METABOLIC STUDIES ON THE TRANSFORMATION OF TRICHODIENE TO TRICHOTHECENE MYCOTOXINS.

by

Andrew R. Hesketh BPharm.

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CONTENTS

LIST OF ABBREVIATIONS

3-AcDON	3-Acetyldeoxynivalenol
CAL	Calonectrin
15-DeacetylCAL	15-Deacetylcalonectrin
15-DeacetylDHC	15-Deacetyl-7α,8α-dihydroxycalonectrin
DHC	7α,8α-Dihydroxycalonectrin
DMAPP	Dimethylallyl diphosphate
EPT	12,13-Epoxytrichothec-9-ene
FPP	Farnesyl diphosphate
7-HydroxyCAL	7α-Hydroxycalonectrin
IPP	Isopentenyl diphosphate
ITD	Isotrichodermin
ITdiol	Isotrichodiol
MVA	Mevalonic acid
NPP	Nerolidyl diphosphate
TDN	Trichodiene
BSTFA	Bis(trimethylsilyl)trifluoroacetamide
*m*CPBA	*m*-Chloroperoxybenzoic acid
PNBP	4-(*p*-nitrobenzyl)pyridine
TEPA	Tetraethylene pentamine
COSY	Correlation Spectroscopy
DEPT	Distortionless enhancement by polarisation transfer
DNOES	Difference nuclear Overhauser enhancement spectroscopy

ABSTRACT

Trichodiene and [^{14}C]trichodiene have been produced in high yields by treatment of *Fusarium culmorum* CMI 14764 cultures with the furanocoumarin xanthotoxin. Smaller amounts of isotrichodermin (ITD) and the unsubstituted trichothecene 12,13-epoxytrichothec-9-ene (EPT) were obtained in the same way. EPT was also produced by semi-synthesis from ITD.

Trichodiene (TDN) was shown to be a precursor of the trichothecene mycotoxins in *F. culmorum*, including EPT, ITD, calonectrin (CAL), 7α-hydroxycalonectrin (7-hydroxyCAL), 15-deacetylcalonectrin, 3-acetyldeoxynivalenol (3-AcDON) and 7,8-dihydroxycalonectrin (DHC). When large amounts of TDN were supplied, a new trichodiene metabolite was found to accumulate which was fully characterised as 12,13-epoxy-2α,11α-dihydroxytrichodiene, and given the trivial name isotrichodiol. A method for the production of ^{14}C-labelled isotrichodiol (ITdiol) was developed, and the incorporation of [^{14}C]ITdiol into 3-AcDON, DHC and 7-hydroxyCAL was demonstrated. Slow, acid-catalysed cyclisation of ITdiol to EPT and pre-sambucoin was demonstrated, and allylic isomerisation to both 9α- and 9β-trichodiol was also detected. Labelled pre-sambucoin was incorporated into sambucoin by *F. culmorum*, and ITdiol is thus proposed as a precursor to both sambucoin and sambucinol, aswel as to the trichothecenes.

A range of semi-synthetic derivatives of TDN were prepared and tested as possible inhibitors of the post-TDN biosynthesic pathway to trichothecenes in *F. culmorum*. In whole-cell systems all the derivatives inhibited the incorporation of labelled TDN into trichothecenes, and also initiated the production of ITdiol.

One derivative, 9β,10β-epoxytrichodiene, was shown to be biotransformed by the fungus, undergoing 12,13-epoxidation with subsequent hydroxylation at C-3 producing 3α-hydroxy-9β,10β;12,13-diepoxytrichodiene.

9β-Trichodiol was isolated from *Trichothecium roseum*, and its slow, acid-catalysed cyclisation to EPT was demonstrated. 9α-Trichotriol, 9β-trichotriol and isotrichotriol were isolated from *F. culmorum* for the first time, and literature assignments for the stereochemistry of the C-9 hydroxyl in trichodiol and trichotriol are reassessed. The incorporation of [^{14}C]ITdiol into trichothecenes in *F. culmorum* was found to be approximately 5 times greater than the incorporation of [^{14}C]-9β-trichotriol, and was shown to be inhibited by isotrichotriol but not by 9β-trichodiol and 9β-trichotriol. It is proposed that trichodiol and trichotriol are not biosynthetic intermediates in the pathway to the trichothecenes, and that they are non-enzymic metabolites produced from ITdiol and isotrichotriol, respectively, by acid-catalysed isomerisations. A new scheme for the biosynthesis of trichothecenes is proposed in which ITdiol and isotrichotriol are intermediates in the production of isotrichodermol from TDN.

Two novel compounds, 15-deacetyl-7,8-dihydroxycalonectrin (15-deacetylDHC) and 8α-hydroxyisotrichodiol were isolated from *F. culmorum*, and 15-deacetylDHC and DHC were shown to be precursors to 3-AcDON. It is proposed that the post-cyclisation biosynthesis of 3-AcDON involves sequential oxygenation of isotrichodermol at C-15, C-7 and C-8 producing DHC, which then undergoes deacylation to 15-deacetylDHC followed by oxidation at C-8 to 3-AcDON.

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INTRODUCTION.

The fungal contamination of plant products destined for human and animal consumption is a global occurrence, and poses a potentially major threat to public health. Mycotoxins produced by moulds which occur naturally on feedstuffs include the aflatoxins, ochratoxins, trichothecenes, zearalenone and ergot alkaloids.

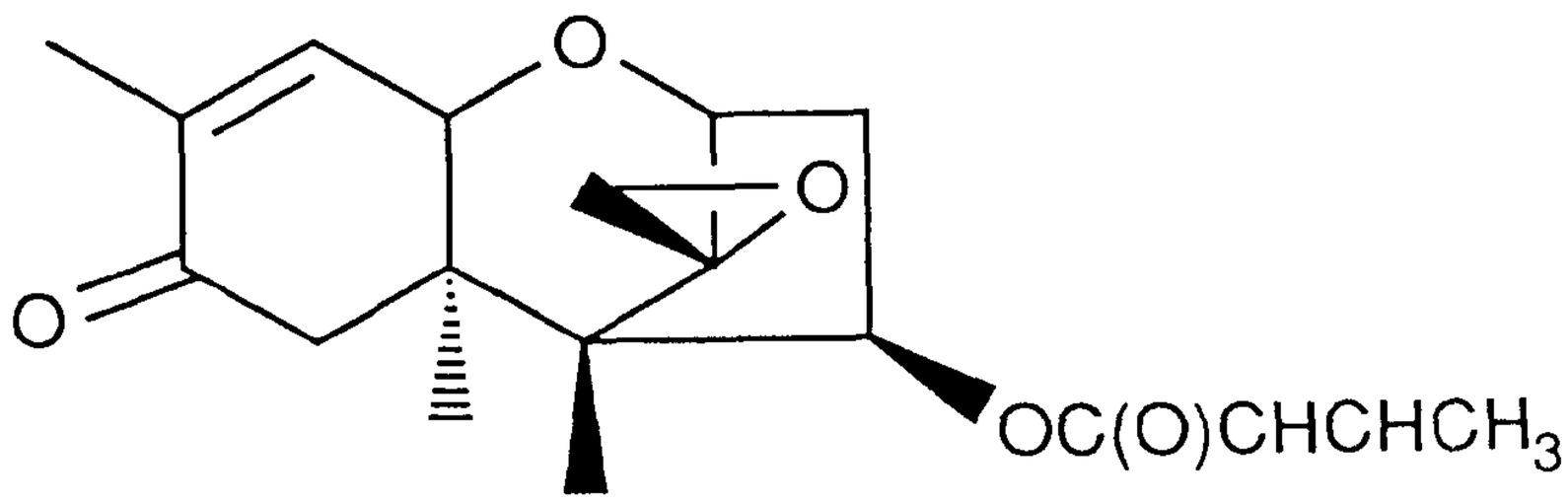
The ergot alkaloids represent the first recognised mycotoxins, and historically have been responsible for the deaths of tens of thousands of people. Consumption of cereals, particularly rye, contaminated by *Claviceps purpurea* caused extensive outbreaks of ergotism in Russia and Europe during the middle ages and up to as recently as the 1920's. Current problems associated with mycotoxins are geographically more widespread, and affect a larger range of plant products. Aflatoxins, carcinogenic metabolites of *Aspergillus flavus*, have been detected in all parts of the world and in a variety of crops ranging from peanuts, beans and oilseeds, to corn, wheat and other cereal crops[1]. The ochratoxins, nephrotoxic metabolites of *Penicillium* spp., and *Fusarium* toxins such as the trichothecenes and zearalenone are equally ubiquitous. Consumption of food contaminated in this way causes a variety of health problems in both human and animal populations, ranging from potentially fatal primary acute toxicoses to longer term chronic effects such as reduced growth rates, immunosuppression and cancer.

The economic impact of mycotoxins is also considerable. Although difficult to quantify, the costs due to health problems in livestock and damage to crops are estimated to be in the order of tens of millions of dollars per annum. Additional expenses are incurred by the sampling and analysis of agricultural produce which is necessary for the detection of possible mycotoxin contamination.

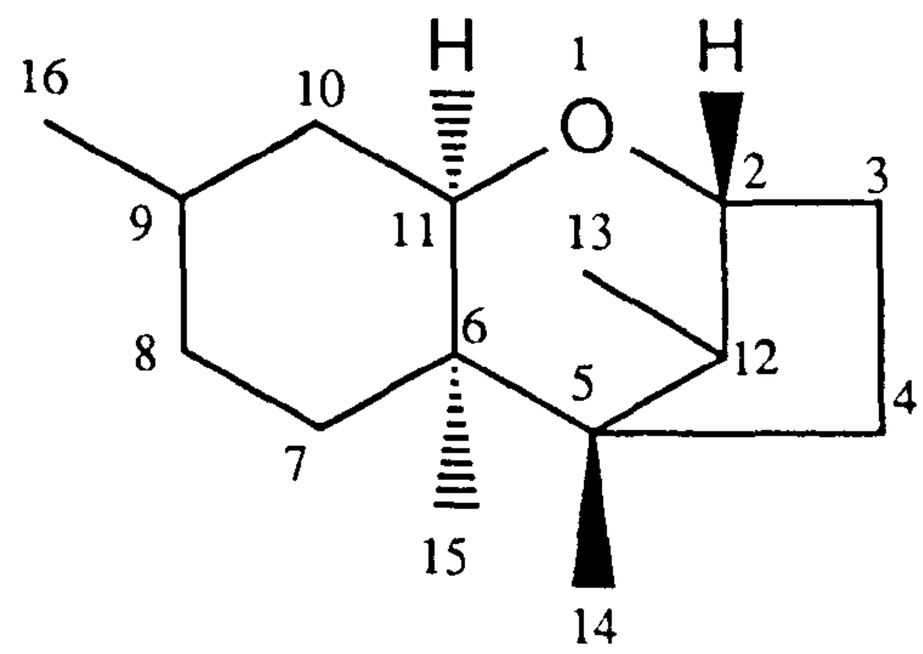
This work is concerned with the trichothecene group of mycotoxins. The trichothecenes are secondary metabolites produced by several genera of the *Fungi Imperfecti* including *Fusarium, Trichothecium, Myrothecium, Stachybotrys* and *Trichoderma.* A small number of trichothecenes, the baccharinoids, have been isolated from the higher plant *Baccharis megapotamica*[2,3] but these are thought to be products of a symbiotic relationship between the plant and a trichothecene producing fungus[4].

Structure and classification.

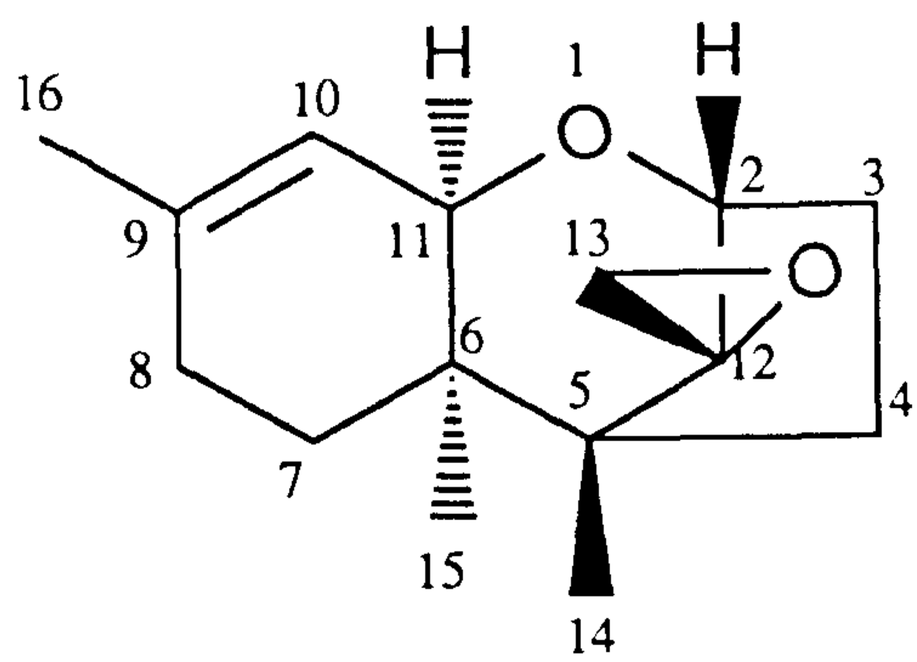
The trichothecenes are named after trichothecin (1), the first example, isolated in 1948 from *Trichothecium roseum* during screening studies for metabolites with antibiotic activity[5]. Around 150 trichothecenes have been isolated from natural sources, all of which are based on the sesquiterpenoid trichothecane skeleton (2). The majority also possess a 9,10-double bond and a 12,13-epoxide function, making 12,13-epoxytrichothec-9-ene (3) the parent structure. They have been classified into four sub-groups according to their structural features[6]. Figures 1-4 give representative examples for each class. Class A (approximately 55 compounds) have hydroxy or acyloxy substituents at any of C-3, C-4, C-7, C-8 and C-15 positions [Fig.1]. Class B (approximately 20 compounds) are characterised by an 8-keto group [Fig.2], class C (5 compounds) possess a 7,8-epoxide [Fig.3], whilst class D (approximately 65 compounds) are the macrocyclic trichothecenes [Fig.4]. Comprehensive lists of trichothecenes isolated, their structure, classification and fungal sources are given in several reviews[7,8,9].

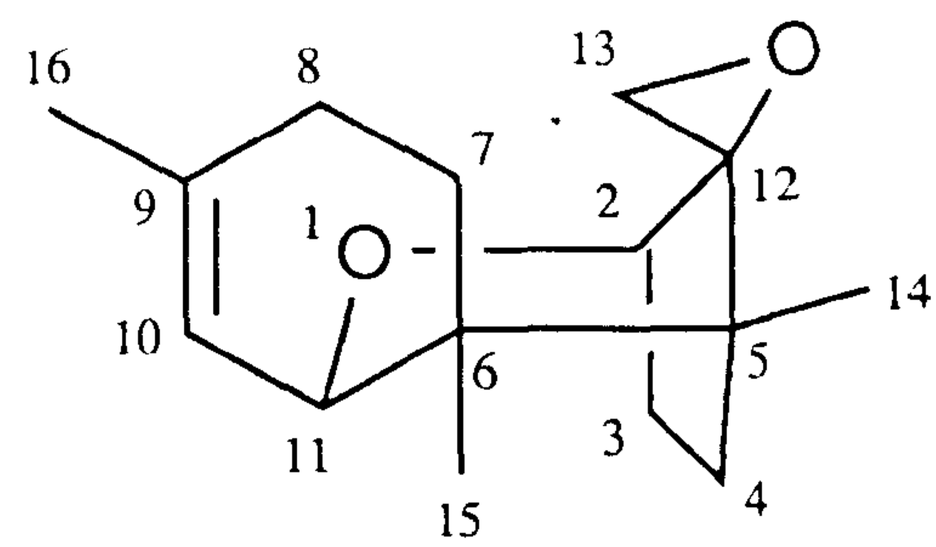


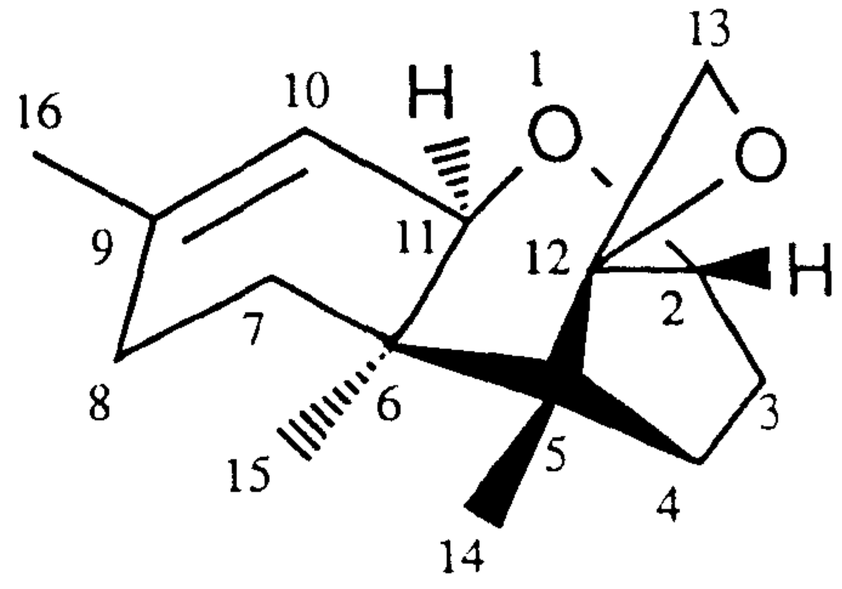
Trichothecin (1)



Trichothecane (2)

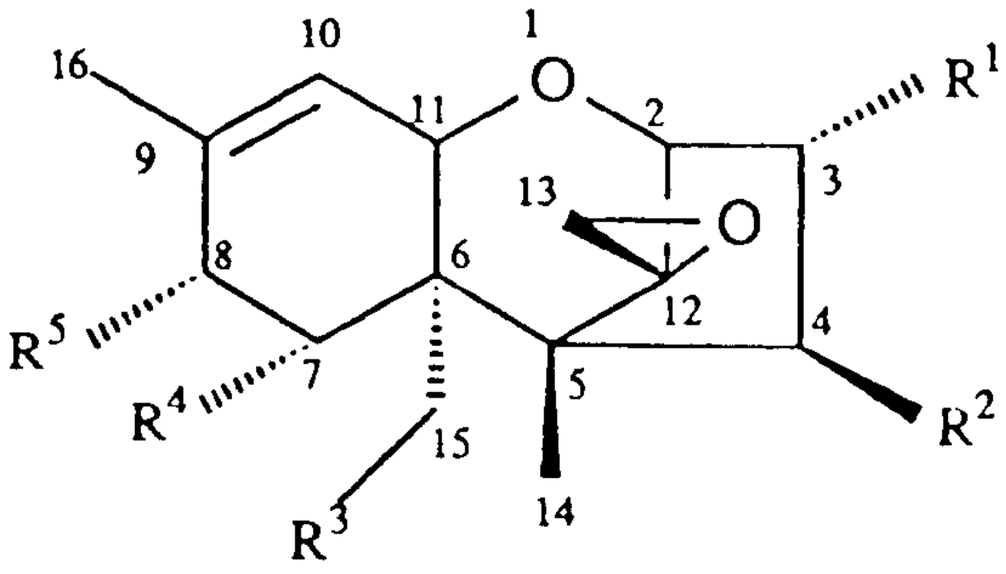






12,13-Epoxytrichothec-9-ene (3)

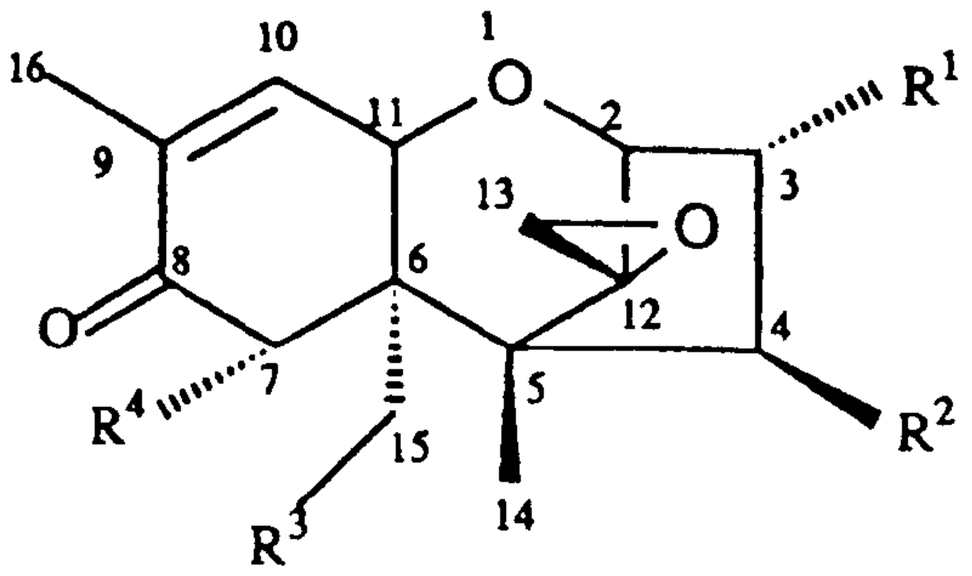
Fig.1: Examples of class A trichothecenes.



NAME	R^1	R^2	R^3	R^4	R^5
(3) 12,13-Epoxytrichothecene	H	H	H	H	H
(4) Trichodermin	H	OAc	H	H	H
(5) Isotrichodermol	OH	H	H	H	H
(6) Calonectrin	OAc	H	OAc	H	H
(7) 7,8-Dihydroxycalonectrin	OAc	H	OAc	OH	OH
(8) Verrucarol	H	OH	OH	H	H
(9) 4,15-Diacetoxyscirpenol	OH	OAc	OAc	H	H
(10) Scirpentriol	OH	OH	OH	H	H
(11) T-2 toxin	OH	OH	OAC	H	X

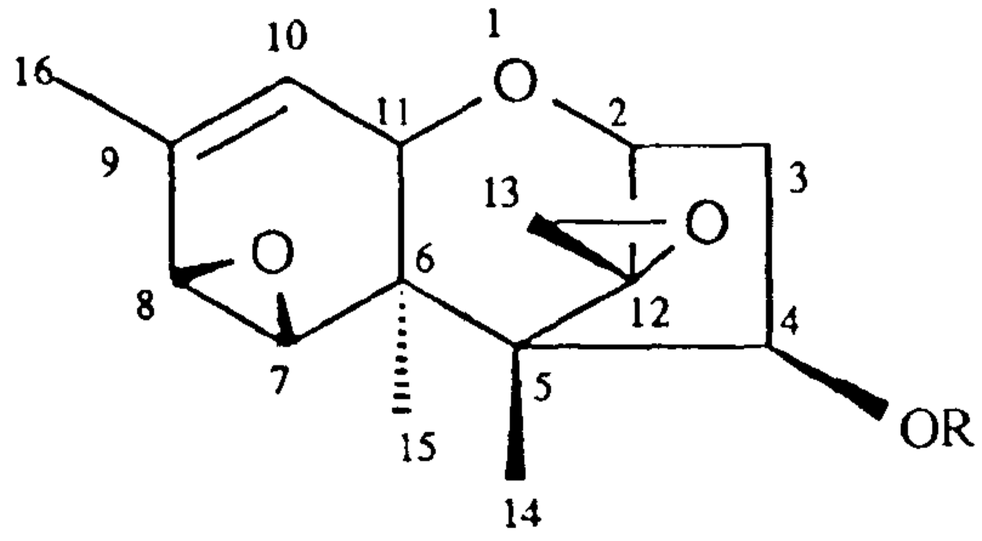
X=$OC(O)CH_2CH(CH_3)_2$

Fig.2: Examples of class B trichothecenes.



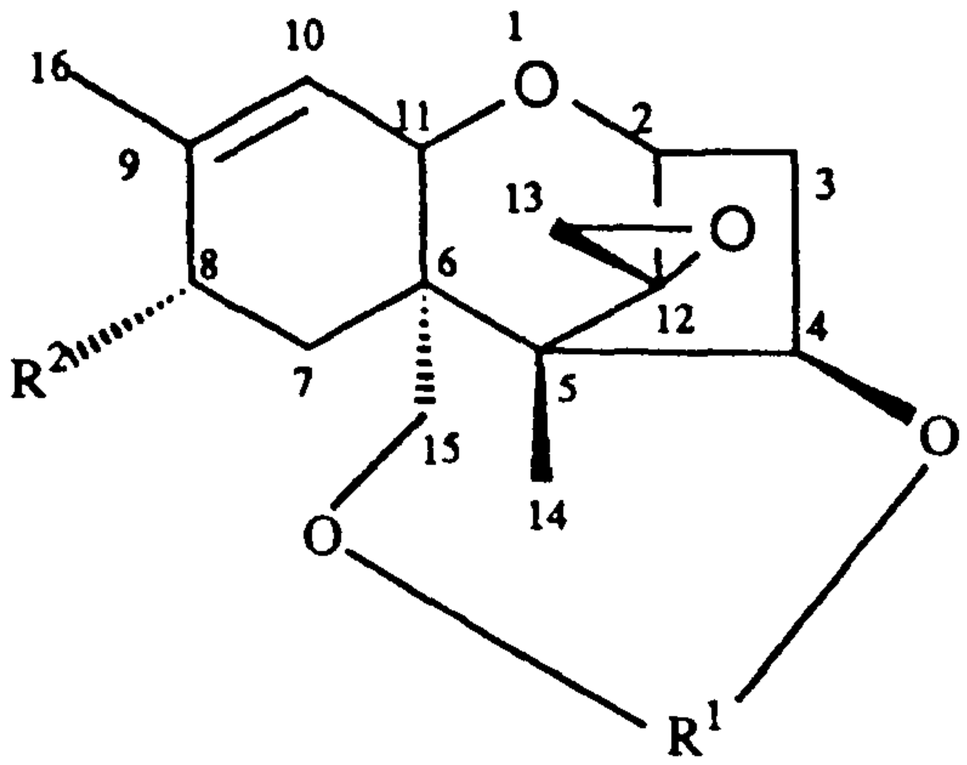
NAME	R^1	R^2	R^3	R^4
(1) Trichothecin	H	$OC(O)CHCHCH_3$	H	H
(12) Trichothecolone	H	OH	H	H
(13) Deoxynivalenol	OH	H	OH	OH
(14) 3-Acetoxydeoxynivalenol	OAc	H	OH	OH
(15) Nivalenol	OH	OH	OH	OH
(16) 15-Acetoxydeoxynivalenol	OH	H	OAc	OH
(17) 3,15-Diacetoxydeoxynivalenol	OAc	H	OAc	OH

Fig.3: Examples of class C trichothecenes.



NAME	R
(18) Crotocol	H
(19) Crotocin	$C(O)CHCHCH_3$

Fig.4: Examples of class D trichothecenes.



NAME	R^1	R^2
(20) Verrucarin A	$-C(O)OHCH(CH_3)CH_2CH_2OC(O)CHCHCHCHC(O)-$	H
(21) Verrucarin L	$-C(O)CHC(CH_3)CH_2CH_2OC(O)CHCHCHCHC(O)-$	OH
(22) Roridin A	$-C(O)CHOHCH(CH_3)CH_2CH_2OCHCHCHCHCHC(O)-$ $CHOH$ CH_3	H
(23) Roridin K	$-C(O)CHC(CH_3)CH_2CH_2OCHCHCHCHCHC(O)-$ $CHOH$ CH_3	OAC
(24) Satratoxin H	$-C(O)CHCCH_2CH_2OCCHCHCHCHC(O)-$ $CHOH$ CH CH_3 OH	H

Mycotoxicoses.

Trichothecene producing fungi are widespread in nature occurring as saprophytes in soil, and as parasites of wild and cultivated plants. It is their occurrence in pastureland and in crops such as barley, corn, wheat and oats which is of major concern. Under suitable environmental conditions of temperature and high humidity, either in the field or in the storage of harvested crops, the fungi can grow and produce their characteristic trichothecene metabolites. Consumption by humans or animals of food contaminated in this way can cause a primary mycotoxicosis. It is also possible that consumed mycotoxins may pass through the food chain into animal products such as meat, milk and eggs. Illnesses arising from these sources are referred to as secondary mycotoxicoses [Fig.5].

Mycotoxicoses associated with trichothecenes are a worldwide phenomenom and have been extensively reviewed[7,9]. Table 1 summarises some of these data, and gives an indication of the scale of the problems caused by trichothecenes. Of particular interest is the outbreak of alimentary toxic aleukia (ATA) in the Soviet Union during World War II, an example of an acute primary mycotoxicosis. ATA was caused by consumption of mouldy grain which had overwintered in the fields, and it was responsible for the deaths of tens of thousands of people. Symptoms included skin lesions, diarrhoea, vomiting, internal haemorrhaging and damage to internal organs and the haematopoietic system. Areas originally affected became necrotic and the dead tissues subsequently became infected with bacteria. Death occurred as a result of the mycotoxins ingested, and sometimes from the secondary bacterial infection[13].

Acute mycotoxicoses arise from consumption of highly contaminated feed.

Fig.5: Mycotoxicoses in humans and animals.

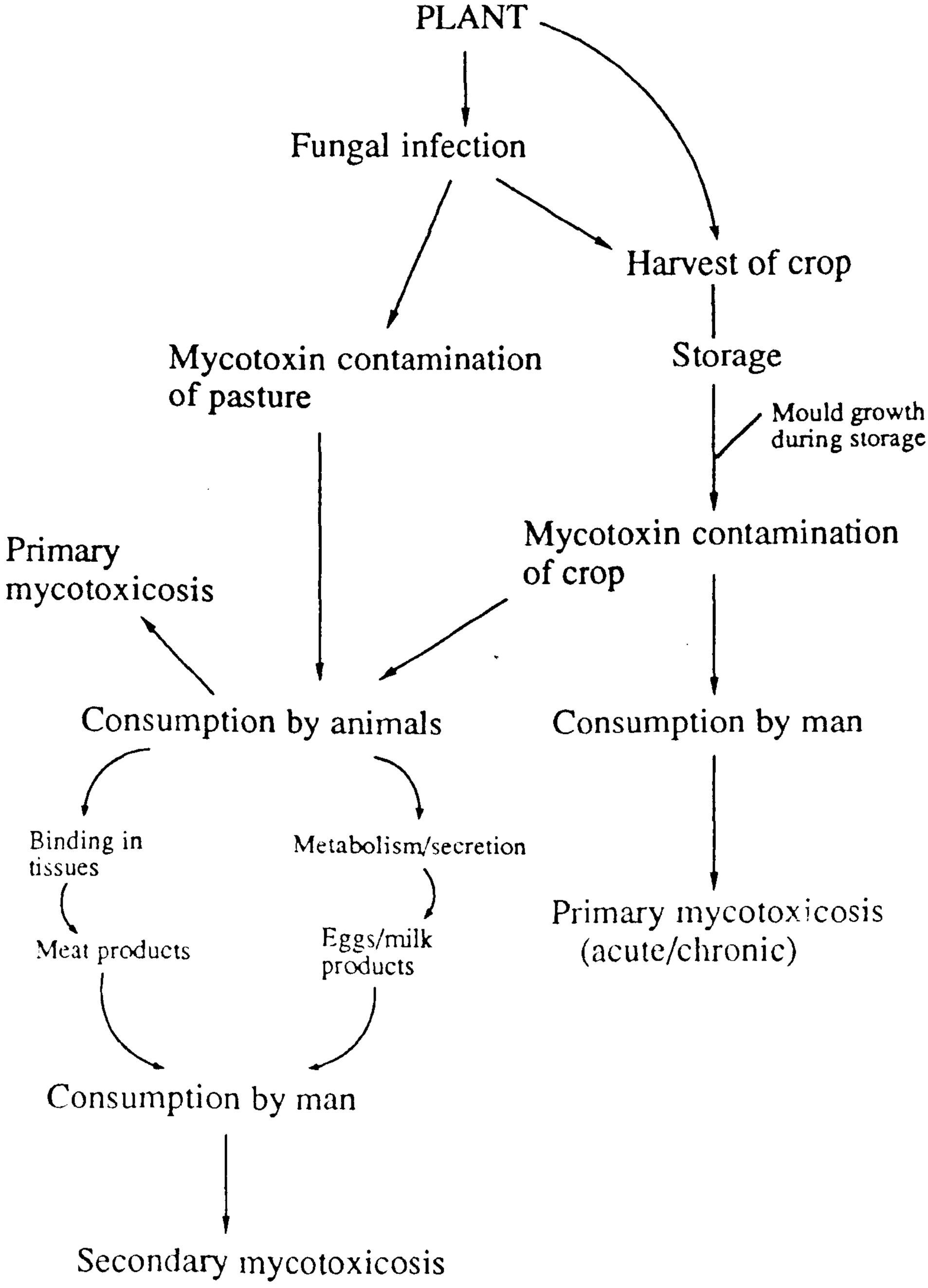


Table 1: Mycotoxicoses associated with trichothecenes.[7,9,10,11,12.]

