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REVISION OF THE STEREOCHEMISTRY IN TRICHODIOL, TRICHOTRIOL AND RELATED COMPOUNDS, AND CONCERNING THEIR ROLE IN THE BIOSYNTHESIS OF TRICHOTHECENE MYCOTOXINS

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Key Word Index—*Fusarium culmorum*; mycotoxin; trichothecene; biosynthesis; trichodiol; trichotriol.

Abstract—Trichodiol and trichotriol are shown to have 9β-hydroxyls (trichothecene numbering), not 9α-hydroxyls as quoted in the literature. The C-9 configurations in other related trichodiene derivatives also need reassignment. Compounds with a 9β-hydroxyl are readily converted into a mixture of 9β- and 9α-hydroxy isomers on treatment with acid, typically in a ratio of about 4 : 1. An allylic carbocation intermediate is postulated, and the same carbocation can be generated from 11-hydroxy analogues such as isotrichodiol. Cyclization to trichothecenes occurs in the presence of a 2-hydroxyl. The natural occurrence of both 9β- and 9α-isomers of trichodiol (in *Trichothecium roseum*) and of trichotriol (in *Fusarium culmorum*) suggests these result from acid-catalysed isomerization of isotrichodiol and isotrichotriol, respectively, via the appropriate carbocations. Accordingly, trichodiol and trichotriol are probably artefacts rather than biosynthetic intermediates on the pathway to trichothecenes. This is borne out by the results of competitive feeding experiments which indicate that the incorporation of isotrichodiol into trichothecenes is not influenced by trichodiol, and only marginally by trichotriol. Isotrichotriol did repress incorporations, so the pathway to trichothecenes is now proposed to proceed via isotrichodiol and isotrichotriol, not via trichodiol and trichotriol. Pre-sambucoin, a further product from acid-catalysed cyclization of isotrichodiol, is an efficient precursor of sambucoin in *Fusarium culmorum*. Isotrichotriol and the new metabolite 8α-hydroxyisotrichodiol were isolated from xanthotoxin-inhibited cultures of *F. culmorum*.

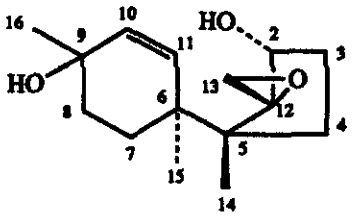
INTRODUCTION

For many years, trichodiol has been postulated to be an intermediate in the biosynthesis of the trichothecene mycotoxins [1–3]. More recently, the observed acid-catalysed cyclization of trichotriol to isotrichodermol (**24**) [4] has led to further proposals involving this intermediate as well as trichodiol. Trichodiol was first isolated from cultures of *Trichothecium roseum* [5] and assigned a structure **1** without characterizing stereochemistry at C-9 (trichothecene numbering), though assuming the stereochemistry at C-2 was analogous to that in the natural trichothecenes. In subsequent publications, this structure has been misrepresented as implying a 9α-hydroxyl [3, 4, 6–8], and the stereochemsitry of trichotriol, a metabolite from *Fusarium sporotrichioides* [4], was also assigned a 9α-hydroxyl analogous to that of trichodiol. This paper demonstrates that trichodiol and trichotriol both contain a 9β-hydroxyl, and that the configuration of a number of related natural products needs revision. Furthermore, there seems no evidence to support any involvement of trichodiol and trichotriol in the biosynthesis of trichothecenes, and a pathway via isotrichodiol (**18**) [9] and isotrichotriol (**23**) [10] is proposed to operate.

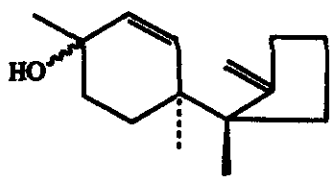
RESULTS AND DISCUSSION

Stereochemistry of trichodiene derivatives

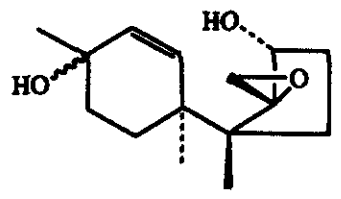
We have synthesized a range of trichodiene derivatives, including the 9β-hydroxy derivative **2b**, for testing as potential inhibitors of trichothecene biosynthesis in *Fusarium culmorum* [8]. Compound **2b** was synthesized according to a literature procedure [11] from 9β,10β-epoxytrichodiene (**14**), whose stereochemistry has been rigorously proven by conversion to the 10β-alcohol **15** (Scheme 1) [11]. Reaction of the epoxide with sodium phenyl selenide gave **16**, which on sodium periodate oxidation and thermolysis yielded the 9β-alcohol **2b** (Scheme 1), spectroscopically identical to that reported earlier [11]. NMR assignments (Tables 1 and 2) were confirmed by ^1H–^1H and ^1H–^{13}C COSY. If thermolysis was carried out by heating the reaction mixture in a sealed container at 75° instead of refluxing at 60°, then two products were formed, **2b** as the major (63%) and a minor product (11%). The minor product was identified as the 9α-alcohol **2a** by comparison of spectral data with those of **2b**. The only significant differences in spectral



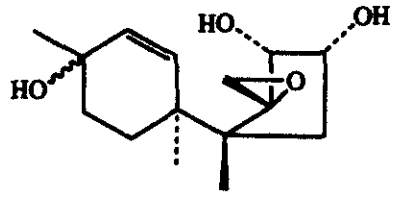
1 trichodiol (ref [1])



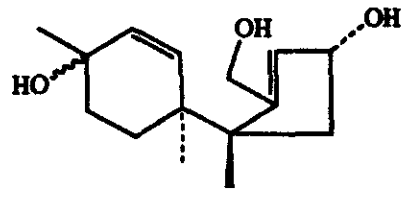
2a 9α-OH
2b 9β-OH



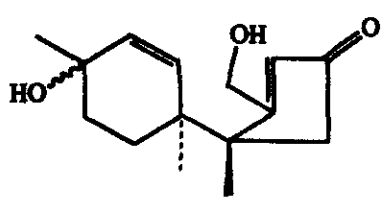
3a 9α-OH
3b 9β-OH } trichodiol



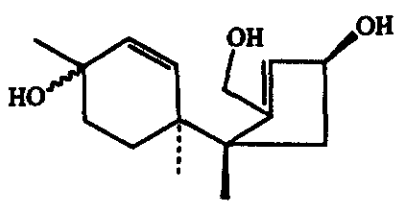
4a 9α-OH
4b 9β-OH } trichotriol



5a 9α-OH
5b 9β-OH } FS-2



6a 9α-OH
6b 9β-OH } FS-4



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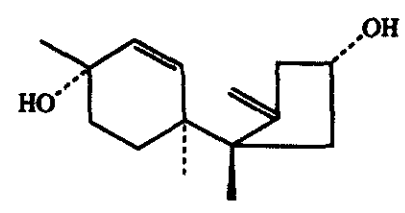

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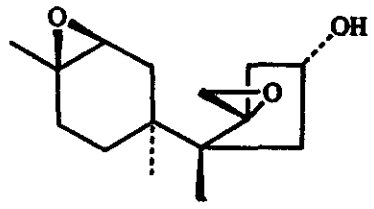

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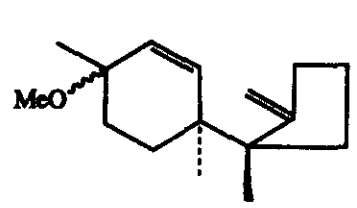
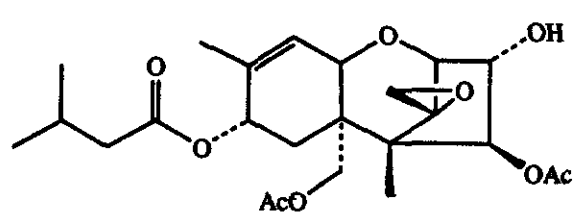
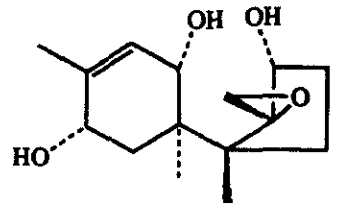


10a 9α-OMe
10b 9β-OMe



11 T-2 toxin



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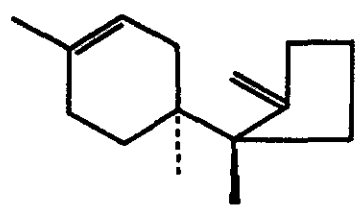
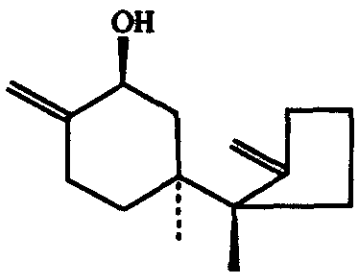


13 trichodiene

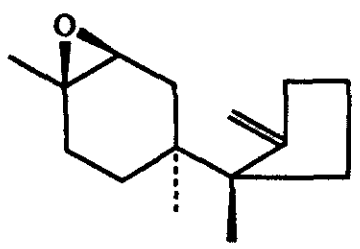
MCPBA



15

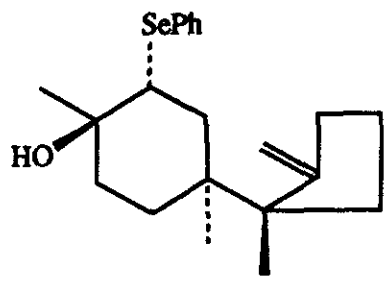
diethylaluminium tetramethylpiperidide

ref [10]



14

NaSePh



16

$NaIO_4$

2b

Δ

syn-elimination

Scheme 1. Synthesis of 9β-alcohol (**2b**).

