Tetrahedron Vol. 45, No. 8, pp. 2277 to 2305, 1989
Printed in Great Britain.

0040-4020/89 $3.00+.00
Pergamon Press plc

BIOSYNTHESIS OF 3-ACETYLDEOXYNIVALENOL AND SAMBUCINOL

LOLITA O. ZAMIR
Department of Applied Microbiology, University of Quebec
Institute Armand-Frappier, 531 boulevard des Prairies,
Laval des Rapides, H7N 4Z3, Canada

(*Received in South Africa* 5 *July* 1988)

CONTENTS

1. ABSTRACT

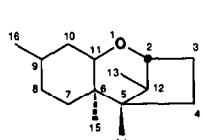
A critical review on the progress in the biosynthesis of 3-acetyldeoxynivalenol is presented. In addition, recent results on the biosynthesis of sambucinol are reported. Late intermediates have been identified and characterized. Radiolabeled and ^{13}C- or ^{2}H-labeled putative precursors were synthesized and fed to the appropriate fungal strain. The resulting 3-acetyldeoxynivalenol and sambucinol were purified and analyzed. These conventional biosynthetic methodologies were supplemented with a kinetic pulse-labeling approach. The latter technique discovered transient metabolites as well as dead-end shunt products.

2. INTRODUCTION

In this review we are focusing on the biosynthesis of 3-acetyldeoxynivalenol and sambucinol. This constitutes an updated version of the previous symposium on mycotoxins where only 3-acetyldeoxynivalenol was discussed (1). The metabolites co-isolated with 3-acetyldeoxynivalenol or sambucinol can give indication about biosynthetic pathways. We will therefore first discuss the different trichothecenes characterized either in the same fungal strain or some similar species. We will then try to briefly discuss the dual biological properties of trichothecenes (toxicity and medicinal). A short account on the acid lability of trichothecenes is given, since the resulting derivative (albeit with different stereochemistry) has been recently demonstrated in our laboratory to be a natural product. We will then proceed with a discussion of each known biosynthetic step. The last section represents very new data from our laboratory, presently in press, submitted or in preparation.

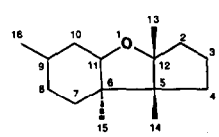
2.1 Trichothecene metabolites

The trichothecene metabolites are sesquiterpenes of fungal origin. There is only one reported trichothecene found in higher plants: baccharin (2). It might be a case of symbiosis between fungi and plants. The trichothecane (3) basic skeleton and stereochemistry is shown in Fig. 1 with its conventional numbering. Most trichothecenes have a double bond at C-9-C-10 (hence the name) and an epoxide at C12-C13. The stereochemistry of this basic skeleton is characteristic with an α-hydrogen at C-11, an α-methyl group at C-15, a β-hydrogen at C-12 and a β-methyl group at C-14. In fig. 1 a different trichothecane structure named apotrichothecane is shown. This structure originates from acid rearrangement of the trichothecane. The apotrichothecane numbering derives from the original carbons of the trichothecane. The stereochemistry of the apotrichothecane has retained an α-hydrogen at C-11, an α-methyl at C-15 and has two β-methyl groups at C-13 and C-14.



TRICHOTHECANE

1



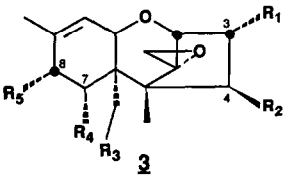
APOTRICHOTHECANE

2

Figure 1

The trichothecenes have been classified by Ueno (4,5) to four groups differing in their biological properties: i) the A-type (shown in Fig. 2 as 3) where the substituents at C-3, C-4, C-7, C-8 and C-15 could be H, OH or acetate, ii) the B-type characterized by a ketone

at C-8 (shown in Fig.2 as 4), iii) the C-type with an additional epoxide group at C_7-C_8 (shown in Fig.2 as 5) and iv) the macrocyclic trichothecenes where the methyl at C-15 and the β-OH are linked by a large group. It is interesting that recently (6) a non-macrocyclic trichothecene without an epoxide was found in Fusarium graminearum (6 in Fig.2). In the non-macrocyclic trichothecenes shown in Fig.2, 3-acetyldeoxynivalenol, trichothecin and trichothecolone are represented by 4 with the appropriate substituents.



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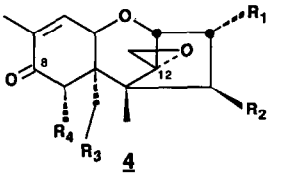
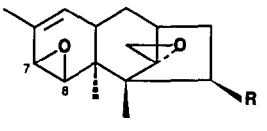


4

3-ACETYLDEOXYNIVALENOL: R_1=OAc ; R_2=H
R_3=OH ; R_4=OH

TRICHOTHECIN: R_2=OCOCHCHCH$_3$; R_1=R_3=R_4=H

TRICHOTHECOLONE: R_2=OH ; R_1=R_3=R_4=H



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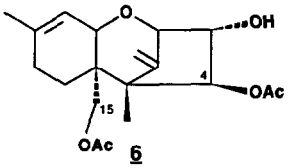
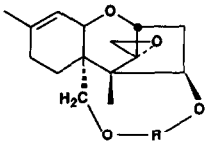


6

Figure 2

We prefer to enter the macrocyclic trichothecenes in a separate group (7, 8, shown in Fig. 3). A macrocyclic trichothecene without an epoxide at C_{12}-C_{13} was isolated from Myrothecium verrucaria (7).



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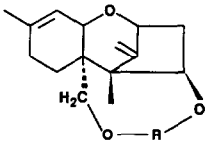


8

Figure 3

In 1984, two modified trichothecenes structures were isolated from cultures of Fusarium sambucinum (8). Their structures were rigorously proven from spectroscopic data and X-ray diffraction as sambucinol and sambucoin (9 and 10 respectively in Fig. 4). Sambucinol and sambucoin were the first non-macrocyclic trichothecenes which lack the epoxide group at C_{12}-C_{13}. Very recently (9) two new derivatives of sambucoin have been isolated and characterized in Fusarium sporotrichioides (11 and 12 in Fig.4). These new derivatives are diastereomeric alcohols at position 8 of sambucoin. A different cyclization product, sporol (13B in Fig.4) lacking the C_9-C_{10} double bond was also characterized in F. sporotrichioides (10a,b). Initially the structure 13A (Fig.4) was assigned to the natural product but was

revised to 13B. Compound 13A (neosporol) has been synthesized via a diastereoselective Claisen rearrangement (10c).

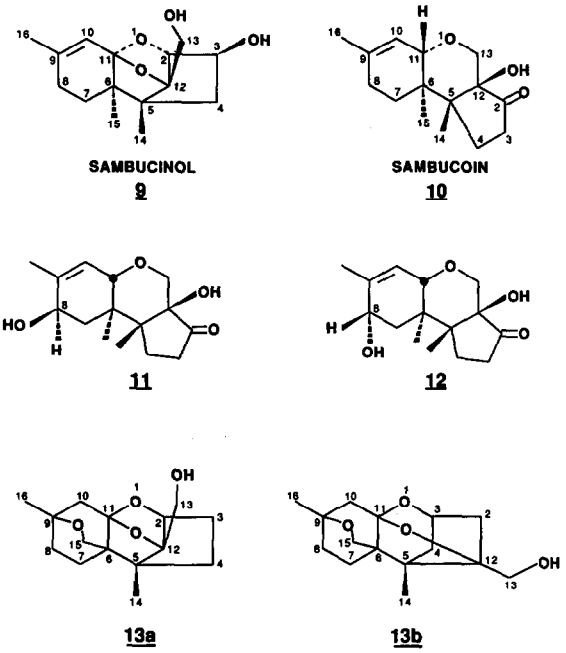


Figure 4

The first natural metabolite with an apotrichothecane structure and a cis junction between rings A and B was reported at a symposium (11,12). The two diastereomeric alcohols at C-3 were detected (14, 15 in Fig.5). The authors stated that the structures were proven by X-ray crystallography. The data was however not shown and the paper has not been published. In our laboratory we found in Fusarium culmorum a new apotrichothecane structure (16 in Fig. 5). The stereochemistry is however very different: it is the first apotrichothecane known with a trans junction between rings A and B. The structure and stereochemistry has been rigorously proved (13,14). The trans stereochemistry found ensures that this compound is not an artefact since non-enzymatic acid rearrangement of trichothecenes gives only cis-apotrichothecane with an α-hydrogen at C-11.