TOXICOSIS	LOCATION	ANIMALS AFFECTED	FUNGUS	CROP	TRICHOTHECENE
Red mould	Japan	Horses, pigs, cows, man	*Fusarium* spp.	Wheat, barley, rice	Deoxynivalenol Nivalenol
Alimentary toxic aleukia	USSR	Man	*F.sporotrichioides*	Millet	T-2 toxin
Oesophageal cancer	South Africa	Man	*F.graminearum* *F.moniliform*	Corn	Deoxynivalenol
Stachybotryotoxicoses	Hungary Europe	Sheep, horses	*Stachybotrys atra*	Hay	Satratoxins G & H
Bean-hull	Japan	Horses	*F.solani*	Beans	T-2 toxin
Mouldy corn	Canada USA	Ducks, geese, horses, pigs, cows	*Fusarium* spp.	Wheat, corn	T-2 toxin Deoxynivalenol Diacetoxyscirpenol
--------	USA	Sandhill cranes	*F.compactum*	Peanuts	Isoneosolaniol

More commonly, lower levels of trichothecenes are present which can cause feed refusal by livestock[14], or chronic primary mycotoxicoses if the feed is consumed. The effects of chronic mycotoxicoses are slower growth rates, reduced reproductive efficiency and a general lack of well being. The immune system is also impaired which predisposes the animals to other secondary microbial infections[15]. Natural mycotoxicoses are rarely caused by a single toxin but are the result of several trichothecenes produced by one or more species of fungi. Potentiation of their toxic effects by other fungal metabolites as well as pesticides and environmental pollutants is therefore a possibility.

Toxicity.

The trichothecenes are toxic to animals[16], plants[4,17], insects[18], fungi[5], tumour cells[19], and cultured cells[20]. Their high toxicity towards animals is reflected in the LD_{50} values of pure compounds [Table 2] and their role as causative agents of primary mycotoxicoses in nature. The biological mode of action of trichothecenes is via inhibition of protein synthesis[21] which means that actively dividing tissues such as bone marrow, spleen, testis and intestinal mucosa are particularly affected. More specifically the trichothecenes have been shown to inhibit protein synthesis in eukaryotic cells by binding to the 60S subunit of ribosomes[7]. Depending on their structure and concentration, this causes inhibition of either the initiation or elongation/termination steps of protein synthesis[22,23]. The 12,13-epoxide group is essential for this activity[22,23] and is thought to be responsible for covalent binding to a receptor located on the ribosomes. Mammalian systems have been observed to remove the epoxide as part of a detoxification mechanism[24,25], and this can also be accomplished by bovine-rumen microorganisms[26,27]. The C-9,10 double bond is

Table 2: LD_{50} values for a range of trichothecenes.[21]

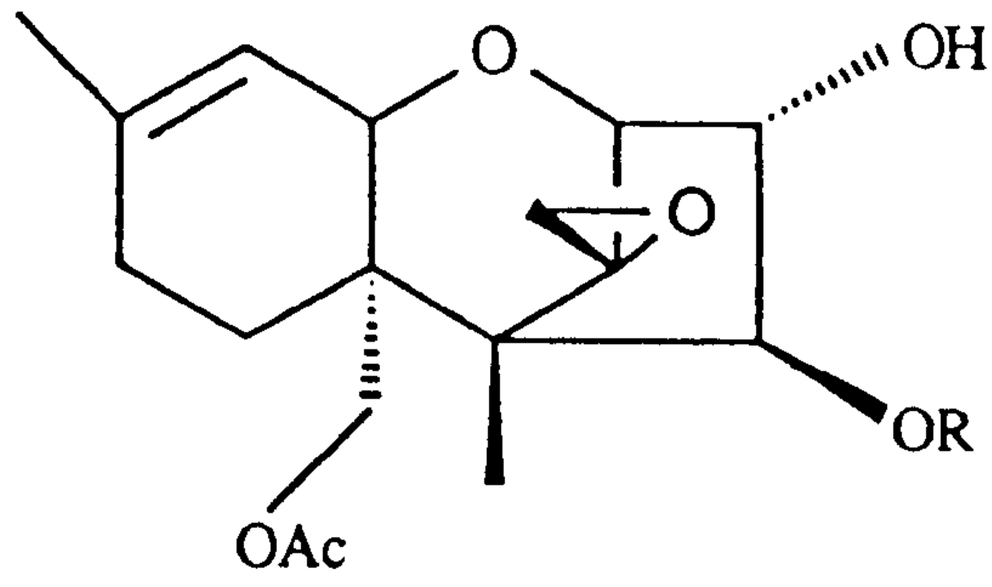
Trichothecene	Class	LD_{50} in mice (mg/kg)
T-2 toxin	A	5.2 (ip)
HT-2 toxin	A	9.0 (ip)
4,15-Diacetoxyscirpenol	A	23 (ip)
Nivalenol	B	4.1 (ip)
Deoxynivalenol	B	70 (ip)
Crotocin	C	700 (iv)
Verrucarin A	D	1.5 (iv)
Roridin A	D	1.0 (iv)

Table 3: LD_{50} values for trichothecenes with different levels of oxygenation.[28]

Trichothecene	Positions of oxygenation	LD_{50} (µg/rat) Direct cerebral application
Verrucarol	4,15	>400
Diacetoxyverrucarol	4,15	>800
Scirpentriol	3,4,15	30
Monoacetoxyscirpendiol		64
Diacetoxyscirpenol		55
Triacetoxyscirpene		36
Neosolaniol	3,4,8,15	14

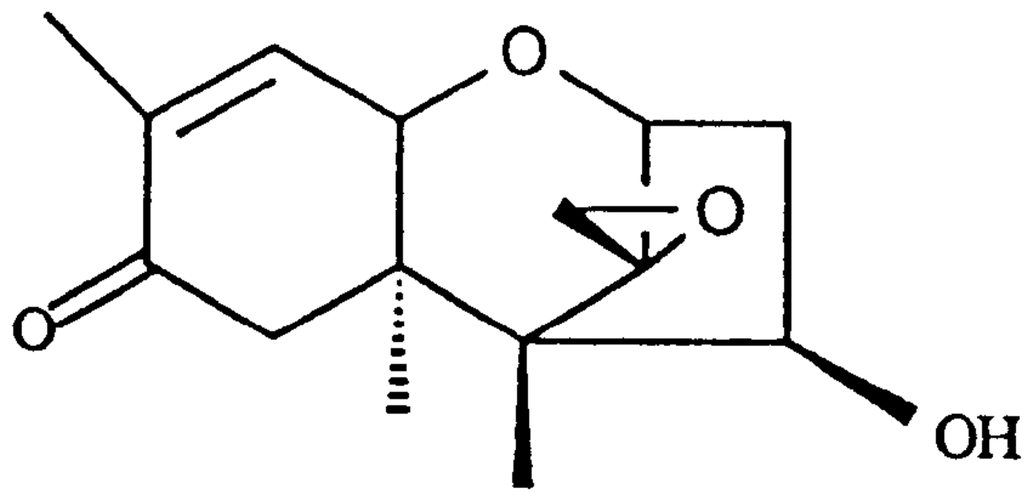
important for activity since reduction or epoxidation of this group leads to a marked decrease in cytotoxicity[22,23]. The presence and nature of substituents at C-3, C-4, C-7, C-8 and C-15 in trichothecenes has also been found to influence biologcal activity. Cytotoxicity and acute toxicity towards animals are highest in the macrocyclic trichothecenes (class D), followed by the class A and B toxins, and then the class C toxins[21] [Table 2]. In the case of the non-macrocyclic compounds, an increase in the level of oxidation of the trichothecene nucleus generally causes an increase in activity [Table 3][28]. The presence of a 3α-hydroxyl group has been found to have a particularly important influence upon toxicity[29], e.g. 3-acetyl T-2 toxin is ten times less cytotoxic than T-2 toxin (11) itself. This is thought to be due to the formation of a hydrogen bond between the 3α-hydroxyl and the oxygen in the B ring by intercalation of a molecule of water. This could protect the 12,13-epoxide from rearside nucleophilic attack before it reaches its site of action. This protective mechanism is missing when the 3α-hydroxyl is acetylated. Esterification of hydroxyl groups at C-4 and C-15 generally causes an increase in activity, but these effects may be due in part to changes in lipophilicity.

The potent cytotoxicity of trichothecenes has led to considerable interest in them as potential chemotherapeutic agents, in particular as anticancer agents. 15-Monoacetoxyscirpendiol (MAS) (25) and 4,15-diacetoxyscirpenol (DAS) (9) display marked anti-tumour activity against P-388 leukaemia in mice[19]. DAS has undergone phase II clinical trials as an anticancer agent, but with limited success due to high toxicity towards healthy tissues. Trichothecolone (12), however, has been observed to be selectively cytotoxic towards cultured tumour cells, and is currently creating interest as a potential anticancer drug[30].

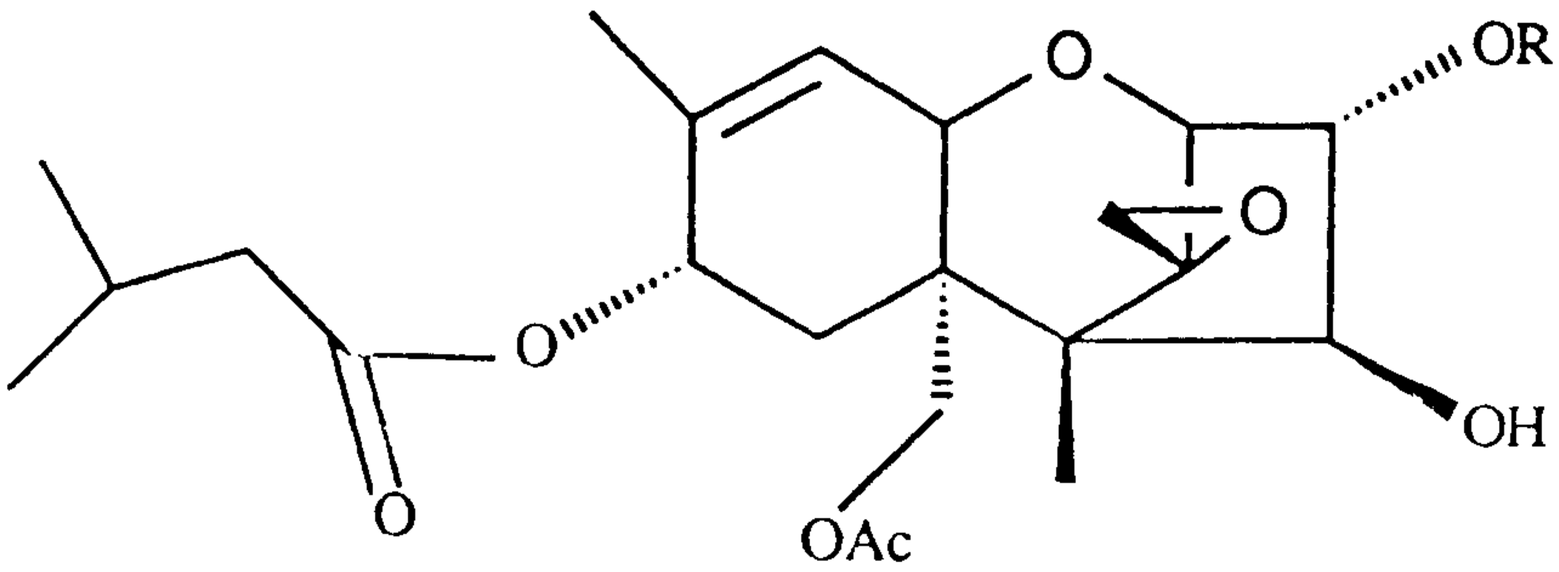


R=H, 15-monoacetoxyscirpendiol (25)

R=Ac, 4,15-diacetoxyscirpenol (9)



Trichothecolone (12)



R=H, T-2 toxin (11)

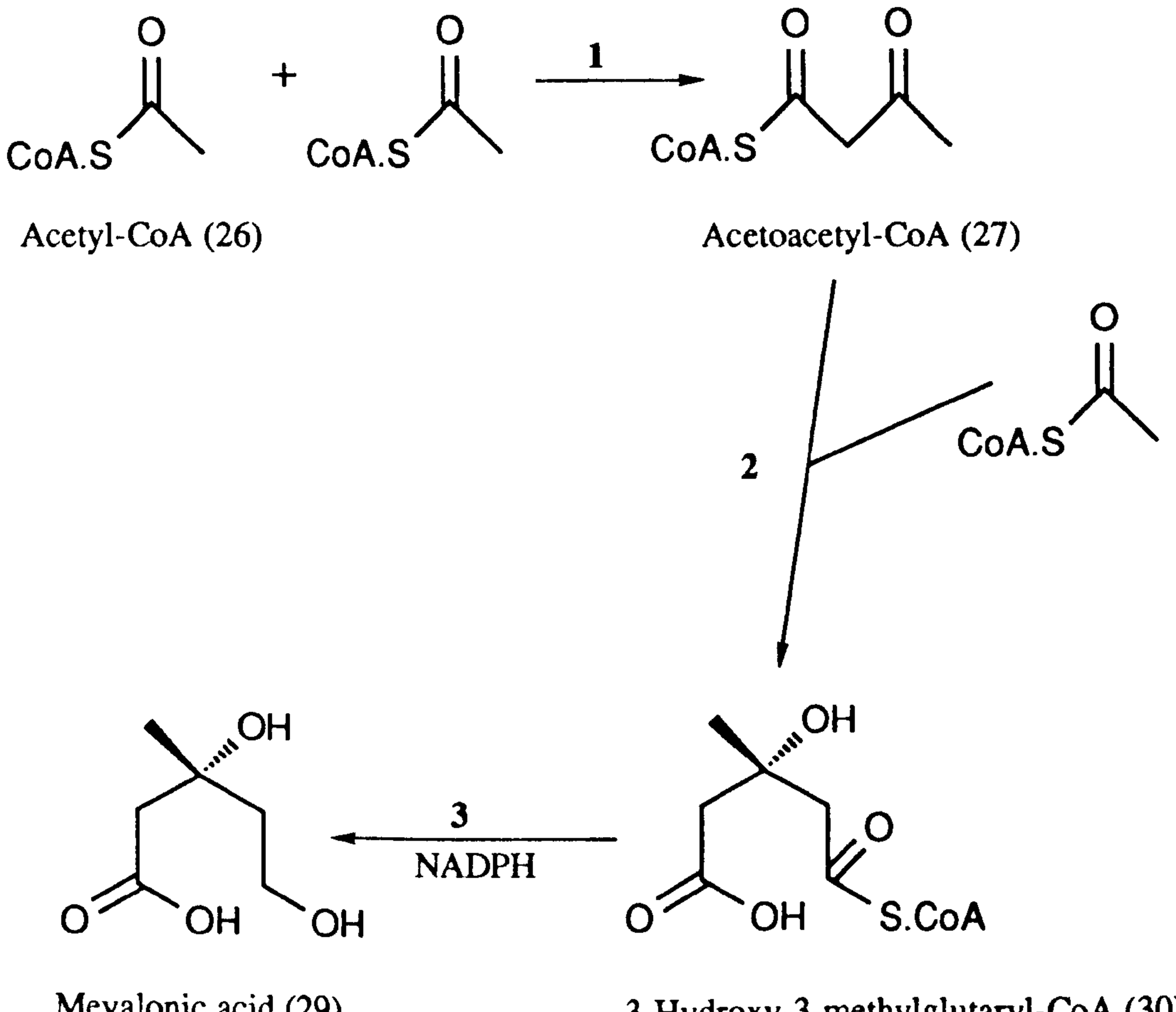
R=C(O)CH_3, 3-Acetyl-T-2 toxin

BIOSYNTHESIS.

The trichothecenes are sesquiterpenoid derivatives and thus part of one of the largest groups of secondary metabolites, the terpenoids. The biosynthesis of terpenoids has been studied in detail[31,32,33] and a sequence from mevalonate via isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) is known to be operative.

Mevalonic acid (MVA) (29) is synthesised from acetyl coenzyme-A (acetyl-CoA) (26) via 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) (28) [Fig.6]. Claisen condensation of two acetyl-CoA units yields acetoacetyl-CoA (27) which, on aldol-like addition of another unit of acetyl-CoA produces HMG-CoA. This is then reduced with NADPH to form MVA. The formation of IPP from MVA involves three enzymic reactions each requiring ATP [Fig.7]. MVA is phosphorylated twice to form the 5-diphosphate ester (30) which then undergoes decarboxylation and dehydration to produce IPP (31). IPP can then be isomerised to DMAPP (32), with the isomerase enzyme selectively removing a mevalonate 4-*pro*-S proton (H_S in Fig.7).

Condensation of IPP with DMAPP produces geranyl diphosphate (GPP) (33). Farnesyl diphosphate (FPP) (34), the precursor to the sesquiterpenes, is formed from GPP by a similar addition of IPP [Fig.8A]. The mechanism for the condensations is known to be ionic[34] [Fig.8B], and occurs with inversion of configuration at C-1 of DMAPP. Initial ionisation of the diphosphate generates an allylic cation which undergoes an electrophilic attack onto the double bond of IPP. Elimination of the 2-*pro*-R hydrogen of the incoming IPP forms the new double bond.



Acetyl-CoA (26)

Acetoacetyl-CoA (27)

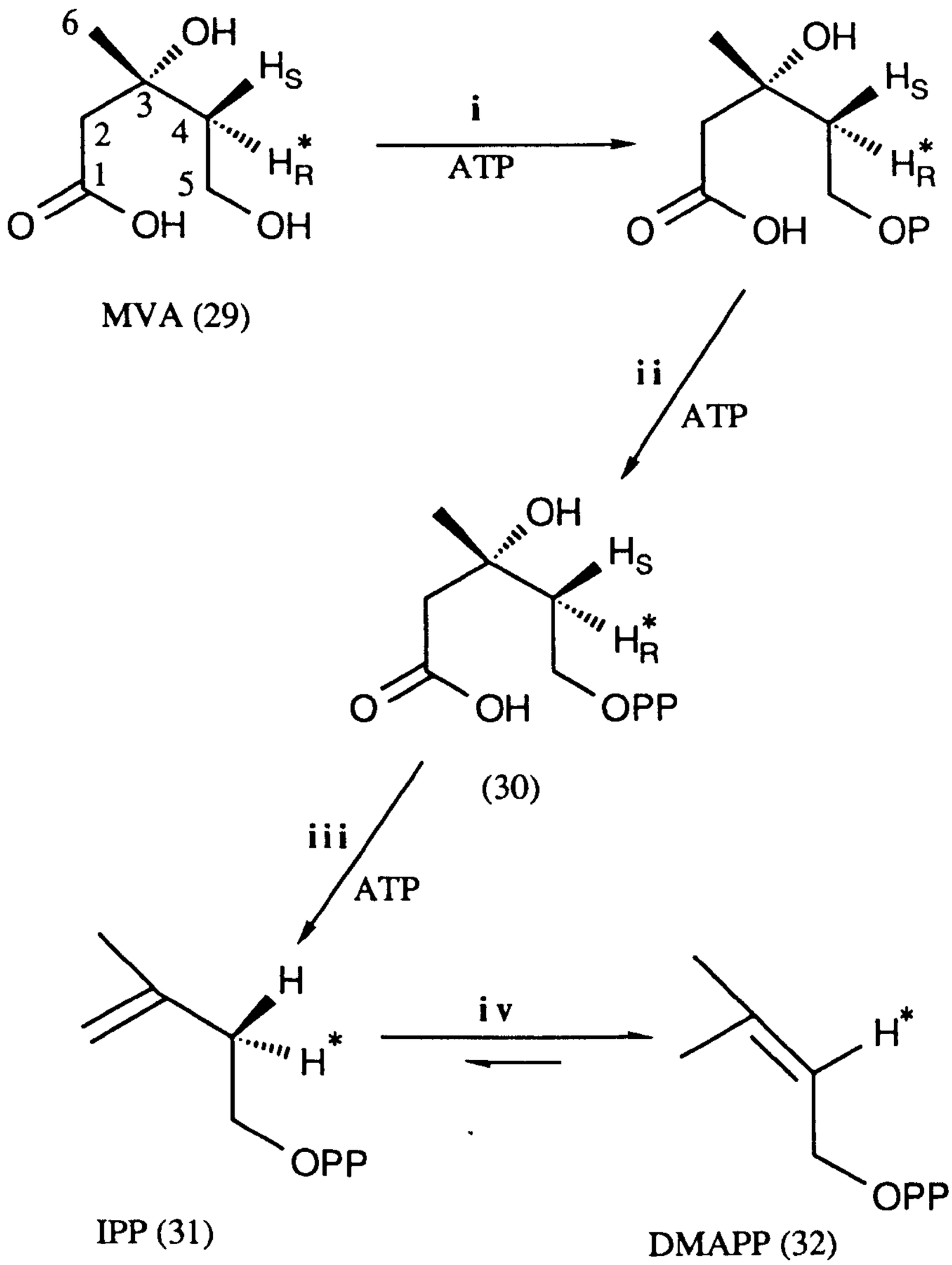
Mevalonic acid (29)
(MVA)

3-Hydroxy-3-methylglutaryl-CoA (30)
(HMG-CoA)

ENZYMES:

1. β-Ketoacylthiolase.
2. HMG-CoA synthetase.
3. HMG-CoA reductase.

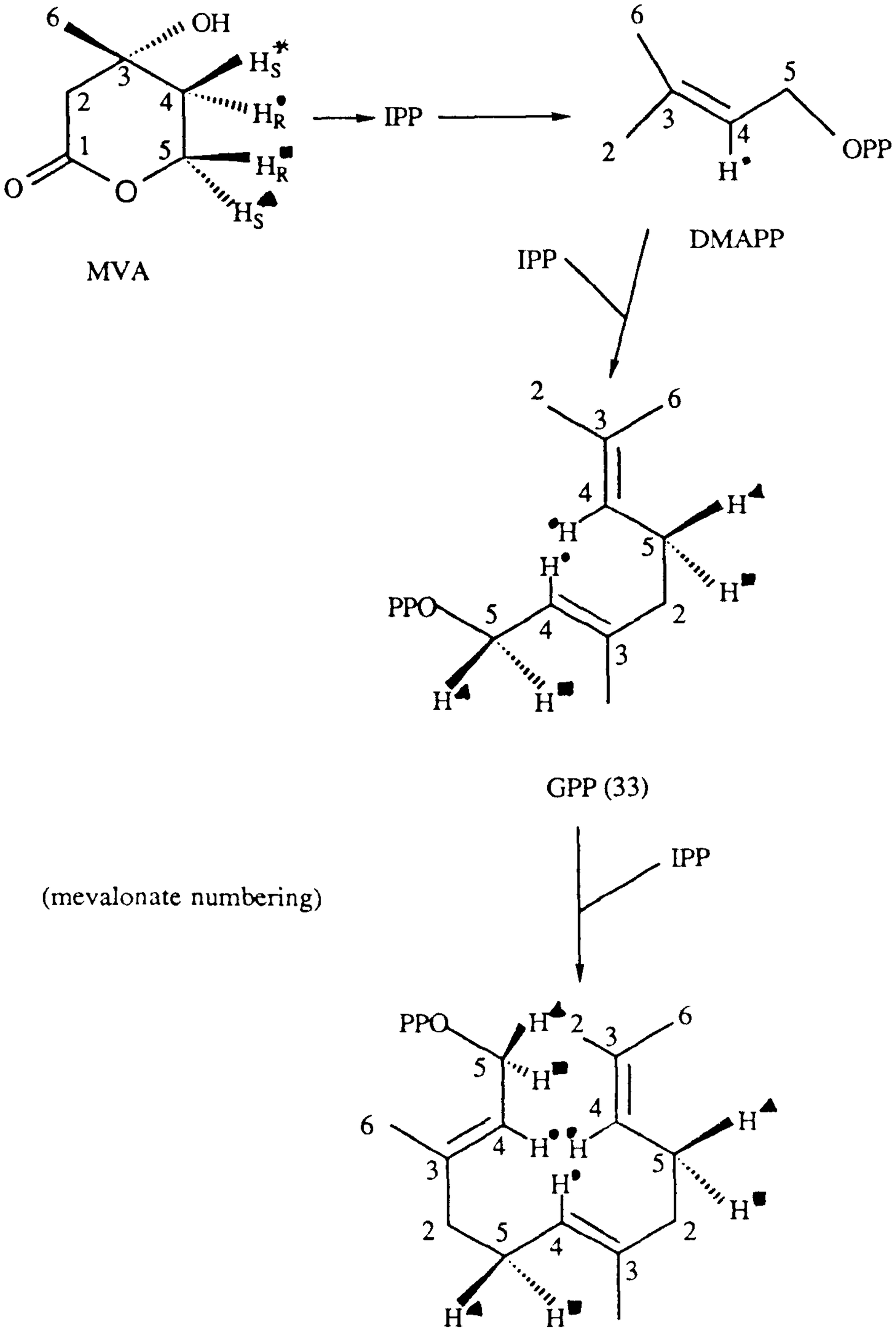
Fig.6: Biosynthesis of mevalonic acid.



ENZYMES:

i. MVA kinase.
ii. 5-PhosphoMVA kinase.
iii. DiphosphoMVA kinase.
iv. Isoprenyldiphosphate isomerase.

Fig.7: Biosynthesis of IPP and DMAPP from MVA.



FPP (34)

Fig.8A: Biosynthesis of FPP.

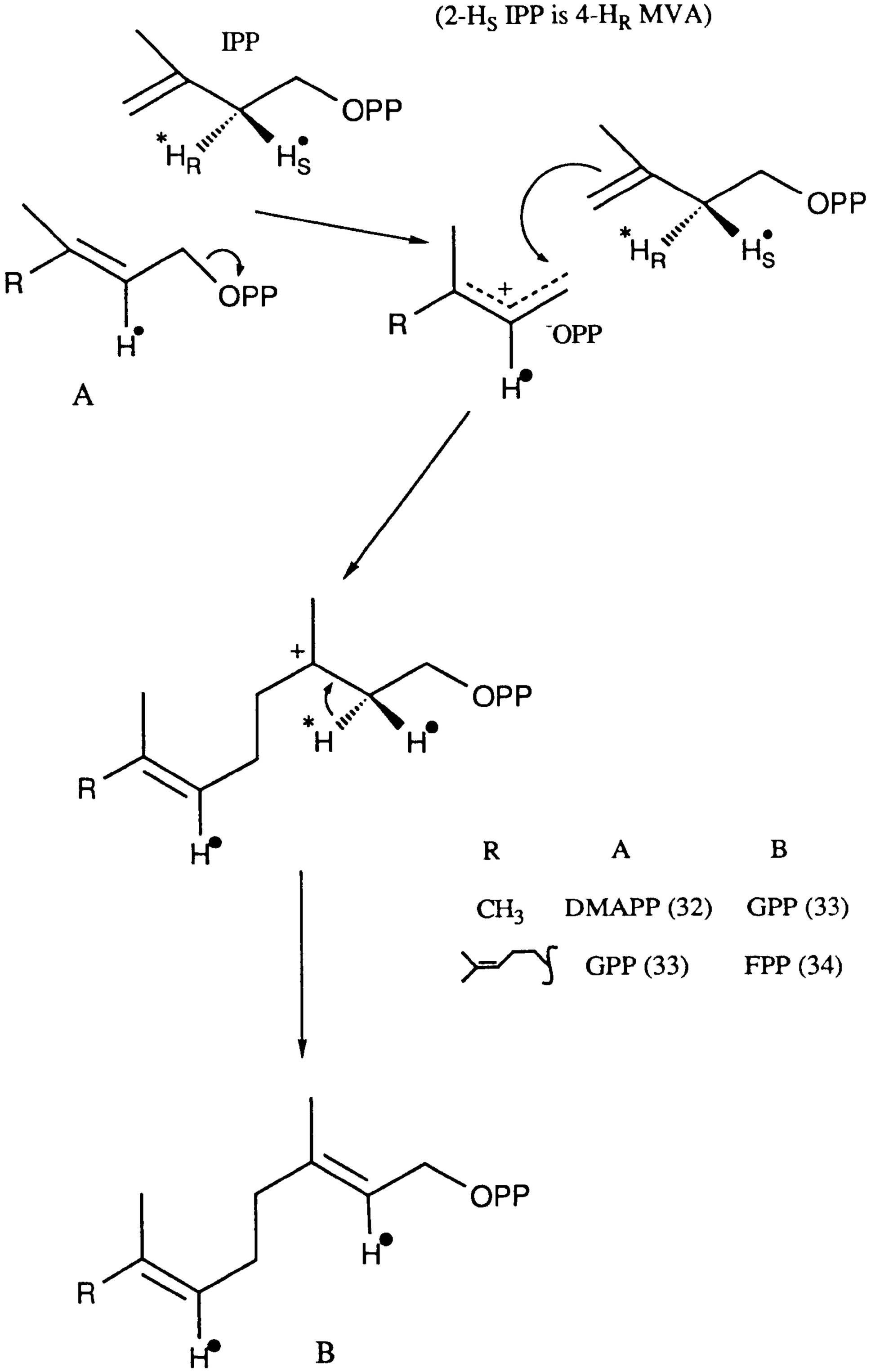


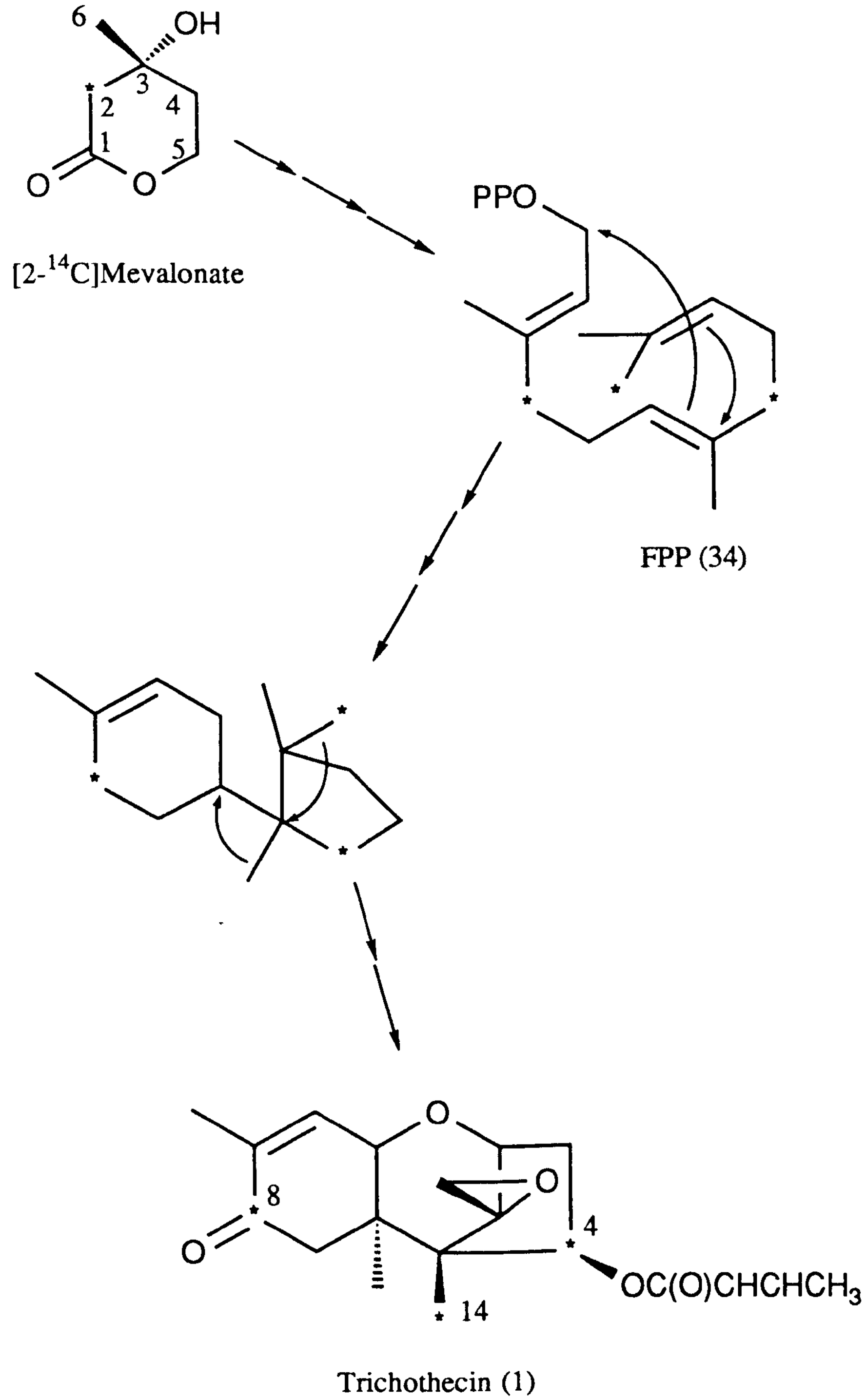
Fig.8B: Ionic condensation of IPP with allyl diphosphates.

The sesquiterpenoid origin of trichothecenes was first established in 1960 by Jones and Lowe[35] who observed the incorporation of three molecules of [2-^{14}C]mevalonate into the trichothecene nucleus of trichothecin (1) in *Trichothecium roseum*. The demonstration of FPP as a precursor to trichothecenes[36] provided confirmation of this. Extensive chemical degradations indicated that the carbons at positions 4, 8 and 14 of the trichothecene skeleton were derived from [2-^{14}C]mevalonate. This labelling pattern was consistent with a double 1,2-methyl shift occurring during the biosynthesis [Fig.9].