Table 1. ^{13}C NMR chemical shift assignments for 9-hydroxytrichodiene derivatives

C	9β-Alcohol (**2b**)	9α-Alcohol (**2a**)	9β-Trichodiol (**3b**)	9β-Trichotriol (**4b**)	9α-Trichotriol (**4a**) [8]	FS-2 (**5b**) [4]	FS-4 (**6b**) [12]	'Trichodiol' (**8**) [4]
2	38.6	38.6	78.9	77.7	77.2	133.0	128.1	68.6
3	23.2	23.2	30.0	69.1	68.6	73.4	205	46.2
4	37.2	37.1	34.1	41.7	41.5	47.4	49	45.8
5	49.5	49.6	45.6	43.8	43.9	54.6	no	no
6	40.5	40.8	39.3	39.5	39.7	40.0	no	no
7	26.2	28.8	27.1	26.9	29.5	27.8	28.2	27.7
8	34.1	35.7	34.0	33.9	34.9	35.0	32.5	35.3
9	66.5	69.7	66.6	66.6	69.0	65.7	no	no
10	131.7	134.0	131.1	131.2	133.8	135.6	133.6	133.8
11	136.5	133.3	136.4	136.3	133.3	133.2	130.9	133.5
Δδ (C-10/C11)	4.8	0.7	5.3	5.1	0.5	2.4	2.7	0.3
12	159.8	159.4	71.5	69.4	69.2	154.0	183	no
13	106.6	106.7	50.1	50.0	50.0	60.7	62	50.7
14	24.3*	24.5*	18.9*	20.4*	20.4	21.3	19.2	19.6
15	20.2*	21.5*	21.2*	21.2*	22.0	22.0	18.0	22.0
16	29.7	27.7	30.7	30.7	27.3	30.9	29.0	29.6

Chemical shifts in ppm from TMS; spectra recorded in $CDCl_3$ soln at 100 MHz.
no = Not observed.
*Tentative assignments and may be interchanged.

data were the ^{13}C NMR resonances for ring A carbons, with C-9 appearing at 66.5 ppm in the 9β-alcohol and at 69.7 ppm in the 9α-alcohol, together with a rather greater separation of the C-10 and C-11 resonances (Δδ 4.8 ppm for 9β-alcohol as against 0.7 ppm for 9α-alcohol) (Table 1).

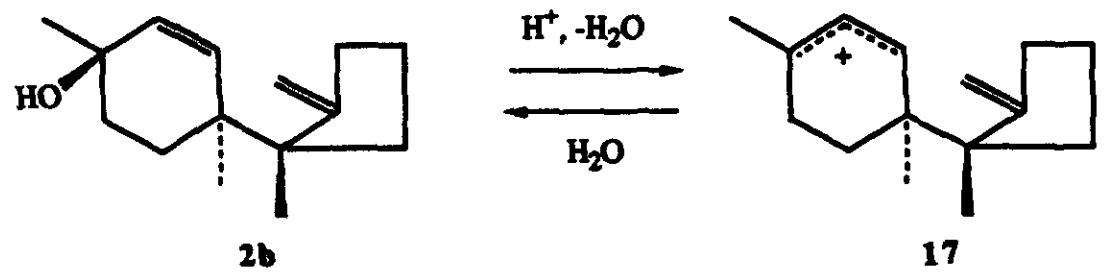
Samples of trichotriol (from *F. culmorum*) and trichodiol (from *T. roseum*) were found to have ^{13}C NMR signals clearly consistent with a 9β-hydroxyl (Table 1). Again, all assignments were confirmed by means of ^{1}H–^{1}H and ^{1}H–^{13}C COSY. These compounds, assigned with 9α-hydroxyls as **4a** and **3a**, respectively, according to the literature [4], must now be reformulated with 9β-hydroxyls, i.e. trichodiol (**3b**) and trichotriol (**4b**). Literature NMR data for other related structures are also presented in Tables 1 and 2. Based on ^{13}C NMR data for C-9, C-10 and C-11, there again is the indication that the wrong C-9 stereochemistry has been assigned. Thus the '9β-trichotriol' from *F. sporotrichioides* [8] is now reassigned as the 9α-hydroxy compound **4a**, whereas FS-2 (formulated as **5a**) from *F. sporotrichioides* [4] and FS-4 (formulated as **6a**) from *F. sambucinum* [12] are most likely the 9β-hydroxy derivatives **5b** and **6b**, respectively. In the case of FS-4, the chemical shift for C-9 is unreported, but the separation of C-10/C-11 resonances (2.7 ppm) is similar to that (2.4 ppm) for FS-2, where the C-9 stereochemsitry is defined by the shift for C-9 (65.7 ppm). A related metabolite 3-epi-FS-2 (**7**) isolated from *F. sporotrichioides* [12] has been assigned with a 9α-hydroxyl, though spectroscopic data have not been reported. A further inaccuracy in the literature is the identity of 'trichodiol' isolated from *F. sporotrichioides* [4]. ^{13}C and ^{1}H NMR data [4], though incomplete, fully characterize this compound as the 3α,9α-diol **8**. The substitution in the B ring is analogous to that of 3α-hydroxy-9β,10β;12,13-diepoxytrichodiene (**9**) isolated as a biotransformation product from 9β,10β;12,13-diepoxytrichodiene [13], and gives exactly equivalent NMR signals. Whilst the ^{13}C NMR data for the 9β- and 9α-alcohols are distinctive, ^{1}H NMR signals for compounds **2–6** show few pointers to the critical stereochemistry at position 9. Where both alcohols are available for study, signals for H-10 and H-11 appear to be spaced rather further apart in the 9β-alcohols than in the 9α-alcohols.

The formation of the 9α-alcohol **2a** during the thermolysis reaction is likely to be the result of solvolysis of the 9β-alcohol to the allylic cation **17**, followed by regiospecific addition of water from either of the two faces (Scheme 2). Indeed, when the 9β-alcohol **2b** was heated in aqueous acid [HCl (pH 4)/75°/90 min], a mixture of **2b** and **2a** in a proportion approximately 3:1 similarly resulted, indicating preferential attack of the nucleophile from the less-hindered β-face. When **2b** was treated in acidic methanol, two 9-methoxy analogues **10b** and **10a** in the proportion approximately 4:1 were similarly formed. Solvolysis of the 11α-alcohol isotrichodiol (**18**) under acid conditions (phosphate buffer pH 5.8, six days) produced the 9β- and 9α-alcohols **3b** and **3a** in the proportion 4:1, demonstrating formation of an analogous allylic cation (**19**) and then regiospecific attack of water nucleophile (Scheme 3). This regiospecificity may arise from the tertiary/secondary nature of C-9/C-11, respectively, influencing relative cationic character, together with the bulky B ring hindering approach of nucleophile to C-11. In the reaction with isotrichodiol, the cyclized trichothe-

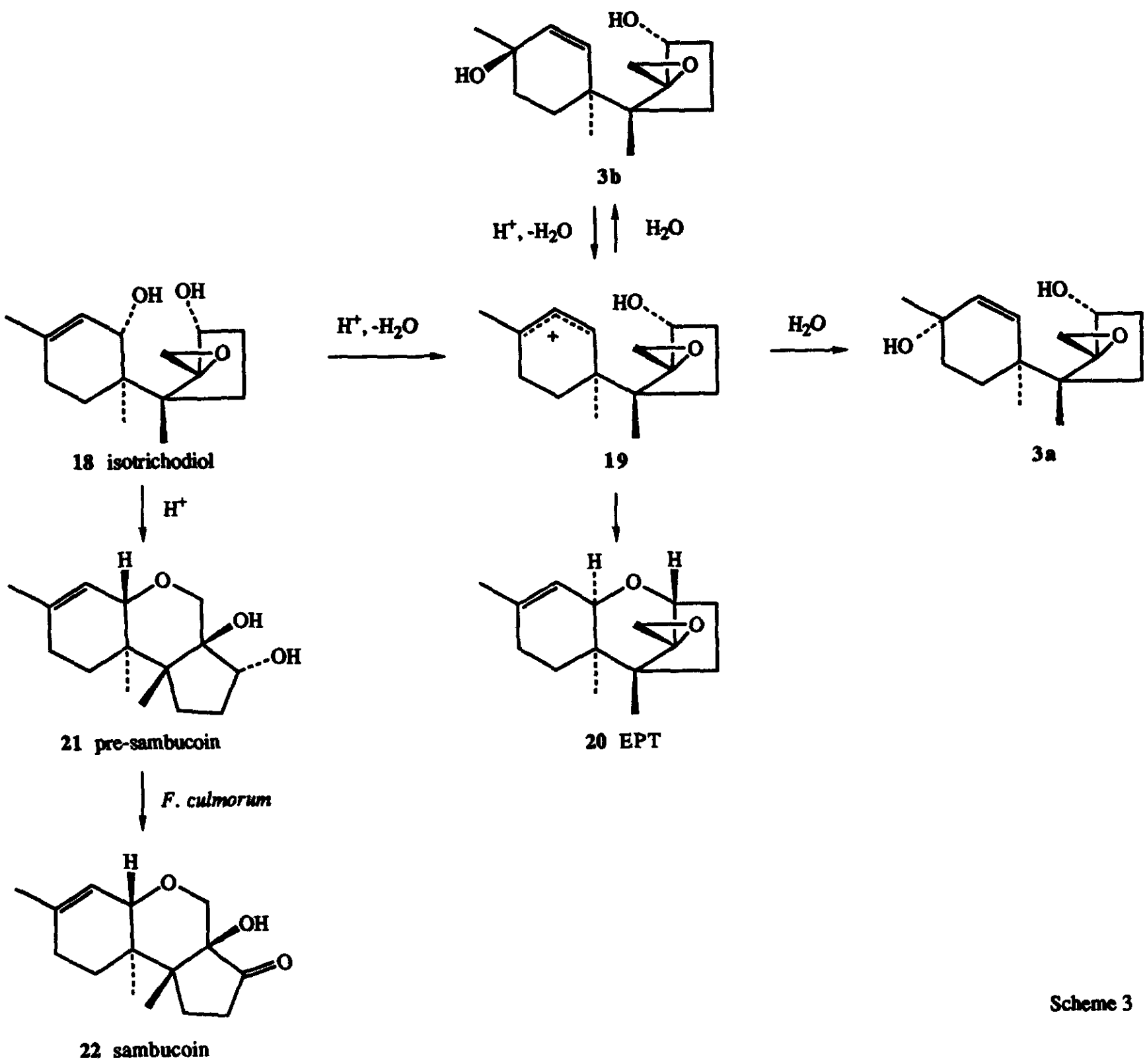
Table 2. ^{1}H NMR chemical shift assignments and coupling constants for 9-hydroxytrichodiene derivatives

