C-6	105.6	55
C-4	111.0	32
C-17	145.0	30

Table 3. ^{2}H-Retention in sterigmatocystin(6a) labelled with [2-^{13}C,2-$^{2}H_3$]-acetate

* ^{2}H-Decoupled ^{13}C-NMR spectra were measured with a 25.2 MHz spectrometer with ^{19}F lock.

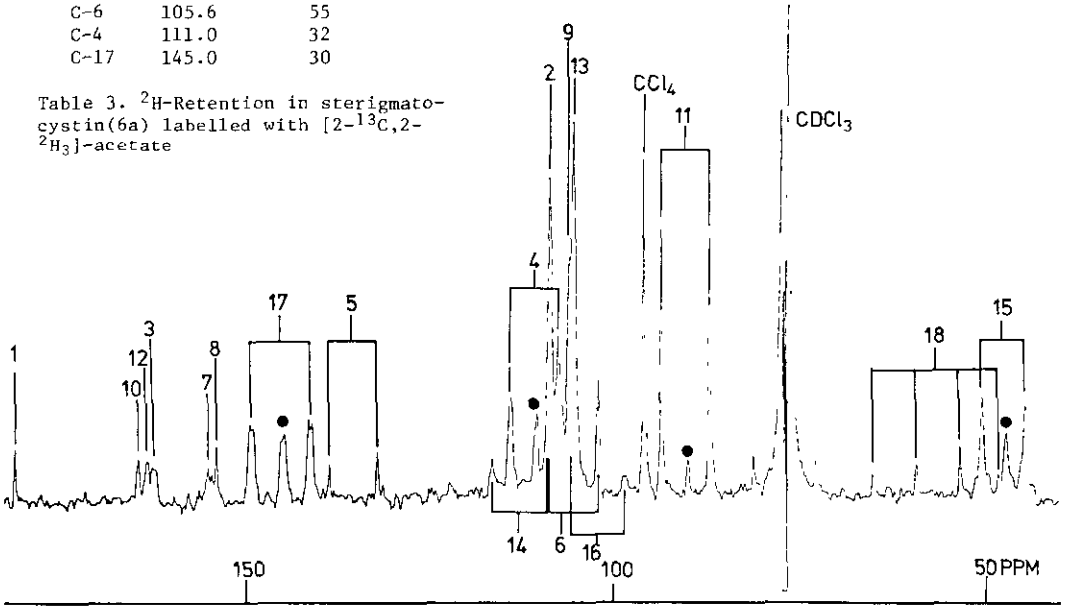

Fig.1 ^{2}H-Decoupled ^{13}C-NMR spectrum of sterigmatocystin(6a) labelled with [2-^{13}C,2-$^{2}H_3$]-acetate

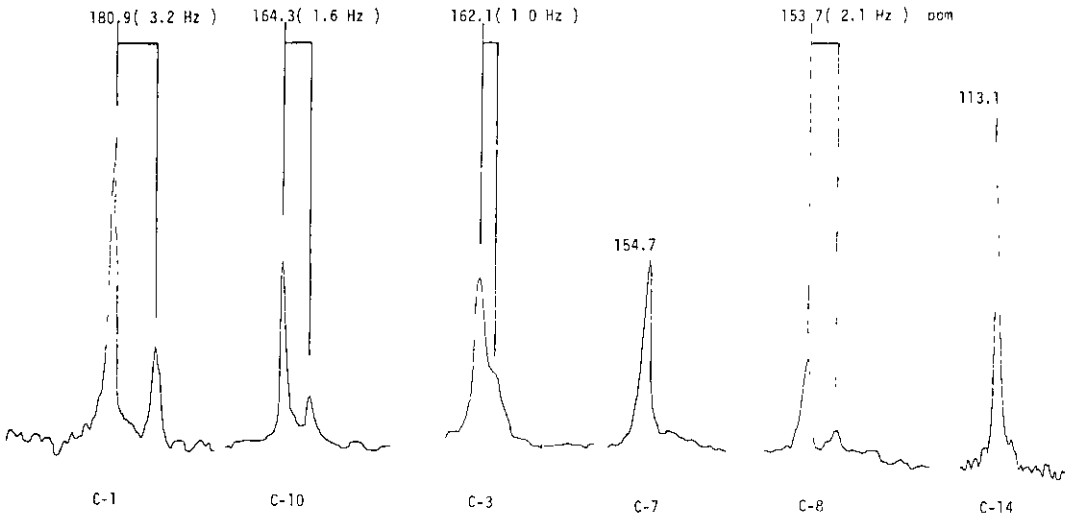

Fig.2 ^{13}C-Signals of sterigmatocystin(6b) labelled by [1-^{13}C,$^{18}O_2$]-acetate(100.7 MHz)