Biosynthesis of trichothecenes from FPP.

Intramolecular attack to the carbon bearing the diphosphate ester by either the central or distal double bonds of FPP can produce a range of cyclic sesquiterpenes[37]. For cyclisation to occur the π-orbitals of FPP must be properly aligned, and in order to produce the trichothecene skeleton there are a limited number of conformations of FPP which meet this condition. Extensive investigations into the incorporation of [^{3}H]- and [^{3}H,^{14}C]-mevalonates into trichothecolone (12) in *Trichothecium roseum*[36,38,39] and verrucarol (8) in *Myrothecium verrucaria*[40] indicated that FPP must be in the 6E configuration, and ruled out the 6Z isomers [Fig.10]. This was deduced from the observation that C-8 of the trichothecene nucleus is derived from C-2 of mevalonate, and that C-10 is derived from C-4 of the precursor. Studies into the incorporation of [1-^{3}H,2-^{14}C]FPP and [2-^{3}H,2-^{14}C]FPP[36] into trichothecin (1) confirmed (6E)-FPP as a precursor.

The first postulated biosynthetic sequences for the cyclisation of (6E)-FPP to trichothecenes involved γ-bisabolene (35)[40,41,42] and trichodiene (36)[43] as



Trichothecin (1)

Fig.9: Double 1,2-methyl shift required to label trichothecene carbons 4, 8 and 14 from [2-^{14}C]mevalonate.

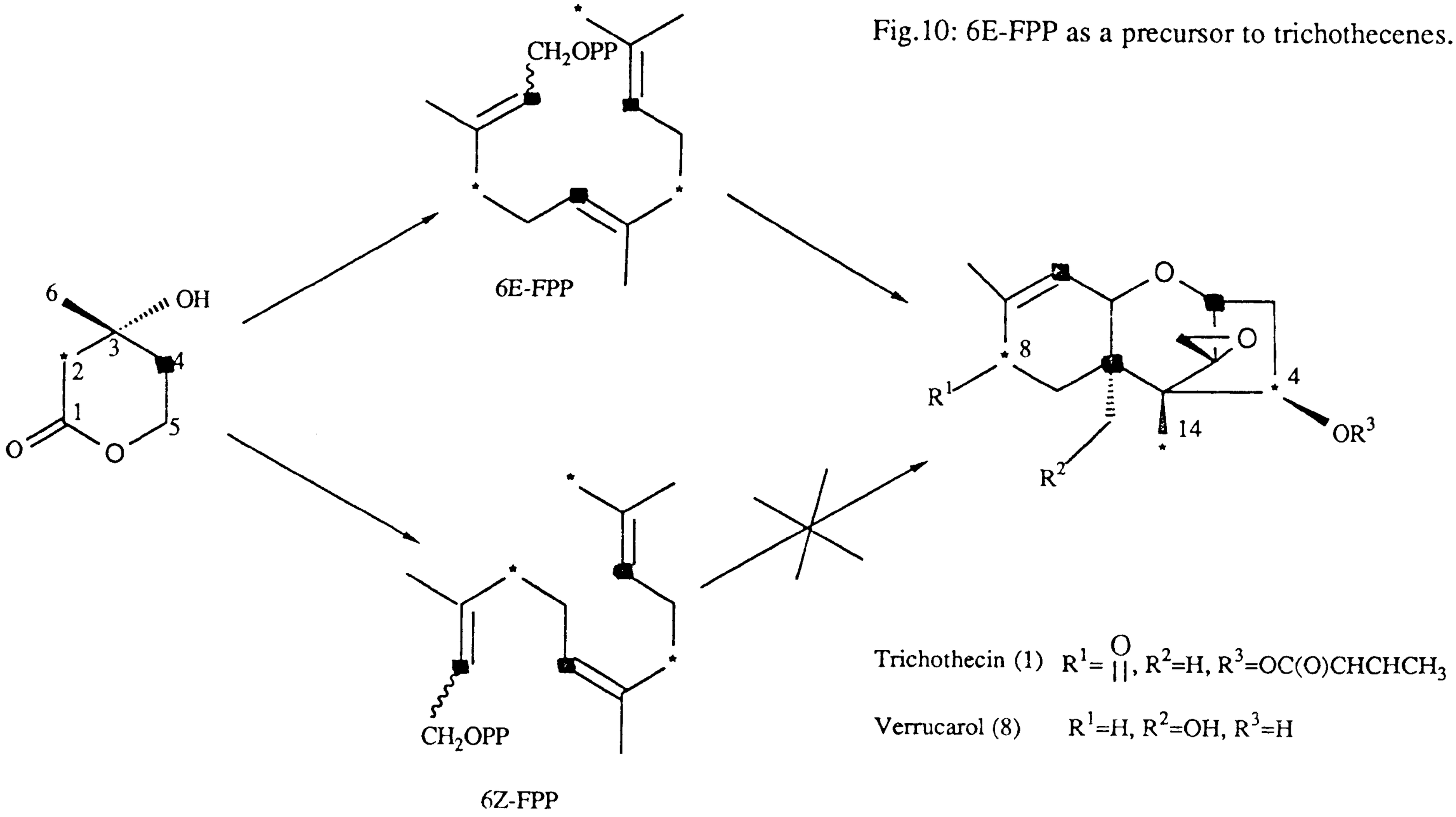


Fig.10: 6E-FPP as a precursor to trichothecenes.

Trichothecin (1) $R^1=$, R^2=H, R^3=OC(O)CHCHCH$_3$

Verrucarol (8) R^1=H, R^2=OH, R^3=H

intermediates [Fig.11]. It was proposed that cyclisation of (6E)-FPP could yield γ-bisabolene via a monocyclic intermediate. Formation of a five-membered ring produces trichodiene which on subsequent oxygenation gives the trichothecenes. The intermediacy of γ-bisabolene was ruled out from the results of a series of feeding experiments using a variety of labelled substrates[36,44,45]. In one study (4R)-[4-^{3}H]-MVA was chosen as a precursor because it is known that in the formation of FPP from MVA it is the *pro*-4S mevalonate hydrogen which is lost, whilst the *pro*-4R is retained (see Fig.7). The incorporation into trichothecin (1) of two of the possible three tritium atoms was observed [Fig.12a]. Degradations located the labels at C-10 and C-2, with the label at C-10 subsequently being shown to originate from the tritium at C-2 of (6E)-FPP (34). Retention of the tritium label in trichothecin when [2-^{3}H,2-^{14}C]GPP was fed [Fig.12b] demonstrated that it was the (4R)-[4-^{3}H]-mevalonate hydrogen incorporated at C-6 of FPP (as opposed to C-10) which provided the label at C-2 of trichothecin[44]. Hence, during the biosynthesis there must be a hydride shift from C-6 of FPP which then becomes H-2 in the final product. This shift was also observed in the biosynthesis of verrucarol (8) from [6-^{3}H]FPP[45] [Fig.12c]. This clearly precludes the intermediacy of γ-bisabolene, for if it had been an intermediate, only one label from (4R)-[4-^{3}H]-MVA would have been incorporated into the trichothecene product [Fig.12d]. A feeding experiment using labelled γ-bisabolene subsequntly confirmed that it was not a precursor to the trichothecenes[46].

Trichodiene (TDN) (36) was first isolated from *Trichothecium roseum*[47] together with two other new metabolites identified as trichodiol (37)[48] and 12,13-epoxytrichothec-9-ene (EPT) (3)[39]. TDN was shown to be a precursor to the trichothecenes when feeding experiments resulted in high incorporation (3.2%) into

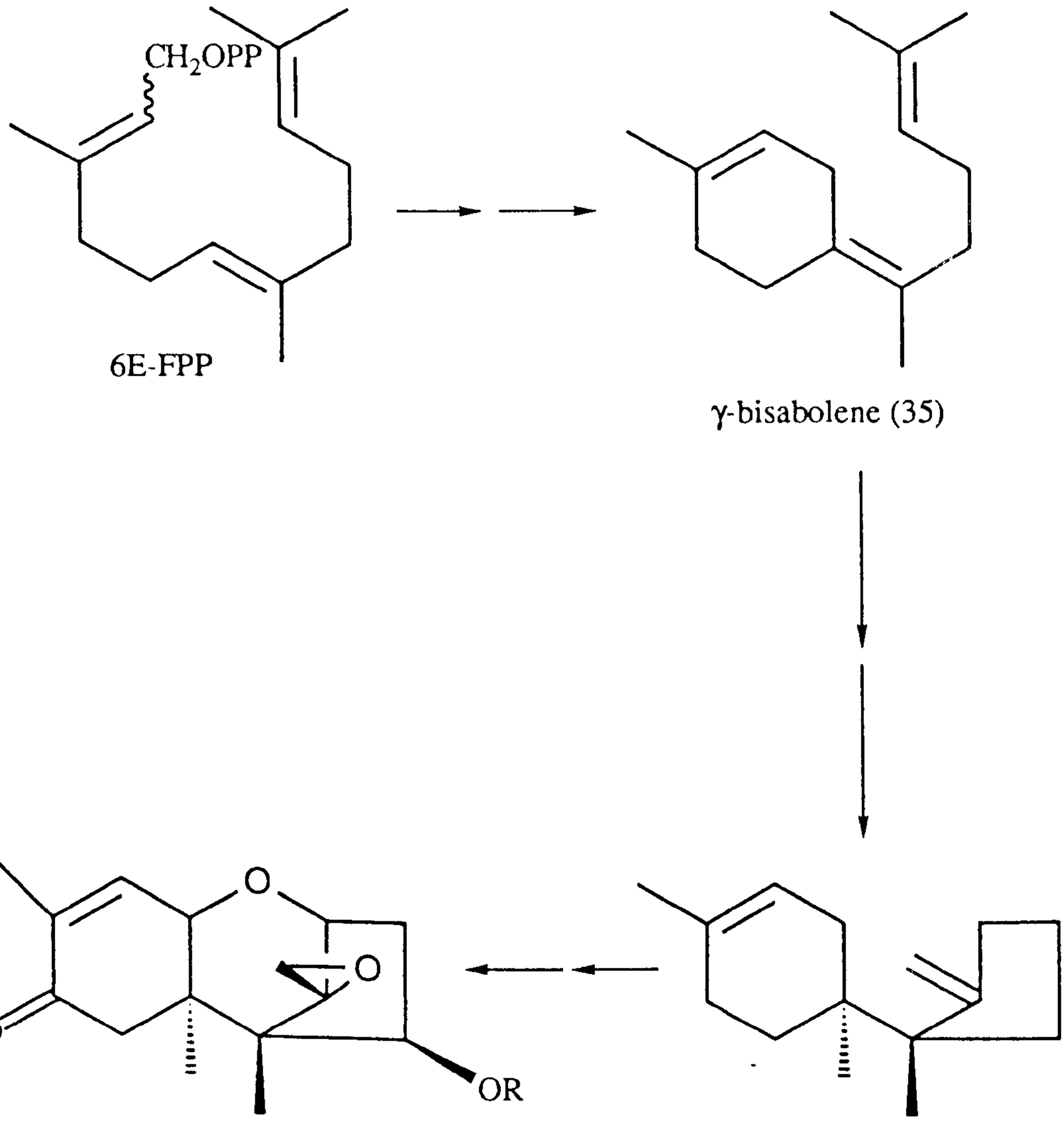
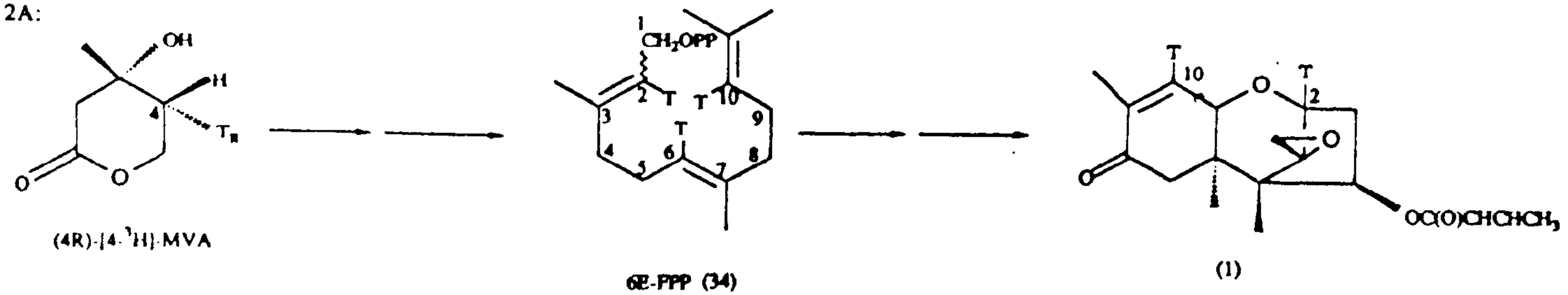


Fig.11: Postulated intermediacy of γ-bisabolene and trichodiene in the biosynthesis of trichothecenes.

Fig.12: Proof for a hydride shift in the cyclisation of FPP, precluding the intermediacy of γ-bisabolene.

Fig.12A:

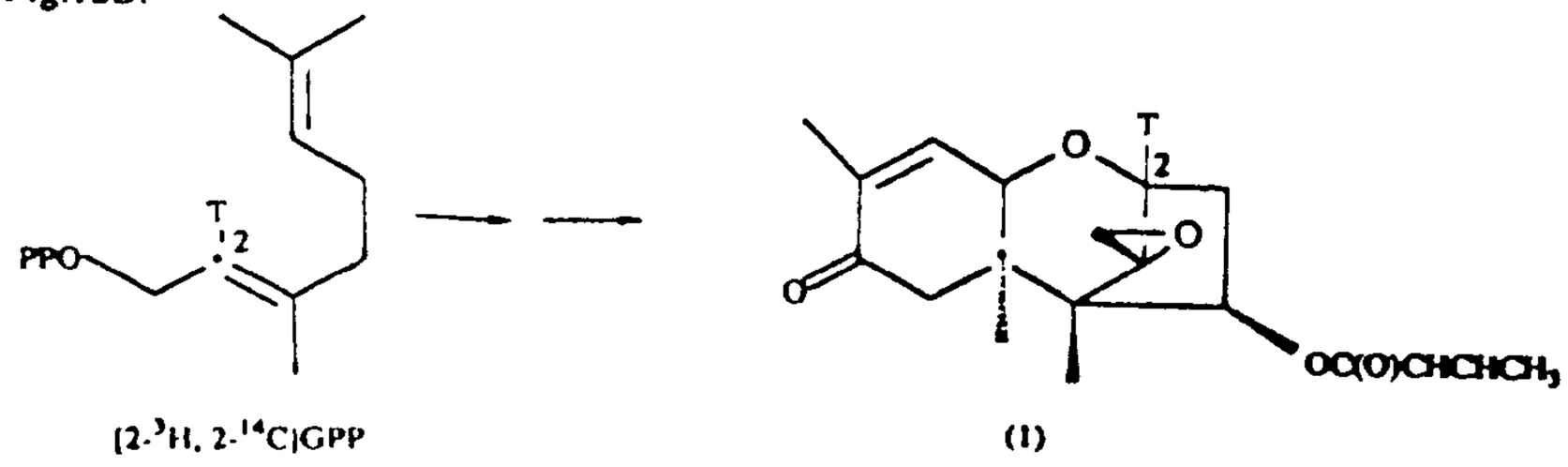


(4R)-[4-^{3}H]-MVA

6E-FPP (34)

(1)

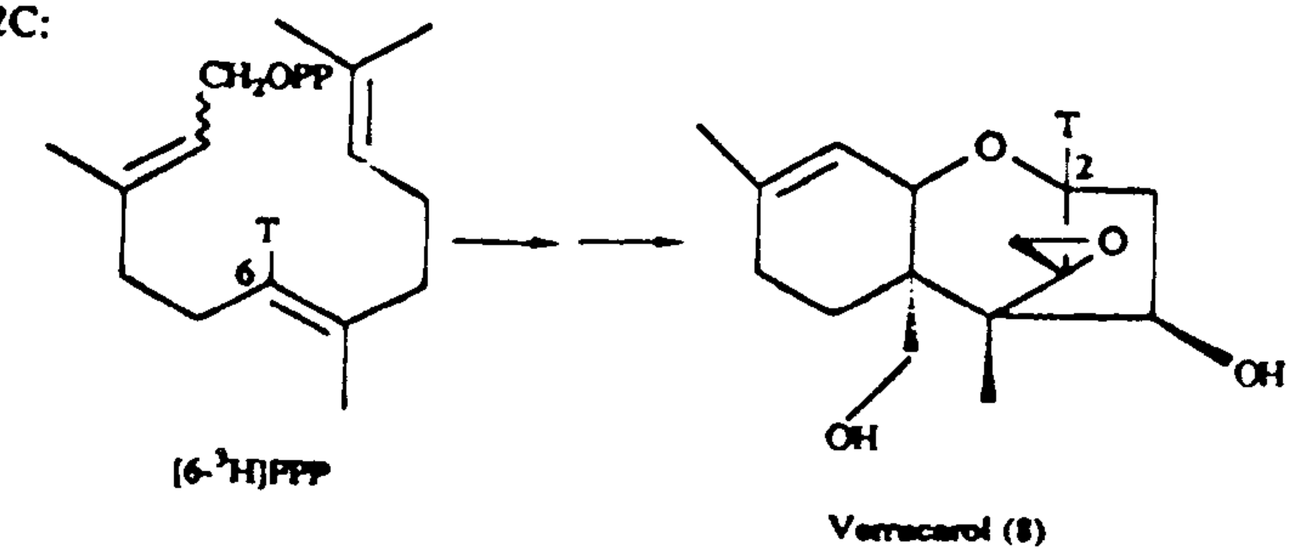
Fig.12B:



[2-^{3}H, 2-^{14}C]GPP

(1)

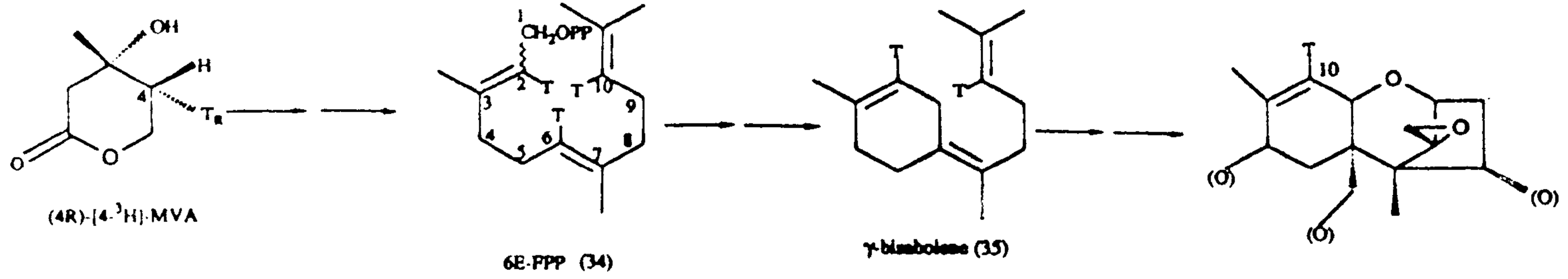
Fig.12C:



[6-^{3}H]FPP

Verrucarol (8)

Fig.12D:



(4R)-[4-^{3}H]-MVA

6E-FPP (34)

γ-bisabolene (35)

trichothecolone (12)[49]. Trichodiol and EPT were also found to be labelled. The conversion of (2E,6E)-FPP into TDN by a cell-free preparation of *T. roseum* demonstrated it to be a key intermediate in the biosynthesis of trichothecenes from FPP[50] and a revised biosynthetic scheme was put forward [Fig.13][38,44]. This proposed a concerted cyclisation sequence initiated by the attack of an enzyme at C-10 of FPP. TDN is then formed by a 1,4-hydride shift followed by two 1,2-methyl migrations and abstraction of a proton. Oxidation to trichodiol with subsequent cyclisation to EPT could produce the trichothecene skeleton. The more complex trichothecenes might then be formed by appropriate hydroxylations and esterifications of EPT.

Biosynthesis of TDN from (2E,6E)-FPP.

The direct formation of six-membered rings from (2E,6E)-FPP is geometrically prohibited since the resultant cyclohexene derivative must contain a *cis*, not a *trans*, double bond. It was therefore evident that a 2E-2Z isomerisation must occur prior to cyclisation. Further investigations into this area were facilitated by the development of a cell-free preparation from *T. roseum* which mediated the conversion of (2E,6E)-FPP into TDN[50,51]. Using this system, cyclisation of [1,5,9-^{3}H, 4,8,12-^{14}C]FPP (^{3}H:^{14}C ratio = 6:3) to TDN was shown to occur with loss of one equivalent of tritium (^{3}H:^{14}C in TDN = 5.2:3)[50]. The other significant product formed was identified as (2Z,6E)-farnesol (41), indicating that *trans-cis* isomerisation of the C-2,3 double bond had indeed taken place. The tritium lost in both of these transformations was found to originate from C-1 of FPP[51]. These results are consistent with a redox mechanism for the isomerisation process prior to cyclisation [Fig.14]. Such a redox mechanism had been demonstrated in orange

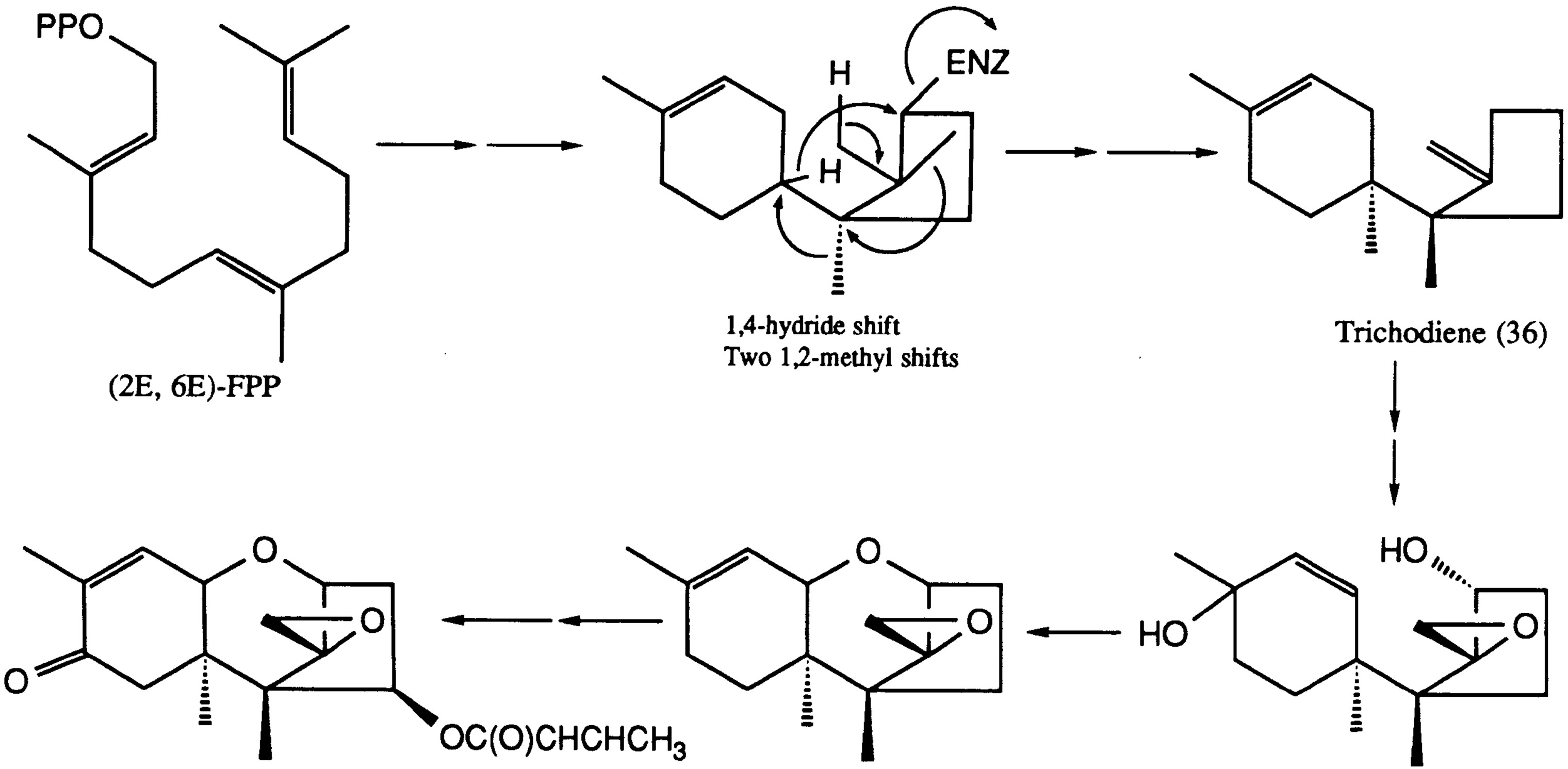
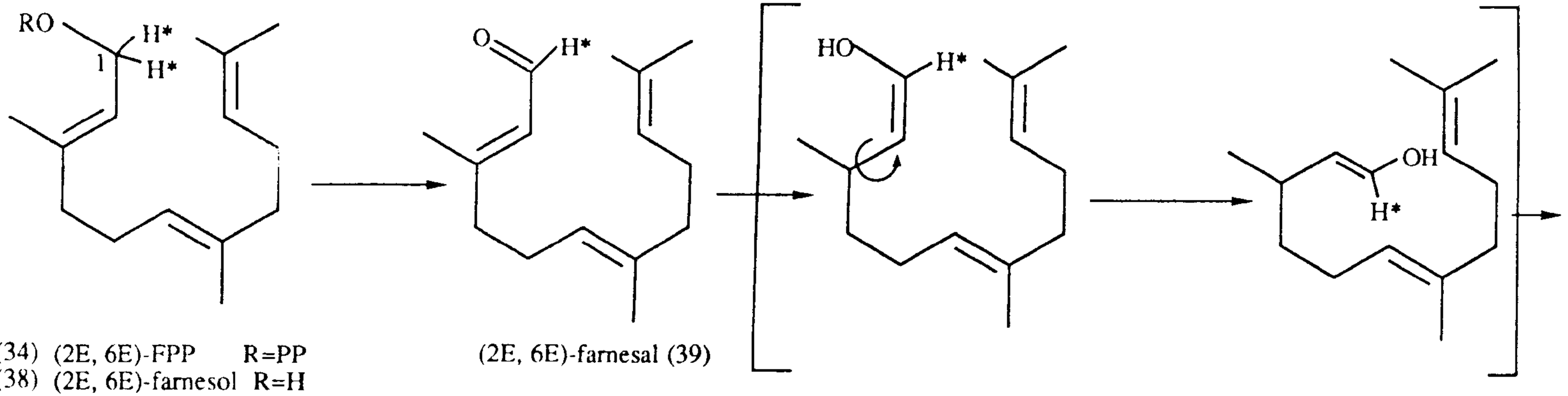
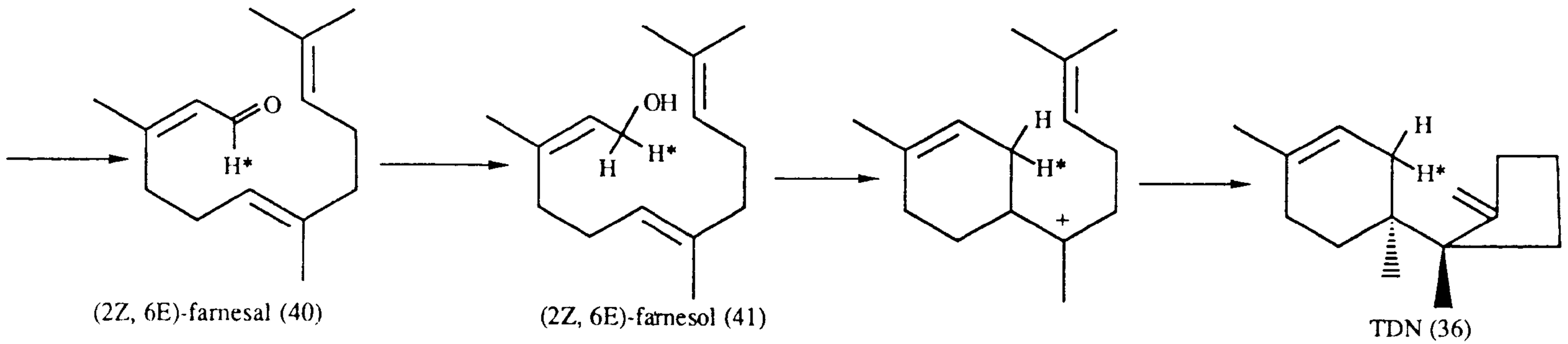


Fig.13: Trichodiene as a key intermediate in the biosynthesis of trichothecenes from FPP.



(34) (2E, 6E)-FPP R=PP
(38) (2E, 6E)-farnesol R=H

(2E, 6E)-farnesal (39)



(2Z, 6E)-farnesal (40)

(2Z, 6E)-farnesol (41)

TDN (36)

Fig.14: Redox mechanism for the isomerisation-cyclisation of (2E, 6E)-FPP to TDN accompanied by loss of a proton from C-1 of FPP

(*Citrus sinensis*). In a time course study using an enzyme preparation derived from orange flavedo, the order of appearance of products when feeding GPP and IPP was found to be *trans*-farnesol (38), *trans*-farnesal (39), 2-*cis*-farnesal (40) and 2-*cis*-farnesol (41)[52]. However, no isomerisations of the corresponding phosphates were observed, and it was later demonstrated that farnesol was not a substrate for the fungal system of *T. roseum*. Also, no significant radioactivity was found to be associated with farnesal when labelled (2E,6E)-FPP was used as a substrate[51].

Re-examination of the cell-free biosynthesis of (2E,6E)-FPP demonstrated conclusively that the isomerisation-cyclisation process in fact takes place without loss of tritium from C-1 of the substrate[53]. This clearly excluded all redox mechanisms and favoured an alternative scheme involving isomerisation to the tertiary allylic isomer nerolidyl diphosphate (NPP) (42) [Fig.15]. Rotation about the C-2,3 single bond allows NPP to adopt a conformation suitable for cyclisation to the bisabolyl cation (43). Further intramolecular cyclisation followed by a 1,4-hydride shift, two methyl migrations, and deprotonation generates TDN. All these operations are assumed to take place at a single active site of the enzyme trichodiene synthase. Purification of this enzyme[54] facilitated verification of this proposed mechanism.

Cyclisation of (2E,6E)-FPP, already known to occur without loss of either hydrogen from C-1, was also shown to take place with net retention of configuration at this centre. This was determined by experiments in which (1S)- and (1R)-[1-^{3}H,12,13-^{14}C]-FPP were incubated separately with trichodiene synthase (TS)[54] which established that the 1-*pro*-R proton of FPP (H_B in Fig.15) becomes H-11β of TDN, whilst the 1-*pro*-S proton (H_A in Fig.15) becomes H-11α of TDN. This is consistent with isomerisation to NPP prior to cyclisation, and

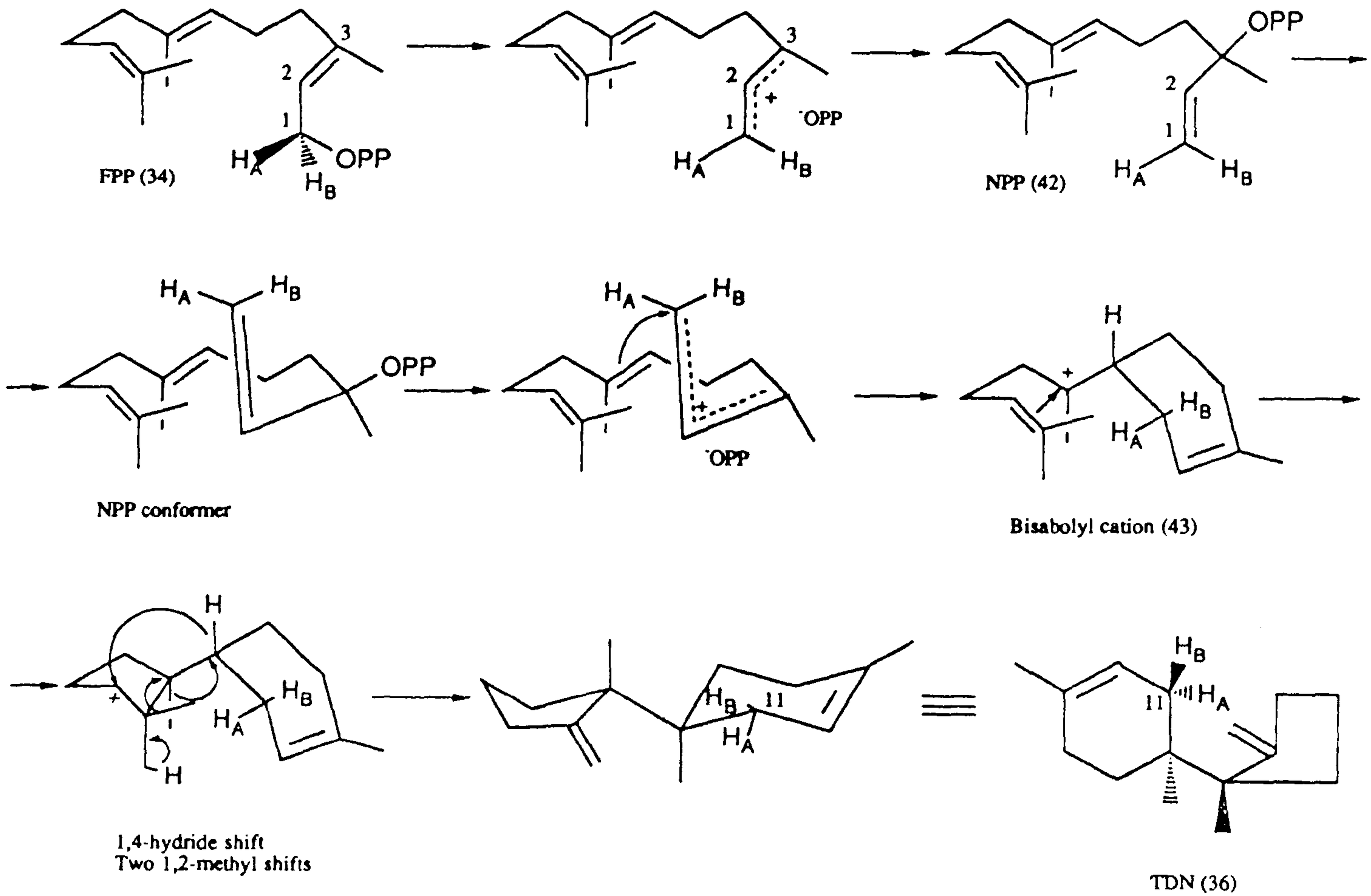


Fig.15: Isomerisation-cyclisation of (2E,6E)-FPP to TDN *via* NPP.

contrasts with the inversion of configuration observed in the synthesis of FPP from IPP and DMAPP (see Fig.8).