H	9β-Alcohol (**2b**)	9α-Alcohol (**2a**)	9β-Trichodiol (**3b**)	9β-Trichotriol (**4b**)	9α-Trichotriol (**4a**)	FS-2 (**5b**) [4]	FS-4 (**6b**) [12]	'Trichodiol' (**8**) [4]
2α	2.27 *m*					5.75 *dd* (3.3, 1.5)	6.27 *t* (1.5)	
2β			3.78 *dd* (5.1, 3.0)	3.68 *d* (4.6)	3.73 *d* (4.7)*			4.49 *m*
3α	1.66 *m*		1.62 *dddd* (13.3, 7.2, 3.1, 3.0)					2.06 *dd* (14, 5)
3β	1.42 *m*		1.97 *dddd* (13.2, 11.2, 7.2, 5.2)	4.25 *ddd* (8.2, 6.4, 4.6)*	4.31 *m*	4.79 *m*		1.85 *m*
4α	1.90 *m*	1.34–1.43 (3H, *m*)	2.24 *ddd* (12.0, 12.0, 7.3)	2.12 *dd* (13.2, 8.0)	2.05 *dd* (13.2, 8.1)	1.89 *m*	2.15 *d* (19.0)	1.45–1.85 *m*
4β	1.42 *m*	1.65–1.80 (5H, *m*)	1.48 *ddd* (12.9, 7.2, 2.9)	1.75 *dd* (13.2, 6.3)		2.10 *m*	2.73 *d* (19.0)	
7α	1.40 *m*	2.20–2.33 (2H, *m*)	1.32 *m*	1.32 *m*		1.31 *m*	1.40 *m*	
7β	1.92 *m*		1.68–1.81 *m*	1.80 *m*	1.4–1.8 *m*	1.75 *m*	1.70 *m*	1.46–1.85 *m*
8α	1.66 *m*		1.68–1.81 *m*	1.67–1.73 *m*		1.73 *m*	1.70 *m*	
8β						1.98 *m*	1.85 *m*	
10	5.59 *dd* (10.2, 1.2)	5.52 *d* (10.4)	5.55 *d* (10.2)	5.54 *d* (10.2)	5.5 *m*	5.58 *d* (10.2)	5.52 *d* (10)	5.53 *d* (10)
11	5.77 *dd* (10.2, 1.4)	5.65 *dd* (10.4, 1.8)	5.71 *br d* (10.2)	5.71 *br d* (10.2)	5.5 *m*	5.67 *dd* (10.2, 1.7)	5.61 *dd* (10, 1.5)	5.47 *dd* (10, 1.5)
13	4.83 *br s*	4.80 *br s*	2.92 *d* (4.0)	2.99 *d* (4.0)	3.06 *d* (4.3)	4.35 *br d* (14.5)	4.69 *d* (17)	3.25 *d* (4)
	4.99 *br s*	4.98 *br s*	3.57 *d* (4.0)	3.53 *d* (4.0)	3.29 *d* (4.3)	4.23 *br d* (14.5)	4.35 *d* (17)	2.76 *d* (4)
14	1.07 *s*	1.02 *s*	1.04 *s*	1.08 *s*	1.07 *s*	1.09 *s*	1.29 *s*	1.04 *s*
15	0.97 *s*	1.04 *s*	0.94 *s*	0.89 *s*	1.01 *s*	0.95 *s*	0.98 *s*	0.98 *s*
16	1.27 *s*	1.27 *s*	1.29 *s*	1.28 *s*	1.28 *s*	1.27 *s*	1.25 *s*	1.28 *s*

Chemical shifts in ppm from TMS; coupling constants (in parentheses) in Hz; spectra recorded in $CDCl_3$ solution at 250 or 400 MHz.
*After MeOD shake.



Scheme 2. Isomerization of 9β- and 9α-alcohols via allylic carbocation.



Scheme 3

Scheme 3. Acid-catalysed transformations of isotrichodiol and trichodiol.

cene system 12,13-epoxytrichothec-9-ene (EPT, **20**) was also formed via intramolecular attack of the 2-hydroxyl in the B ring system, together with compound **21** (presambucoin, see below). 9β-Trichodiol (**3b**) in phosphate buffer (pH 5.0/28°/five days) similarly gave EPT in 64% yield. The acid-catalysed formation of isotrichodermol (**24**) from isotrichotriol (**23**) has also been noted [8], and the appearance/disappearance of trichotriol (9β-hydroxy: **4b**) during the reaction was interpreted as indicating an intermediate role for **4b**. The present results argue strongly for generation of an allylic cation from **23**, with **4b** and **24** arising by nucleophilic attack, **4b** predominating initially due to the large excess of solvent, but then being converted into **24** via the same cation.

Biosynthesis of trichothecenes

These results now call into question the natural occurrence and biosynthetic roles of the 9β-alcohols trichodiol (**3b**) and trichotriol (**4b**). Logically, isotrichodiol (**18**) and isotrichotriol (**23**) could be converted enzymically into EPT (**20**) or isotrichodermol (**24**), respectively, by a one-step process, for which an allylic cation is transiently formed, though probably not released from the enzyme. Trichodiol (**3b**) and trichotriol (**4b**) could then be formed as shunt metabolites as a direct result of pH changes in culture media (which typically become more acidic as growth progresses) leading to transformations of **18** or **23**. To support this hypothesis, it is pertinent to note that both 9β- and 9α-trichotriols **4b** and **4a** have been isolated from *F. sporotrichioides* [8, 10], and the trichodiol isolated from *Trichothecium roseum* can be demonstrated by GC-mass spectrometry to contain a small amount (approx. 5%) of the isomeric 9α-trichodiol (see Experimental). Furthermore, trichotriol levels in cultures of *F. culmorum* are observed to diminish markedly if the pH of the culture medium is maintained at 7.0. Isotrichotriol and both isomers of trichotriol can all be isolated from cultures of *F. culmorum*, amounts of each material varying according to culture conditions (see Experimental).

In a series of supplementation experiments using a *F. sporotrichioides* mutant [8], isotrichotriol (**23**) and both trichotriols **4a** and **4b** have been shown to lead to a resumption of T-2 toxin (**11**) production. All three compounds are capable of yielding the same allylic cation, or alternatively, both **4a** and **4b** could be incorporated via chemical cyclization to isotrichodermol (**24**), a demonstrated precursor of T-2 toxin [8]. Significantly, supplying trichodiol (**3b**) did not restore T-2 toxin production. Although similar chemical cyclization of trichodiol to EPT is possible (see above), EPT is typically metabolized by species of *Fusarium* to trichothecene-related materials such as sambucinol, and oxygenated trichothecenes arise via isotrichodermol [14]. 3-Hydroxylation thus occurs before cyclization to the trichothecene. The inability of trichodiol to restore trichothecene biosynthesis indicates neither trichodiol nor the derived carbocation could be 3-hydroxylated in the biological system.