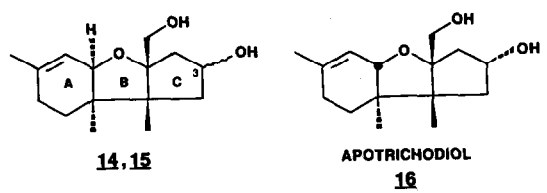


Figure 5

The bicyclic intermediate (trichodiene 17 in Fig.6) which has been postulated for a long time to be the precursor to trichothecenes was never found in Fusarium species. It was originally found in Trichothecium roseum(15). Addition of ancymidol (which blocks cytochrome P450-monooxygenases) to F. sporotrichioides led to accumulation of trichodiene (16). This interesting work certainly supports the intermediacy of trichodiene in the pathway. Trichodiol, an oxygenated version of trichodiene, initially demonstrated in Trichothecium roseum (15) was recently found in Fusarium (16) (Fig.6). In addition three other trichodiene type metabolites with three-four hydroxyl groups were also demonstrated in Fusarium (16,17) (19, 20, 21 in Fig.6). These interesting structures offer many possible biogenetic schemes.

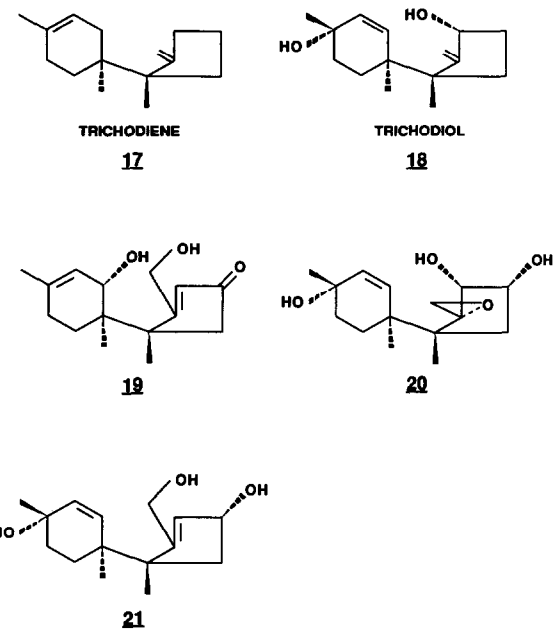


Figure 6

This account of new trichothecene structures in Fusarium is by no means exhaustive. I have only reported the trichothecenes found in Fusarium species similar to F. culmorum in order to discuss their biosynthesis.

2.2 Acid lability of trichothecenes

Trichothecenes are labile to acid conditions (18,19) as well as basic conditions (20,21). We will discuss here only the acid lability. Indeed the structures obtained are similar to natural metabolites, albeit with different stereochemistry. An extensive study of the effects of the substituents on the acid rearrangement has been done by Grove (18,19). The key functional group in these rearrangements is the epoxide. Therefore, as soon as the epoxide is opened in acid media, it is followed by intramolecular rearrangement and addition of the nucleophile at the least hindered centre. Grove found that trichothecenes with no substituent at ring A (Fig. 7) could easily rearrange to 10,13-cyclotrichothecane (boiling with water). Since this reaction depends only on the conformations of rings A and B to

enable the proximity of the double bond and the epoxide, substituents on ring C have no effect on this rearrangement. On the other hand, a ketone or an alcohol at C-8 will prevent the transformation to the 10,13-cyclotrichothecane because of the reduced nucleophilicity of the 9,10 double bond. In strongly acidic media, the attack from the oxygen bridge of ring B on the protonated epoxide prevails with concomitant attack of the nucleophile on C-2. Therefore, if the reaction is done with HCl/MeOH (or $HCl/CHCl_3$ which can give a cleaner product), the chlorine atom is found in C-2 (Fig. 7). On the other hand, treatment of the trichothecene with CF_3CO_2H and H_2O will give a hydroxyl group at C-2.

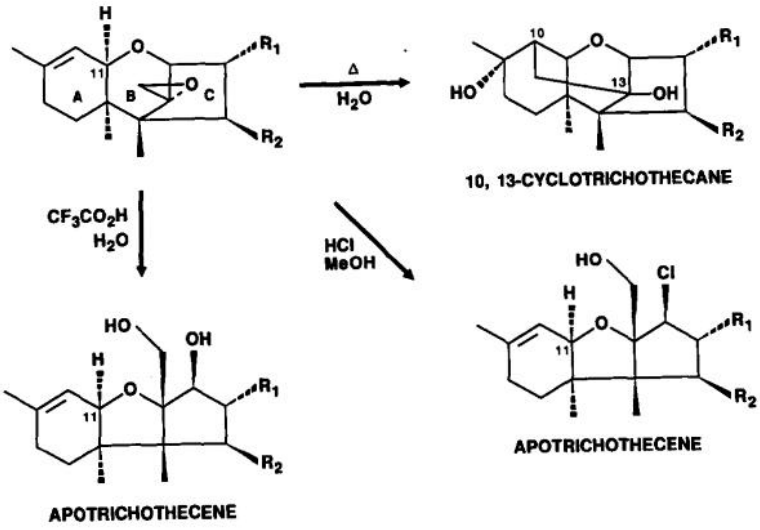


Figure 7

A ketone function at C-8 of the trichothecene with no other substituent on ring A will not prevent the rearrangement to the apotrichothecene as expected since the 9,10 double bond is not involved (Fig. 8). A highly functionalized molecule such as 3-acetyldeoxynivalenol (3-acetylvomitoxin) yields a mixture of chlorohydrin and hemiacetal (Fig. 8).

It is important to notice that in all the acid catalyzed rearrangements of the trichothecenes to the apotrichothecenes the cis stereochemistry between the rings A and B is always retained. Indeed, there is no reason to cleave the C-11-O-1 bond and change that stereochemistry.

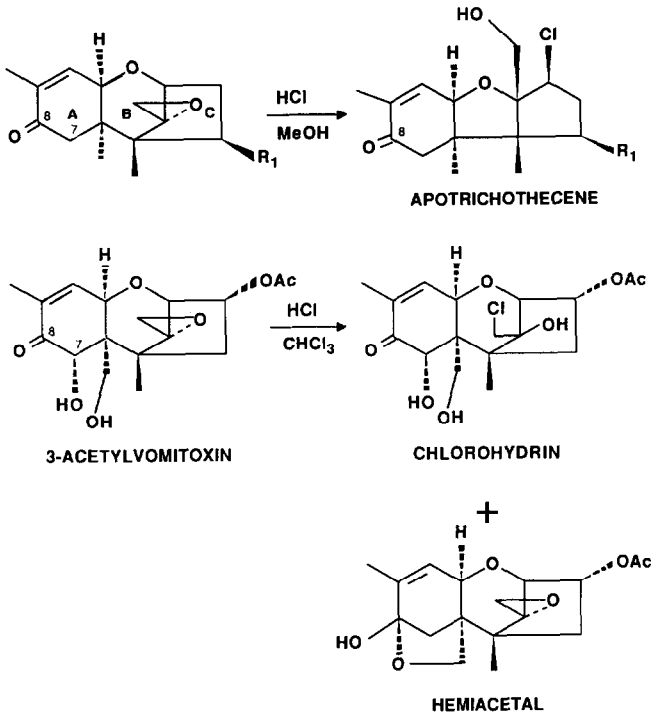
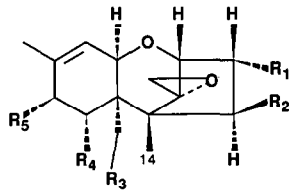


Figure 8

2.3 Toxic and medicinal properties of trichothecenes

A severe disease called alimentary toxic aleukia (ATA) was widespread in the USSR during World War II and until 1947 (22). The cause of this disease was found to be certain Fusarium species which develop on grain overwintered in the field. It was only in the 1970's that it was found that trichothecenes are the toxic metabolites in the Fusarium species. In particular, T-2 toxin (Fig. 9) was the major cause for ATA in humans. Joffe (23) reports the respective degrees of toxicity of Fusarium species isolated. He found that F. sporotrichioides and F. poal are associated with overwintered cereal grains and outbreaks of ATA. There are other human infections associated with Fusarium species: in the nails (onychomycosis) and in the cornea (keratomycosis) (23). Since 1975, the United States obtained reports that lethal chemical and biological warfare weapons, the so-called "Yellow Rain" were used against civilian populations in Southeast Asia. The "Yellow Rain" weapon was associated with trichothecene mycotoxins (T-2 toxin, deoxynivalenol, etc.) (24) (Fig.9). There has been much controversy about "Yellow Rain" (25) and it is not clear that the trichothecenes are the principal factor of "Yellow Rain". More recently, a new trichothecene was the major factor of feed refusal, emesis and decreased weight gains in swine which had ingested corn contaminated with Fusarium spp. This factor was called vomitoxin and was characterized as 4-deoxynivalenol (26,27). Deoxynivalenol is produced by Fusarium species. Fusaria are widely distributed throughout the world. The widespread occurence of deoxynivalenol as contaminant of grains has caused large economic problems and serious concern in agriculture, mainly in the northern United States and Canada.