NPP was then proven to be a precursor of TDN by incubating (1Z)-[1-^{3}H,12,13-^{14}C]-NPP (44) with TS, which produced TDN labelled at H-11β[55,56] [Fig.16a]. TS was shown to utilise only the (3R)-enantiomer of NPP by incubating a mixture of (3S,1Z)-[1-^{3}H]-NPP (45) and (3RS)-[12,13-^{14}C]-NPP (46,47) with the enzyme. This yielded TDN labelled only with ^{14}C [Fig.16b][55,56]. The intermediacy of NPP in the biosynthesis of TDN from FPP was further demonstrated in a competition feeding experiment involving [1-^{3}H]FPP and [12,13-^{14}C]NPP[55]. Measurement of the ^{3}H/^{14}C ratio in the resulting TDN indicated that both compounds compete for the same active site in TS. However, the calculated values of V_{max}/K_M for (3R)-NPP was found to be approximately twice that for FPP, implying that the initial isomerisation to NPP is the rate determining step in the production of TDN from FPP.

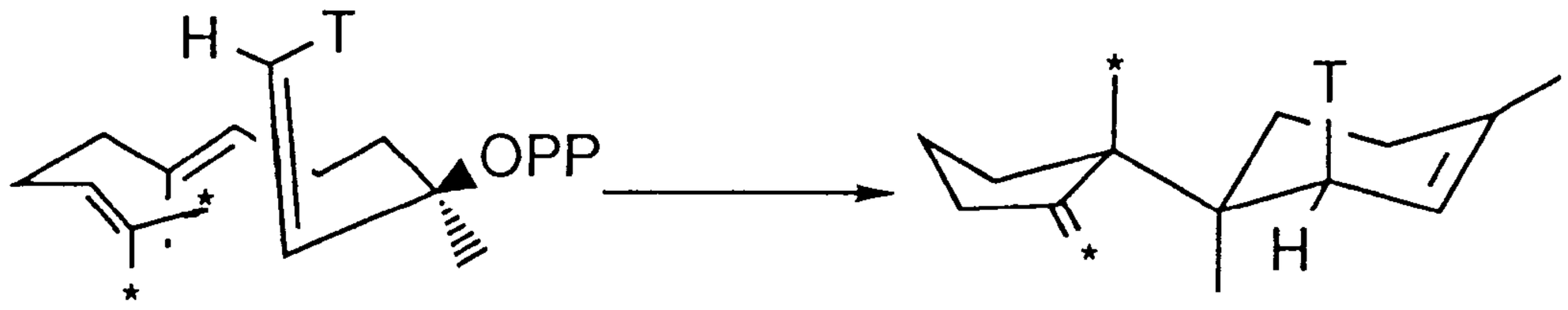
TS has since been isolated from *Fusarium sporotrichioides*[57], purified and the protein amino acid sequence deduced. The TS gene has also been isolated and the nucleotide sequence determined[58].

Post-trichodiene biosynthesis of trichothecenes.

TDN is generally accepted to be the hydrocarbon precursor to all the trichothecenes, but the nature of its conversion and the intermediates involved are largely unknown. Trichodiol (37) and EPT (3) were isolated from the same source as TDN and proposed as intermediates in the observed formation of trichothecin from TDN (see Fig.13), but their involvement has never formally been demonstrated. EPT has subsequently been isolated from *Fusarium culmorum*[59]

Fig.16.

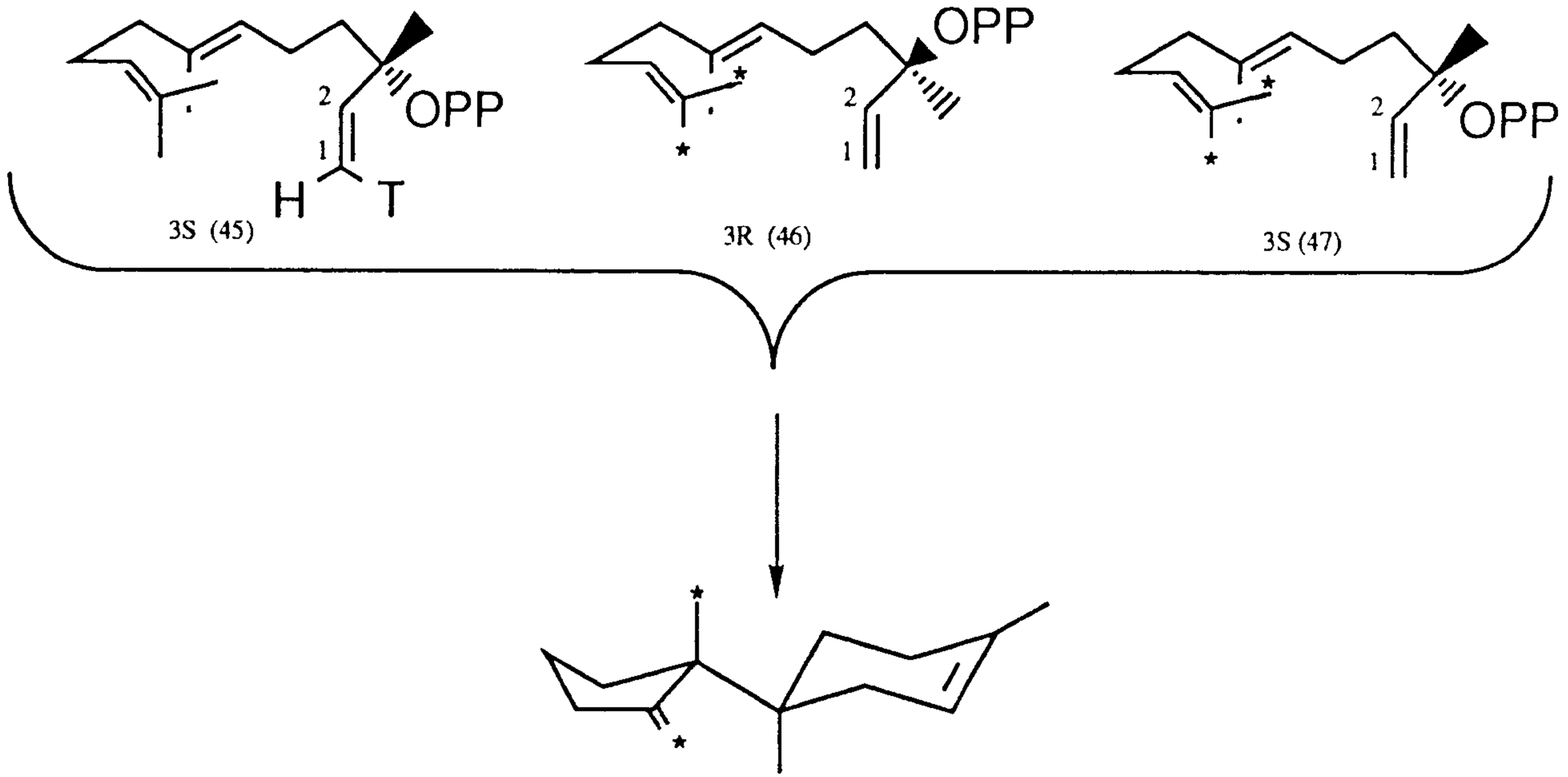
A



(1Z)-[1-^{3}H, 12,13-^{14}C]NPP (44)

TDN

B



3S (45)

3R (46)

3S (47)

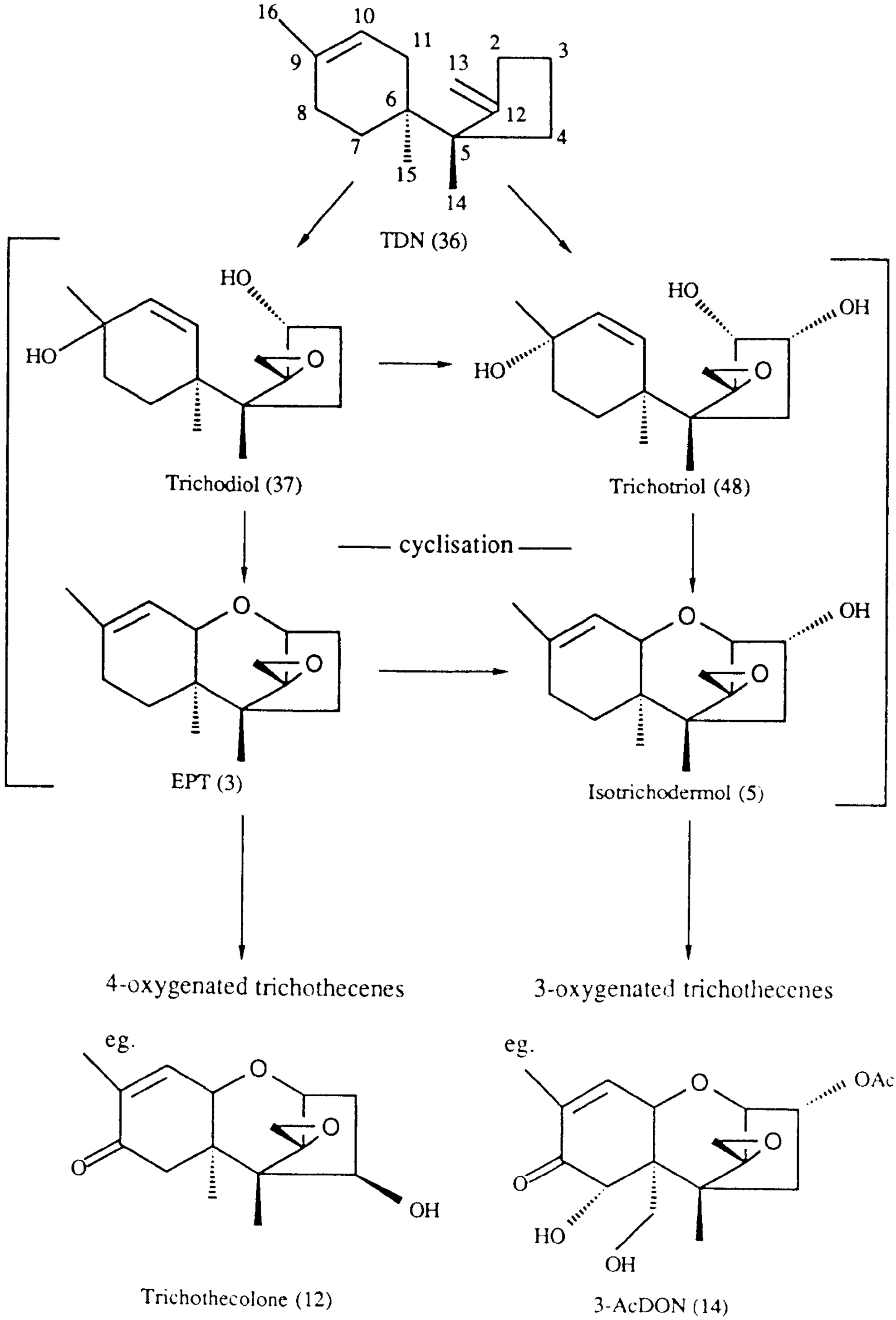
TDN

supporting the hypothesis that it may be a preursor in the biosynthesis of all trichothecenes. The isolation of trichotriol (48) from *Fusarium sporotrichioides*[60], and its observed non-enzymatic acid-catalysed cyclisation to isotrichodermol (5), suggests an alternative (and complementary) sequence leading to the 3-oxygenated trichothecenes in which introduction of the 3α-hydroxyl occurs prior to cyclisation [Fig.17]. A concerted S_N2' mechanism has been formulated for the cyclisation of both trichodiol and trichotriol involving attack by the C-2α hydroxyl onto C-11, with transfer of the C-10,11 double bond to C-9,10, and expulsion of the C-9 hydroxyl group.

More recently, the isolation of a number of novel uncyclised trichothecenes from mutant strains of *F. sporotrichioides* has been reported[61]. Five strains were produced which were all blocked in their ability to synthesise T-2 toxin (11) but were found to accumulate a number of novel metabolites based on TDN [Fig.18]. Their appearance in blocked mutants suggests that these compounds may have a role in trichothecene biosynthesis. This aspect is discussed in detail in the results and discussion section (see p. 150).

In order for TDN to give rise to the trichothecenes several oxygenation steps are required. The oxygen atoms in T-2 toxin (11) were shown to originate from either water or molecular oxygen[62,63] [Fig.19]. Labelling of T-2 toxin produced in the presence of either $^{18}O_2$ or $H_2{}^{18}O$ was analysed using GC-MS. Up to three ^{18}O atoms per molecule of T-2 were detected when the toxin was biosynthesised in the presence of $H_2{}^{18}O$, whereas up to six were incorporated from $^{18}O_2$. Hydrolysis to T-2 tetraol (49) showed that the ^{18}O atoms derived from $H_2{}^{18}O$ were associated with side-chain carbonyl oxygens. Similarly, the oxygens in position 1, the 12,13-epoxide and the hydroxyl groups at C-3, C-4, C-8 and C-15 were shown to

Fig.17: Proposed intermediates in the biosynthesis of trichothecenes from TDN.

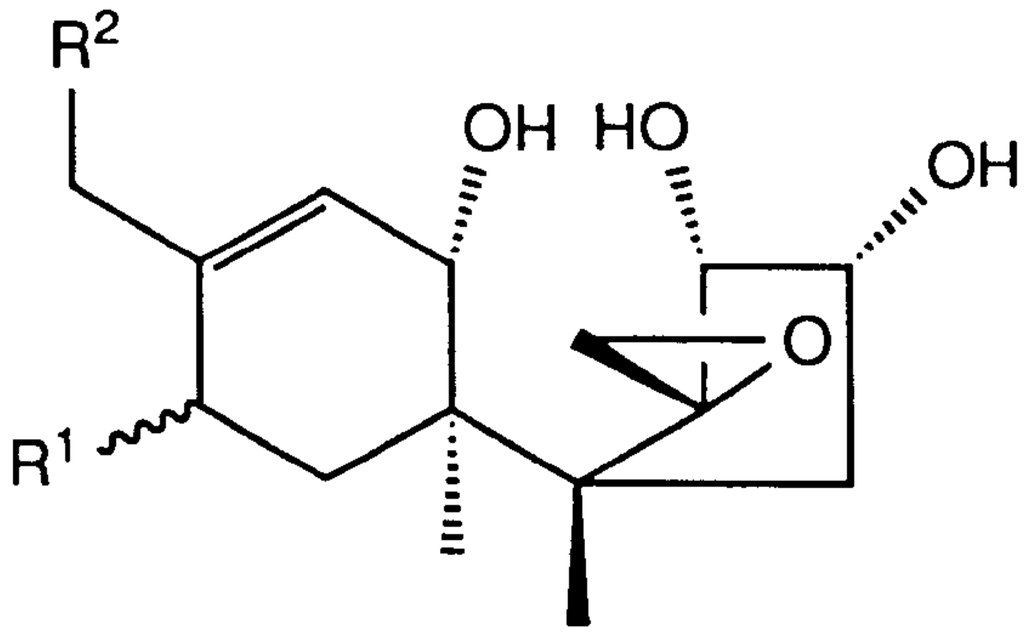


eg.

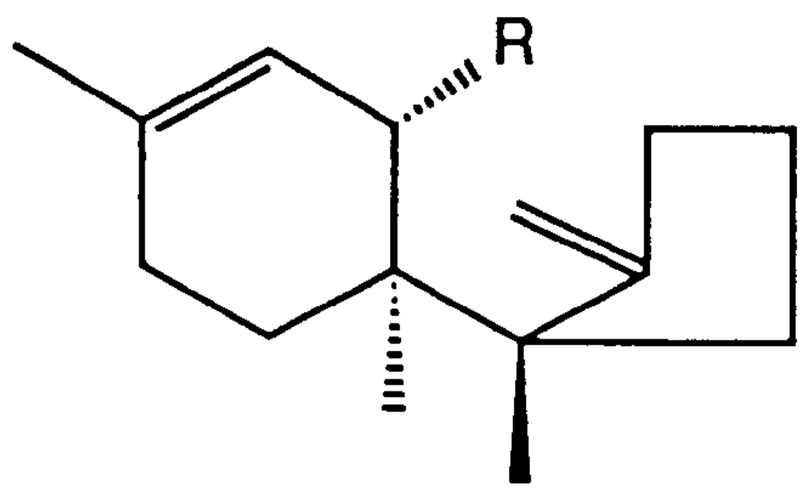
Trichothecolone (12)

eg.

3-AcDON (14)



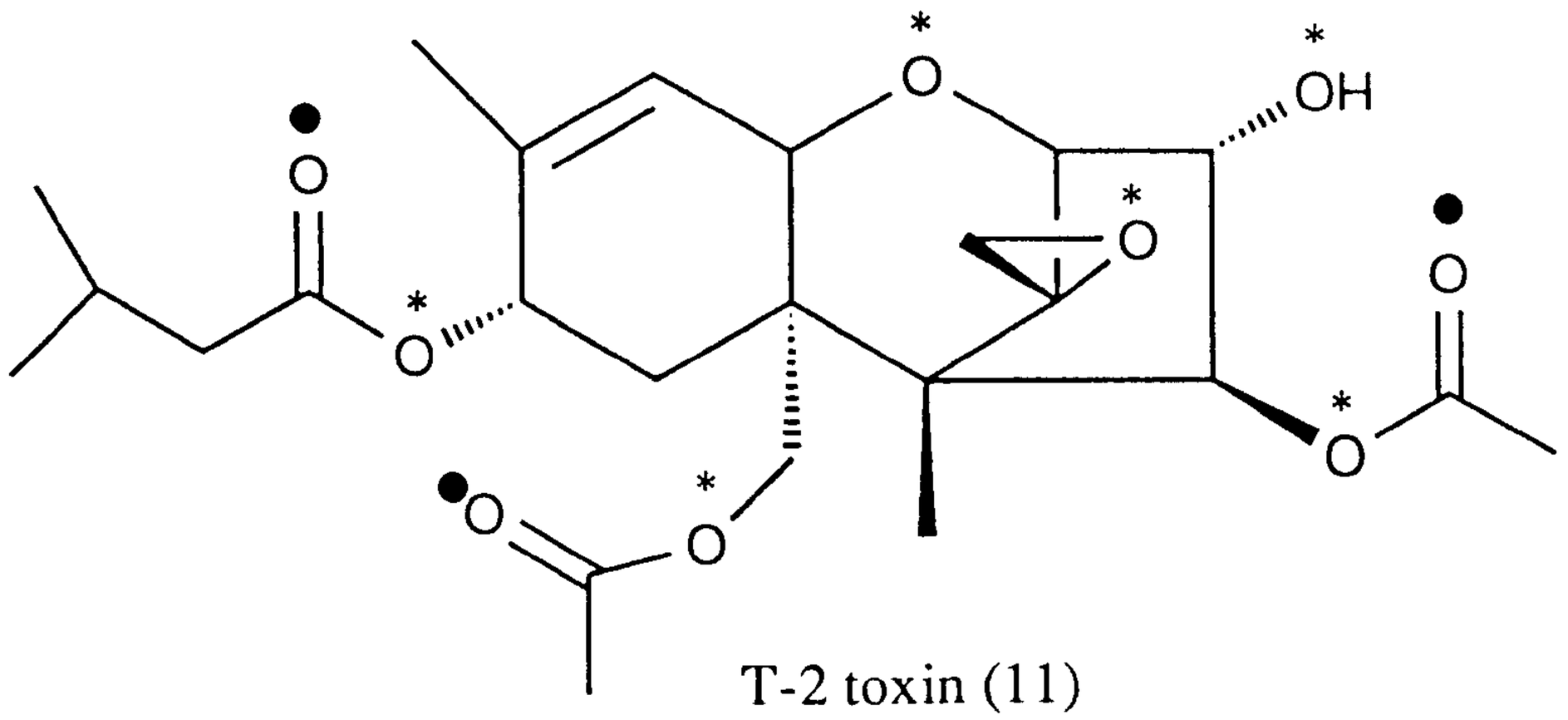
R^1	R^2	
H	H	Tricho-9-ene-2α,3α,11α-triol (85)
αOH	H	Tricho-9-ene–2α,3α,8α,11α-tetraol (93)
βOH	H	Tricho-9-ene-2α,3α,8β,11α-tetraol (94)
H	OH	Tricho-9-ene-2α,3α,11α,16-tetraol (95)



R=OH, 11α-Hydroxytrichodiene (92)
R=H, Trichodiene (36)

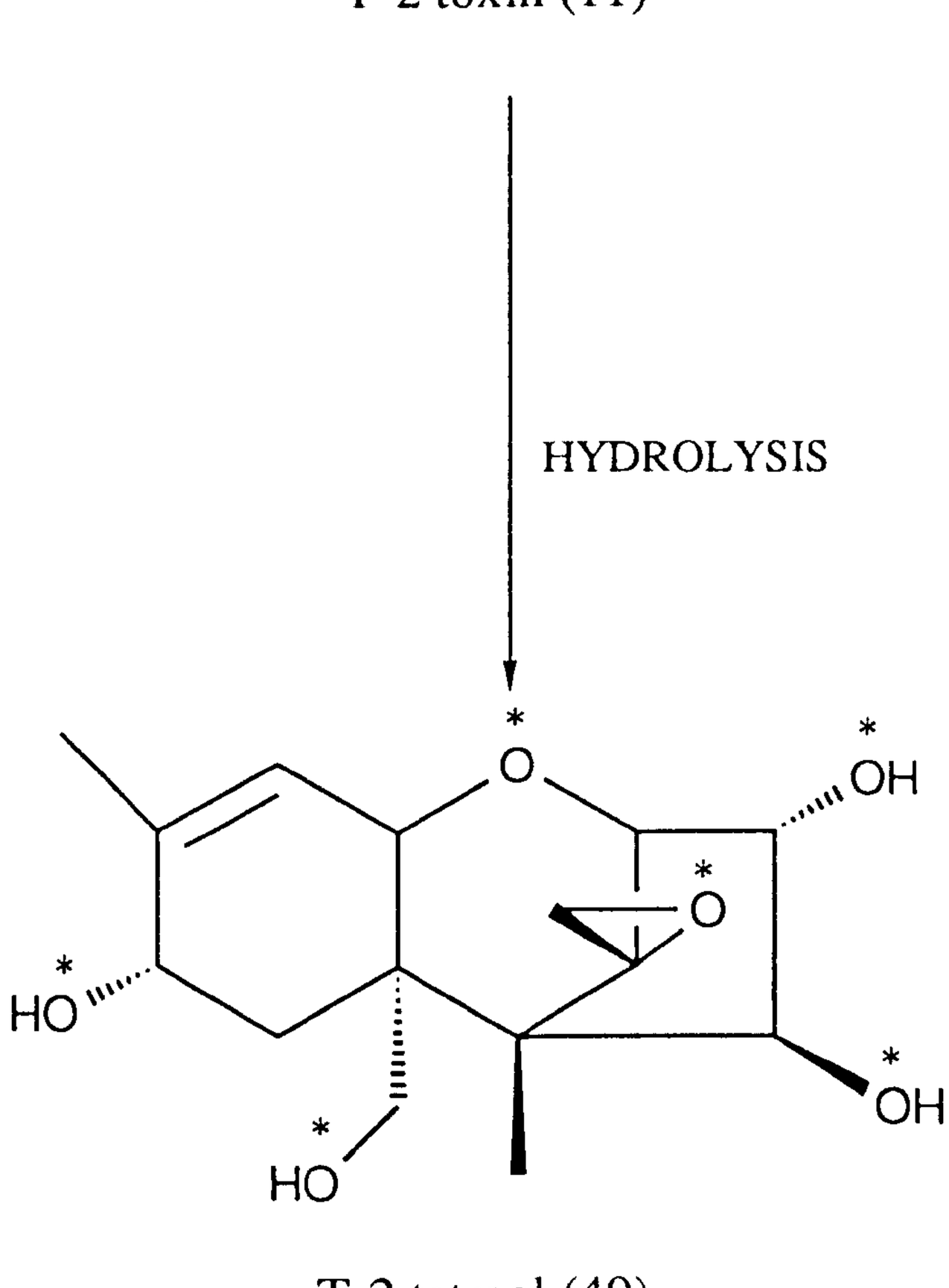
Fig.18: Uncyclised trichothecenes isolated from mutant strains of *Fusarium sporotrichioides*[61].

Fig.19: Origin of the oxygen atoms of T-2 toxin.



T-2 toxin (11)

HYDROLYSIS



T-2 tetraol (49)

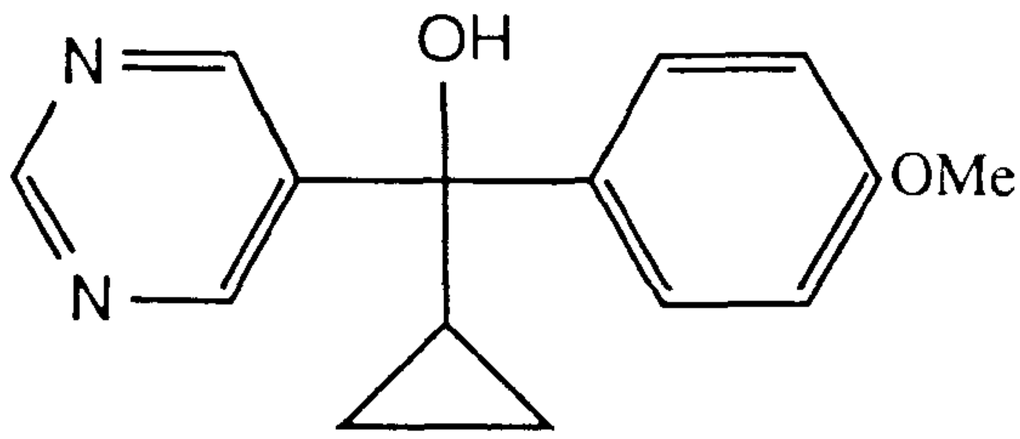
* = oxygen atoms originating from O_2
● = oxygen atoms originating from H_2O

be derived from $^{18}O_2$. Trichothecenes are therefore synthesised from TDN by a series of oxygenations requiring molecular oxygen, and mediated by oxygenase enzymes. This process is known to be inhibited by cytochrome P-450 mono-oxygenase inhibitors such as ancymidol (50)[63] and xanthotoxin (8-methoxypsoralen) (51)[64]. These compounds suppressed the production of the highly oxygenated trichothecenes T-2 toxin and 4,15-diacetoxyscirpenol (9) in *F. sporotrichioides*, and caused the accumulation of significant amounts of TDN, presumably the last non-oxygenated precursor.

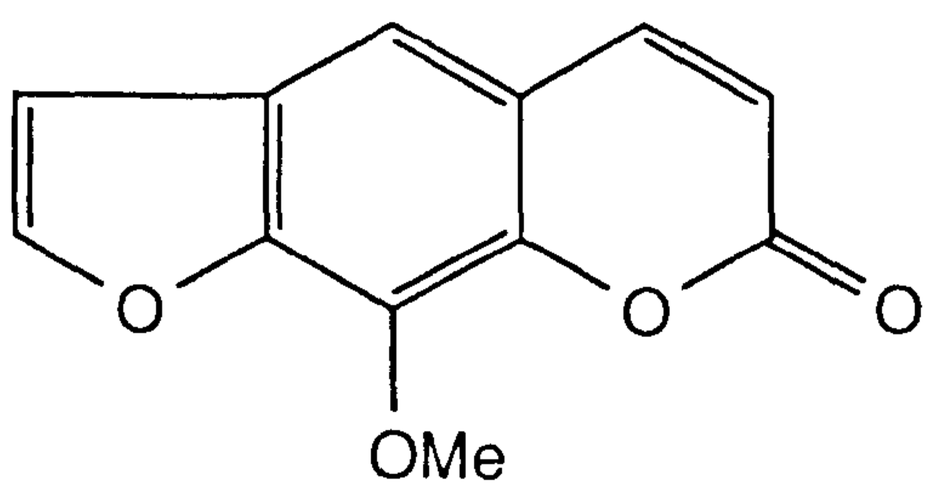
After formation of the trichothecene nucleus in the form of either EPT or isotrichodermol, hydroxylations at any of positions C-3, C-4, C-7, C-8 and C-15 can produce a range of trichothecenes. The proposed pathway for the production of trichothecin from EPT[65] is a good example of these modifications [Fig.20].

Biosynthetic studies using stable isotopes.