To test these proposals, comparative feedings of [^{14}C]isotrichodiol and [^{14}C]9β-trichotriol to *F. culmorum* cultures were performed. TLC-autoradiograms of the toxin fraction (Fig. 1, lane 2) showed almost complete conversion of isotrichodiol into trichothecenes, primarily 3-acetyldeoxynivalenol (3-AcDON, **26**) and dihydroxycalonectrin (DHC, **25**), as demonstrated earlier [9]. Some 37% of the applied activity was associated with these two compounds. Trichotriol was also incorporated, primarily

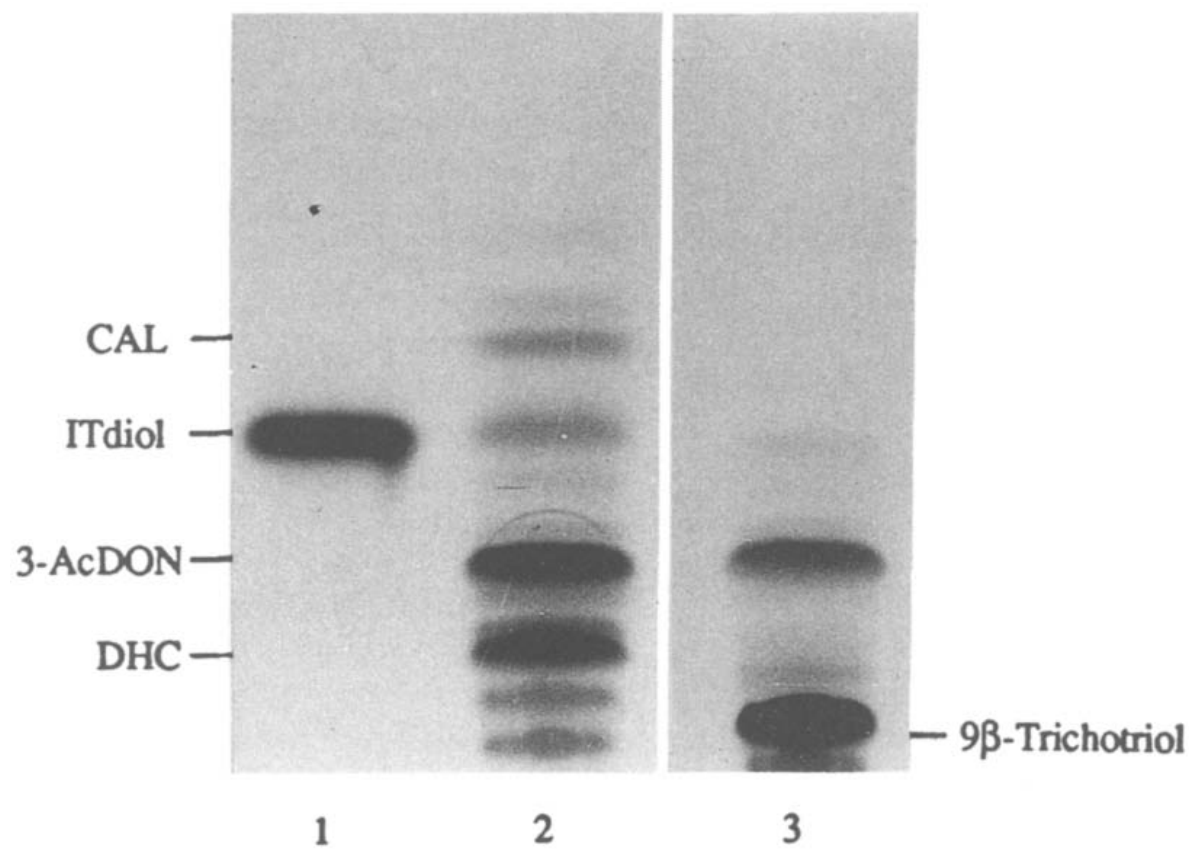


Fig. 1. TLC-autoradiograms showing conversion of [^{14}C]isotrichodiol and [^{14}C]9β-trichotriol into trichothecenes. Lane 1, ısotrichodiol standard; lane 2, fed ısotrichodiol; lane 3, fed 9β-trichotriol. Solvent: hexane–EtOAc (1 : 1).

into 3-AcDON, but over the feeding period (7 hr), conversion was markedly less (Fig. 1, lane 3), with only 6.9% of the applied activity incorporated. Most of the applied material was recovered unchanged. Whilst this difference could be due to transport difficulties, it is more likely to be the result of slow chemical conversion to isotrichodermol before incorporation. Indeed, when [^{14}C]trichotriol was incubated with the crude cell-free system from *F. culmorum* capable of mediating the epoxidation of 9β,10β-epoxytrichodiene [15], the amount of cyclization to isotrichodermol observed over 2 hr (5.0%) was almost identical to that recorded (5.2%) when a boiled extract was incubated under the same conditions. Some of the transformation must be attributed to chemical cyclization during the work up, but the results clearly show no additional contribution from a possible enzymic cyclization via the cell-free system.

In the absence of suitable labelled samples of trichodiol and isotrichotriol, and with only limited amounts of these materials available, their role was established in a different manner. Their ability to suppress the incorporation of [^{14}C]isotrichodiol into trichothecenes was examined. Samples (0.5 mg) of the unlabelled material were fed to cultures of *F. culmorum* 30 min before the labelled isotrichodiol (0.5 mg). Cultures were worked up after 7 hr, and the labelling profiles examined by TLC-autoradiography (Fig. 2). These showed no significant changes relative to the control isotrichodiol incorporation (lanes 2 and 5) when trichodiol was fed (lane 3), and a small inhibition when trichotriol was used (lane 4). With isotrichotriol (lane 6), however, there was a distinct change in labelling pattern so that DHC became more prominent than in the control, and the amount of labelled trichotriol was enhanced. Since DHC is a demonstrated precursor of 3-AcDON [13], this indicates the unlabelled isotrichotriol is entering the biosynthetic pathway, thus delaying the incorporation of isotrichodiol and allowing a build-up of labelled DHC rather than 3-AcDON. Percentage incorporations for isotrichodiol into 3-AcDON and DHC were measured and are given in Table 3. These show that the presence of trichodiol and trichotriol produced relatively small effects on incorporations, but isotrichotriol caused a marked increase in DHC incorporation relative to 3-AcDON. Furthermore, TLC analysis also showed that whereas the added unlabelled trichodiol and trichotriol were largely recovered unchanged from these feedings, all the unlabelled isotrichotriol had been metabolized. The smaller effects associated with trichotriol can probably be ascribed to chemical cyclization to isotrichodermol during the experiment. In a further set of experiments, the amount of unlabelled substrate was increased to give a 6:1 ratio over labelled isotrichodiol, and thus intensify any inhibitory effects. Again, trichodiol caused no

Table 3. Effects of isotrichotriol, trichotriol and trichodiol on incorporation of [^{14}C]isotrichodiol into trichothecenes*

Inhibitor	Lane (Fig. 2)	% Incorporation isotrichodiol 3-AcDON	DHC
Experiment 1			
None	2	23.0	14.3
9β-Trichodiol	3	29.4	16.2
9β-Trichotriol	4	23.9	12.3
Experiment 2			
None	5	21.8	7.2
Isotrichotriol	6	18.5	17.7

*[^{14}C]Isotrichodiol (0.5 mg) fed 15 min after 0.5 mg substrate; experiment worked up after 7 hr.