	R_1	R_2	R_3	R_4	R_5
T-2 TOXIN	OH	OAc	OAc	H	O CO $CH_2CH(CH_3)_2$
DEOXYNIVALENOL	OH	H	OH	OH	O
ANGUIDINE	OH	OAc	OAc	H	H

Figure 9

The Fusarium species are not only toxic to grains but also to other plants. In addition to their toxic effects, trichothecenes show also antibacterial, antiprotozoal, and insecticidal effects (22). The most impressive medicinal properties related to trichothecenes derived from the mechanism of toxicity. Studies of trichothecene toxicity in laboratory animals have shown that they rapidly destroy dividing cells (28). This effect is not seen with other mycotoxins. Since anguidine (diacetoxyscirpenol, fig. 9) was the most active it was tested as an anticancer agent in experimental tumor models (29,30). The compound was dropped from clinical trials at the National Cancer Institute since it was too toxic. Anguidine could be used as an antineoplastic agent if compounds were identified that protected normal tissues against its toxicity (31).

3. MEVALONATE IS A PRECURSOR TO 3-ACETYLDEOXYNIVALENOL

The key role of mevalonate in the biosynthesis of terpenes and sterols has induced the development of various syntheses of mevalonolactone as well as labeled derivatives. I will review very briefly the recent available syntheses of mevalonolactone labeled with isotopes of carbon, hydrogen and oxygen.

3.1 Syntheses of labeled mevalonate

[3S]- and [3R]- unlabeled mevalonolactone was prepared by a combination of enzymatic and chemical procedures (32). Syntheses of (3R,S)-mevalonolactone radiolabelled with ^{14}C has been accomplished with ^{14}C at all six positions: [1-^{14}C]- (33,34),[2-^{14}C]- (33,35,36), [3-^{14}C]- (37), [4-^{14}C]- (33,38), [4,5-$^{14}C_2$]- (39), [5-^{14}C]- (39,40), [6-$^{14}C^3H_3$]- (39,41). (3RS)-mevalonate labeled with deuterium has been prepared recently at positions: [2-$^2H_2$]- (42); [6-C^2H_3]- (43) as well as stereospecifically at position 5:(5R)[5-^2H]- and (5S)[5-^2H]- (44). [5-^{18}O]-mevalonalactone has also been synthesized (45). Singly- and double labeled mevalonates with ^{13}C have been prepared with almost every combination: [2-^{13}C]- (46); [3-^{13}C]- (47);[4-^{13}C]- (48);[2,3-$^{13}C_2$]- (49);[3,4-$^{13}C_2$]- (48,50);[2,4-$^{13}C_2$]- (51);[4,6-$^{13}C_2$]- (52); [3,6-$^{13}C_2$]- (53).

3.2 Mevalonate ⟶ 4-Oxytrichothecenes

Several elegant studies and in particular those of Arigoni, Cane, Hanson, Nozoe and Tamm have largely contributed to our understanding of the first stages of trichothecene biosynthesis leading to trichodiene: mevalonate ⟶ farnesylpyrophosphate ⟶ trichodiene. For a general review on the biosynthesis of trichothecenes see Tamm and Breitenstein (1980) and Cordell (1976) (54,55). For the first steps mevalonate ⟶ farnesylpyrophosphate detailed investigations can be found in the Biosynthesis of Isoprenoid Compounds (56). The mevalonate origin of 4-oxytrichothecenes was based on studies done with verrucarin (a macrocyclic trichothecene) and trichothecin. Verrucarin is made by Myrothecium species (verucaria and roridum) and trichothecin is produced by Trichothecium roseum. Trichothecin was the first sesquiterpene whose biosynthesis was investigated in 1959 (57). The initial studies with [2-^{14}C]mevalonate were revised due to the wrong structure of the product, trichothecolone (trichothecin with the isocrotonate moiety at C-4 replaced by an-OH). In the 1970's the [2-^{14}C]mevalonate label distribution in trichothecin was correctly assigned to C-4, C-8 and C-14 (Fig. 10)(54,1). This label distribution gave a strong indication that a double 1,2- methyl shift must have occured in the formation of the trichothecane skeleton. (4R)[4-^{3}H][2-^{14}C]mevalonate was fed to T. roseum. During the conversion of isopentenyl pyrophosphate to dimethyllallyl pyrophosphate the 4S-hydrogen is eliminated (56), we would therefore expect three tritiums to be retained in farnesyl pyrophosphate and in trichodiene (see section 4). We would expect only two tritiums in the resulting trichothecin, after the oxidation of trichodiene. Indeed, the (4R)[4-^{3}H][2-^{14}C]mevalonate-derived trichothecin was shown to retain two tritiums at C-2 and C-10 (Fig. 10) by isotopic ratios as well as by chemical degradations. [5-$^{3}H_2$][2-^{14}C]mevalonate was fed to T. roseum and after preliminary erroneous results (only four tritium atoms were found in trichothecin), the expected five tritium labels were retained in trichothecin. The isocrotonate moiety in trichothecin is acetate-derived (Fig. 10).

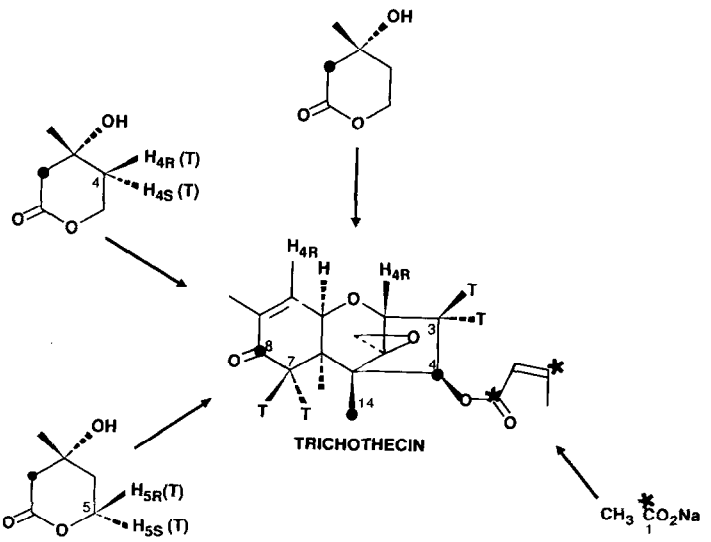


Figure 10

The stereochemistry of the hydroxylation at C-4 of verrucarin A was established with the separate feedings of (2R)[2-^{3}H][2-^{14}C]- and (2S)[2-^{3}H][2-^{14}C]mevalonates feeding to Myrothecium spp (Fig. 11). The specific activities of the resulting verrucarols and their oxidized keto-aldehydes (Fig. 11) were measured. The result was supportive for retention of configuration during hydroxylation, i.e., the hydroxyl group at C-4 replaces [2R][2-^{3}H]mevalonate. The same conclusion was reached for trichothecin: hydroxylation at C-4 proceeds with retention of configuration. One experiment done with (5R)[5-^{3}H][2-^{14}C]mevalonate feeding to Fusarium culmorum showed from the ^{3}H/^{14}C ratio that the tritium was lost from C-3 in calonectrin. This result indicates that likely also the hydroxylation at C-3 occurs with retention.

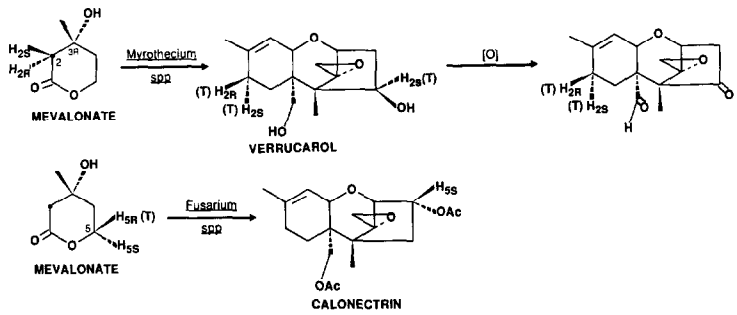


Figure 11

Most of the work done on 4-oxytrichothecene was based on radiolabeled compounds or double labeled ^{3}H and ^{14}C precursors. ^{3}H/^{14}C ratios can be reliable if accompanied by chemical degradations to locate the label. The advantage of ^{13}C or ^{2}H precursors is that they give rigorous results with no need for chemical degradations. [1-^{13}C]-, [2-^{13}C]- and [1,3-$^{13}C_2$]acetate precursors and [2-^{13}C]mevalonate precursors (54,58-60) were fed to Trichothecium roseum and the distribution of label of trichothecin was confirmed.