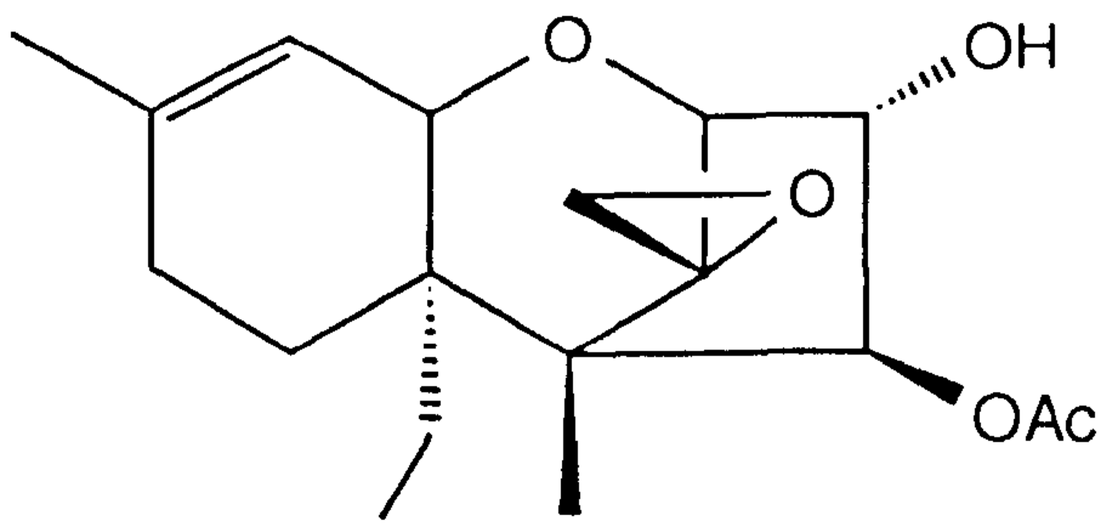
The studies so far mentioned investigated trichothecene biosynthesis using feeding experiments with radiolabelled substrates, and often involved determination of $^{3}H/^{14}C$ isotopic ratios in the final product and subsequent degradations to locate the label(s). The advent of ^{13}C and ^{2}H NMR spectroscopy meant that precursors labelled with stable isotopes could be used. Both these techniques allow direct determination of the positions at which labels are incorporated into the product, foregoing the need for complex chemical degradations. With ^{13}C NMR this is achieved by comparison of spectra from ^{13}C-enriched samples with natural abundance spectra. Distortionless enhancement by polarisation transfer (DEPT) together with techniques such as $^{1}H/^{13}C$ correlation spectroscopy (COSY) can be used to help assign the ^{13}C-resonances to specific positions in the molecular



Ancymidol (50)



Xanthotoxin (51)



4β,15-Diacetoxyscirpenol (9)

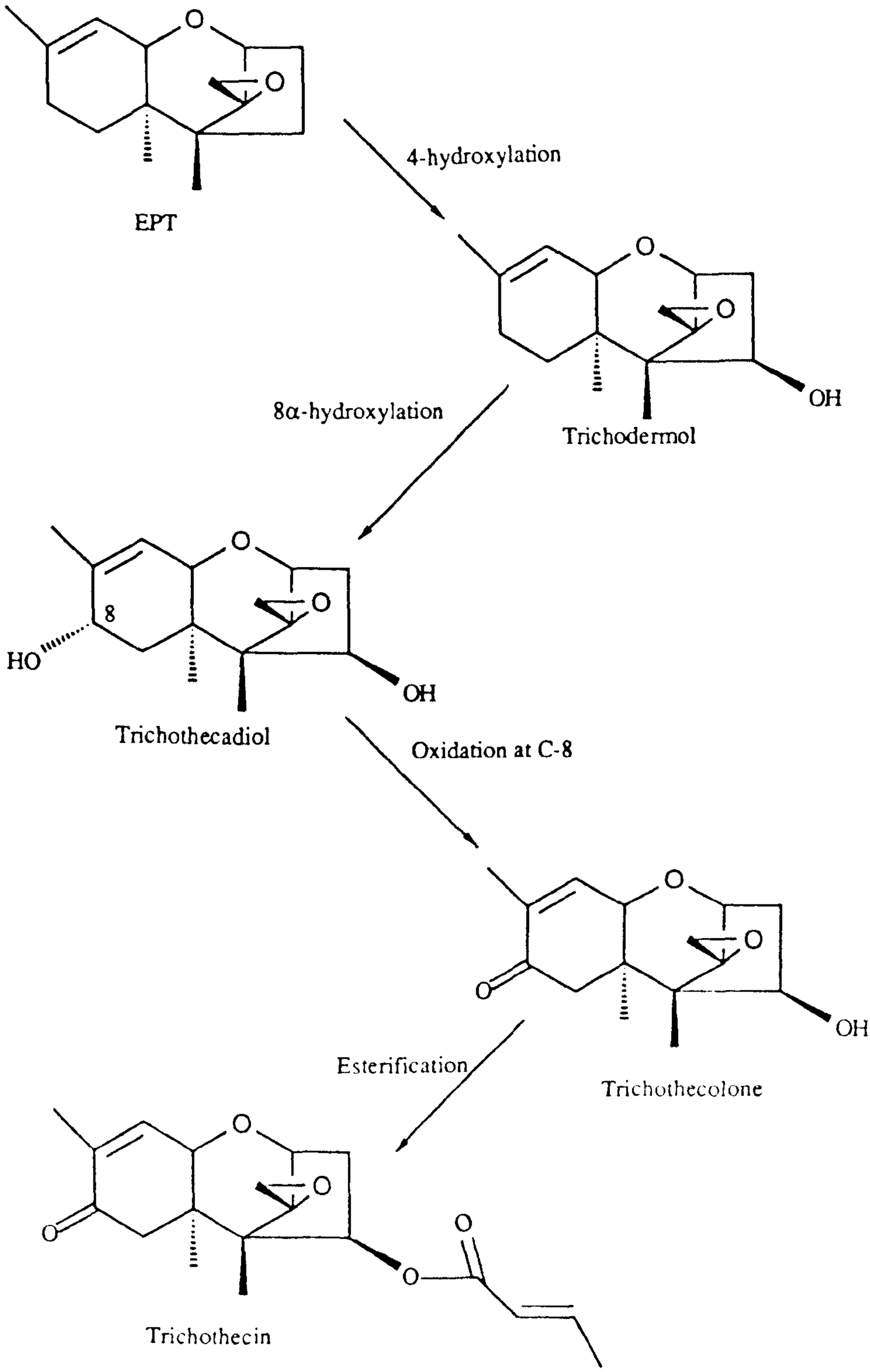


Fig.20: Proposed biosynthesis of trichothecin from EPT.

structure. Measurement of ^{13}C-^{13}C coupling constants allows location of adjacent ^{13}C labels and so can be used to observe the incorporation of intact precursors, e.g. [1,2-^{13}C]acetate, into the trichothecenes. Results from studies using stable isotopes have provided supporting evidence for the biosynthetic findings obtained previously with radiolabels.

A) Biosynthesis of trichothecenes from acetate and mevalonate.

Trichothecolone produced from [2-^{13}C]MVA showed enrichment only at positions C-4, C-8 and C-14[66] which is in accord with earlier results (see Fig.9). When using [1,2-^{13}C]acatate as a precursor, ^{13}C-^{13}C couplings identified the incorporation of intact acetate units into trichothecolone [Fig.21a][67]. This pattern, in particular the lack of coupling involving C-8 and the coupling between C-6 and C-7, and C-10 with C-11 confirms the proposed folding of the intermediate (6E)-FPP (see Fig.10). The observed labelling pattern in enriched 3-acetyldeoxynivalenol (3-AcDON) (14) derived from [3,4-^{13}C]MVA also supported the involvement of (6E)-FPP [Fig.21b][68].

Feeding experiments to *Fusarium graminearum*[69] using [1-^{13}C]- and [2-^{13}C]-acetates produced enrichments in 15-acetoxydeoxynivalenol (15-AcDON) (16) in accordance with the suggested biosynthetic sequence [Fig.22]. ^{13}C-^{13}C couplings observed between C-5 and C-12, and C-6 and C-15 are consistent with the occurrence of two 1,2-methyl migrations. Similar feeding experiments to *Fusarium culmorum* produced 3-AcDON labelled in the same way[71]. One such methyl shift was subsequently demonstrated when [4,6-^{13}C]MVA was fed to *F. culmorum* [Fig.23][70]. The 3-AcDON obtained showed direct ^{13}C-^{13}C coupling (J=38.5Hz) between C-6 and C-15, proving that there had been a 1,2-methyl

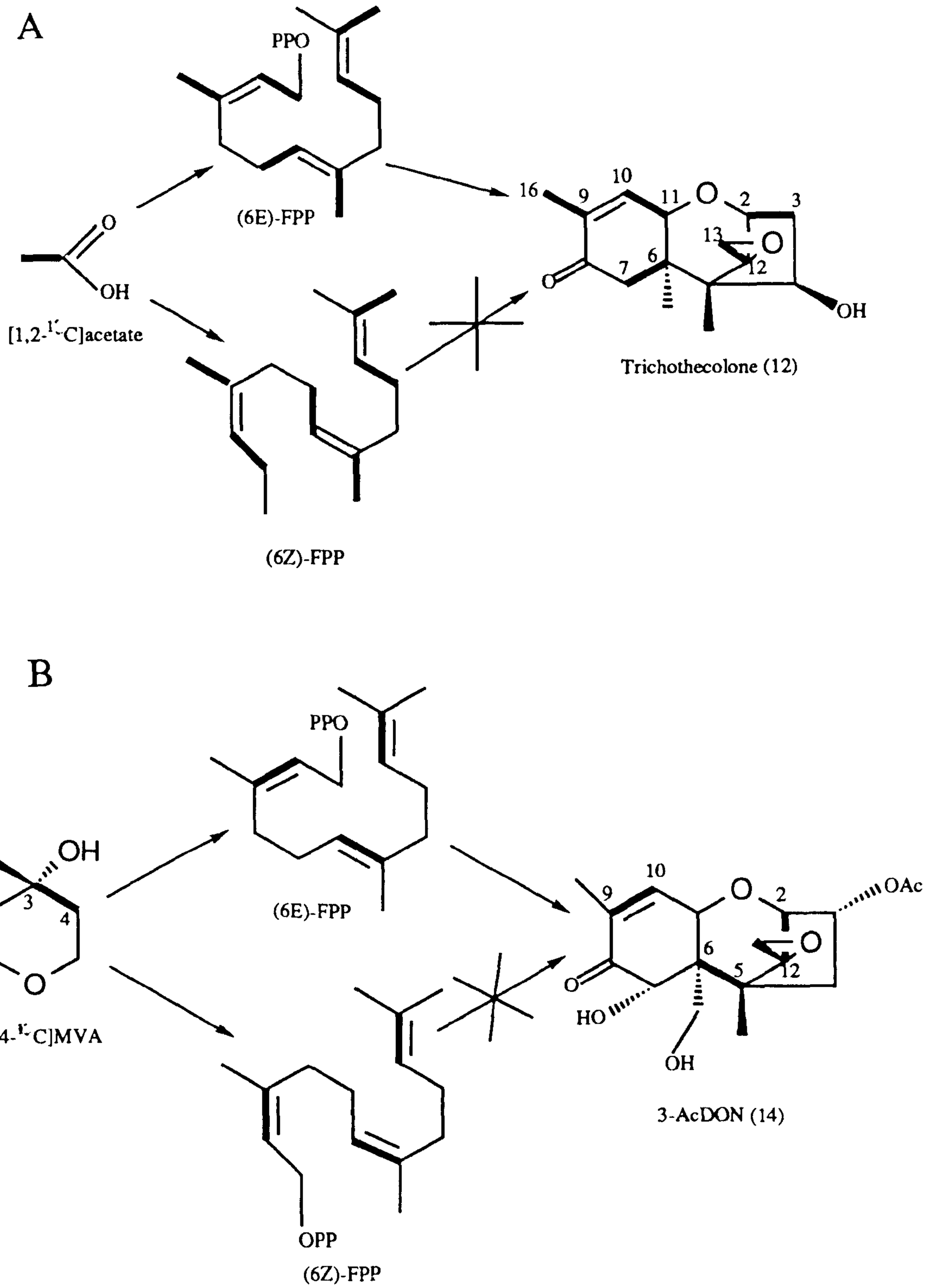


Fig.21: Proof for the (6E)- folding of FPP in the biosynthesis of [A] Trichothecolone, and [B] 3- AcDON.

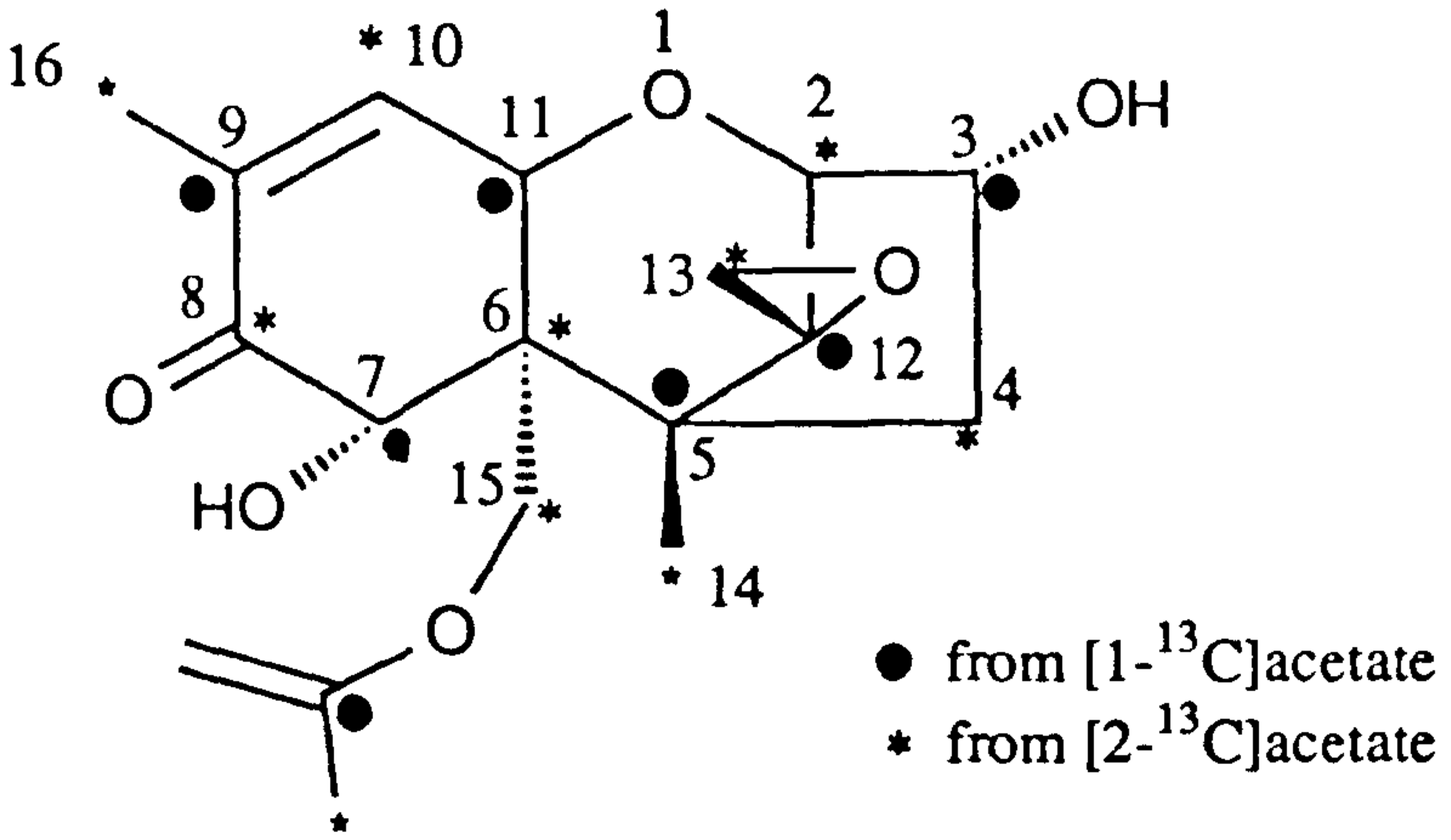


Fig.22: Sites of enrichment in 15-AcDON (16) derived from [1-^{13}C]- and [2-^{13}C]-acetates.

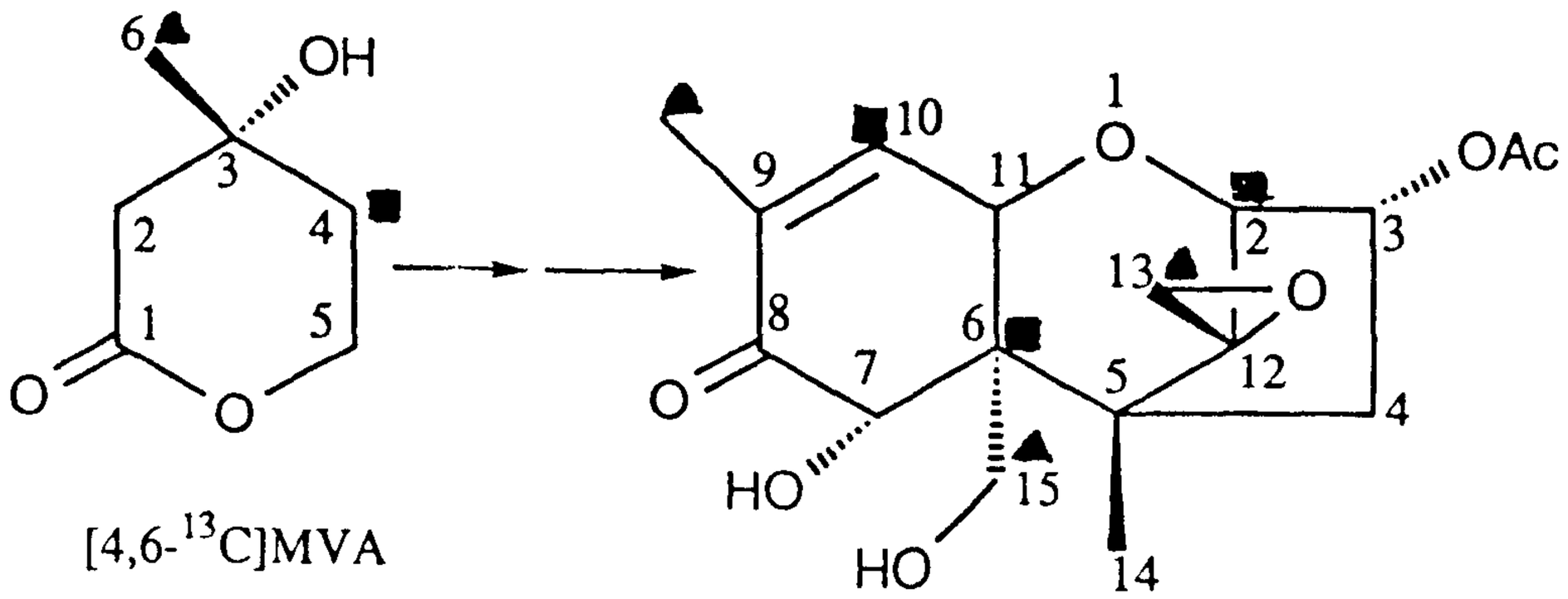


Fig.23: A 1,2-methyl shift in the biosynthesis of 3-AcDON.

migration involving the transfer of C-6 to C-4 of mevalonate.

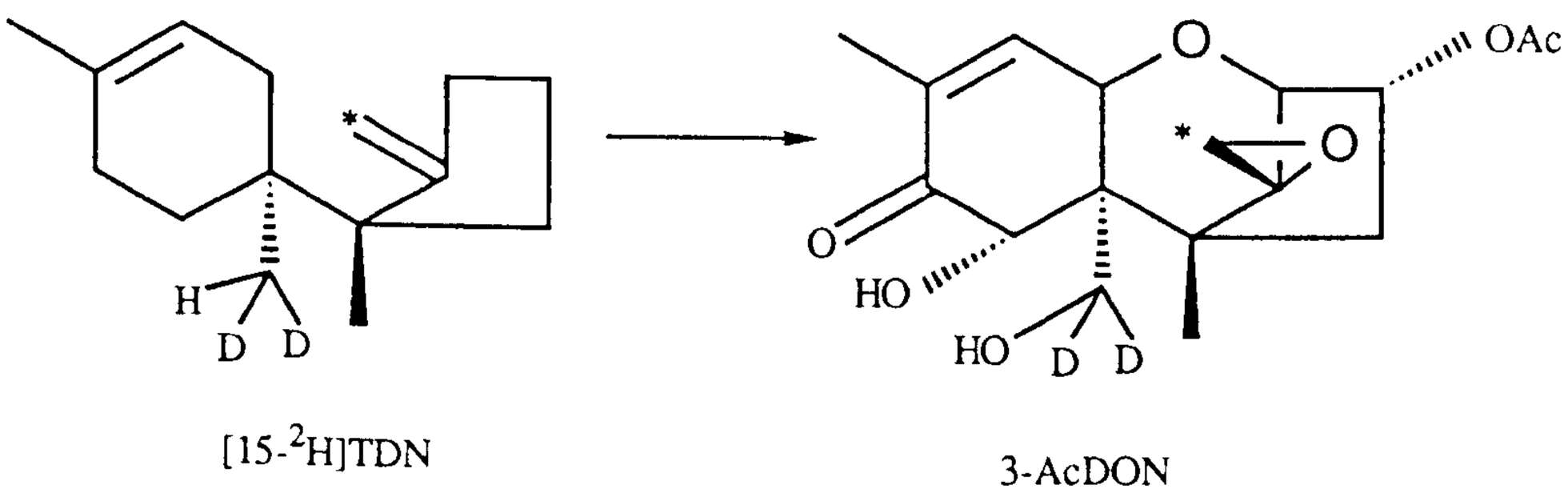
B) Biosynthesis of trichothecenes from TDN.

Although the incorporation of tritiated-TDN into trichothecin has been demonstrated[49,51], no degradations were performed to locate the label in the product. Recent studies involving specifically labelled [^{2}H]TDN[72] and [^{13}C]TDN[73], using NMR to confirm the location of the label(s) in the product, have conclusively proven TDN to be the precursor to the trichothecenes [Fig.24]. Labelled bazzanene (52), the epimer of TDN, was not incorporated into 3-AcDON (14) indicating the specificity of the enzyme(s) involved at this stage of the biosynthesis.

Trichothecene-related metabolites.

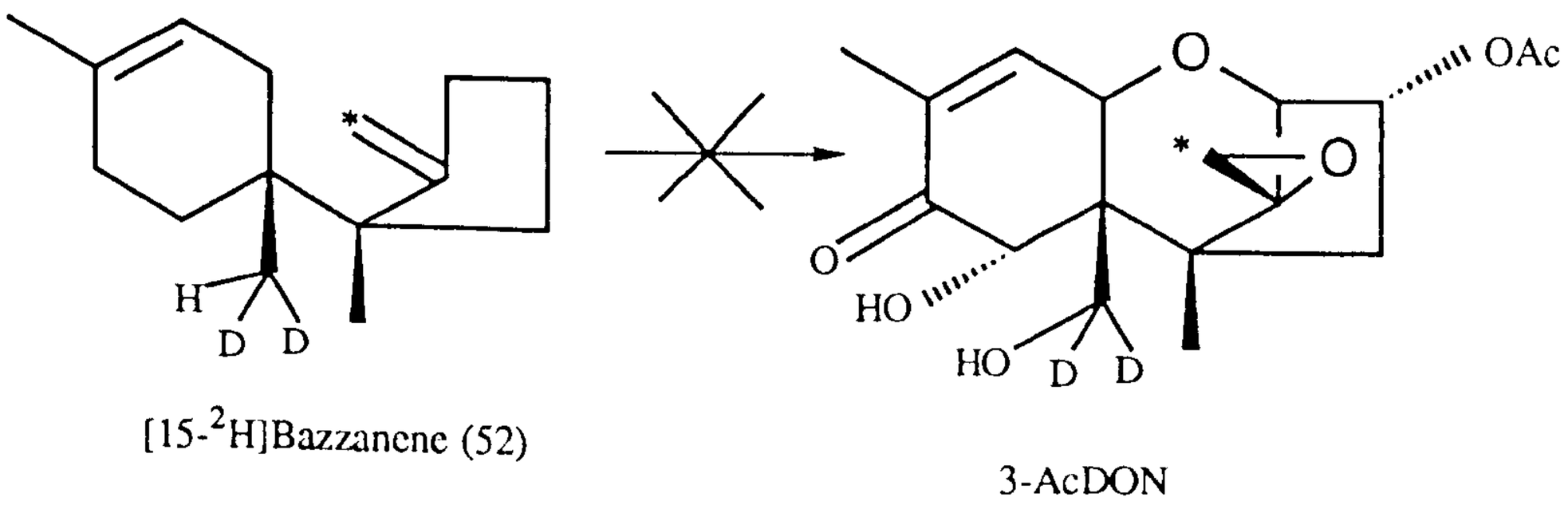
In addition to the trichothecenes, *Fusarium* fungi produce a variety of other secondary metabolites [Fig.25]. Some of these compounds are structurally related to the trichothecenes (e.g. sambucoin (53) and sambucinol (54)), whilst others are based on TDN (e.g. FS-1 (55) and FS-2 (56)). Unrelated metabolites include zearalenone (57), an oestrogenic mycotoxin, and also culmorin (58) and culmorone (59). The latter compounds are derived from FPP but via a different cyclisation process to that which produces the trichothecenes[74].

Sambucoin, sambucinol and the 11-epi-apotrichothecenes are thought to be produced by all *Fusarium* species[75]. They differ from the trichothecenes in the nature and stereochemistry of the ring system. They possess a *trans*-fused A/B ring system with H-11 β, as compared with the *cis*-fused A/B system with H-11 α in the trichothecenes. Trichothecenes can rearrange to form apotrichothecenes in acidic conditions [Fig.26][8] but the A/B rings in the product remain *cis*-fused. The



[15-^{2}H]TDN

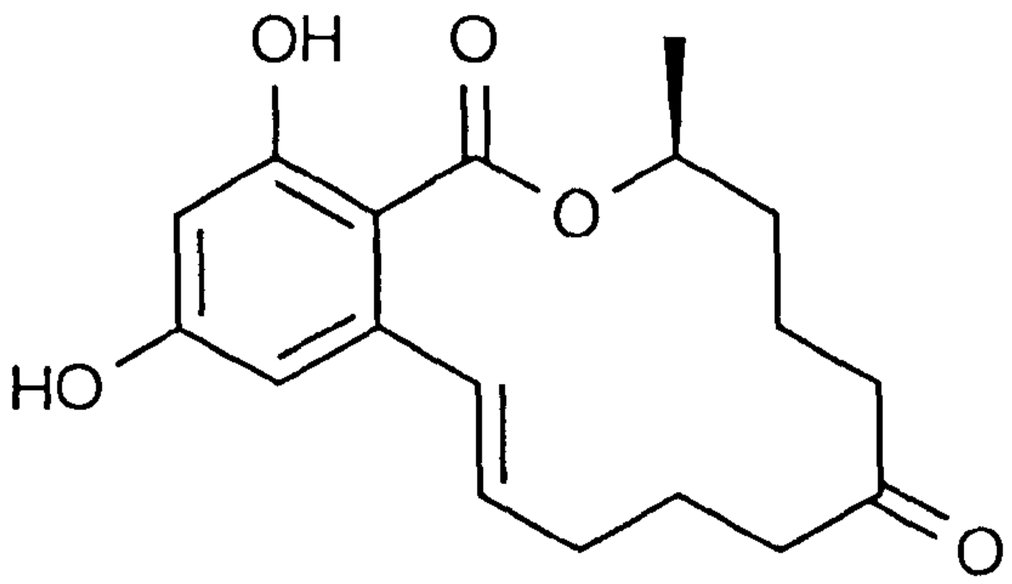
3-AcDON



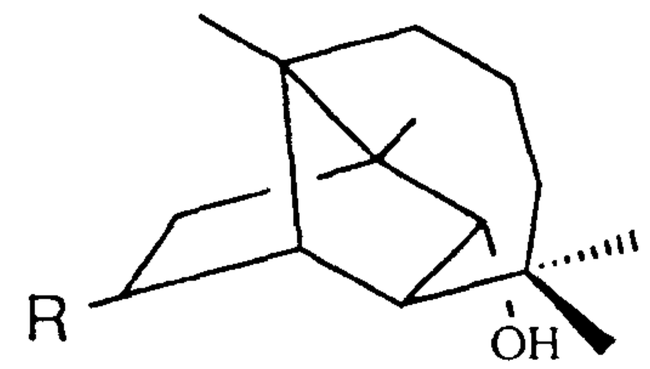
[15-^{2}H]Bazzanene (52)

3-AcDON

Fig.24: Trichodiene as a precursor to 3-AcDON.



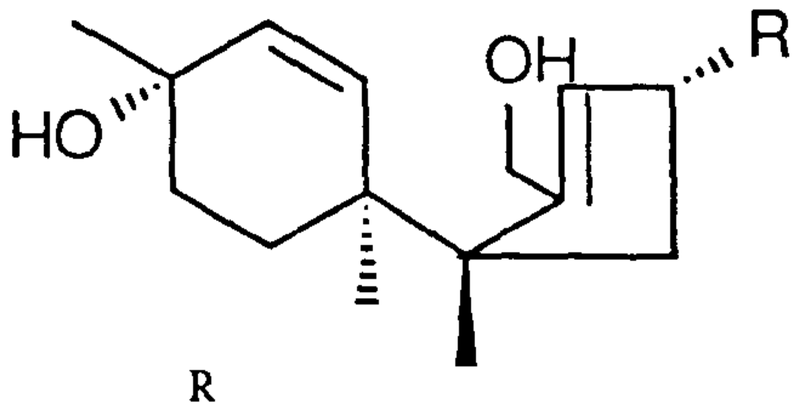
Zearalenone (57)



R

OH Culmorin (58)

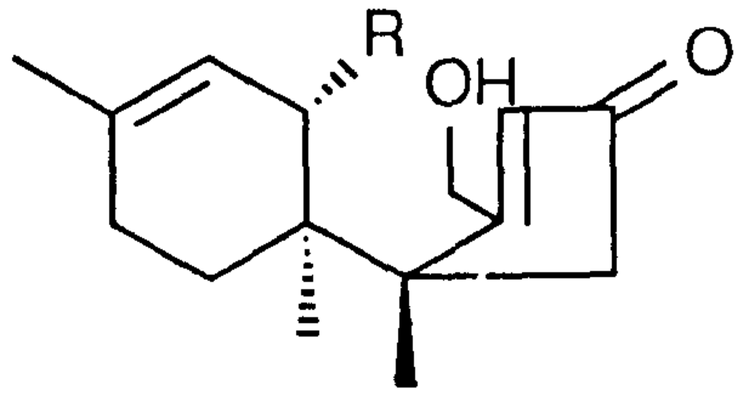
=O Culmorone (59)



R

OH FS-2 (56)[60]

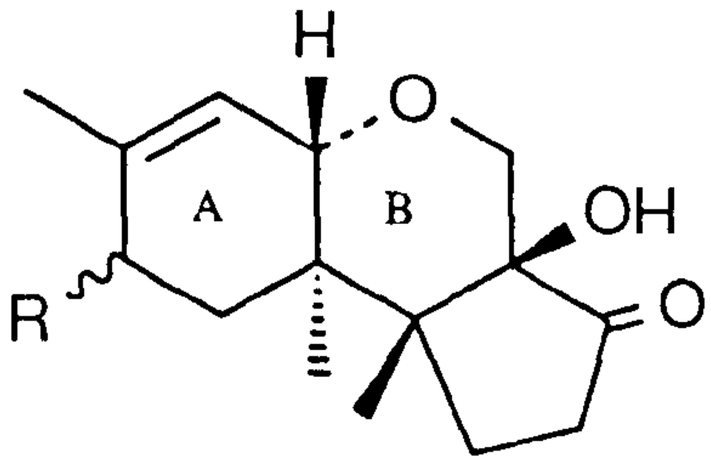
=O FS-4 (60)[81]



R

OH FS-1 (55)[83]

=O FS-3 (61)[81]

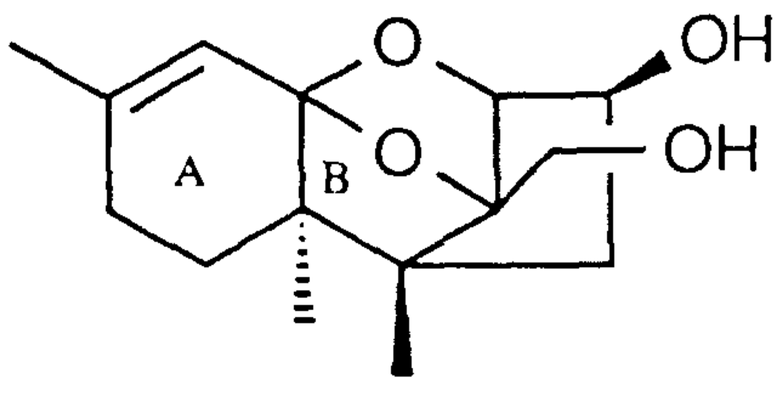


R

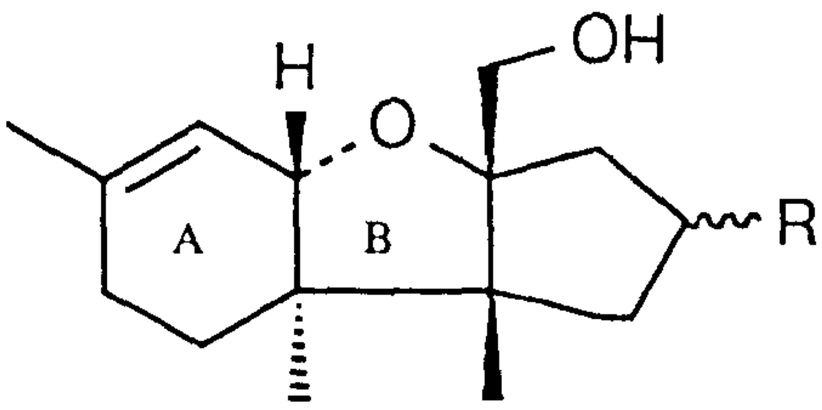
H Sambucoin (53)[77]

αOH 8α-OH-Sambucoin (62)[82]

βOH 8β-OH-Sambucoin (63)[82]



Sambucinol (54)[77]

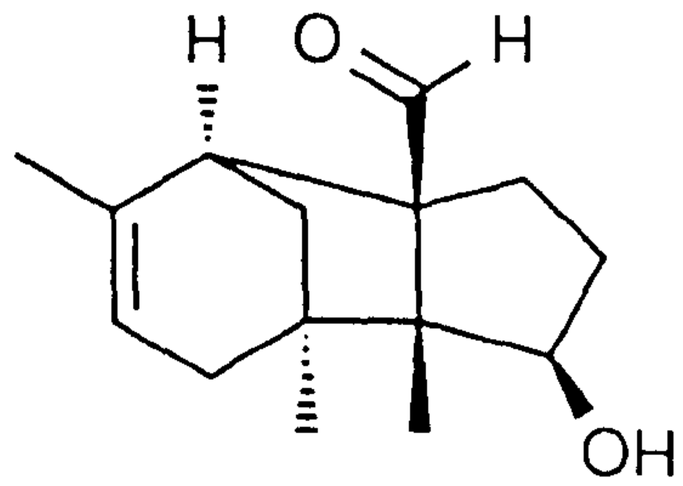


R

αOH 3α,13-dihydroxy-11-epi-apotrichothecene (64)[79]

βOH 3β,13-dihydroxy-11-epi-apotrichothecene (65)[79]

=O 3-keto-13-OH-11-epi-apotrichothecene (66)[79]



Sambucinic acid (67)

Fig.25: Some secondary metabolites from *Fusarium* species.