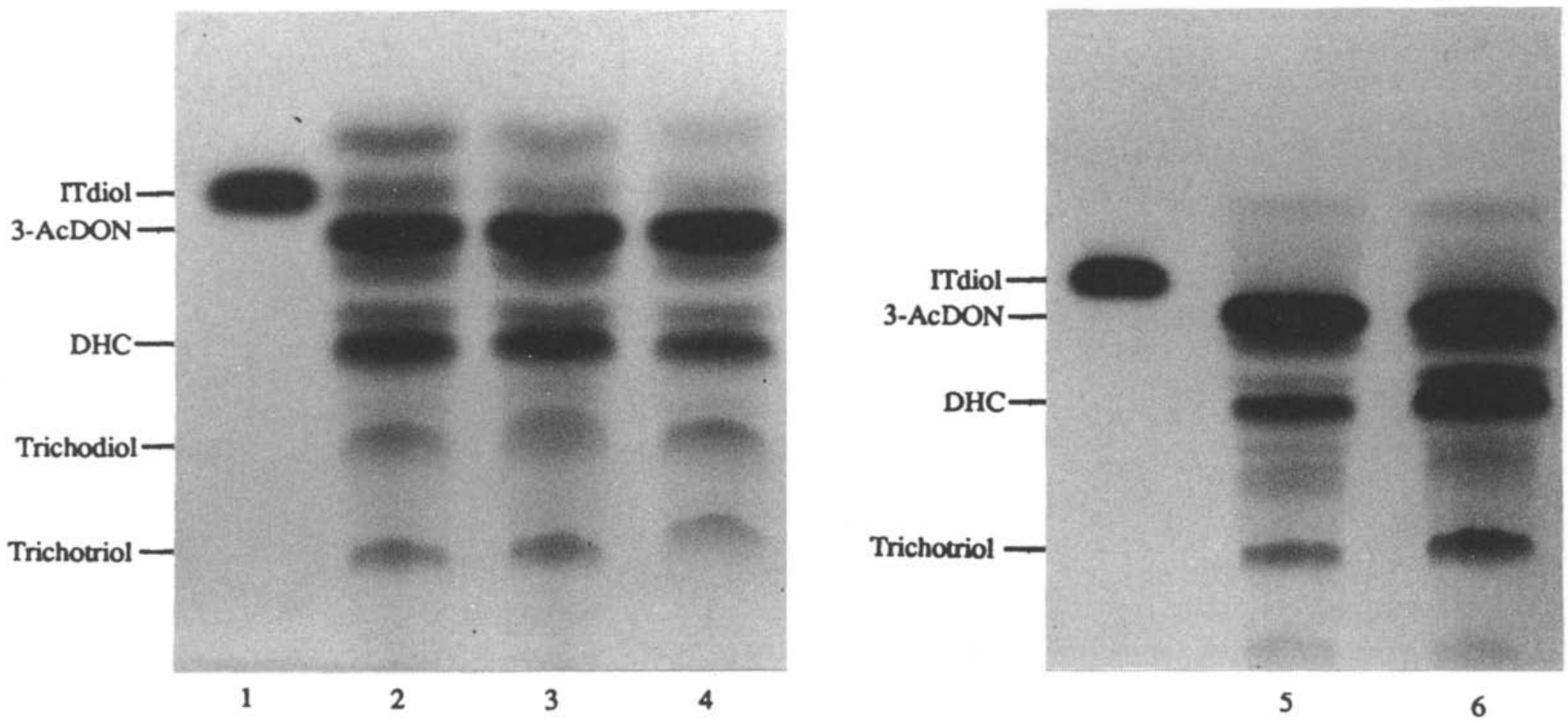


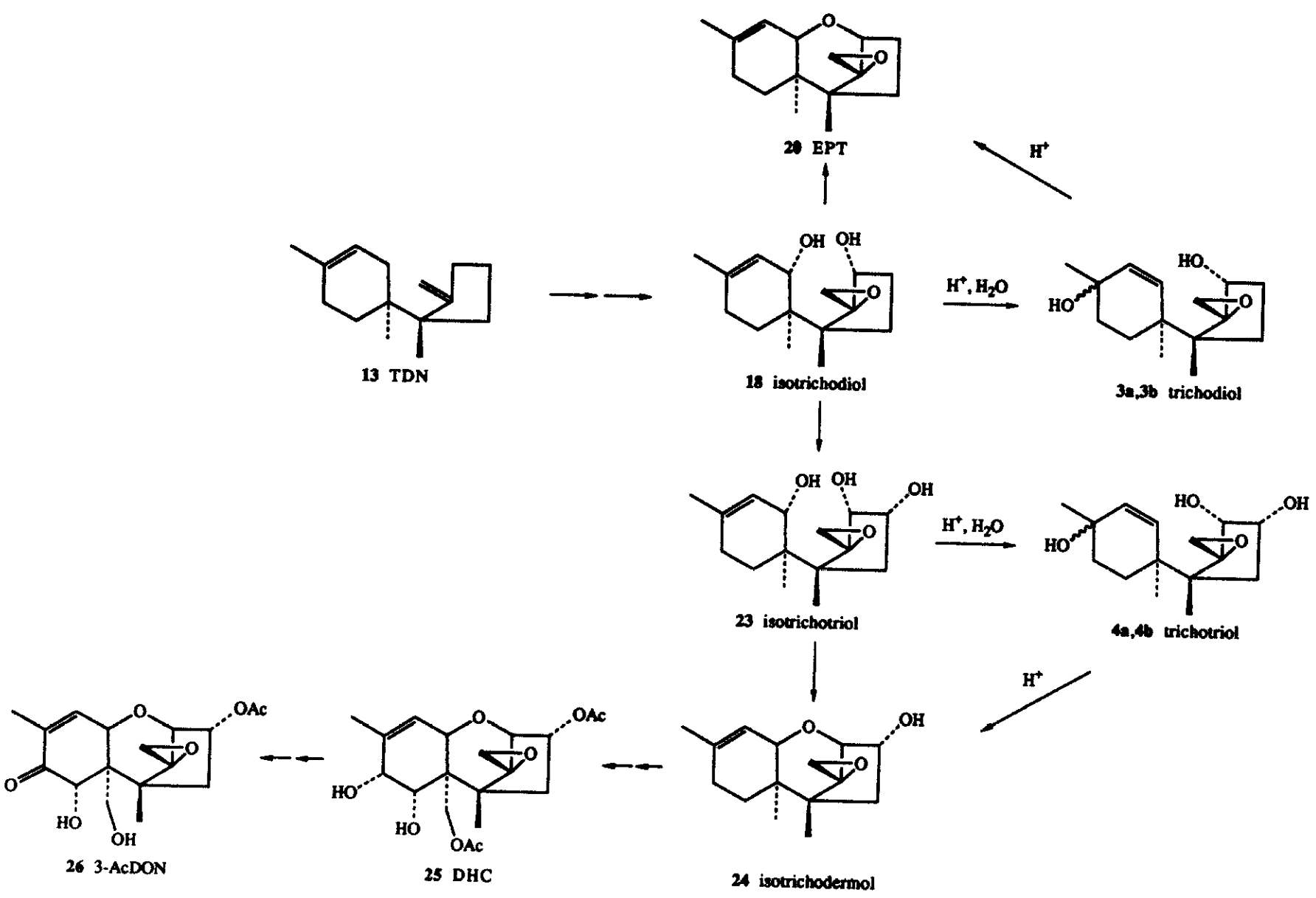
Fig. 2. TLC-autoradiograms showing effects of 9β-trichodiol, 9β-trichotriol and isotrichotriol on incorporation of [^{14}C]isotrichodiol into trichothecenes. Lane 1, isotrichodiol standard; lane 2, control; lane 3, 9β-trichodiol; lane 4,9β-trichotriol; lane 5, control; lane 6, isotrichotriol. Solvent: Et_2O–Me_2CO (9 : 1).

changes relative to the uninhibited control, whilst a small inhibition occurred with trichotriol. Isotrichotriol caused much more inhibition however, with considerably weaker bands for 3-AcDON and DHC in the autoradiogram, and significantly, an increase in activity associated with isotrichodiol, comparable to the effects of unlabelled isotrichodiol itself. Whilst these experiments cannot be as definitive as direct feeding experiments with labelled precursors, or the appropriate enzyme studies, they do support the argument that neither trichodiol nor trichotriol are intermediates in the biosynthesis of trichothecenes, and a pathway via isotrichodiol and isotrichotriol is both more plausible and more probable. Therefore, we now propose that isotrichodiol is transformed into oxygenated trichothecenes in *F. culmorum* via the pathway shown in Scheme 4. In combination with the Schemes given in the previous paper [13], this now provides a plausible complete sequence to 3-AcDON from trichodiene.

Pre-sambucoin (**21**) *as a precursor of sambucoin* (**22**)

GC-mass spectral analysis of the products obtained by incubation of isotrichodiol with phosphate buffer (pH 5.9) showed the formation of 9β- and 9α-trichodiols, EPT and one further compound. In a larger scale experiment, this material was isolated in 17% yield and was characterized as **21**.

The EI mass spectrum showed a molecular ion at *m/z* 252 consistent with a formula of $C_{15}H_{24}O_3$, and indicating that, unlike EPT, it had been formed from isotrichodiol (also $C_{15}H_{24}O_3$) without loss of H_2O. The base peak in the spectrum was at *m/z* 124, with no peak at *m/z* 84, the base peak in isotrichodiol produced by retro Diels-Alder fragmentation in ring A. Comparison of the ^{1}H NMR spectrum with that of isotrichodiol indicated that both compounds have similar A ring structures. The C-16 methyl (trichothecene numbering) is a broad singlet at δ 1.68 indicating that the 9,10-double bond is still present, and H-10 and H-11 are broad singlets at δ 5.25 and 4.15, respectively, demonstrating retention of α-oxygenation at C-11. H-11 is shifted upfield from δ 4.60 in isotrichodiol and this, together with the absence of a peak at *m/z* 84 in the EI mass spectrum, indicates that the 11α-hydroxyl group is no longer present and is consistent with the formation of an ether link from C-11α to the B ring of isotrichodiol. The epoxide protons in isotrichodiol at δ 3.06 and 3.32 are no longer present, and the C-13 protons now appear as AB doublets upfield at δ 3.75 and 4.11 (J_{AB} = 12.5 Hz) indicating that the 12,13-epoxide has been opened, and is consistent with the formation of an oxygen bridge between C-11α and C-13, i.e. structure **21**. The resonance at δ 4.09 is due to H-2, indicating that the 2α-hydroxyl group has been retained. A full assignment of the proton NMR data was achieved using COSY. The ^{13}C NMR data, although incomplete due to the small



Scheme 4. Proposed biosynthetic pathway to trichothecene mycotoxins via isotrichodiol and isotrichotriol.

sample size, are in full agreement with the structure **21**. They show two methines next to oxygen at δ 80.7 (C-2) and 71.6 (C-11), a vinylic methine at δ 122.9 (C-10), and a methylene next to oxygen at δ 67.8 (C-13). The three methyl resonances, and four methylene resonances could not be fully assigned from the data available, and only three of the four quaternary carbons were located.

Confirmation of the structure was obtained when a sample was oxidized using dimethylsulphoxide–trifluoroacetic anhydride [16] yielding sambucoin (**22**) (40%), spectrally (^{1}H NMR and EI mass spectrometry) identical to the natural product isolated from *Fusarium sambucinum* [17, 18] and *F. culmorum* [19]. Because of its structural and biosynthetic relationship to sambucoin, **21** has been given the trivial name pre-sambucoin. Its formation from isotrichodiol is a result of cyclization by intramolecular attack of the 11α-hydroxyl on to C-13 of the epoxide (Scheme 3).

In order to investigate the biosynthesis of sambucoin, a ^{14}C-labelled sample of pre-sambucoin was prepared from [^{14}C]isotrichodiol by cyclization at pH 5.0. This material (1.0 mg) was fed to a growing culture of *Fusarium culmorum* (72 hr after subculture) and incubated for 18 hr. The culture was then harvested by extraction of the filtrate with ethyl acetate, and the extract spiked by addition of unlabelled sambucoin to facilitate isolation of product. An incorporation of [^{14}C]pre-sambucoin into [^{14}C]sambucoin of 7.1% was recorded. This indicates that pre-sambucoin is most probably a biosynthetic precursor of sambucoin, and supports a sequence from isotrichodiol via cyclization to pre-sambucoin (compare ref. [17]).