3.3 Mevalonate ⟶ 3-Acetyldeoxynivalenol

The structure of 3-acetyldeoxynivalenol (3-ADN) a major metabolite of F. culmorum was established in 1984 by X-ray Crystallography (61). [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]acetates were fed to Fusarium graminearum and the label distribution of its major metabolite 15-acetyldeoxynivalenol was established (68). We have rigorously established (1,63) the mevalonate distribution of label of 3-acetyldeoxynivalenol and the proofs for the folding of farnesyl pyrophosphate, the methyl shifts and the stereochemistry of the hydroxylations. These results were obtained from the feeding of ^{13}C-double labeled mevalonates as well as stereospecifically deuterium labeled mevalonates. The mevalonate label distribution obtained for 3-acetyldeoxynivalenol is shown in Fig. 12.

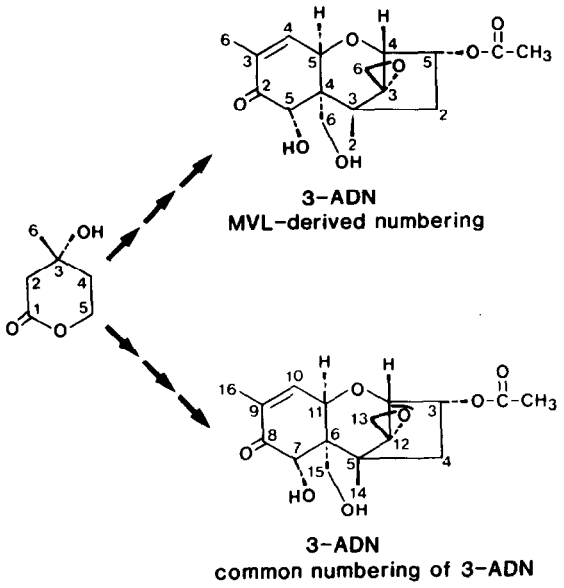


Figure 12

The methyl shifts and the stereochemistry of hydroxylations will be discussed in this section. The folding of farnesyl pyrophosphate will be dealt with in the next section. The methyl shifts were always implied in the biosynthesis of trichothecenes with no rigorous experimental proof. One of the methyl shifts originates from C-6 of mevalonate and the transfer is from its attachment on C-3 to C-4. Mevalonolactone was synthesized with two simultaneous ^{13}C labels on the alternate carbons C-4 and C-6. The 3-ADN derived from the feeding of [4,6-$^{13}C_2$] mevalonolactone showed ^{13}C-^{13}C direct coupling between C-6 and C-15 (38.5 H_z) (Fig. 13) demonstrating conclusively that there was a 1,2-methyl shift involving the transfer of carbon 6 to carbon 4 of mevalonolactone. In addition, the ^{13}C-NMR of the (3R)[4,6-$^{13}C_2$]mevalonolactone-derived-3-ADN showed enrichment at positions 2,6,10,13,15 and 16 as expected from the biogenetic scheme. Proof of the second methyl shift is indirect and derives from the distribution of mevalonate label of 3-ADN.

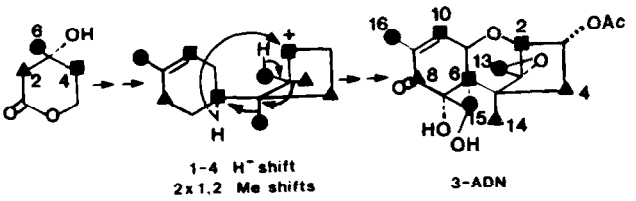


Figure 13

In order to analyze the positions 3,7 and 11 of 3-ADN, stereospecifically deuterated (3RS)(5R)[5-^{2}H]-, (3RS)(5S)[5-^{2}H]- and (3RS)[5-$^2H_2$]mevalonolactones (MVL) were synthesized. The correct stereochemistry of mevalonolactone at position 5 was obtained by enzymatic reduction (horse liver dehydrogenase) of an appropriately labeled aldehyde (48). The assignment of the ^{1}H-NMR spectrum of 3-ADN has been reported (64). The ^{1}H-NMR spectra of 3-ADN derived from (5R)[5-^{2}H]-, (5S)[5-^{2}H]- and (5RS)[5-$^2H_2$]mevalonolactones (MVL) are identical to the one published (64). The only difference is seen from the integration of the signals where deuterium is incorporated. Whereas the signal due to H_{11} in 3-ADN shows a clear result (7% deuterium incorporation in 3-ADN-(5R)[5-^{2}H]MVL-derived, 9% deuterium incorporation in 3-ADN(5RS)[5-$^2H_2$]MVL-derived, 0% deuterium incorporation in 3-ADN-(5S)[5-^{2}H]MVL-derived), other proton integrations were found unreliable. It is not surprising since the incorporation of deuterium at each site is of the order of 8-10% and the accuracy of the integral measurements is 5%. ^{2}H-NMR was found to be the best technique to locate the deuteriums unambiguously, despite its wider line width. Due to the very low natural abundance of deuterium, we observed only the signals from the enriched sites. The ^{2}H-NMR observed prove unambiguously that (5R)(5S)[5-$^2H_2$]MVL is incorporated at two positions of 3-ADN: H-3 and H-7. The signal observed at 4.672 ppm proves unambiguously that (5R)[5-^{2}H]MVL is incorporated only at C-11 of 3-ADN, whereas (5S)[5-^{2}H]MVL is incorporated at two positions of 3-ADN: H-3 and H-7. Interestingly, the 3-ADN derived from the feeding of (5RS)[5-$^2H_2$]MVL shows only two signals in the ratio 1:2. This finding results from the fact that deuterium signals at positions 7 and 11 are very close in shift and since deuterium signals are characterized by broad line width, these two signals overlap. These results show that the hydroxylation of 3-ADN at C-3 and C-7 replaced a 5-pro-R mevalonoid hydrogen atom whereas the hydroxylation at C-11 replaced a 5-pro-S mevalonoid hydrogen atom (Fig. 14). The folding of farnesyl pyrophosphate shows that in all the three positions the new carbon-oxygen bonds possess the same relative stereochemical orientation as the carbon-hydrogen bond it replaced, i.e., there is retention of configuration during oxygenations.

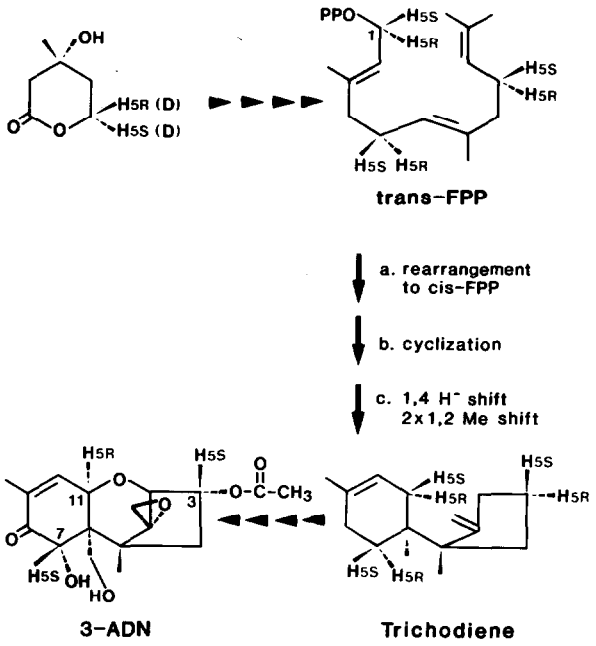


Figure 14

4. FARNESYL PYROPHOSPHATE IS A PRECURSOR TO TRICHODIENE

4.1 Biosynthesis of farnesyl pyrophosphate

The biosynthesis of farnesyl pyrophosphate is shown in Fig. 15. Mevalonic acid is phosphorylated twice to form mevalonic 5-pyrophosphate which is then dehydrated and decarboxylated to give isopentenyl pyrophosphate, the biological isoprene unit which was discovered by K. Bloch and F. Lynen (65,66). The stereochemistry of the next steps have been elegantly worked out by Popjak and Cornforth (67). In the isomerisation of isopentenyl pyrophosphate to dimethylallyl pyrophosphate it is the H_S which is removed. The first condensation of isopentenyl pyrophosphate with dimethylallyl pyrophosphate gives geranyl pyrophosphate. Addition of a second isopentenyl pyrophosphate gives farnesyl pyrophosphate. The enzymes involved shown in Fig. 15 are: 1: mevalonic acid kinase; 2: 5-phosphomevalonic acid kinase; 3: pyrophosphomevalonic acid decarboxylase; 4: isopentenyl pyrophosphate isomerase; 5: geranyl pyrophosphate synthetase; 6: farnesyl pyrophosphate synthetase. The condensation of two isopentenyl pyrophosphate and dimethylallyl pyrophosphate occurs with inversion of configuration at C-1 of dimethylallyl pyrophosphate. The condensation has been elegantly shown by Poulter and Rilling to be ionic: initial ionization of the pyrophosphate to generate an allylic cation which undergoes an electrophilic attack on the 3,4 double bond of isopentenyl pyrophosphate (68).