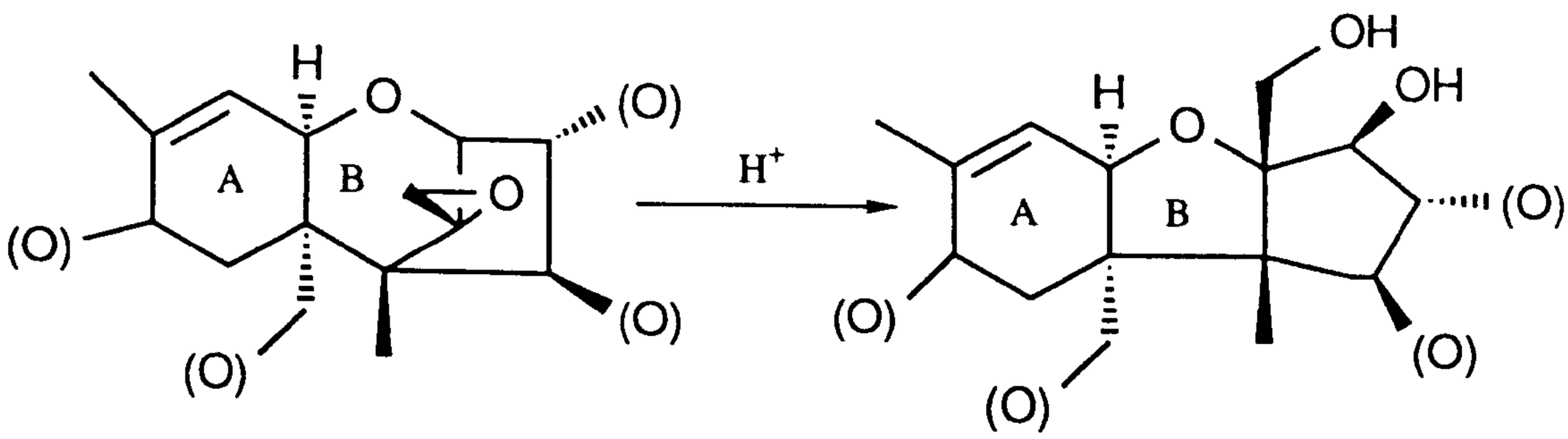
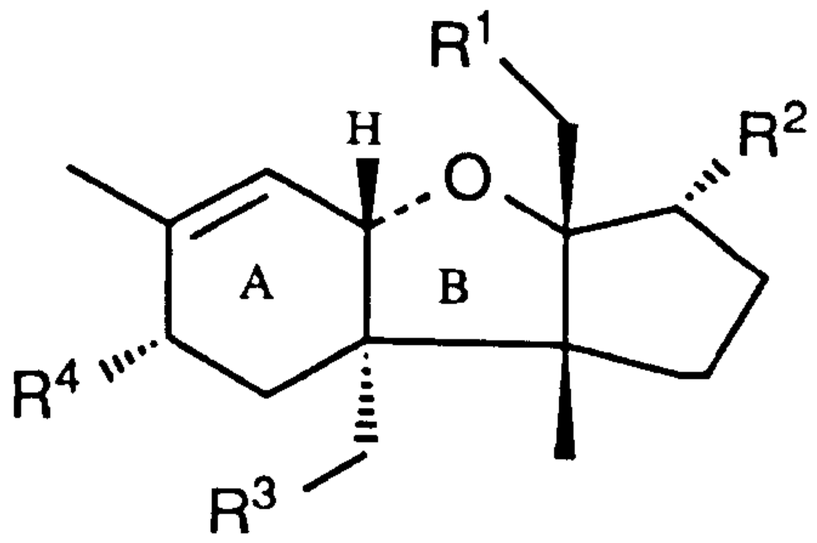
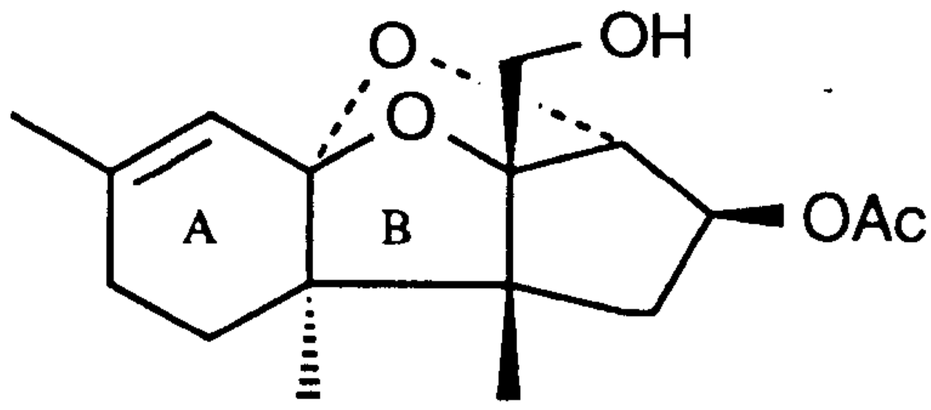
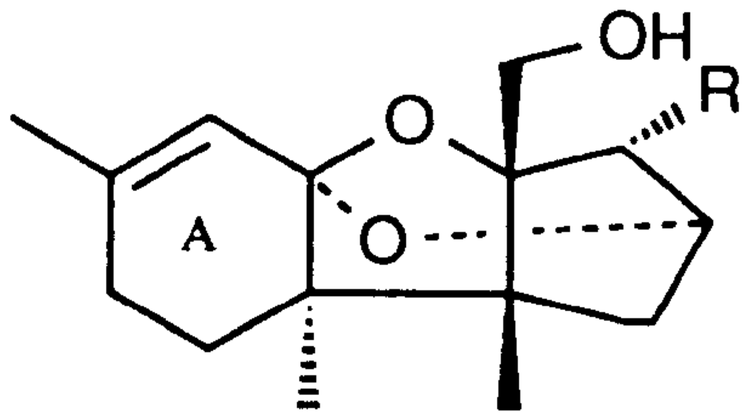


Fig.26: Acid rearrangement of trichothecenes to apotrichothecenes[8].





R^1	R^2	R^3	R^4
OH	OAc	OAc	H
OAc	OAc	H	OH
OH	OAc	H	OH
OAc	OAc	H	OAc



R=H or OH

Fig.27: 11-epi-apotrichothecenes ex *F.sporotrichioides*.

11-epi-apotrichothecenes isolated are therefore natural metabolites and do not stem from the chemical rearrangement of pre-formed trichothecenes. The isolation of a number of new compounds with structures based on the 11-epi-apotrichothecene nucleus has recently been reported [Fig.27][76], making this the most common type of trichothecene-related metabolite.

Biosynthesis of trichothecene-related metabolites.

TDN is presumed to be the common biosynthetic precursor to the 11-epi-apotrichothecenes, sambucoin, sambucinol and the trichothecenes [Fig.28][77,78,79,80]. Cyclisation via different oxygenated intermediates can account for the differences in A/B ring stereochemistry between the trichothecene-related metabolites and the trichothecenes. Central to the scheme is oxidation of TDN at C-11 to give an 11α-hydroxyl which could subsequently form an oxygen bridge to C-13 producing the sambucoin ring structure, or to C-12 which could lead to sambucinol and the 11-epi-apotrichothecenes. The 11α-hydroxyl is essential to produce the *trans*-fused A/B ring system and the correct stereochemistry at C-11. Isolation of FS-1 (55) from *Fusarium sporotrichioides*[83] indicates that 11α-hydroxylation of TDN can occur.

The mevalonate origin of sambucinol and 3α,13-dihydroxy-11-epi-apotrichothecene (64) was demonstrated when [3,4-^{13}C]MVA was fed to *Fusarium culmorum*[84]. The results also indicated that the mode of folding of the intermediate FPP is the same as for the trichothecenes (see Figs.10 and 21). TDN was proven to be a precursor to sambucinol and the 11-epi-apotrichothecenes in feeding experiments using specifically labelled material[72,73].

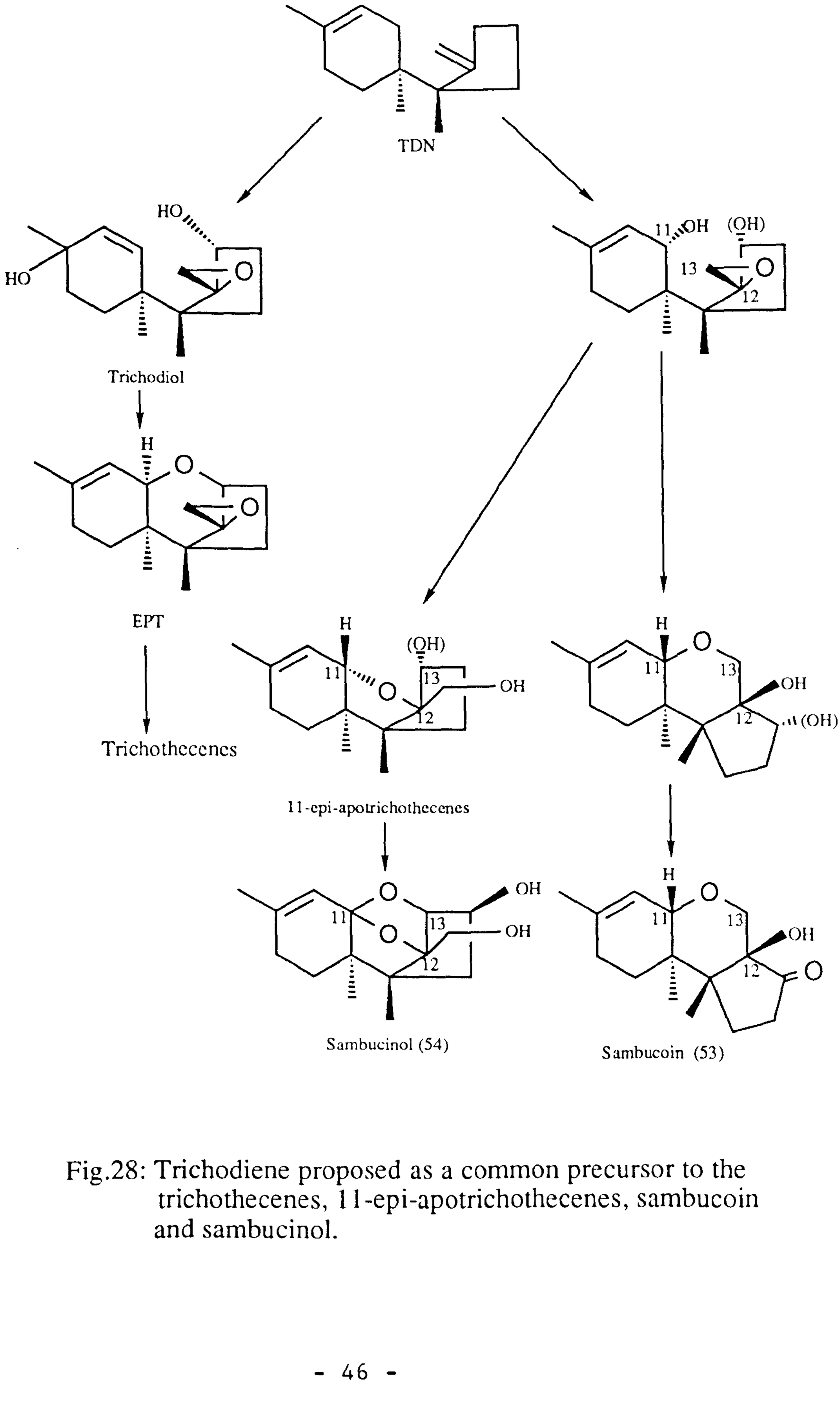


Fig.28: Trichodiene proposed as a common precursor to the trichothecenes, 11-epi-apotrichothecenes, sambucoin and sambucinol.

Toxicity of trichothecene-related metabolites.

The toxic activity of the trichothecenes is associated with the 12,13-epoxide group. The trichothecene-related metabolites lack this function and are therefore considered to be much less active. The values determined for their relative cytotoxicity in baby hamster kidney (BHK-21) cells support this assumption [Table 4].

Table 4: Relative cytotoxicity of a range of *Fusarium* metabolites in cultured BHK-21 cells[20].

	LC_{100} ($ngml^{-1}$)
T-2 toxin	5
8β-OH sambucoin	2×10^3
FS-1	5×10^3
3-keto-11-epi-apotrichothecene	6×10^4
8α-OH sambucoin	8×10^4
FS-2	1×10^5
FS-3	1×10^5

LC_{100}=concentration required to cause cell death in 100% of the wells tested.

These figures indicate the relatively low cytotoxic activity of pure trichothecene-related metabolites as compared to a pure trichothecene (T-2 toxin). However, in natural mycotoxicoses these compounds are present in a mixture, and there is a possibility of synergistic toxicity effects. As evidence for this it has been observed that a mixture of deoxynivalenol (DON) (13) with either sambucinol or culmorin leads to markedly enhanced toxicity of DON towards caterpillars[85].

Aims of the project.

The interest in trichothecene mycotoxins is due to their widespread occurrence and potent biological activity. Their presence in a range of cereal crops poses a potential risk to human health, and causes considerable economic losses within the agricultural industry. Health problems in livestock, feed-refusal, and the disposal or decontamination of affected crops result in significant financial losses to farmers worldwide. There is therefore a need for a detailed understanding of trichothecene production. This is now especially important because *Fusarium* species are being utilised to produce protein for human consumption.

The biosynthesis of trichothecenes as far as TDN is fully understood whilst the sequence thereafter is completely unknown. Detailed studies have identified the intermediates involved in the production of TDN, the enzyme responsible, trichodiene synthase (TS), has been purified, and the TS-gene has been isolated and sequenced. A similar level of understanding of the post-TDN biosynthesis is desirable since this is the stage at which toxicity is conferred.

The aim of this project was to provide further information on the production of trichothecenes from TDN, and ultimately to provide a basis for the isolation and characterisation of the enzymes involved. It was proposed that this could be achieved by producing semi-synthetic derivatives of TDN which could then be used as inhibitors in competitive feeding experiments to fungal cultures. By producing derivatives analogous to natural intermediates it was hoped that the post-TDN biosynthesis of trichothecenes could be inhibited leading to the accumulation of novel biosynthetic intermediates.

RESULTS AND DISUSSION.

Production of trichothecenes in cultures of *Fusarium culmorum* CMI 14764.

Culture of fungus.

The production of trichothecenes from liquid cultures of *F. culmorum* was achieved by a shake culture method which had already been used previously to produce good yields of mycotoxins[71]. Erlenmeyer flasks containing sterile production medium were inoculated with the fungus, each flask receiving a mycelial homogenate prepared from one agar plate of *F. culmorum*. The flasks contained only 20% volume of medium in order to allow for good aeration of the culture, an essential requirement for strong fungal growth. Typically the volume of a fermentation batch was 8L, comprising twenty 2L flasks each containing 400ml of culture. These were incubated in the dark at 28°C on an orbital shaker (250rpm) for 7 days and then harvested. This involved filtration of the culture, saturation of the filtrate with salt, followed by repeated extraction with ethyl acetate. The combined organic extracts were then dried and concentrated *in vacuo* to yield a viscous brown oil (approximately $1gL^{-1}$ of culture) containing the trichothecenes.

Detection.

A range of analytical techniques have been employed in the detection of trichothecenes including thin layer chromatography[86] (TLC), gas chromatography[87] (GC), combined gas chromatography-mass spectrometry[88] (GC-MS), and high performance liquid chromatography[89] (HPLC). Of these, TLC represents the most convenient method and this was therefore employed for the routine analysis of

culture extracts. Separation using a suitable solvent system and subsequent visualisation with appropriate spray reagents provides a qualitative assay which is both rapid and reasonably sensitive. An indication of the relative quantities of trichothecenes present can be obtained from the size and intensity of the spots produced.

Table 5: TLC visualisation procedures for trichothecenes[90].

Reagent	Trichothecene	Colour	Limit of detection (µg/spot)
20% H_2SO_4	Deoxynivalenol	Yellow	0.05
	Diacetoxyscirpenol	Purple	0.20
	T-2 toxin	Grey	0.20
	HT-2 toxin	Grey	0.50
PNBP/TEPA	All epoxytrichothecenes	Blue or purple	0.02-0.2
10% $AlCl_3$	8-Ketotrichothecenes	Blue (fluorescent)	0.10
p-Anisaldehyde	Deoxynivalenol	Yellow	0.05
	Diacetoxyscirpenol	Purple	0.10
	T-2 toxin	Brown	0.10
	HT-2 toxin	Brown	0.20

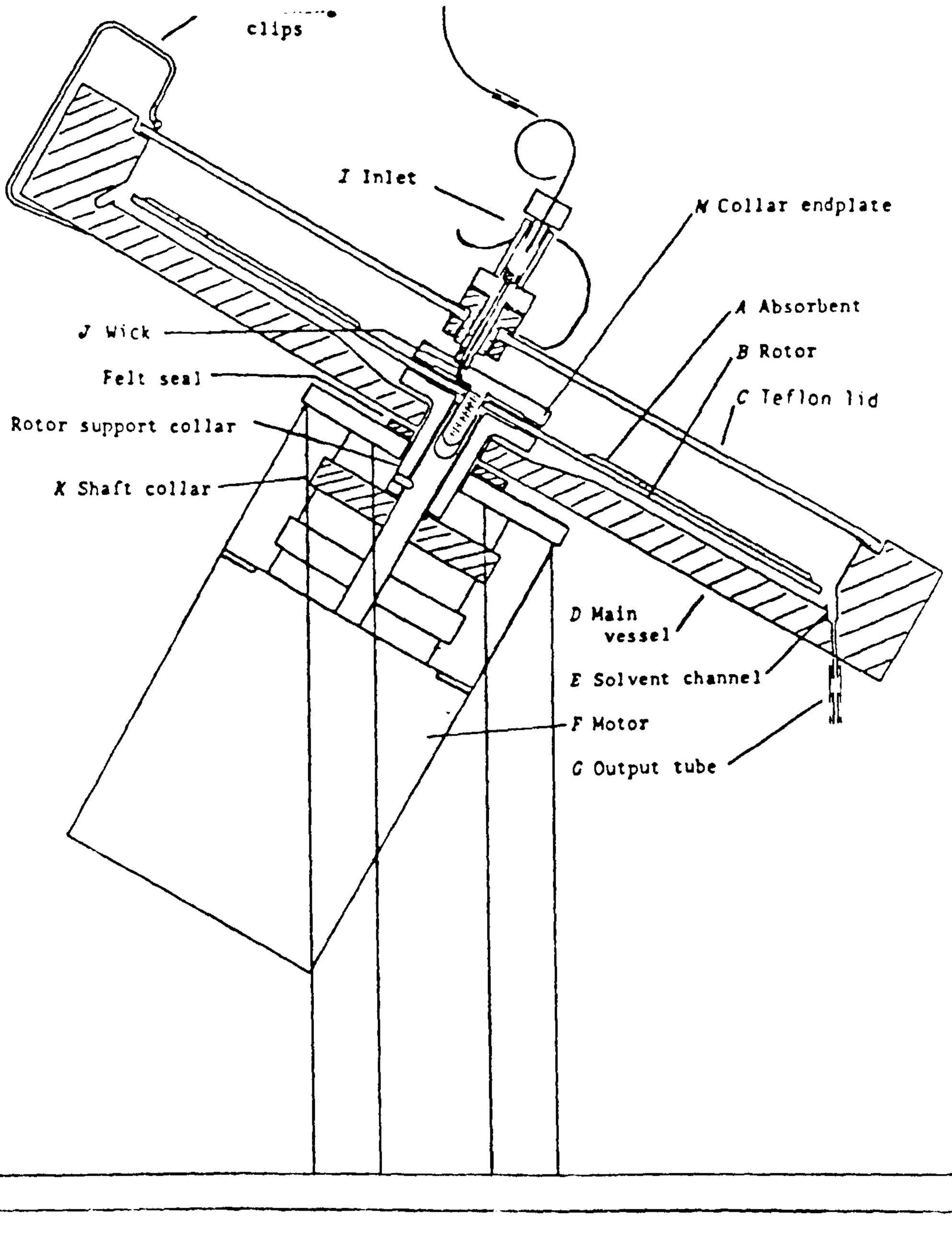
In this project, two plates were typically run per analysis with a different visualisation method being applied to each. One plate was sprayed with 20% aqueous sulphuric acid and heated at 140°, which procedure causes all organic compouds present to char. This is therefore a non-specific test, although some trichothecenes do exhibit characteristic colours upon development [Table 5]. The

second plate was sprayed with 4-(*p*-nitrobenzyl)pyridine (PNBP, 1% solution in chloroform), heated at 140° for 5-10 mins, and then oversprayed with tetraethylene pentamine (TEPA, 10% solution in chloroform). This causes trichothecenes to show up as blue or purple spots on a pale background. PNBP/TEPA is a chromogenic reagent for alkylating agents, organophosphates and epoxides, and therefore affords a specific test for the presence of 12,13-epoxytrichothecenes in these extracts. Aluminium chloride (10% solution in chloroform) was occasionally used to identify the presence of 8-ketotrichothecenes.

Purification.

TLC analysis of the *F. culmorum* extracts with reference standard compounds, indicated the presence of 3-acetyldeoxynivalenol (3-AcDON) and 7,8-dihydroxycalonectrin (DHC) as the major trichothecene products. The purification of these compounds was completed by using centrifugal thin layer chromatography[91] (CTLC), which has superceded column chromatography as the method of choice in this laboratory. Although both systems were based on silica gel as adsorbent, CTLC has proven to be a much quicker procedure than column chromatography, and it consistently produces a more effective separation of compounds. Separations are also highly reproducible since the silica plates can be used time and time again, unlike silica gel columns which tend to be used only once. In CTLC, separations are performed using a thin layer of adsorbent coated onto a circular glass plate which is placed into a chromatotron [Fig.29]. The plate is rotated in an inert atmosphere at 750rpm and a solution of the mixture to be separated is applied to a clear area at the centre of the plate via the inlet and wick. The eluent is then admitted by the same route at a fixed rate. Elution produces

Fig.29: Chromatotron[91].



U.S. Patent no. 4139458
Patents pending in other countries.

concentric bands of the separated substances which leave the edge of the plate together with the solvent. Fractions are then collected from the output tube. Plates with different adsorbent layer thicknesses can be cast (1mm, 2mm and 4mm) to cope with a range of sample loads up to several grams [Table 6].

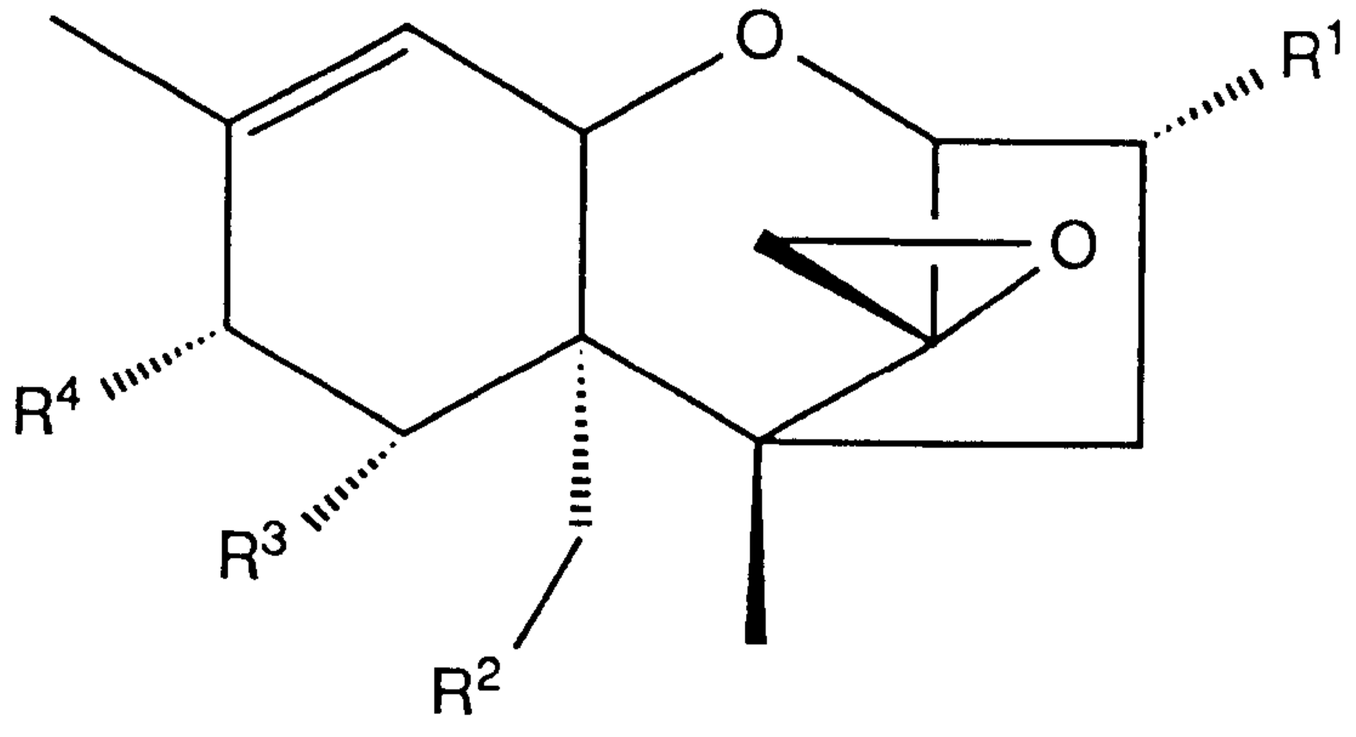
Table 6: Reccomended sample loads for different thicknesses of absorbent[91].

Thickness of plate	Sample size
1mm	100mg per component. 250mg total sample
2mm	300mg per component. 750mg total sample
4mm	1.5g total sample
	(up to 9g with gradient elution)

Sequential use of progressively thinner plates using a variety of solvent systems proved to be an extremely fast and effective method of producing high purity trichothecenes from culture extracts. To achieve good resolution of compounds it was essential to equilibrate the plates by development with the solvent system prior to chromatographing the sample.

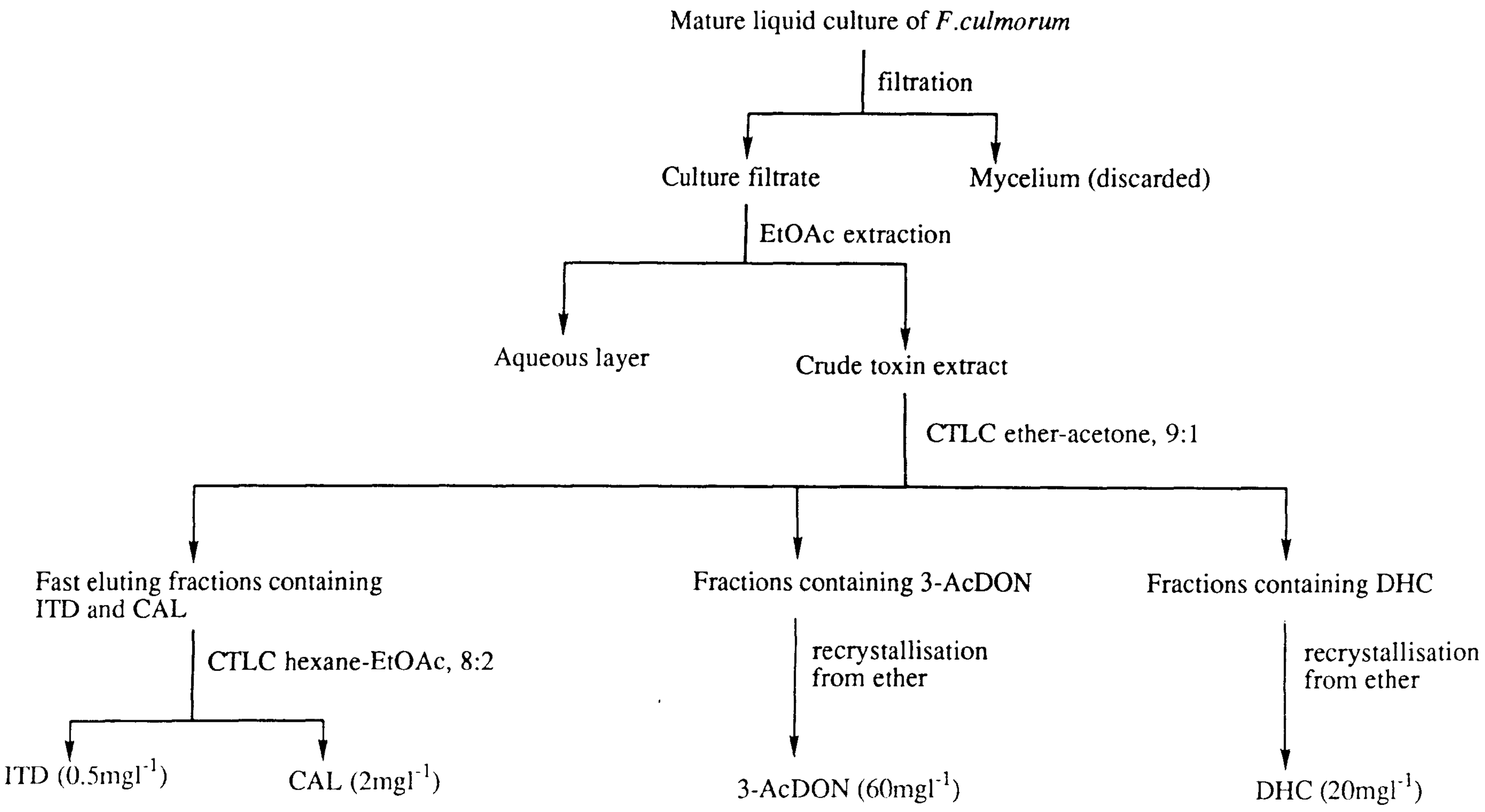
In this way four trichothecenes were purified from the ethyl acetate extracts derived from cultures of *F. culmorum* [Fig.30]: 3-AcDON (14) (60mgL^{-1}), DHC (7) (20mgL^{-1}), calonectrin (CAL) (6) (2mgL^{-1}) and isotrichodermin (ITD) (68) (0.5mgL^{-1}). These were fully characterised using ^{1}H NMR and MS, which produced data in accordance with earlier published results[59].

In previous work at this laboratory, a total of seven trichothecenes have been isolated from this strain of *F. culmorum*[59]. In addition to the four trichothecene products reported here, low yields of 3,15-diacetoxydeoxynivalenol (17),



	R^1	R^2	R^3	R^4
EPT (3)	H	H	H	H
ITD (68)	OAc	H	H	H
CAL (6)	OAc	OAc	H	H
DHC (7)	OAc	OAc	OH	OH
DON (13)	OH	OH	OH	=O
3-AcDON (14)	OAc	OH	OH	=O
3,15-DiAcDON (17)	OAc	OAc	OH	=O

Fig.30: Scheme for the isolation of trichothecenes from *Fusarium culmorum*.



deoxynivalenol (DON) (13), and 12,13-epoxytrichothec-9-ene (EPT) (3) were also obtained. A number of other trichothecenes with various levels of substitution have also been isolated as minor metabolites from this strain by other groups of workers[92,93] (see Table 19).

Production of trichodiene from cultures of *F. culmorum.*

The main aim of this project was to investigate the production of trichothecenes from trichodiene (TDN), and thus it was essential to have access to relatively large amounts of TDN, preferably in radiolabelled form.

In the time since TDN was first isolated in 1970[47] a number of methods have been used to produce material suitable for use in biosynthetic studies. Labelled samples have been prepared by synthetic modification of TDN isolated from *Trichothecium roseum*[49], by cyclisation of [^{14}C]FPP using an enzyme system prepared from *T. roseum*[51], and more recently via total chemical synthesis[72,73]. Preparation of TDN by synthetic means is complex and time-consuming, whilst the procedures involving fungal production yield only very small quantities of material. An alternative to these methods was therefore sought.

Treatment of liquid cultures of *Fusarium sambucinum* with the cytochrome P-450-dependent monooxygenase inhibitor ancymidol (50) was reported to cause a decrease in the amounts of trichothecenes produced, accompanied by an accumulation of small quantities of TDN ($\approx$2mgL^{-1})[63]. The same group in further work investigated the effect of a variety of naturally occurring flavonoids and furanocoumarins on trichothecene biosynthesis in *Fusarium sporotrichioides*[64]. These compounds had previously been shown to inhibit cytochrome P-450-dependent monooxygenases, and were added to cultures of *F. sporotrichioides* in

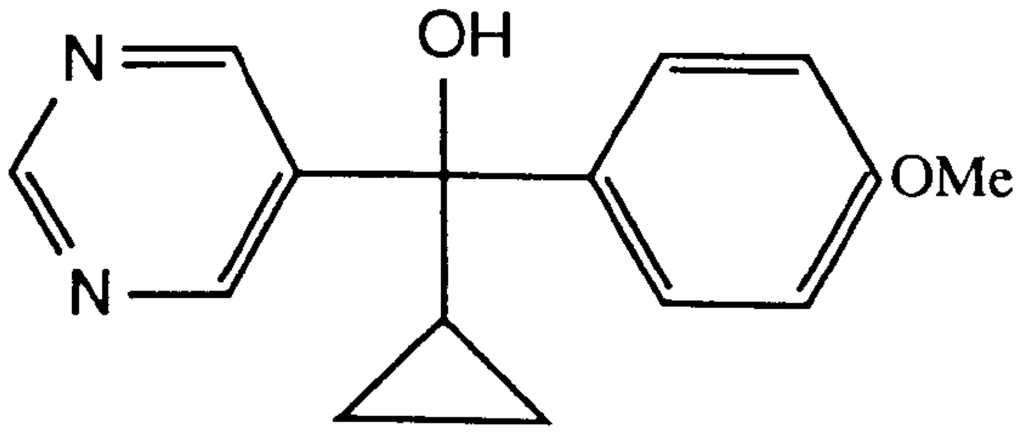
concentrations ranging from 1.5-5mM. Analysis of culture extracts indicated that toxin prodution had been suppressed, and that TDN had accumulated [Table 7]. The greatest amount of TDN detected (≈500mgL^{-1}) was in the cultures treated with xanthotoxin (8-methoxypsoralen) (51).