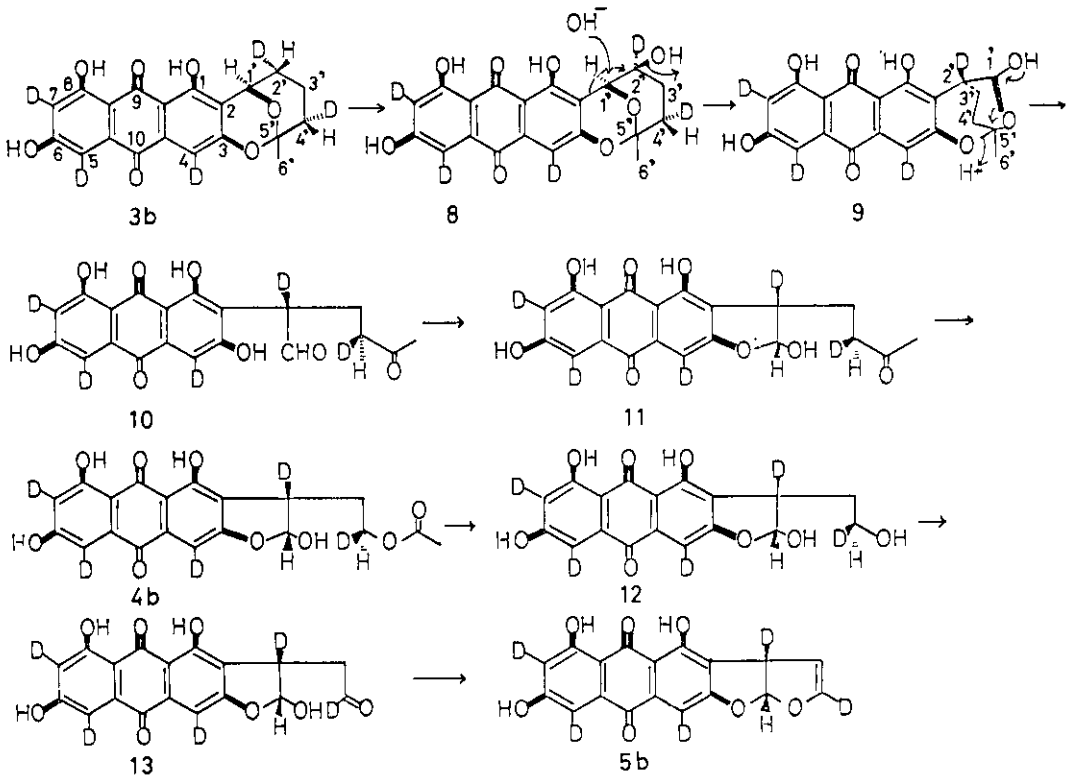

Chart 2 Scheme for the conversion of averufin(3b) into versicolorin A(5b) in Aspergilli

indicates that the C_6 linear side chain of averufin(3) undergoes migration of the aromatic ring and also the Baeyer-Villiger oxidation of the terminal acetyl group. The terminal alcoholic carbon of versiconal(12) should be further transformed into an aldehyde(13) to form dihydrobisfuran ring. This oxidation is thought to be catalyzed by alcohol dehydrogenase (Chart 2). Since alcohol dehydrogenases are known to remove pro-R carbinol hydrogen of primary alcohol in stereospecific manner, the chiralities of C-2' and -4' of averufin(3a) are presumed to be R and S configurations, respectively.[15]

As ^{13}C-NMR can be used for the detection of ^{2}H incorporation from [2-^{13}C, 2-$^{2}H_3$]-acetate, the incorporation of ^{18}O from [1-^{13}C,$^{18}O_2$]-acetate can be detected by ^{18}O induced isotopic shift in ^{13}C-NMR.[16] This method was successfully applied in biosynthetic studies by Vederas et al.[17] and also by us.[18] Vederas et al. reported the intact incorporation of ^{13}C-^{18}O from [1-^{13}C,$^{18}O_2$]-acetate into averufin(3b).[17] In order to clarify the origin of oxygen atoms of sterigmatocystin(6), we administered [1-^{13}C,$^{18}O_2$]-acetate to A. versicolor and the ^{13}C-NMR spectrum of enriched sterigmatocystin(6b) was measured with a 100.7 MHz spectrometer. The spectra shown in Fig. 2 were recorded with 1500 Hz spectral width and 32 K data points. Expanded signals of C-1, -3, -8 and -10 were accompanied by ^{13}C-^{18}O signals which were observed at 1.0 - 3.2 Hz upfield, while those of C-7 and -14 showed no shifted siganls. The absence of ^{13}C-^{18}O signal at C-14 clearly demonstrates that C-14 and adjacent oxygen atoms of sterigmatocystin(6b) are not derived from the same acetate molecule, and the ^{13}C-^{18}O bond between C-1' and an acetal oxygen in averufin(3a) was cleaved during the transformation of the side chain into bisfuran ring. Several mechanisms have been proposed to account for the