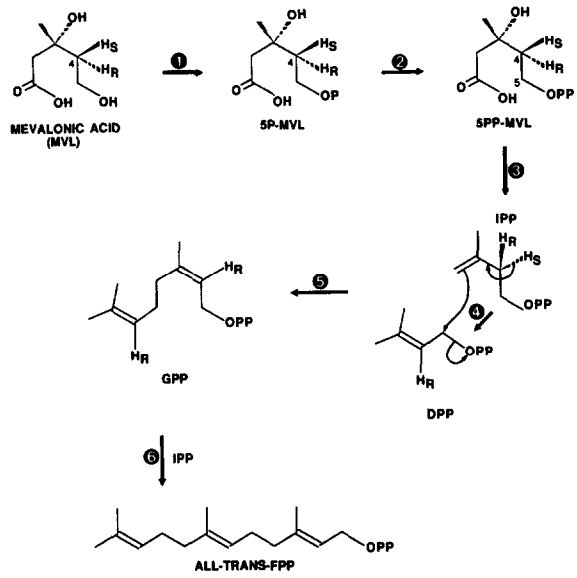


Figure 15

4.2 Folding of farnesyl pyrophosphate

[2^{13}C]Mevalonate-derived trichothecolone (69) indicated a preference for the B folding of farnesyl pyrophosphate. In order to ensure that in the biosynthesis of 3-ADN this is indeed the case (Fig. 16, the middle bond designated by an arrow is trans), [3,4-$^{13}C_2$]mevalonate was synthesized (48) and fed to F. culmorum. The intact 3,4 bond of mevalonate could be followed in the derived 3-ADN by ^{13}C-NMR. The "A" folding of farnesyl pyrophosphate would result in 3-ADN with ^{13}C-^{13}C couplings between the carbons C-2 and C-12; C-5 and C-6; C-8 and C-9. The experimental data showed couplings between the carbons C-2 and C-12; C-5 and C-6, C-9 and C-10. The "B" folding orientation of farnesyl pyrophosphate (Fig. 16) is therefore rigorously proven. Due to the high incorporation of (3RS)[3,4-$^{13}C_2$]mevalonolactone into 3-ADN (10%) we could also observe an additional coupling between C-12 and C-5 which shows the preferred conformation of farnesyl pyrophosphate for the cyclization of the five-membered ring. The same cyclization folding of farnesyl pyrophosphate was demonstrated in the biosynthesis of sambucinol and apotrichodiol (14).

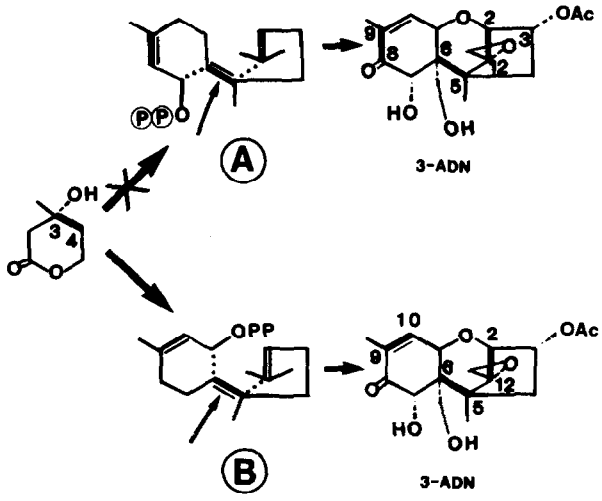


Figure 16

4.3. Cyclization of farnesyl pyrophosphate.

In the 1970's Hanson and co-workers found that all-trans farnesyl pyrophosphate was a precursor to trichodiene (69). The cyclization step necessitates the cis-isomer. A trans-cis isomerization step must occur. The cyclization of farnesyl pyrophosphate was followed by a novel, 1,4-hydride shift. This hydride shift was elegantly proved by Hanson's and Arigoni's groups (1,54) (Fig. 17). Hanson *et al.* showed the incorporation of [2-^{3}H][2-^{14}C]geranyl pyrophosphate into trichothecin with no loss of tritium. The 2 position in geranyl pyrophosphate corresponds to C-4 of mevalonate. 4H$_{R}$ of mevalonate has been shown to be incorporated into H-2 and H-10 of trichothecin, hence there must have been a hydride shift. Arigoni's group fed [6-^{3}H][12,14-$^{14}C_{2}$]trans-farnesyl pyrophosphate to *M. roridium* and showed the same $^{3}H/^{14}C$ ratio in the derived verrucarol.

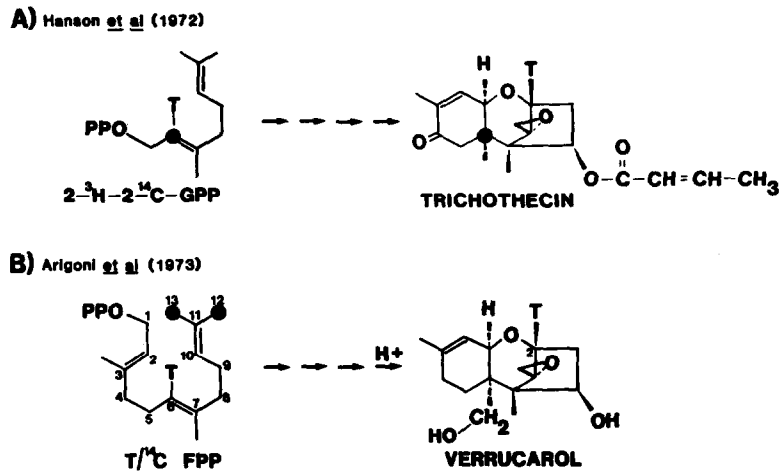


Figure 17

In addition, chemical degradation showed rigorously that the tritium was on C-2 of verrucarol. The product of the cyclization of farnesyl pyrophosphate is trichodiene which was first isolated by Nozoe from mycelial extract of T. roseum (15). A cell free system from T. roseum which could catalyze the conversion of trans-trans-farnesyl pyrophosphate to trichodiene was developed by Hanson's group. (70,71). There was much controversy on the mechanism of cyclization of farnesyl pyrophosphate and many erroneous results based on isotopic ratios (without verification by chemical degradations) were published. The elegant and rigorous work of Cane's research group (72) proved conclusively that the two hydrogens of carbon-1 of farnesyl pyrophosphate were retained in the conversion to trichodiene at C-11. They prepared (1S)[1-^{3}H][12,13-$^{14}C_2$]farnesyl pyrophosphate and (1R)[1-^{3}H][12,13-$^{14}C_2$]farnesyl pyrophosphate and incubated them separately with a cell free preparation of trichodiene synthetase from T. roseum. Degradation of the derived-trichodienes established conclusively that the cyclization on C-1 occurs with retention of configuration. This was explained by the possible intermediacy of nerolidyl pyrophosphate (Fig. 18). The first step would be conversion of all trans farnesyl pyrophosphate (FPP) to trans-trans-nerolidyl pyrophosphate (NPP). An isomerization to cis-trans nerolidyl pyrophosphate is then essential for the cyclization leading to the bicyclic intermediate. A 1,4 hydride shift and two methyl shifts give rise to trichodiene. Trichodiene synthetase has been purified from Fusarium sporotrichioides by hydrophobic interaction, anion exchange and gel filtration (73). The specific activity of the purified enzyme is fifteen fold higher than previously reported.