In view of these findings, it was decided to investigate the possibility of using xanthotoxin to stimulate the production of TDN in *F. culmorum*[94]. Xanthotoxin was added to liquid cultures of *F. culmorum*, 2 days after subculture, in concentrations ranging from 0.0-1.0mM. Cultures were then incubated for a further 6 days before harvesting. Analysis of culture extracts using GC-MS indicated that TDN had accumulated, and the concentration of inhibitor required for maximum TDN production was found to be 0.1mM[94] [Graph 1].

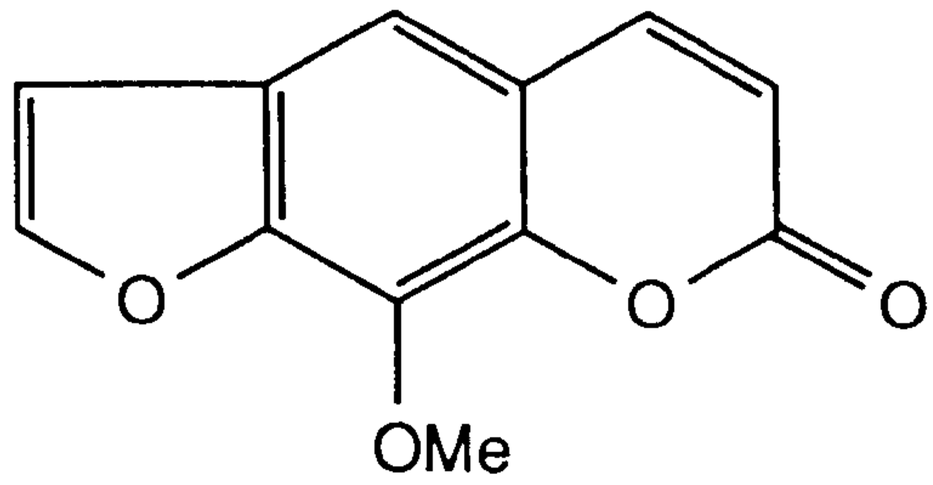
From these results, it was decided that this would be a suitable method for obtaining TDN in reasonable quantities from cultures of *F. culmorum*.

Consequently, sufficient xanthotoxin (as a solution in dimethylsulphoxide) to produce a concentration of 0.1mM was added to a liquid culture of *F. culmorum* (8.0L), 2 days after subculture. Incubation (27°C,250rpm) was continued for a further 6 days during which time the cultures developed a milky appearance due to the accumulation of large amounts of trichodiene. The cultures were then harvested by filtration and extraction with ethyl acetate. Purification of the extract by column chromatography, eluting with hexane, yielded TDN (270mgL^{-1}) as a colourless low viscosity oil. EI-MS and ^{1}H NMR measurements produced spectral data in accordance with published values[48,73].

The use of xanthotoxin to inhibit the cytochrome P-450-dependent monooxygenase enzymes involved in trichothecene biosynthesis has therefore been shown to offer a rapid and straightforward method for producing large quantities of



Ancymidol (50)



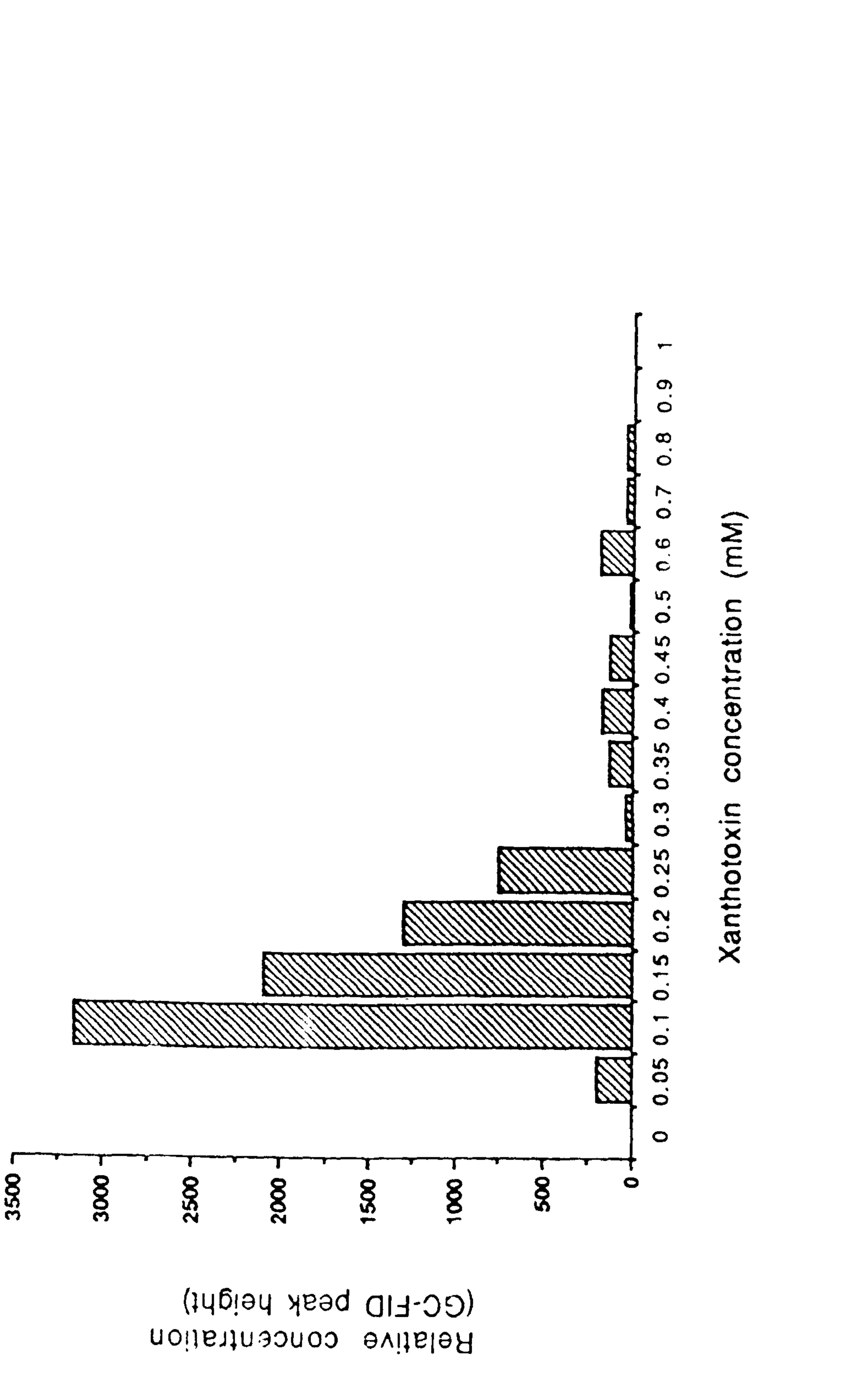
Xanthotoxin (51)

Table 7: Effect of flavone and furanocoumarins on T-2 toxin and trichodiene production by *Fusarium sporotrichioides*[64].

Compound	Concentration (mM)	% Inhibition of T-2 toxin production	Trichodiene (μM)
Flavone	5	100	7
	4	100	6
	2	100	59
Heraclenin A	5	96	42
B	5	100	0
Imperatorin A	5	97	64
B	5	100	1
Xanthotoxin A	1.5	95	265
B	1.5	100	304
Bergapten	5	100	0
Pimpinellin	5	82	14
Isopimpinellin	5	100	39
Psoralen	5	100	74

A and B designate replicate experiments.

Graph 1: Accumulation of TDN in xanthotoxin-inhibited cultures of *Fusarium culmorum*.



pure TDN. In this way amounts in excess of 2 grams of material can be obtained within the space of 8 days.

Production of labelled TDN.

Radiolabelled TDN was required for use in biosynthetic studies. This would allow the incorporation of TDN into trichothecenes to be monitored using scintillation counting procedures or autoradiography. Previous work in this laboratory had demonstrated satisfactory production of ^{14}C-labelled 3-AcDON by feeding [2-^{14}C]acetate to growing fungal cultures of *F. culmorum*[71]. The excellent yields of TDN obtained from xanthotoxin-inhibited cultures of *F. culmorum* meant that it should be feasible to produce [^{14}C]TDN using a similar method.

A preliminary experiment was carried out to determine whether the labelled precursors should be added to the cultures in a single batch or in several smaller batches over a period of time. A solution of sodium [2-^{14}C]acetate (925Bq) was added to a 2 day old culture of *F. culmorum* together with sufficient xanthotoxin to give a concentration of 0.1mM. A similar culture received the same total quantity of sodium [2-^{14}C]acetate but this was added in five equal portions at times 0, 24, 48, 72 and 120 hours after addition of the xanthotoxin. The experiments were carried out in duplicate, and the cultures were worked up after 124 hours. The [^{14}C]TDN present was isolated, purified and weighed, and its specific activity determined by scintillation counting. The results showed that the multiple batch addition produced [^{14}C]TDN with a higher specific activity than the single batch addition [Table 8]. It also gave a greater percentage incorporation, although this figure is dependent on the yield of TDN obtained, so is therefore subject to any biological variations between the inhibited cultures.

Table 8: Production of [^{14}C]TDN from [2-^{14}C]acetate using xanthotoxin-inhibited cultures of *F.culmorum*.

[2-^{14}C]acetate added as:-		Culture volume (ml)	[^{14}C]TDN produced (mg)	[^{14}C]TDN produced specific activity* ($KBqmM^{-1}$)	Total activity (KBq)	Incorporation (%)
Single batch (1 x 925KBq)	A	50	29.9	197	0.96	3.1
	B	50	40.1	233	1.14	4.9
Multiple batch (5 x 185KBq)	A	50	46.3	290	1.42	7.1
	B	50	36.1	284	1.39	5.4

A and B designate replicate experiments

$$\% \text{ Incorporation} = \frac{\text{Total activity of TDN}}{\text{Total activity added to culture}} \times 100.$$

* specific activities are an average of four separate determinations.

Table 9: Large scale production of [^{14}C]TDN by multiple batch addition of [2-^{14}C]acetate to xanthotoxin-inhibited cultures of *F.culmorum*.

[2-^{14}C]acetate	Culture volume (L)	Trichodiene yield (mg)	Trichodiene specific activity ($MBqmM^{-1}$)
5 x 7.4MBq	0.4	440	4.33
5 x 7.4MBq	0.2	164	8.45

When this method was applied to xanthotoxin-inhibited cultures on a larger scale (0.2 and 0.4L), [^{14}C]TDN yields of up to 1100mgL^{-1} were obtained. The specific activity of the material varied depending on the activity of the precursor used [Table 9], but was suitably high for use in biosynthetic studies.

Isolation of EPT from cultures of *F. culmorum.*

It has been proposed that all the naturally occurring oxygenated trichothecenes are produced from TDN via a common intermediate, 12,13-epoxytrichothec-9-ene (EPT). It was therefore desirable to obtain a supply of EPT, preferably in radiolabelled form, for use in experiments attempting to confirm this pathway. The isolation of EPT (0.7mgL^{-1}) from *F. culmorum* cultures has previously been reported[59], but during this study EPT was not detected in the extracts from such cultures (see p. 53). Even so, with yields at such low levels this would not offer a practicable source for the production of ^{14}C-labelled EPT. Attempts to obtain EPT (and hence [^{14}C]EPT) semi-synthetically from major trichothecene products such as DAS and trichothecolone, which are available in relatively large quantities (>200mg), have also proven unsucessful[94].

Treatment of fungal cultures with xanthotoxin was considered to be a possible alternative method for the production of EPT. Inhibiting the trichothecene biosynthetic pathway in this way should lead to the accumulation of less oxygenated trichothecenes in addition to the observed production of large amounts of TDN. The GC-MS traces from the experiment in which *F. culmorum* cultures were exposed to a range of xanthotoxin concentrations (see p. 57) were therefore re-examined. Both EPT and isotrichodermin (ITD) were detected, with EPT present in significantly higher yields than in the uninhibited cultures. The

concentration of EPT varied with the amount of inhibitor used, and peaked at xanthotoxin concentrations betweem 0.2 and 0.8mM [Graph 2]. ITD levels remained reasonably constant [Graph 3]. When a large scale culture of *F. culmorum* was exposed to a xanthotoxin concentration of 0.7mM, EPT and ITD were isolated by CTLC in yields of $1mgL^{-1}$ and $3mgL^{-1}$ respectively. These were identified by ^1H NMR and EI-MS analysis which produced data in agreement with literature values[59].

The increased accumulation of EPT in the inhibited culture adds support to the proposed biosythetic sequence to the trichothecenes via EPT and ITD. However, the yields of EPT were not sufficiently large for this to offer a feasible method for the production of [^{14}C]EPT.

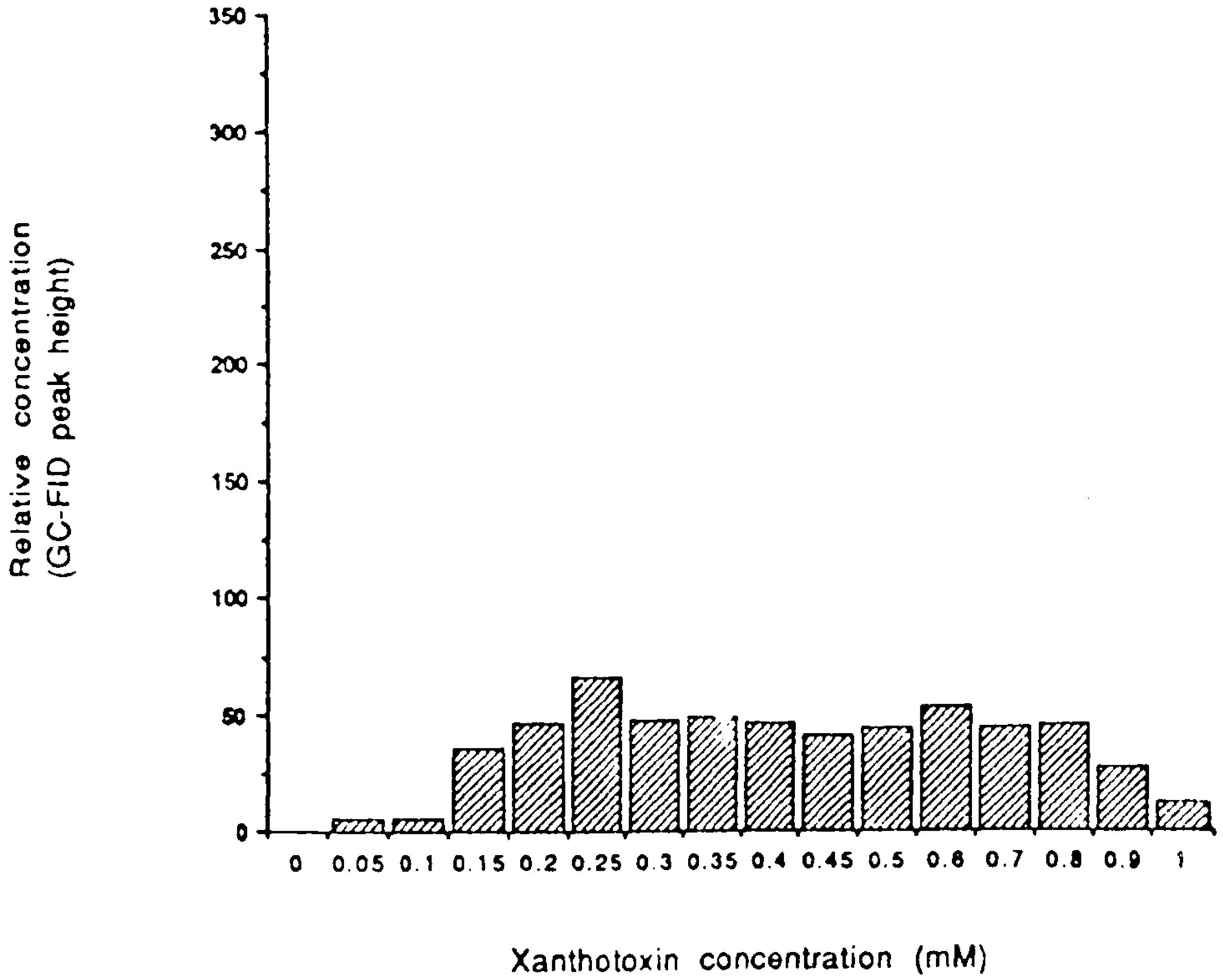
Semi-synthesis of EPT from ITD.

ITD was produced from the xanthotoxin-inhibited cultures in higher yields than EPT, and thus offered a source from which more EPT could be obtained by semi-synthesis.

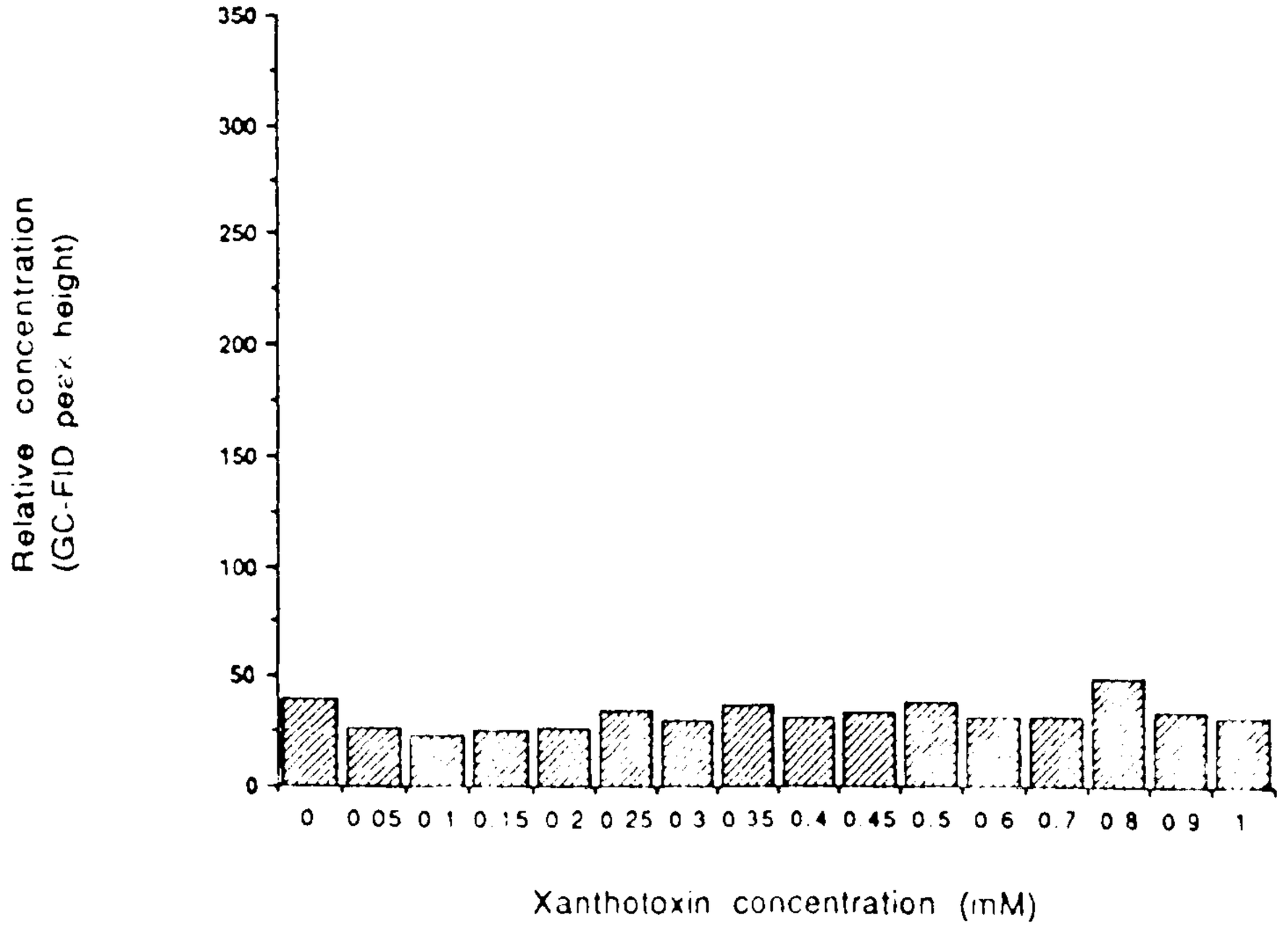
The conversion of ITD to EPT was achieved using a reaction sequence based upon the Barton-McCombie deoxygenation procedure for secondary alcohols[95] [Scheme 1]. This method had successfully been applied for the removal of oxygen substituents at both positions C-3 and C-4 in the trichothecenes by previous groups of workers[96,97].

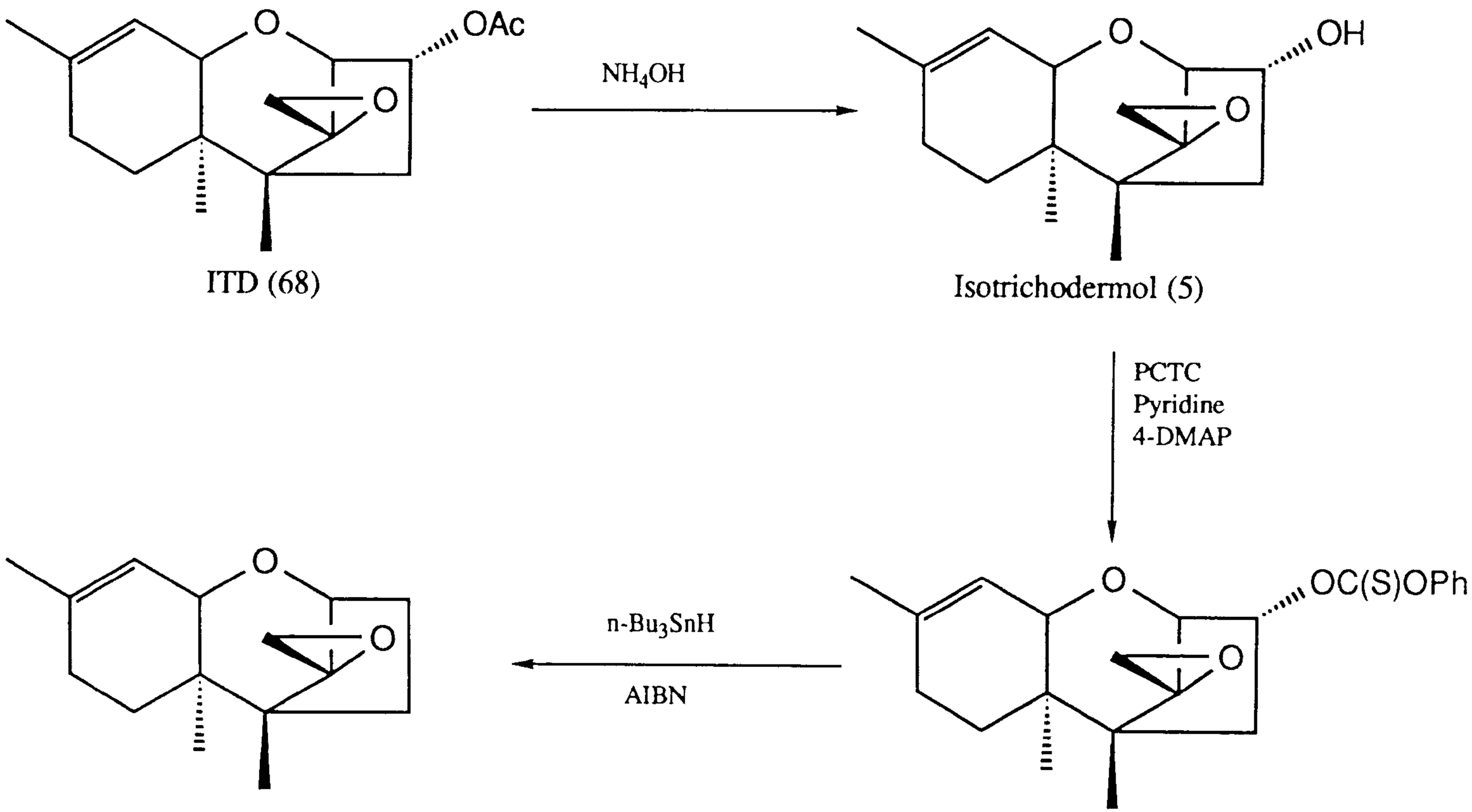
Base hydrolysis of ITD using 2M aqueous ammonia produced isotrichodermol (35%) as white crystals with ^1H NMR data in agreement with published values[60]. Esterification by reaction with phenylchlorothionocarbonate in the presence of pyridine-4-dimethylaminopyridine yielded the 3-phenylthionocarbonate (69) (48%)

Graph 2: Accumulation of EPT in xanthotoxin-inhibited cultures of *Fusarium culmorum*.



Graph 3: Presence of ITD in xanthotoxin-inhibited cultures of *Fusarium culmorum*.





Scheme 1: Semi-synthesis of EPT from ITD by chemical deoxygenation at C-3.

upon work-up. Reduction of this ester with tributyltin hydride afforded EPT (42%) as a colourless viscous oil, spectrally identical to the natural material.

Therefore further EPT can be obtained from the xanthotoxin-inhibited cultures via semi-synthesis from the ITD produced. However, the total yields of EPT are still too low to make production of [^{14}C]EPT in this way worthwhile.

Metabolic studies on the production of trichothecenes from trichodiene.

Having developed a method by which [^{14}C]TDN can be obtained in high yields, it was now possible to investigate the biosynthetic sequences involved in the transformation of TDN to trichothecenes. It was decided that this could best be achieved using two complementary research approaches. The first formed the basis of a separate project and aimed ultimately to identify, isolate and characterise the enzymes involved in the production of trichothecenes from TDN. This was to be achieved via development of cell-free systems capable of transforming TDN and other intermediates in the trichothecene pathway[98]. The second approach, undertaken in this project, was to probe the biosynthetic pathway using semi-synthetic TDN derivatives. By producing analogues of the natural intermediates we hoped to inhibit the post-TDN biosynthesis of trichothecenes, and perhaps cause the accumulation of novel intermediates. Any biotransformations of the analogues used would also provide information on the enzymic reactions taking place.

Semi-synthetic TDN analogues.

The transformation of TDN to trichothecenes is known to require molecular oxygen, and involves hydroxylation and epoxidation reactions which are thought to be mediated by monooxygenase enzymes[62]. Trichodiol (37) and trichotriol (48) are

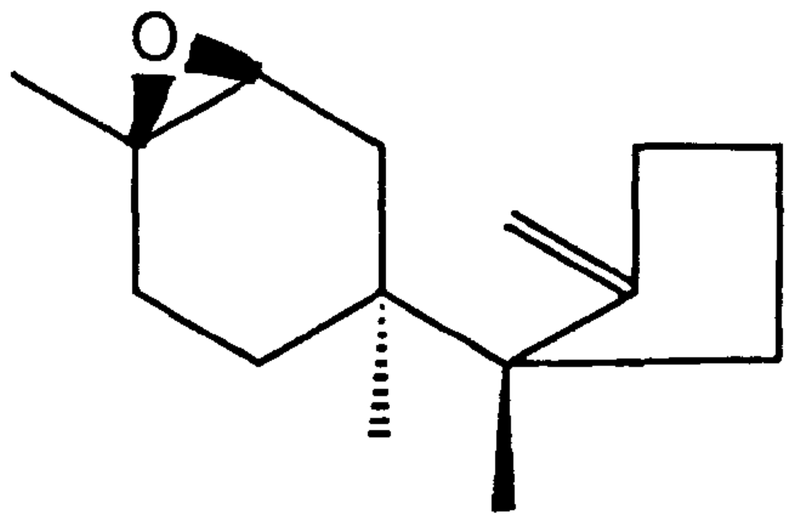
oxygenated metabolites of TDN, and are believed to be precursors to the trichothecenes (see Fig.17). Consequently, suitable oxygenated TDN analogues for use as inhibitors of this biosynthetic sequence were prepared as in Fig.31. TDN (36) is numbered according to the positions the carbons would occupy when incorporated into the trichothecene skeleton, and all compounds based on TDN are numbered in the same way.

9β-Hydroxytricho-10,12-diene (70) has the same A ring structure as both trichodiol and trichotriol (except perhaps for the stereochemistry at C-9), but has the B ring structure of TDN. 12,13-EpoxyTDN (71) and 9β,10β;12,13-diepoxyTDN (72) both possess the 12,13-epoxide function which is present in all the natural trichothecenes, whilst 9β,10β-epoxyTDN (73) and 9β,10α-dihydroxyTDN (74) are similar to TDN except for unnatural oxidation in the A ring.

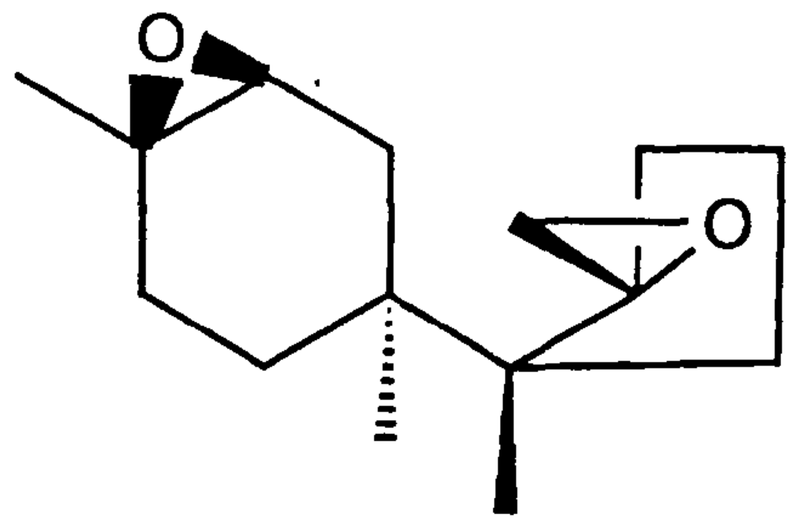
1. 9β,10β-EpoxyTDN (73).

9β,10β-EpoxyTDN was prepared according to the procedure of Cane *et al*[54]. A solution of TDN in dichloromethane was treated with one equivalent of *m*-chloroperoxybenzoic acid (*m*CPBA) with disodium hydrogenphosphate present as a buffer[99]. TLC analysis (hexane-ethyl acetate, 8:2) showed that all the starting material had reacted after 10 mins, and that a single major product had been formed. This was more polar than TDN, and produced a blue colour with PNBP/TEPA indicating the presence of an epoxide. Purification yielded the 9,10-epoxide (73) (68%) as a viscous colourless oil.