8α-*Hydroxyisotrichodiol* (**12**)

This novel compound was isolated from a xanthotoxin-inhibited culture of *F. culmorum* along with trichodiene (TDN, **13**), isotrichotriol (**23**), and 9α-trichotriol (**4a**) (see Experimental). Characterization was achieved by comparison of EI mass spectral and ^{1}H NMR data with those previously recorded for isotrichodiol [9]. The EI mass spectrum shows a molecular ion at *m/z* 268 consistent with a molecular formula $C_{15}H_{24}O_4$, 16 amu heavier than isotrichodiol. The base peak at *m/z* 100 is consistent with a retro Diels-Alder fragment ion $C_5H_8O_2$ and indicates the presence of two hydroxyl groups in the A ring. The ^{1}H NMR spectrum is similar to that for isotrichodiol with H-2β as a doublet ($J_{2\beta,3\beta}$ = 4.0 Hz) at δ 3.65, and H-4α a doublet of triplets at δ 2.72 ($J_{4\alpha,4\beta} \approx 13$ Hz, $J_{4\alpha,3\alpha} \approx 7$ Hz, $J_{4\alpha,3\beta} \approx 13$ Hz) showing a 2α-hydroxyl in the B ring. The significant differences are the appearance of a resonance at δ 3.98, and downfield shifts for the H-10 signal from δ 5.14 to 5.33 and the C-16 methyl resonance from δ 1.66 to 1.81, all of which are consistent with the introduction of a hydroxyl group at C-8. The two A ring hydroxyls are therefore at C-11α and C-8, with the stereochemistry of the hydroxyl at C-8 being assigned as α from the multiplicity of the H-8 resonance. It is a broadened doublet with $J_{8\beta,7\beta}$ = 5.1 Hz, and $J_{8\beta,7\alpha} \approx 0$ Hz as predicted from molecular models, and similar to the coupling found in 8α-hydroxyisotrichotriol [8]. Both 8α- and 8β-hydroxyisotrichotriols have been reported as metabolites from a mutant strain of *F. sporotrichioides* [8]. Neither of these compounds could restore T-2 toxin production when fed to a trichodiene-accumulating *F. sporotrichioides* mutant, suggesting they are not on a direct pathway to trichothecenes. Since 8-hydroxyisotrichodermin has been found in several species of *Fusarium* including *F. culmorum* [20], 8α-hydroxyisotrichodiol may be involved in the biosynthesis of this compound, or of minor trichothecene-related metabolites such as 8α-hydroxysambucoin [18] or the range of 2α,8α-dihydroxy-11-epi-apotrichothecenes isolated from *Fusarium* species [21]. Alternatively, it may be a dead-end metabolite produced simply by hydroxylation of isotrichodiol.

EXPERIMENTAL

General. General techniques for culture of *Fusarium culmorum* CMI 14764, isolation and centrifugal TLC (CTLC) of metabolites are described in the previous paper [13]. GC was carried out using a capillary column (J & W, DB-5; 30 m × 0.25 mm), with He carrier gas (flow rate 1 ml min^{-1}), an injection temp. of 300°, and a temp. programme of 1 min at 100°, then 10° min^{-1} to 270°. Detection was by FID or EIMS (70 eV). Peak heights were used to assess relative amounts of metabolites in GC analyses. Production of [^{14}C]isotrichodiol [9] and [^{14}C]9β-trichotriol [13] have been reported earlier.

9β-Hydroxy- and 9α-hydroxy-tricho-10,12-*diene* (**2b**)/(**2a**). A soln of 9β-hydroxy-10α-phenylselenotrichodiene (**16**, 120 mg) [11, 13] in THF (10 ml) at 0° was treated with a soln of $NaIO_4$ (202 mg) in H_2O (4 ml). The reaction mixt. was stirred at 0° for 1.5 hr, then heated in a sealed Reactivial at 75° for a further 3 hr. After cooling, the mixt. was treated with satd aq. NaCl (30 ml) and extracted with Et_2O (3 × 40 ml). The combined extracts were washed with satd aq. NaCl (50 ml), dried over $MgSO_4$ and evapd to an oil under red. pres. Purification by CTLC (1-mm plate) in hexane–Et_2O (1:3) collecting 1-ml fractions yielded **2b** (44.5 mg, 63%) in frs 10–16 as waxy crystals, mp 33–35°. EIMS *m/z* (rel. int.): 202 [M $-H_2O]^+$ (7), 187 (5), 125 (28), 107 (100), 96 (42), 95 (44), 91 (29), 81 (37). Frs 18–22 yielded **2a** (8.0 mg, 11%) as crystals, mp 87–89°. EIMS *m/z* (rel. int.): 202 [M $-H_2O]^+$ (17), 125 (72), 107 (100), 96 (39), 95 (59), 91 (33), 81 (55).

9α-Hydroxytricho-10,12-*diene* (**2a**). A soln of 9β-hydroxytricho-10,12-diene (3.1 mg) in THF–H_2O (1:1; 4 ml) was adjusted to approx. pH 4 by addition of HCl (1 M, 20 μl) and the mixt. heated in a Reactivial at 75°. TLC analysis (hexane–Et_2O, 1:3) indicated that after 1.5 hr 9α-tricho-10,12-diene was present, with the ratio of β:α being approx. 3:1. The reaction was monitored periodically for up to 15 hr, but no observable change in this ratio was detected.

9β-Methoxy- and 9α-methoxy-tricho-10,12-*diene* (**10b**/**10a**). (a) A soln of 9β-hydroxy-10α-phenylselenotrichodiene (100 mg) in THF (5.4 ml) at 0° was treated with a soln of $NaIO_4$ (183 mg) in 70% aq. MeOH (6 ml).

The reaction mixt. was stirred at 0° for 1.5 hr then heated in a sealed Reactivial at 75° for a further 2.5 hr, then worked up as above. TLC analysis (hexane–Et_2O, 1:3) indicated the formation of a major product (R_f 0.6) and two minor products (R_f 0.45 and 0.35). These were purified by CTLC (1-mm plate) eluting with hexane–Et_2O (1:3) and collecting 0.5-ml fractions. Frs 29–32 gave 9β-hydroxytricho-10,12-diene (5.5 mg, 9%), and frs 34–36 yielded 9α-hydroxytricho-10,12-diene (3 mg, 5%). Frs 17–20 yielded 9-methoxytricho-10,12-diene (15 mg, 24%) which was shown by ^{1}H NMR to be a mixture of 9β-methoxy- and 9α-methoxy-tricho-10,12-diene in a ratio of 17:3. The two methoxy compounds were resolved by TLC in hexane–Et_2O (17:3). 9β-Methoxytricho-10,12-diene: ^{1}H NMR ($CDCl_3$): δ 5.87 (1H, *dd*, J = 10.4, 1.7 Hz, H-11), 5.56 (1H, *dd*, J = 10.4, 1.7 Hz, H-10), 4.97 (1H, *br s*, H-13), 4.82 (1H, *br s*, H-13), 3.20 (3H, *s*, OMe), 2.2–2.3 and 1.2–2.0 (2H and 8H, *m*, H-2, H-3, H-4, H-7, H-8), 1.19 (3H, *s*, H-16), 1.06 and 0.95 (each 3H, *s*, H-14, H-15); EIMS *m/z* (rel. int.): 219 [M − Me]$^+$ (1), 202 (12), 187 (5), 156 (5), 139 (52), 108 (40), 107 (100), 96 (38), 95 (51). 9α-Methoxytricho-10,12-diene: ^{1}H NMR ($CDCl_3$) [additional signals visible in spectrum of **10b**] δ: 5.83 (1H, *dd*, J = 10.4, 1.7 Hz, H-11), 5.52 (1H, *dd*, J = 10.4, 1.7 Hz, H-10), 3.22 (3H, *s*, OMe).

(b) A soln of 9β-hydroxytricho-10,12-diene (2 mg) in THF–70% aq. MeOH (1:1, 2 ml) was adjusted to approx. pH 4 by addition of HCl (1 M, 15 μl) and the mixt. heated in a Reactivial at 75° for 1.5 hr. TLC analysis (hexane–Et_2O, 1:3) indicated that about 50% of the starting material had reacted producing 9-methoxytricho-10,12-diene. Further TLC analysis (hexane–Et_2O, 17:3) indicated that this was a mixt. of the 9β- and 9α-methoxy isomers in a ratio of approx 4:1.