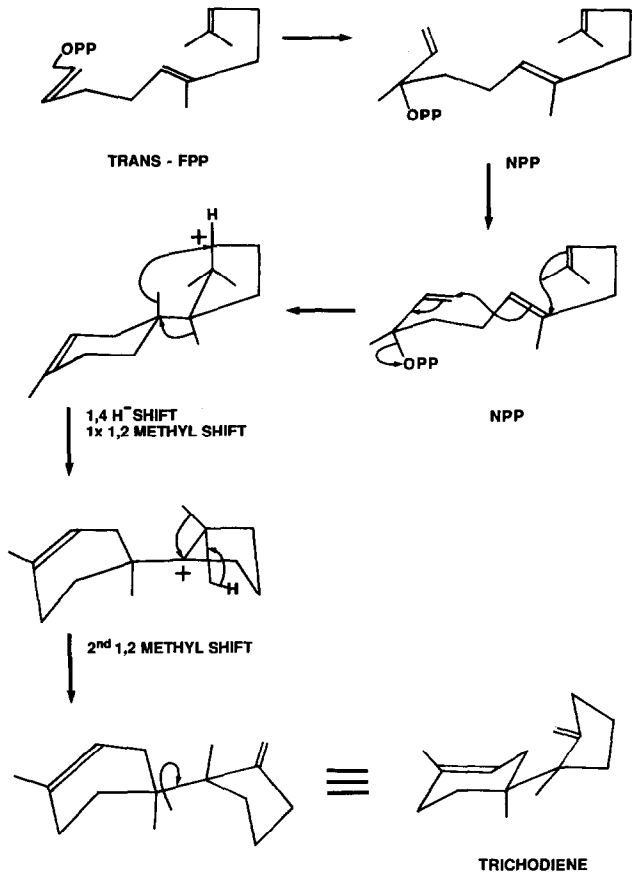


Figure 18

5. HAS TRICHODIENE BEEN RIGOROUSLY PROVEN TO BE THE PRECURSOR TO TRICHOTHECENES?

5.1. Incorporation of trichodiene into trichothecenes.

Nozoe and Machida (15) were the first to characterize trichodiene and trichodiol (Fig. 6) as metabolites from T. roseum. It is interesting to note that seven years earlier Bu'Lock (74) had suggested that the structure of trichodiene might be a natural metabolite and a putative precursor to trichothecin. In order to determine if trichodiene is a precursor to trichothecin, Nozoe and Machida (15) prepared tritiated trichodiene on the olefinic methyl group. They fed it to T. roseum and upon saponification isolated trichothecolone and crystallized it to constant specific activity (3.2% incorporation). They also found radioactivity in the TLC band corresponding to 12,13-epoxytrichothec-9-ene and in a band corresponding to trichodiol A which is an artefact produced from trichodiol during alkaline saponification. The first result (incorporation of trichodiene into trichothecolone) gives an indication that trichodiene is probably a precursor to trichothecin. However, since there was no degradation to locate the label with certainty, this experimental result does not constitute an unambiguous proof. The second result (radioactivity on the TLC band) cannot be considered. Indeed, R_f on TLC in one dimension does not constitute a proof of

identity. ^{13}C-Labeled trichodiene (with ^{13}C on the exomethylene group) was reported (75) to be incorporated into all the trichothecenes produced by Fusarium culmorum. They did not isolate the trichothecenes but showed the ^{13}C-NMR of the crude extract. This result also suggests that trichodiene is a precursor to trichothecenes.

5.2 Accumulation of trichodiene

Incubation of Fusarium sporotrichioides in the presence of $H_2{}^{18}O$ or $^{18}O_2$ determined the origin of the oxygens in T_2-toxin (76) (Fig. 9). Mass spectral analysis of $H_2{}^{18}O$-derived-T_2-toxin showed incorporation up to three ^{18}O atoms per molecule. After hydrolysis of $H_2{}^{18}O$-derived-T_2-toxin the resulting T-2 tetraol mass spectra showed no labelled ^{18}O. The authors concluded that the oxygens of the three acyl groups in T-2 toxin are H_2O derived. No rigorous proof was given that the acyl groups do not exchange with water of the media via hydrate. Mass spectral analysis of T-2 toxin formed in the presence of $^{18}O_2$ incorporated up to six atoms of ^{18}O per T-2 molecule. This result implies that the oxygens at position 1, the 12,13 epoxide and the hydroxyl groups at C-3,C-4, C-8 and C-15 are all derived from molecular oxygen as would be expected. Since the six oxygens of T-2-toxin-tetraol are derived from oxygenases, addition of an inhibitor of oxygenases should prevent its production. Indeed, this elegant result was obtained by the USDA research group (77,78): ancymidol-treated cultures of Fusarium sporotrichiodes accumulated trichodiene.

According to the author of this chapter, all these interesting results do suggest that trichodiene is a precursor to trichothecenes but do not prove it. A rigorous proof is therefore needed.

6. LATE INTERMEDIATES TO TRICHOTHECENES AND DEAD-END METABOLITES

We have seen in the previous sections that the steps from mevalonate to trichodiene have been very well worked out. The stereochemistry and the mechanisms are well defined and most enzymes have been isolated and purified. Trichodiene has been suggested in 1965 to be an intermediate to trichothecenes prior to its discovery as a natural metabolite. A rigorous proof of its intermediacy and the exact position of its incorporation is still needed. The only other large intermediate which has been shown to be incorporated to trichothecin (3.4%) is trichodermol (79) (Fig. 19). This result is based however only on isotopic $^3H/^{14}C$ ratio with no chemical degradation to locate the radiolabel.

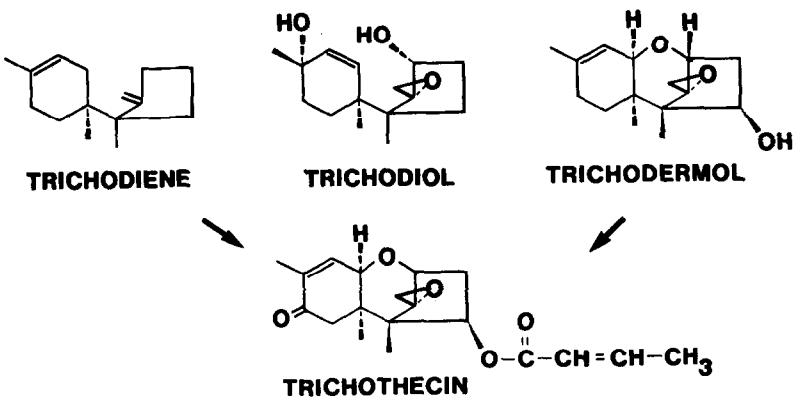


Figure 19

6.1 Kinetic Pulse-Labeling Study of Fusarium culmorum.

I will briefly summarize here the work that we recently published (13) which allows us to detect plausible biosynthetic intermediates and differentiate them from dead-end metabolites. The original technique of kinetic pulse-labeling was developed by Calvin in his studies on photosynthesis. The method as we have applied it to F. culmorum is shown in Fig. 20.

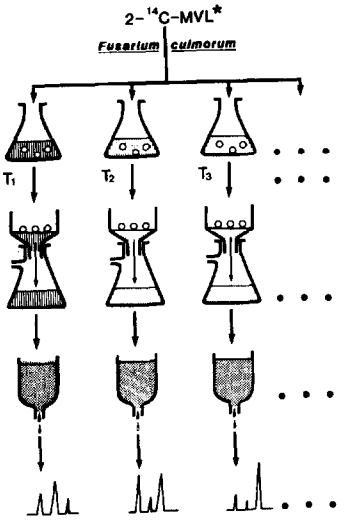


Figure 20

We feed an early radiolabeled precursor to liquid cultures of the microorganism and at various time intervals we analyze the sequential appearance of the newly synthesized radiolabeled metabolites. The radiolabeled precursor [2-^{14}C]mevalonate was fed to 50ml of production medium which contained seed culture homogenate. At different time intervals two cultures were removed and filtered under vacuum. The filtrate was extracted with ethyl acetate using Chemtubes. The extract was analyzed by HPLC connected to a radioactivity monitor. The radiolabeled peaks formed with time are then easily detected. The radioactive HPLC tracings of a kinetic pulse-labeling experiment 30 min, 1,2,4,6 and 24 h after the feeding of [2-^{14}C] mevalonate are shown in Fig. 21.