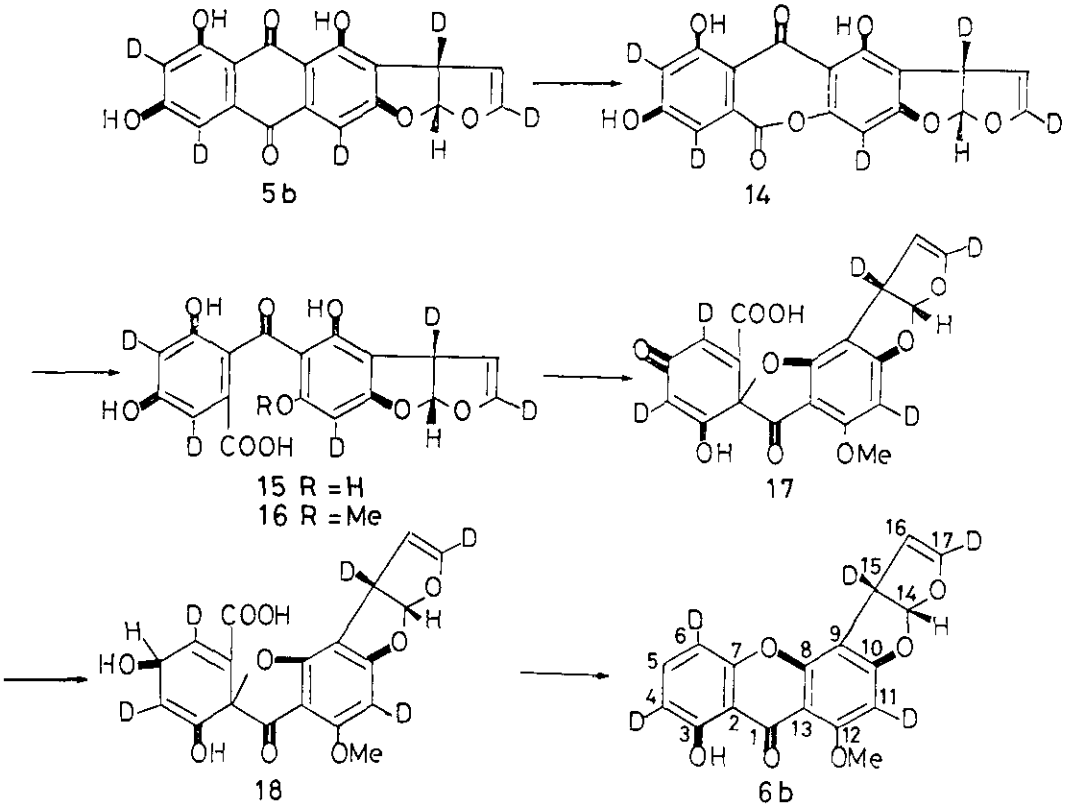

Chart 3 Scheme for the conversion of versicolorin A(5b) into sterigmatocystin(6b) in Aspergilli

migration of an aromatic ring to the adjacent carbon. Kingston et al. suggested a pinacol-type rearrangement of open chain nidrufin(8)[19], whereas Steyn et al. favored a rearrangement of an epoxide intermediate.[9a]

It is unreasonable to assume the loss of ^{18}O by an equillibrium between an aldehyde(11) and a hemiacetal(12), because, if this is the mechanism, ^{2}H at C-15 of sterigamtocystin(5b) should be lost by racemization as in versiconal acetate(4). The obtained results suggest that a pinacol-type rearrangement of nidrufin(8) itself is more appropriate, however we have to wait for the result of incorporation study with $^{18}O_2$, which is undertaken in our laboratory. Integrity of acetate C-O bond in xanthone skeleton is observed in four C-O bonds of sterigmatocystin(6b). The transformation of anthraquinone into xanthone has been mainly discussed in ergochrome biosynthesis[20], but not in the biosynthesis of sterigmatocystin(6) and aflatoxin(7). It was especially difficult to give a reasonable explanation for the loss of C-6 phenolic hydroxyl of versiocolorin A(5). The scheme of transformation, which accounts for all the experimantal evidences hitherto obtained is shown in Chart 3. The loss of oxygen function is accounted for by the elimination of an alcoholic group at the step of xanthone skeleton formation. Significant exchange of ^{2}H at C-11 of sterigmatocystin(6) (Table 3) during the transformation reactions to give xanthone skeleton is also accounted for by the presence of a phloroglucinol ring in a benzophenone intermeidate(15), which is expected to suffer an extensive exchange of aromatic hydrogen.

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