Acid-catalysed cyclization of isotrichodiol (**18**). (a) Isotrichodiol (2.0 mg) [9] in EtOAc (0.3 ml) was added to KPi buffer (100 mM; pH 5.8: 12 ml) and incubated at 28° for 6 days. The products were extracted with EtOAc (20 ml), and the extract dried over $MgSO_4$ and evapd to an oil under red pres. Following trimethylsilyation (BSTFA) this was analysed by GC-MS, demonstrating the presence of EPT (R_t 3.41 min) {EIMS *m/z* (rel. int.): 234 [M]$^+$ (10), 219 (24), 178 (2), 163 (11), 149 (6), 142 (5), 135 (7), 119 (15), 107 (60), 93 (69), 91 (63), 41 (100)}; the di-TMS ether of residual isotrichodiol (R_t 5.18 min) {EIMS *m/z* (rel. int.): 381 [M − Me]$^+$ (0.3), 339 (0.9), 313 (0.3), 306 (1), 291 (1), 169 (20), 156 (100), 73 (89)}; the di-TMS ethers of 9β- (R_t 4.65 min) and 9α-trichodiols (R_t 5.05 min) {EIMS *m/z* (rel. int.): 9β-trichodiol 396 [M]$^+$ (0.5), 381 (3), 351 (0.5), 306 (0.5), 197 (60), 183 (15), 169 (27), 107 (89), 93 (20), 81 (17), 73 (100); 9α-trichodiol 396 [M]$^+$ (0.5), 381 (3), 341 (1), 306 (0.5), 291 (1), 233 (2), 197 (34), 183 (8), 169 (18), 156 (27), 107 (66), 73 (100)}; and the di-TMS ether of pre-sambucoin (R_t 5.34 min) {EIMS *m/z* (rel. int.): 396 [M]$^+$ (0.5), 381 (0.5), 306 (0.5), 291 (0.5), 266 (1), 257 (1), 237 (1), 182 (7), 146 (6), 124 (100), 93 (17), 73 (55)}.

(b) Isotrichodiol (13.2 mg) in EtOAc (0.5 ml) was incubated with KPi buffer (200 ml) as above for 6 days. After work-up, the products were sepd by prep. TLC (2 0.2-mm plates, 20 × 20 cm) in hexane–EtOAc (1:1) to give EPT (**20**, 2.2 mg, 18%) identical to natural material [22], and pre-sambucoin (**21**, 2.2 mg, 17%) as a viscous oil. ^{1}H NMR ($CDCl_3$): δ 5.25 (1H, *br s*, H-10), 4.15 (1H, *br s*, H-11β), 4.11 (1H, *d*, J = 12.5 Hz, H-13), 4.09 (1H, *dd*, J = 10.7, 6.7 Hz, H-2β), 3.75 (1H, *d*, J = 12.5 Hz, H-13), 1.99 (1H, *br m*, H-8α), 1.98 (1H, *dd*, J = 14.0, 8.5 Hz, H-4α), 1.90 (1H, *ddd*, J = 13.6, 7.0, 6.6 Hz, H-3β), 1.84 (1H, *br dd*, J = 18.0, 6.0 Hz, H-8β), 1.68 (3H, *br s*, H-16), 1.55 (2H, *m*, H-3α, 7β), 1.25 (1H, *ddd*, J = 13.8, 12.3, 8.0 Hz, H-4β), 1.25 (1H, *m*, H-7α), 1.07 (3H, *s*, H-14), 0.98 (3H, *s*, H-15); ^{13}C NMR ($CDCl_3$): δ 135.0 (C-9), 122.9 (C-10), 80.7 (C-2), 71.6 (C-11), 67.8 (C-13), 46.1 (C-5), 37.0 (C-6), 31.1, 30.9, 29.5, 27.7 (C-3, C-4, C-7, C-8), 22.7, 22.3, 16.4 (C-14, C-15, C-16); EIMS *m/z* (rel. int.): 252 [M]$^+$ (26), 237 (34), 234 (11), 149 (12), 124 (100), 107 (55), 93 (67).

Sambucoin (**22**). A soln of trifluoroacetic anhydride (33 μl) in dry CH_2Cl_2 (0.4 ml) at −78° was treated dropwise with a soln of DMSO (27 μl) in dry CH_2Cl_2 (0.4 ml) also at −78°. After stirring at this temp. for 10 min, pre-sambucoin (3 mg) in dry CH_2Cl_2 (0.2 ml) was added, and the reaction mixt. stirred for a further 30 min. Triethylamine (30 μl) was added and the reaction temp. slowly allowed to increase to −25° over 40 min, and then more rapidly to room temp. The mixt. was diluted with CH_2Cl_2 (15 ml), washed with satd aq. NaCl (20 ml), dried over $MgSO_4$, filtered and evapd to an oil under red. pres. Sambucoin (**22**, 1.2 mg, 40%) was obtained as a solid after prep. TLC (1 0.2-mm plate, 20 × 20 cm) in hexane–EtOAc (1:1). ^{1}H NMR ($CDCl_3$): δ 5.20 (1H, *br s*, H-10), 4.20 (1H, *d*, J = 11.3 Hz, H-13), 3.96 (1H, *br s*, H-11), 3.45 (1H, *d*, J = 11.3 Hz, H-13), 2.58 (1H, *ddd*, J = 19.9, 11.8, 2.9 Hz, H-3), 2.28 (1H, *ddd*, J = 19.9, 10.0, 8.0 Hz, H-3), 2.01 (1H, *ddd*, J = 13.5, 10.0, 3.0 Hz, H-4), 1.97–1.90 (2H, *m*, H-7, H-8), 1.78 (1H, *ddd*, J = 13.7, 11.8, 8.0 Hz, H-4), 1.64 (3H, *br s*, H-16), 1.57 (1H, *br dd*, J = 10.8, 6.6 Hz, H-8), 1.46 (1H, *ddd*, J = 13.4, 5.3, 2.3 Hz, H-7), 1.15 (3H, *s*, H-14), 0.65 (3H, *s*, H-15); EIMS *m/z* (rel. int.): 250 [M]$^+$ (40), 235 (30), 180 (18), 125 (36), 124 (32), 108 (100), 93 (72), 84 (32). Spectral data are in agreement with lit. values [17, 18].

Culture of Trichothecium roseum. *Trichothecium roseum* CMI 50661 was maintained on beer wort agar (BWA) (Oxoid) slopes at 26° in the dark. BWA plates were inoculated with a spore suspension obtained by flooding the slope culture with sterile H_2O (1–2 ml). Plates were incubated at 26° in the dark for 5–7 days, to provide a further spore suspension which was used to inoculate Czapek Dox (CD) seed medium (malt extract, 2 g; yeast extract, 2 g; peptone, 2 g; KH_2PO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 2 g; $FeSO_4 \cdot 7H_2O$, 0.2 g; NH_4Cl, 3 g; glucose, 20 g; dist. H_2O to 1 l) at the rate 1 plate to 200 ml medium. Seed cultures were incubated at 27°, 250 rpm for 2 days, then aliquots (50 ml) were used to inoculated 2-l Erlenmeyer flasks containing 400 ml of CD corn steep medium (ammonium tartrate, 2 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; K_2HPO_4, 1 g; KCl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $ZnSO_4$, 0.1 g; corn steep liquor, 10 g; glucose, 50 g; dist. H_2O to 1 l; pH adjusted to 5.5 with 1 M NaOH). Cultures were incubated for a further 21 days.

9β-Trichodiol (**3b**). A 6-l culture of *T. roseum* was filtered under red. pres. and the mycelia transferred to a

metal container and frozen with liquid N_2. Boiling H_2O (500 ml) was added, and when the mycelia had thawed the slurry was extracted by stirring with EtOAc (400 ml). The culture filtrate was satd with NaCl and also extracted with EtOAc (3 × 400 ml). The combined organic extracts were dried over $MgSO_4$ and evapd to an oil (2.5 g) under red. pres. This was chromatographed by CTLC (4-mm plate) in CH_2Cl_2–MeOH (9:1), collecting 7-ml fractions. Frs 9–16, after further CTLC in Et_2O–Me_2CO (9:1) yielded trichothecolone (150 mg) as crystals, mp 180–182° (lit. [23] 183–184°). Frs 17–30 were combined and rechromatographed by CTLC (2-mm plate) in $CHCl_3$–MeOH (9:1), collecting 2-ml fractions, from which frs 36–42 were further purified (CTLC; 1-mm plate, Et_2O–Me_2CO, 9:1; 1-ml fractions) giving 9β-trichodiol (16.1 mg) as crystals from frs 42–49. On TLC analysis ($CHCl_3$–MeOH, 9:1) 9β-triochodiol (**3b**) appeared as a brown spot (R_f 0.37) with H_2SO_4 spray, and a bright sky blue colour with PNBP reagent. EIMS *m/z* (rel. int.): 252 $[M]^+$ (1), 234 (3), 219 (10), 147 (6), 135 (8), 127 (5), 125 (47), 109 (43), 108 (91), 107 (53), 97 (56), 81 (77), 43 (100); GC-MS di-TMS ether: 9β-trichodiol (R_t 4.71 min) and 9α-trichodiol (R_t 5.06 min) in ratio approx 19:1.

Acid-catalysed cyclization of 9β-trichodiol (**3b**). 9β-Trichodiol (3.5 mg) in EtOAc (0.1 ml) was added to KPi buffer (0.1 mM, pH 5.8, 10 ml) and incubated at 27°, 250 rpm for 5 days. The mixture was extracted with EtOAc (20 ml), the extract dried over $MgSO_4$, and evapd to an oil under red. pres. Prep. TLC (1 0.2-mm plate; 20 × 20 cm: hexane–EtOAc, 1:1) gave EPT (2.1 mg, 64%) as a viscous oil spectrally identical to the natural material [22].

9β-Trichotriol (**4b**), *9α-trichotriol* (**4a**), *isotrichotriol* (**23**) *and 8α-hydroxyisotrichodiol* (**12**) *from* F. culmorum. (a) 9β-Trichotriol (**4b**, 11.3 mg) was isolated from the crude toxin extract of *F. culmorum* (1.6-l culture) grown under standard culture conditions as described in the previous paper [13]. On TLC analysis ($CHCl_3$–MeOH, 9:1) it gave a faint yellow coloured spot (R_f 0.23) with H_2SO_4 spray before heating, which became brown on heating at 140°. PNBP reagent produced a bright sky blue colour. EIMS *m/z* (rel. int.): 268 $[M]^+$ (1), 250 (1), 163 (10), 146 (10), 135 (10), 125 (100), 108 (75), 107 (93), 95 (35), 81 (76).