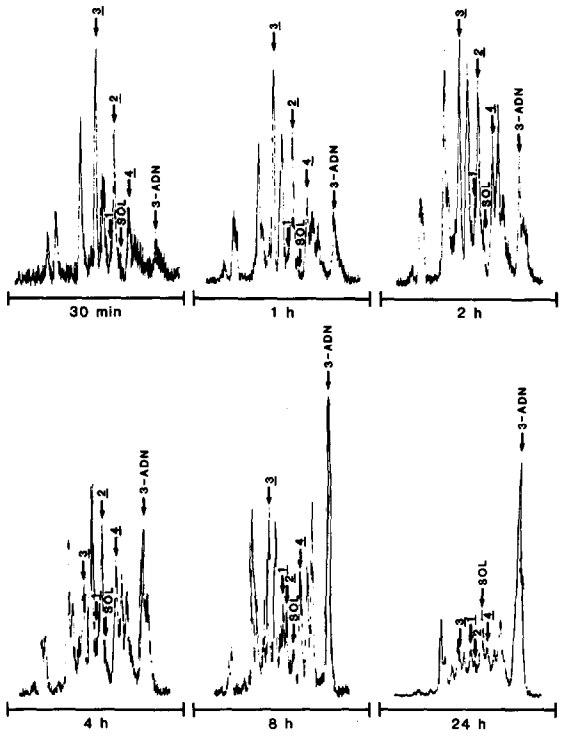


Figure 21

We see the end-products 3-ADN and SOL increasing with time. Some of the newly synthesized peaks shown in Fig.21 (3, 2, and 4) were: i) labeled appreciably by ^{14}C mevalonate ii) formed prior to the end products 3-acetyldeoxynivalenol (3-ADN) and sambucinol (SOL) and iii) they seem to be metabolized while radioactive 3-ADN or SOL are building up. The behavior of these peaks (3, 2, 4) can be ascribed to plausible biosynthetic precursors. On the other hand, some of the newly synthesized peaks behaved differently. They do not seem to be degraded with time (1). After a certain time they reach a constant level. This behavior could be ascribed to dead end metabolites. We can visualize these two types of newly synthesized metabolites (putative intermediates and dead end metabolites) by calculating the areas of the new peaks and expressing them as percent of the total radioactive peak areas. Fig. 22 shows a representation of these values plotting the relative area of a new peak next to the values for 3-ADN or for SOL. Figure 22 shows on the left the pattern of a putative precursor (3, black bar graphs) decreasing while the product 3-ADN (grey bar graphs) increases. On the right of figure 22 a characteristic dead end metabolite is shown (1, apotrichodiol, black bar graphs) where the relative amount of radioactivity with time increases in concert with the end product sambucinol (SOL).

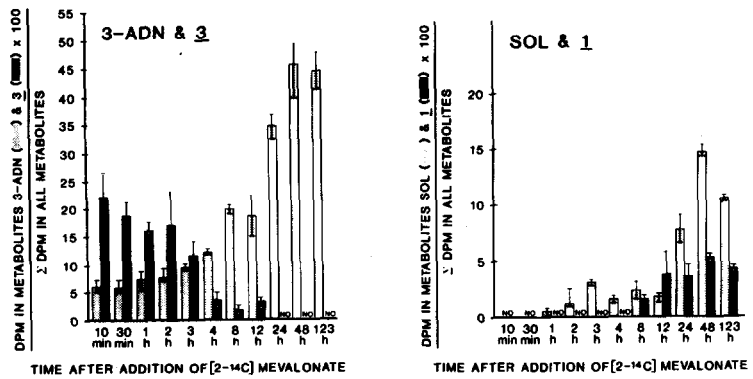


Figure 22

It is important to note that despite the fact that the radioactive peaks considered are not necessarily homogenous (more than one compound can be represented in that peak) the profiles of these peaks with time can indicate if it is worthwhile to look for a new intermediate in this region of the chromatogram. If a radiolabeled peak behaves like a potential intermediate, it is then purified to homogeneity and re-fed to F. culmorum cultures. The resulting 3-ADN and SOL are purified to constant specific activity. If the incorporation is positive, we then search for the unlabeled compound with appropriate short term incubations and characterize it spectroscopically (^{1}H-NMR, ^{13}C-NMR, Mass Spectra etc.). We would like to emphasize that this is a qualitative method and that feeding experiments with very pure metabolites are essential to determine whether a plausible intermediate is indeed a biosynthetic precursor. This method was very successful with the study of the biosynthesis of 3-ADN and SOL and most of the results which will be shown in the next sections were based on it.

6.2 Dead end metabolites

From the kinetic pulse-labeling technique, we had detected a compound (compound 1) which behaved like a dead end metabolite. In order to ascertain the validity of our technique and the interpretation of the accumulation profiles, we fed very "hot" (radiolabeled with high specific activity) compounds 1 to F. culmorum but "cold" (unlabeled) 3-ADN and SOL were derived. With appropriate incubations (corresponding to the kinetic pulse-labeling method) we isolated unlabeled compound 1 and proved its structure by the usual spectroscopic methods (14) as apotrichodiol (ATD) shown in fig. 23 with the structures of 3-ADN and SOL. We have also shown the label distribution in ATD from [3,4-$^{13}C_2$]mevalonate and found the same folding of farnesyl pyrophosphate as shown for 3-ADN and SOL. It is interesting that the stereochemistry of apotrichodiol (established by NOE difference experiments on its diacetate) shows a trans-relationship between rings A and B.

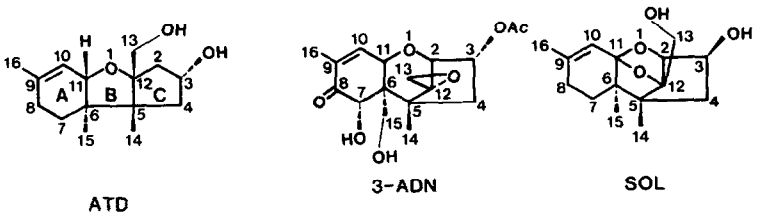


Figure 23

7. VERY RECENT RESULTS ON THE BIOSYNTHESIS OF 3-ACETYLDEOXYNIVALENOL AND SAMBUCINOL

All the results shown in this section are unpublished: some are in press, some only submitted and some are still in preparation for publication.

7.1 Distribution of label of sambucinol.

We fed mevalonates double-labeled with ^{13}C at various positions to ensure that the mevalonate label distribution in sambucinol is the same as 3-acetyldeoxynivalenol. All the results of the feedings are pictured in Fig. 24. [4,6-$^{13}C_2$]-, [2,4-$^{13}C_2$]- and [3,4-$^{13}C_2$]mevalonates were fed to F. culmorum. The sambucinols isolated and purified showed the expected enrichment sites similar to 3-acetyldeoxynivalenol.

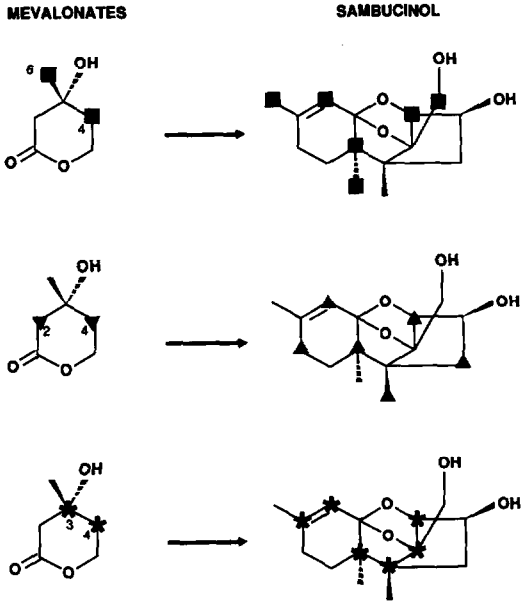


Figure 24

We studied the stereochemistry of the hydroxylation at C-3 with stereospecifically deuterated (5R)[5-^{2}H]-, and (5S)[5-^{2}H]mevalonates as well as with (5RS)[5-$^2H_2$]mevalonates. Here too, we observed retention of configuration during hydroxylation (80). The 3-hydroxyl group in sambucinol replaced 5-H_S of mevalonate. (Fig. 25)

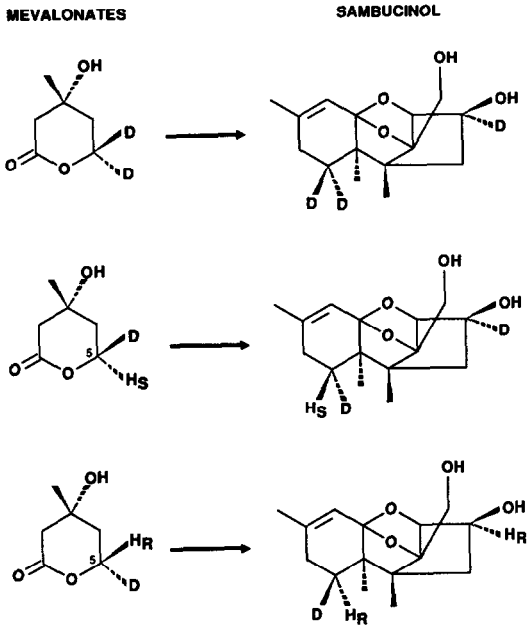


Figure 25

7.2 Trichodiene is a precursor to 3-acetyldeoxynivalenol and to sambucinol.

We have synthesized trichodiene and its isomer bazzanene with tritium as well as with deuterium at position 15 (nomenclature is according to 3-ADN). We fed them separately to F. culmorum and purified 3-ADN and SOL. The ^{2}H-NMR of 3-ADN and of SOL showed conclusively that trichodiene was incorporated into both 3-ADN and SOL at position C-15 as expected. On the other hand, even a very "hot" sample of bazzanene was neither incorporated into 3-ADN nor into SOL. (Fig. 26).

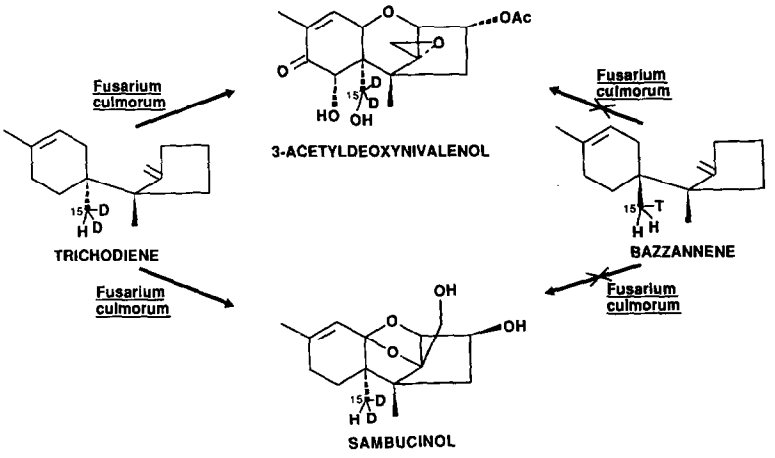


Figure 26

7.3. Isotrichodermin is a major precursor to 3-acetyldeoxynivalenol.

The next three sections represent results based on the kinetic pulse-labeling method. One of the radiolabeled plausible intermediates was purified extensively and was then re-fed to F. culmorum. After purification of the resulting 3-ADN to constant specific activity, this compound showed an incorporation of the order of 30%. On the other hand, the resulting sambucinol was unlabeled. As a further check the derived 3-ADN was deacetylated with base to deoxynivalenol (DON) which has a retention time on HPLC very different from 3-ADN. All the radioactivity was transferred to DON and remained with DON after successive purifications. Short term incubations corresponding to the timing obtained from the kinetic pulse-labeling profiles enabled us to isolate the metabolite corresponding to this radioactive intermediate. Characterization of this compound rigorously proved that it was isotrichodermin (Fig. 27).

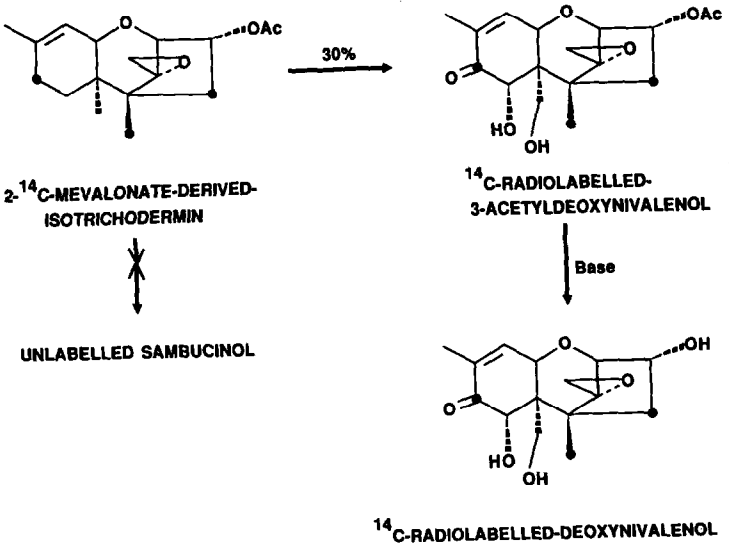


Figure 27

7.4 12,13-Epoxytrichotec-9-ene is a precursor to 3-acetyldeoxynivalenol and sambucinol.

Another intermediate detected from the kinetic pulse-labeling method was incorporated into 3-acetyldeoxynivalenol and sambucinol. Incubation with the timing assessed from the kinetic pulse labeling experiment enabled us to isolate this intermediate in appreciable quantities. The structure of this compound was shown from ^{1}H-NMR, ^{13}C-NMR and mass spectra to be 12,13-epoxytrichotec-9-ene. (Fig. 28).

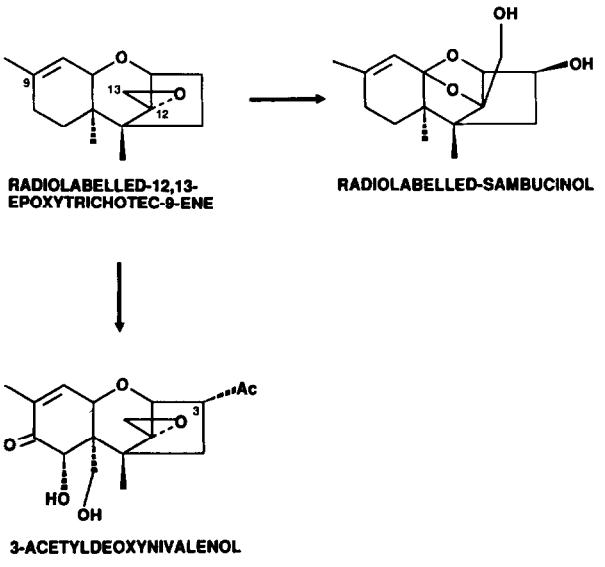


Figure 28

7.5 Apotricho-ol, a new metabolite is incorporated into sambucinol.

Another intermediate detected from the kinetic pulse-labeling method was incorporated into sambucinol albeit to a low extent (~ 2%). This metabolite is not incorporated into 3-acetyldeoxynivalenol. This new intermediate was characterized and shown to belong to the apotrichothecane series. It is the second natural metabolite with an apotrichothecane structure (14). It also has the trans junction between rings A and B. These results are shown in fig. 29.

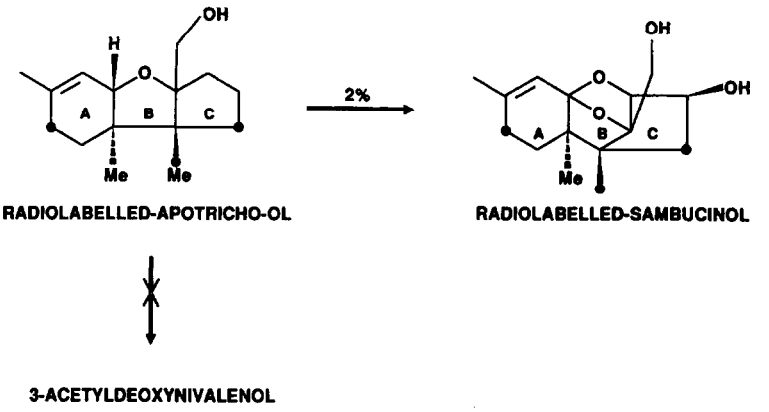


Figure 29

8. CONCLUSIONS

The kinetic pulse-labeling method was very fruitful in our laboratory to identify new intermediates. The main advantages are that it can: i) differentiate between plausible intermediates and dead end metabolites and ii) uncover unpredictable intermediates. Its main disadvantage is that the intermediates detected are present in tracer amounts (radioactive peaks) and therefore are difficult to characterize. However, due to the recent advances in spectroscopic techniques, one can now prove structures of compounds present in minute amounts. There are still unknowns in the biosynthesis of trichothecenes and no enzymes isolated after trichodiene synthetase. The order of the oxygenation steps is interesting and might shed some light on how to inhibit these mycotoxins.

9. ACKNOWLEDGMENTS

I acknowledge the substantial contributions to this research program of Drs K. Devor, Y. Nadeau, C.D. Nguyen, Ms A. Nikolakakis, M.J. Gauthier and L. Cousineau. These studies are supported by the National Science and Engineering Research Council of Canada (a research and HPLC equipment grants to L. O. Z. and a graduate student fellowship to A.N.).

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80. We would like to apologize about the misprints in refs 1 and 14 concerning the stereochemistry of the 3-hydroxy group in sambucinol. It should be β and not α as shown.