(b) The extract from an 8-l culture of *F. culmorum* which had been treated with xanthotoxin in DMSO (to give a concn of 0.1 mM) was loaded on to a silica gel column (90 mm × 65 mm diam.) packed in hexane. The column was eluted first with hexane (400 ml) and then with EtOAc (600 ml), collecting 100-ml fractions. Frs 1–4 gave trichodiene (**13**, 2.04 g) and frs 8–10 were combined to give a yellow oil (413 mg). This was chromatographed by CTLC (2-mm plate) eluting first with $CHCl_3$–MeOH (19:1; 70 ml) and then with $CHCl_3$–MeOH (9:1; 80 ml) and $CHCl_3$–MeOH (4:1; 70 ml), collecting 2-ml fractions.

Frs 71–78 were combined and rechromatographed by CTLC (1-mm plate) in Et_2O–Me_2CO (4:1), collecting 1-ml fractions. Frs 25–29 were further purified by prep. TLC (2 0.2-mm plates; 20 × 20 cm) first in Et_2O–Me_2CO (9:1), and then in CH_2Cl_2–MeOH (9:1), yielding 8α-hydroxyisotrichodiol (**12**, 2.9 mg) as a viscous oil. ^{1}H NMR ($CDCl_3$): δ 5.33 (1H, *br s*, H-10), 4.56 (1H, *br s*, H-11), 3.98 (1H, *br d*, *J* = 5.1 Hz after MeOD shake, H-8), 3.65 (1H, *d*, *J* = 4.0 Hz, H-2), 3.32 (1H, *d*, *J* = 4.1 Hz, H-13), 3.06 (1H, *d*, *J* = 4.1 Hz, H-13), 2.79 (1H, *ddd*, *J* = 13.0, 13.0, 7.0 Hz, H-4α), 1.0–2.0 (5H, *m*, H-3, H-4β, H-7), 1.81 (3H, *br s*, H-16), 1.13 (3H, *s*, H-14), 0.98 (3H, *s*, H-15); EIMS *m/z* (rel. int.): 268 $[M]^+$ (1), 250 (3), 224 (3), 149 (14), 140 (13), 123 (30), 110 (32), 100 (100), 95 (26). Similarly, frs 36–43 were rechromatographed by prep. TLC (2 0.2-mm plates; 20 × 20 cm) in Et_2O–Me_2CO (9:1) to yield isotrichotriol (**23**, 4.6 mg) as a solid. ^{1}H NMR ($CDCl_3$): δ 5.15 (1H, *br s*, H-10), 4.64 (1H, *br s*, H-11), 4.27 (1H, *ddd*, *J* = 9.2, 6.6, 4.0 Hz, H-3), 3.63 (1H, *d*, *J* = 4.0 Hz, H-2), 3.31 (1H, *d*, *J* = 4.3 Hz, H-13), 3.13 (1H, *d*, *J* = 4.3 Hz, H-13), 2.72 (1H, *dd*, *J* = 13.6, 9.3 Hz, H-4α), 1.8–2.0 (2H, *m*, H-8), 1.83 (1H, *dd*, *J* = 13.5, 6.5 Hz, H-4β), 1.43 (1H, *dd*, *J* = 14.3, 8.4 Hz, H-7α), 1.0–1.5 (1H, *m*, H-7β), 1.66 (3H, *br s*, H-16), 1.03 (3H, *s*, H-14), 0.91 (3H, *s*, H-15); EIMS *m/z* (rel. int.): 268 $[M]^+$ (3), 252 (14), 250 (4), 237 (22), 149 (42), 137 (60), 124 (100), 109 (81), 93 (69), 84 (73), in agreement with lit. data [10].

Frs 86–end were combined and rechromatographed by CTLC (2-mm plate) in Et_2O–Me_2CO (9:1), collecting 2-ml fractions. Further purification of frs 48–70 by prep. TLC (1 0.2-mm plate; 20 × 20 cm) in CH_2Cl_2–MeOH (19:1) yielded 9α-trichotriol (**4a**, 1.3 mg) as a viscous oil. EIMS *m/z* (rel. int.): 268 $[M]^+$ (1), 250 (1), 163 (7), 146 (12), 135 (8), 125 (100), 109 (56), 108 (75), 107 (90), 95 (35), 81 (76). On TLC analysis (CH_2Cl_2–MeOH; 9:1) 8α-hydroxyisotrichodiol (R_f 0.40), isotrichotriol (R_f 0.37) and 9α-trichotriol (R_f 0.25) all reacted with PNBP reagent producing sky blue coloured spots.

Feeding experiments. (*a*) *Incorporation of* ^{14}C*-labelled isotrichodiol and 9β-trichotriol.* A filtered and washed mycelial mat from a 200-ml culture of *F. culmorum* (4 days old) was divided into 12 equal portions (*ca* 0.5 g wet wt) and these were transferred to flasks (50 ml) containing distilled H_2O (10 ml). Samples of [^{14}C]isotrichodiol (0.5 mg; 0.63 MBq mM^{-1}) or [^{14}C]9β-trichotriol (0.5 mg; 0.30 MBq mM^{-1}) in Me_2CO (50 μl) were added to 2 cultures. The cultures were incubated at 28°, 250 rpm for 7 hr, filtered (Whatman No. 4) under red. pres, and the mycelia washed with EtOAc (30 ml). The filtrates were extracted with the washings, and the extracts dried over $MgSO_4$ and evapd to dryness under red. pres. The extracts were dissolved in EtOAc (40 μl for trichotriol; 50 μl for isotrichodiol) and aliquots (20 μl/15 μl, respectively) were analysed by TLC (hexane–EtOAc, 1:1, and Et_2O–Me_2CO, 9:1) and autoradiography. Product formation was measured by scintillation counting.

(b) Incubation of [^{14}C]*9β-trichotriol with cell-free extract.* A filtered and washed mycelial mat (12 g wet wt) from 2 × 200-ml cultures of *F. culmorum* was suspended in KPi buffer (0.1 M; pH 8.0; 80 ml) containing 250 mM sucrose. The mixture was cooled to 0° and liquidized for 15 sec in a blender. The fragmented mycelia were then ruptured at a peak pressure of 10–20 000 psi in a microfluidizer (Microfluidic Corp Model 110T; Newton) precooled with ice. The homogenate was centrifuged at

2500 *g* for 15 min at 4° and the supernatant used immediately. Portions (0.75 ml) of the cell-free extract were incubated at 28°, 250 rpm for 2 hr with KPi buffer (0.1 M; pH 7.2; 4.5 ml) and [^{14}C]9β-trichotriol (0.2 mg; 0.30 MBq mM^{-1}) in Me_2CO (40 μl). In a control experiment, the extract was boiled for 10 min, cooled, and incubated as above. The cultures were then extracted with EtOAc (20 ml), the extracts dried over $MgSO_4$, filtered and evapd to dryness under red. pres. Extracts were analysed by TLC (Et_2O–Me_2CO, 9:1), autoradiography, and scintillation counting.

(*c*) *Competitive feeding experiments.* A filtered and washed mycelial mat from a 200-ml culture of *F. culmorum* (4 days old) was divided into 12 equal portions (*ca* 0.5 g wet wt), and portions were resuspended in dist. H_2O (10 ml) in 50-ml flasks. To these cultures were added the substrate being tested (0.5 mg) in Me_2CO (50 μl). The cultures were incubated at 28°, 250 rpm for 15 min, then [^{14}C]isotrichodiol (0.5 mg; 0.63 MBq mM^{-1}) in Me_2CO (50 μl) was added. Incubation was continued for a further 6.75 hr. Cultures were worked up as before, the extracts were dissolved in EtOAc (50 μl), and aliquots (15 μl) analysed by TLC (hexane–EtOAc, 1:1, and Et_2O–Me_2CO, 9:1) and autoradiography. In a further series of experiments, mycelium (0.1 g wet wt) was incubated with unlabelled substrate (0.6 mg) for 15 min and then with [^{14}C]isotrichodiol (0.1 mg) for 5.75 hr.

(*d*) *Biosynthesis of sambucoin.* A filtered and washed mycelial mat from a 100-ml CD production medium culture of *F. culmorum* (3 days old) was resuspended in sterile CD production medium (50 ml). [^{14}C]Presambucoin (1.0 mg; 0.41 MBq mM^{-1}) in Me_2CO (50 μl) was added, and the culture incubated at 28°, 250 rpm for 18 hr, then harvested by filtering under red. pres. and extracting the filtrate with EtOAc (2 × 50 ml). The extract was dried over $MgSO_4$ and evapd to an oil. To this was added unlabelled sambucoin (6.5 mg) [19], and the mixture was sepd by CTLC (1-mm plate) in hexane–EtOAc (1:1), collecting 1-ml fractions. Frs 18–21 were combined to yield crystals (6.9 mg) which were further purified by prep. TLC (2 0.2-mm plates; 20 × 20 cm) in Et_2O–Me_2CO (9:1). Recrystallization to constant sp. act. from EtOAc yielded sambucoin (3.2 mg; 3.83 KBq mM^{-1}).

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