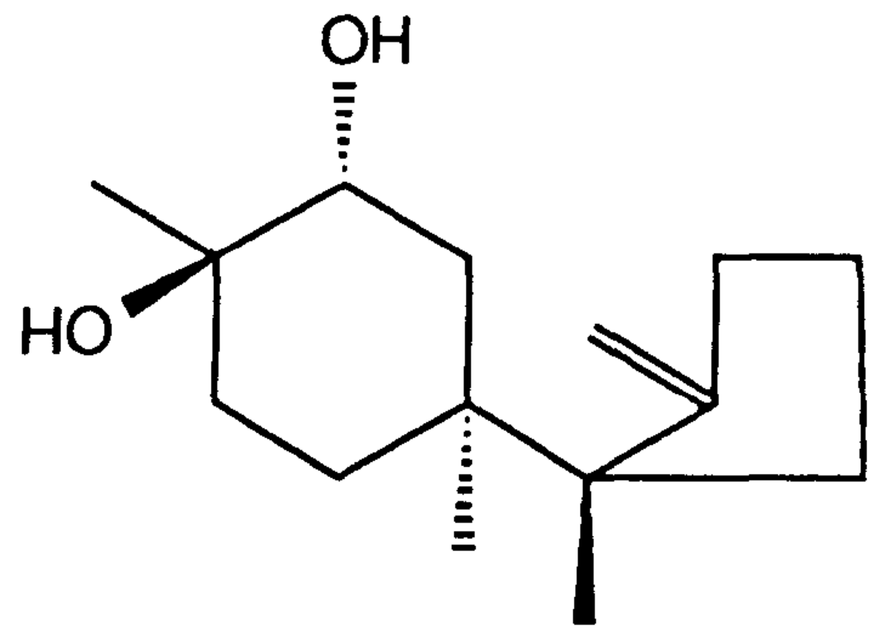
The product was identified by EI-MS, ^{1}H NMR [Table 10, Fig.32] and ^{13}C NMR [Table 11] analysis which produced data in agreement with literature



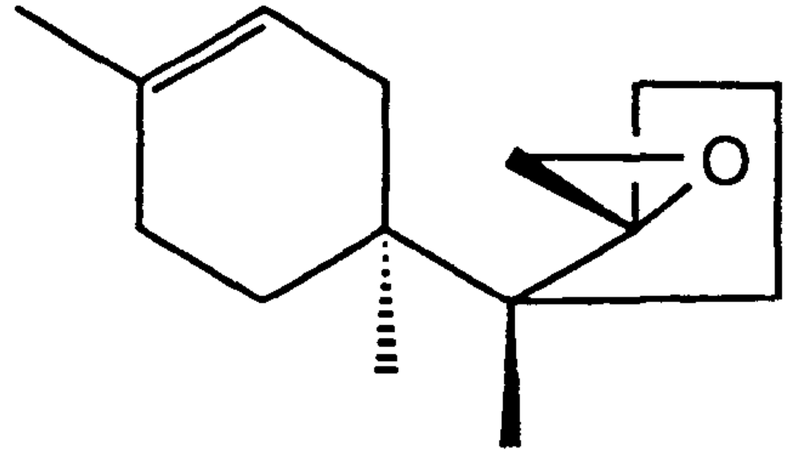
9β,10β-EpoxyTDN (73)



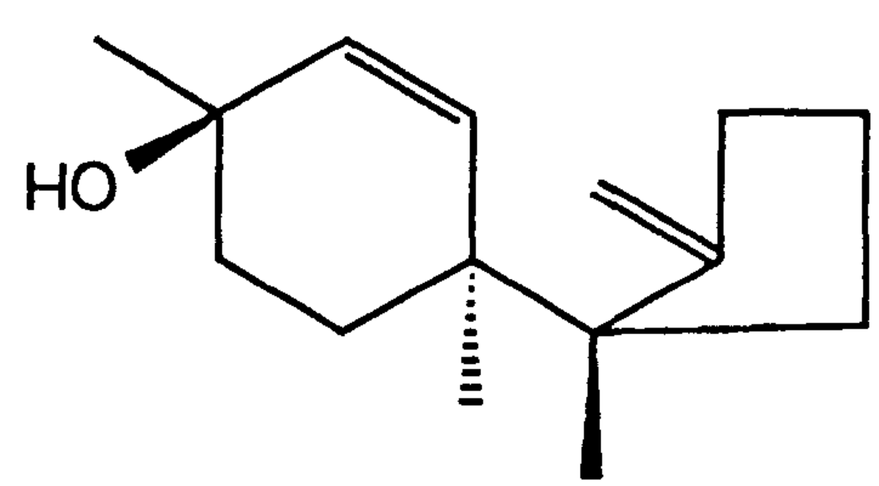
9β,10β;12,13-DiepoxyTDN (72)



9β,10α-DihdroxyTDN (74)



12,13-EpoxyTDN (71)



9β-Hydroxytricho-10,12-diene (70)

Fig.31: Oxygenated TDN derivatives targeted for synthesis for use in inhibition studies.

values[54]. Identification was also aided by comparison with the spectral data obtained for TDN [Tables 10 and 11, Fig.33]. Thus, in the ^{1}H NMR spectrum for TDN, the vinylic proton (H-10) is at δ5.29 and appears as a doublet of quartets due to direct coupling with H-11α ($J_{10,11\alpha}$=5.4Hz) and long range coupling with the C-16 methyl protons ($J_{10,16}$=1.3Hz). Molecular models predict that the dihedral angle between H-10 and H-11β is approximately 90°, and consequently coupling is not observed between these two protons (i.e. $J_{10,11\beta}$≈0Hz). The vinylic protons at C-13 are non-equivalent and resonate at δ4.73 and δ4.96. Methyl resonances, readily identified from integration, are observed at δ1.64, δ0.84 and δ1.04. The signal at δ1.64 is assigned to the C-16 protons from its downfield position due to the deshielding effect of the C-9,10 double bond, and also because it is split by long range coupling to H-10 ($J_{16,10}$=1.4Hz) and to the two protons at C-11 ($J_{16,11}$=0.7 and 0.6Hz). Savard *et al*[73] assigned the signals at δ0.84 and δ1.04 to H-14 and H-15, respectively, by using resolution enhancement. The methylene protons at C-2, C-3, C-4, C-7, C-8 and C-11 are all present in the region δ1.3-2.4 forming a complex overlapping multiplet. Individual assignments of these protons is therefore not readily achieved.

The ^{13}C NMR (with DEPT) shows four quaternary carbons at δ37.0 (C-6), 50.8 (C-5), δ132.3 (C-9) and δ160.7 (C-12). A vinylic methine at δ120.6 can readily be assigned to C-10, and the ethylenic resonance at δ106.7 to C-13. Three methyl resonances at δ18.0, δ24.1 and δ23.2, and the remaining six methylene signals were assigned according to data published by Snowden *et al*[100]. This also agrees with values published by Savard *et al*[73] apart for the assignments for C-3 and C-7 which have been interchanged. ^{13}C NMR analysis of a range of semi-synthetic and natural derivatives of TDN, led us to agree with the assignments made by

Fig.32: ^{1}H NMR spectrum of 9β,10β-epoxytrichodiene (73).

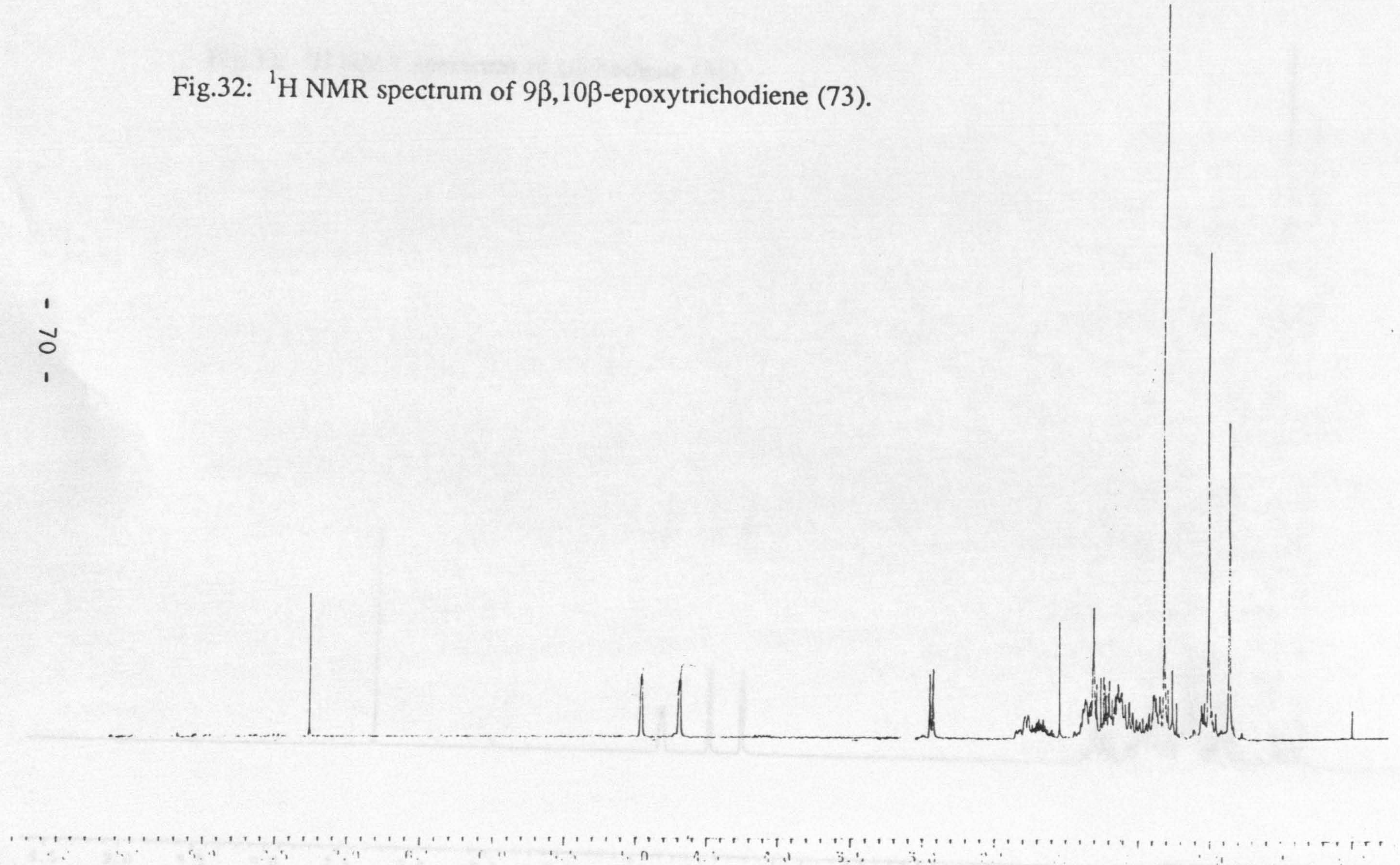


Fig.33: ^{1}H NMR spectrum of trichodiene (36).

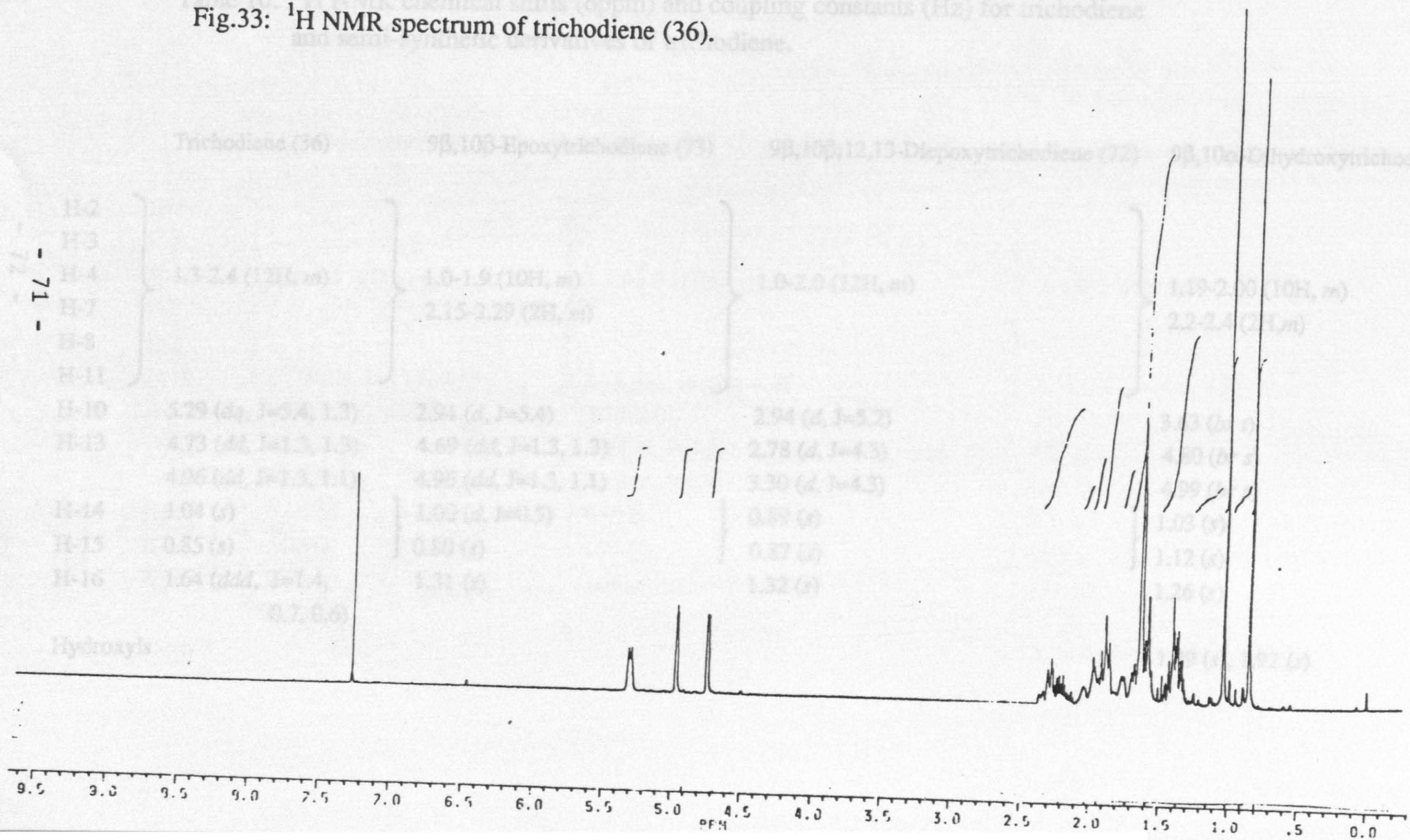


Table 10: ^{1}H NMR chemical shifts (δppm) and coupling constants (Hz) for trichodiene and semi-synthetic derivatives of trichodiene.

	Trichodiene (36)	9β,10β-Epoxytrichodiene (73)	9β,10β;12,13-Diepoxytrichodiene (72)	9β,10α-Dihydroxytrichodiene (74)
H-2 H-3 H-4 H-7 H-8 H-11	1.3-2.4 (12H, *m*)	1.0-1.9 (10H, *m*) 2.15-2.29 (2H, *m*)	1.0-2.0 (12H, *m*)	1.19-2.00 (10H, *m*) 2.2-2.4 (2H,*m*)
H-10	5.29 (*dq*, J=5.4, 1.3)	2.94 (*d*, J=5.4)	2.94 (*d*, J=5.2)	3.63 (*br t*)
H-13	4.73 (*dd*, J=1.3, 1.3)	4.69 (*dd*, J=1.3, 1.3)	2.78 (*d*, J=4.3)	4.80 (*br s*)
	4.96 (*dd*, J=1.3, 1.1)	4.96 (*dd*, J=1.3, 1.1)	3.30 (*d*, J=4.3)	4.99 (*br s*)
H-14	1.04 (*s*)	1.00 (*d*, J=0.5)	0.89 (*s*)	1.03 (*s*)
H-15	0.85 (*s*)	0.80 (*s*)	0.87 (*s*)	1.12 (*s*)
H-16	1.64 (*ddd*, J=1.4, 0.7, 0.6)	1.31 (*s*)	1.32 (*s*)	1.26 (*s*)
Hydroxyls				1.89 (*s*), 1.92 (*s*)

	9,10-Dibromotrichodiene (75)	9,10-Dibromo-12,13-epoxytrichodiene (76)	9β-Hydroxy,10α-phenylselenotrichodiene (78)
H-2	1.0-2.5 (11H, *m*)	1.25-2.0 (11H, *m*)	1.0-2.2 (11H, *m*)
H-3			
H-4			
H-7			
H-8			
H-11	2.75 (*dd*, J=15.6, 4.6)	2.79 (*dd*, J=15.6, 4.6)	2.44 (*dd*, J=14.6, 5.3)
H-10	4.78 (*dd*, J=4.6, 2.2)	4.76 (*m*)	3.50 (*t*, J=5.5)
H-13	4.84 (*br s*)	2.83 (*d*, J=4.0)	4.76 (*br s*)
	5.02 (*br s*)	3.48 (*d*, J=3.8)	4.93 (*br s*)
H-14	1.06 (*s*)	0.94 (*s*)	0.98 (*s*)
H-15	1.23 (*s*)	1.21 (*s*)	1.03 (*s*)
H-16	2.03 (*s*)	2.04 (*s*)	1.28 (*s*)
Phenyls			7.25-7.60 (5H, *m*)

	9β-Hydroxytricho-10,12-diene (70)	9α-Hydroxytricho-10,12-diene (82)	9-Methoxytricho-10,12-diene (81)	
			β	α
H-2	2.27 (*m*)	1.34-1.43 (3H, *m*) 1.65-1.80 (5H, *m*) 2.2-2.33 (2H, *m*)	1.2-2.0 (8H, *m*) 2.2-2.3 (2H, *m*)	
	2.27 (*m*)			
H-3	1.66 (*m*)			
	1.42 (*m*)			
H-4	1.9 (*m*)			
	1.42 (*m*)			
H-7	1.40 (*m*)			
	1.92 (*m*)			
H-8	1.66 (*m*)			
	1.66 (*m*)			
H-10	5.59(*dd* J=10.2, 1.2)	5.52 (*d* J=10.4)	5.56 (*dd*, J=10.4, 1.7)	5.52 (*dd*, J=10.4, 1.7)
H-11	5.77 (*dd*, J=10.2, 1.4)	5.65 (*dd*, J=10.4, 1.8)	5.87 (*dd*, J=10.4, 1.7)	5.83 (*dd*, J=10.4, 1.7)
H-13	4.83 (*br s*)	4.80 (*br s*)	4.82 (*br s*)	
	4.99 (*br s*)	4.98 (*br s*)	4.97 (*br s*)	
H-14	1.07 (*s*)	1.02 (*s*)	1.06 (*s*)	
H-15	0.97 (*s*)	1.04 (*s*)	0.95 (*s*)	
H-16	1.27 (*s*)	1.27 (*s*)	1.19 (*s*)	
Methoxy			3.20 (*s*)	3.22 (*s*)

Table 11: ^{13}C NMR chemical shifts (δppm) for trichodiene and semi-synthetic derivatives of trichodiene.

	Trichodiene (36)	9β,10β-Epoxytrichodiene (73)	9β,10α-Dihydroxytrichodiene (74)	9β,10β;12,13-Diepoxytrichodiene (72)
C-2	38.9	38.6	39.0	37.5
C-3	23.4	23.2	23.2	21.4
C-4	37.4	37.3	37.1	37.4
C-5	50.8	50.3	51.1	47.9
C-6	37.0	36.0	37.1	35.4
C-7	28.3	24.6	26.3	25.2
C-8	27.9	26.5	29.9	26.2
C-9	132.3	57.1	70.9	57.0
C-10	120.6	58.7	75.1	58.5
C-11	33.2	32.2	34.3	32.5
C-12	160.7	159.3	159.9	68.2
C-13	106.7	107.2	106.9	51.1
C-14	24.1	24.2	23.6	21.8
C-15	18.0	20.0	20.4	18.4
C-16	23.2	23.1	27.0	23.1

	9,10-Dibromotrichodiene (75)	9β-Hydroxytricho-10,12-diene (70)	9α-Hydroxytricho-10,12-diene (82)
C-2	38.9	38.6	38.6
C-3	23.2	23.2	23.2
C-4	37.0	37.2	37.1
C-5	51.3	49.5	49.6
C-6	37.1	40.5	40.8
C-7	29.2	26.2	28.8
C-8	33.3	34.1	35.7
C-9	72.1	66.5	69.7
C-10	58.6	131.7	134.0
C-11	36.5	136.5	133.3
C-12	158.9	159.8	159.4
C-13	107.5	106.6	106.7
C-14	23.6	24.3	24.5
C-15	21.5	20.2	21.5
C-16	35.8	29.7	27.7

The EI-MS [Table 12] shows a molecular ion at m/z 204 and a fragment corresponding to $[M\text{-}CH_3]^+$ at m/z 187. The peaks at m/z 109 and m/z 95 correspond to A and B ring fragments produced by the facile cleavage of the C-5/6 bond [Fig.34].

9β,10β-EpoxyTDN gives a similar ^1H NMR spectrum to TDN but H-10 is shifted upfield from δ5.29 to δ2.93 due to the loss of the C-9,10 double bond, and the introduction of the 9β,10β-epoxide group. The signal is no longer split by coupling to the C-16 methyl protons but still couples to H-11α ($J_{10,11\alpha}$=5.3Hz) and is therefore now a doublet rather than a doublet of quartets. Similarly, the H-16 resonance now appears as a singlet and is shifted upfield from δ1.64 to δ1.31. It is still downfield to both the C-14 and C-15 methyls because of the deshielding effect of the C-9,10 epoxide function.

The ^{13}C NMR data shows that only the resonances for C-9 and C-10 are significantly different to those for TDN. The new quaternary carbon C-9 is thus readily identifiable using DEPT, and is shifted upfield from δ132.3 to δ57.1, whilst C-10 is shifted from δ120.6 to δ58.7. This is consistent with the loss of the double bond at this position, and both carbons now being attached to an oxygen atom.

The EI-MS [Table 12] sows a molecular ion at m/z 220 which is 16 mass units higher than for TDN, indicating the addition of an oxygen atom. Cleavage of the C-5/6 bond produces A and B ring fragments at m/z 125 and m/z 95 respectively. This indicates that oxygenation is in the A ring since it is 16 mass units higher than for the A ring fragment for TDN (m/z 109), whereas the B ring fragment at m/z 95 is the same as for TDN and therefore has the same structure. The base peak in the spectrum is at m/z 107 and corresponds to the loss of H_2O from the A

Fig.34: EI-MS fragmentation of TDN by cleavage of the C5/C6 bond.

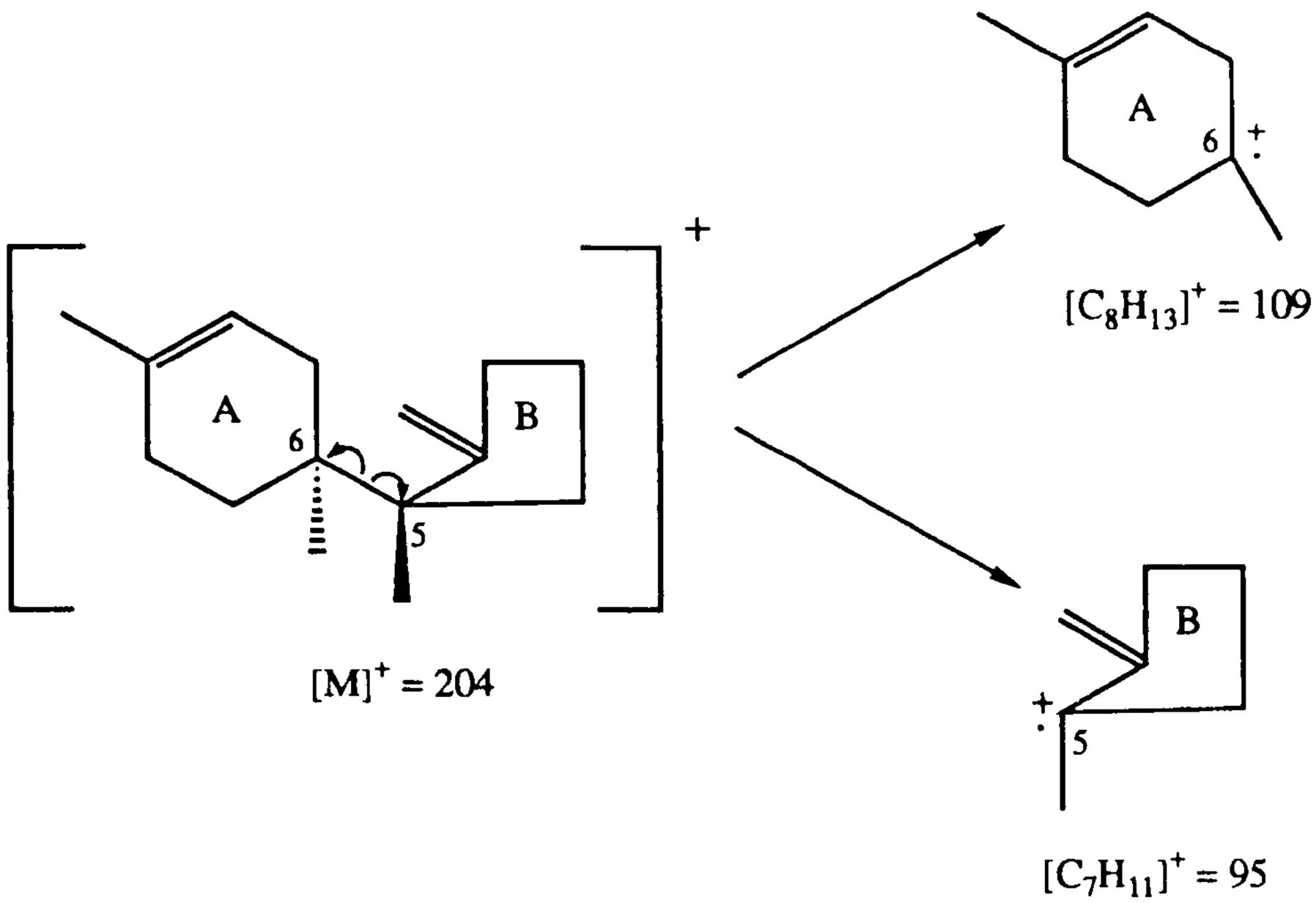


Table 12: Molecular ions and A and B ring fragment ions in the mass spectra of TDN derivatives.

a) EI-MS

	$[M]^+$	$[M-H_2O]^+$	$[A]^+$	$[A-H_2O]^+$	$[B]^+$
TDN	204	NR	109	NR	95
9β,10β-EpoxyTDN	220	202	125	107	95
9β,10β;12,13-DiepoxyTDN	236	218	125	107	111
9β,10α-DihydroxyTDN	238	220	--	125	95
9α/9β-OH-Tricho-10,12-diene	--	202	125	107	95
9-Methoxytricho-10,12-diene	--	NR	139	NR	95

b) FAB-MS

	$[M-H]^-$	$[A]^-$	$[B]^-$
9,10-DibromoTDN	361/3/5	267/9/71	95
9,10-Dibromo-12,13-epoxyTDN	377/9/81	267/9/71	111

NR = not relevant
-- = not observed

ring fragment.

2. 9β,10α-DihydroxyTDN (74).

9β,10α-DihydroxyTDN was synthesised from 9β,10β-epoxyTDN (73) by acid hydrolysis of the epoxide using two equivalents of 3% perchloric acid[54]. Work-up and purification yielded pure 9,10-diol (74) (60%) as white crystals. EI-MS, ^{1}H NMR [Table 10] and ^{13}C NMR [Table 11] spectra were in agreement with published values[54].

Comparison with the NMR spectral data for (73) assisted in identification of the product. The ^{1}H NMR spectrum shows that the H-10 resonance is shifted downfield from δ2.94 to δ3.63, and now appears as a triplet. This indicates that the conformation of the A ring has been altered allowing H-10 to couple with both protons at C-11, and is consistent with the opening of the epoxide ring to produce a hydroxyl group at C-10. Other minor differences include slight changes in the shifts for the methyl protons at C-16, and the vinylic C-13 protons. Two additional signals are present in the upfield region at δ1.89 and δ1.92 which are shown by integration to each be equivalent to one proton. These signals disappear on shaking with D_2O, and can therefore be assigned to the C-9 and C-10 hydroxyl protons.

The ^{13}C NMR spectrum also shows significant changes in the resonances for C-9, C-10 and C-16. C-9 is shifted downfield from δ57.1 to δ70.9, and C-10 from δ58.7 to δ75.1 indicating that these carbons are now attached directly to hydroxyl groups, which are more deshielding than the epoxide oxygen. Similarly, C-16 is shifted, although to a lesser extent, from δ23.1 to δ27.0.

Comparison of the EI-MS [Table 12] with that of TDN also helped confirm the

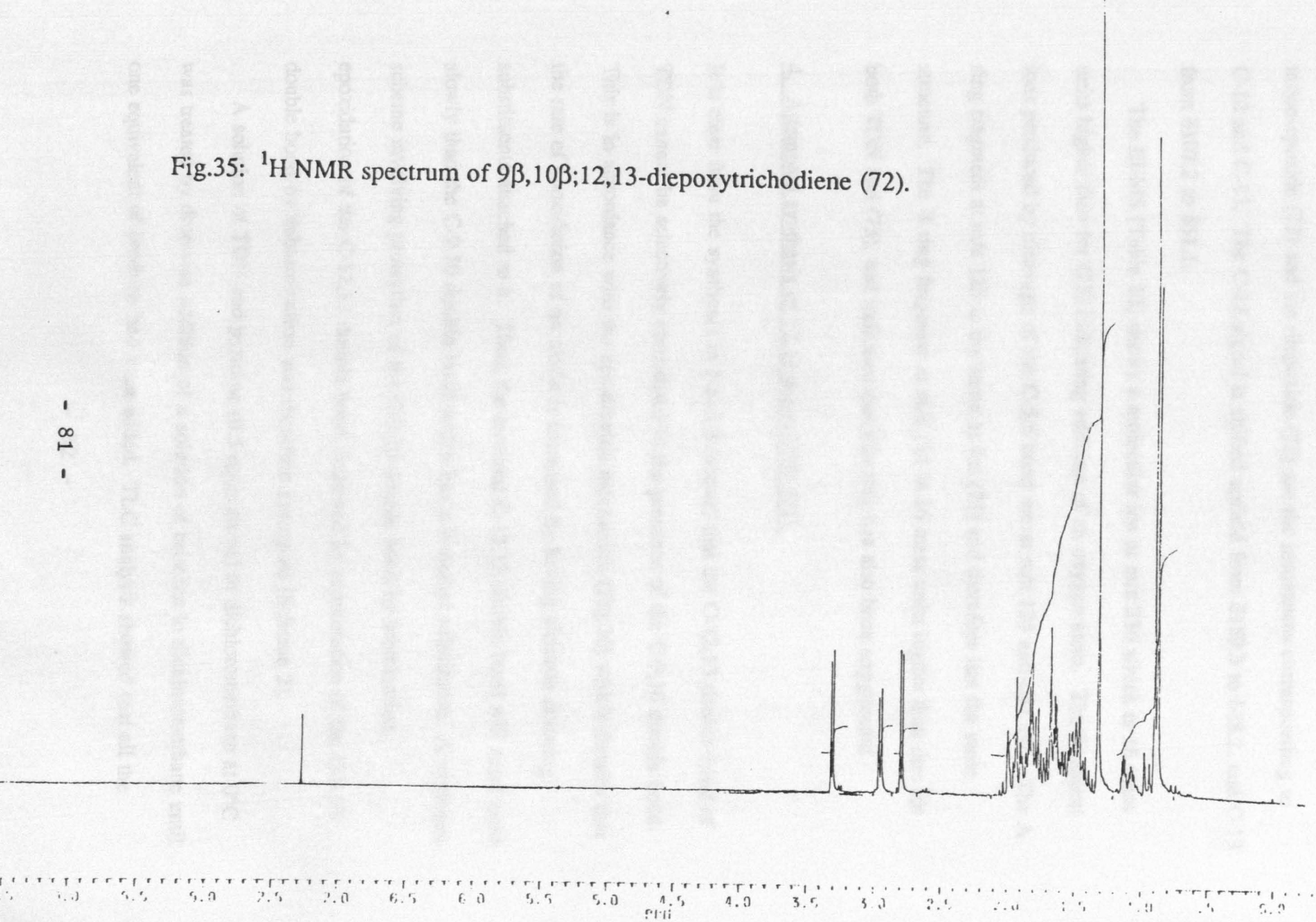
structure of the product. This shows the molecular ion at m/z 238 which is 34 mass units heavier than TDN, and consistent with the addition of two hydroxyl groups. The A and B ring fragments produced by cleavage of the C-5/6 bond are at m/z 125 and m/z 95 respectively. The B ring fragment at m/z 95 is the same as for TDN indicating a similar structure, and suggesting that the hydroxyl groups are in the A ring. The fragment at m/z 125 is 16 mass units higher than for the corresponding A ring fragment for TDN (m/z 109) demonstrating the presence of at least one hydroxyl in this ring. It is also 18 units less than the expected A ring fragment (m/z 143), and corresponds to this fragment minus H_2O. A further loss of water produces the base peak at m/z 107.

3. 9β,10β;12,13-DiepoxyTDN (72).

9β,10β;12,13-DiepoxyTDN was synthesised from TDN by the method used for the production of the mono-epoxide (73), except that two equivalents of *m*CPBA were employed, and the reaction took 6 hours to run to completion. Purification yielded the diepoxide (72) (54%) as a viscous colourless oil.

Comparison of the ^{1}H NMR data [Table 10, Fig.35] with the spectrum for (73) [Table 10, Fig. 32] clearly demonstrated that it was the expected product. The spectra are similar except for the resonances of the C-13 protons. In (73) these are non-equivalent and appear as broad singlets at δ4.69 and δ4.96 due to their position on an unsaturated carbon. In the diepoxide (72) the C-13 protons are shifted upfield to δ2.78 and δ3.30, with geminal coupling yielding well resolved doublets (J_{gem}=4.3Hz). This AB system centred at about δ3.0-3.2 with J_{AB}=4.2Hz is characteristic of the C-13 protons of the 12,13-epoxide group, and these signals are present in all the trichothecene spectra (see Table 18).

Fig.35: ^{1}H NMR spectrum of 9β,10β;12,13-diepoxytrichodiene (72).



The ^{13}NMR data [Table 11] also confirms that epoxidation of the C-12,13 double bond has taken place. The only differences between the spectra for the mono-epoxide (73) and the diepoxide (72) are the resonances corresponding to C-12 and C-13. The C-12 signal is shifted upfield from δ159.3 to δ68.2, and C-13 from δ107.2 to δ51.1.

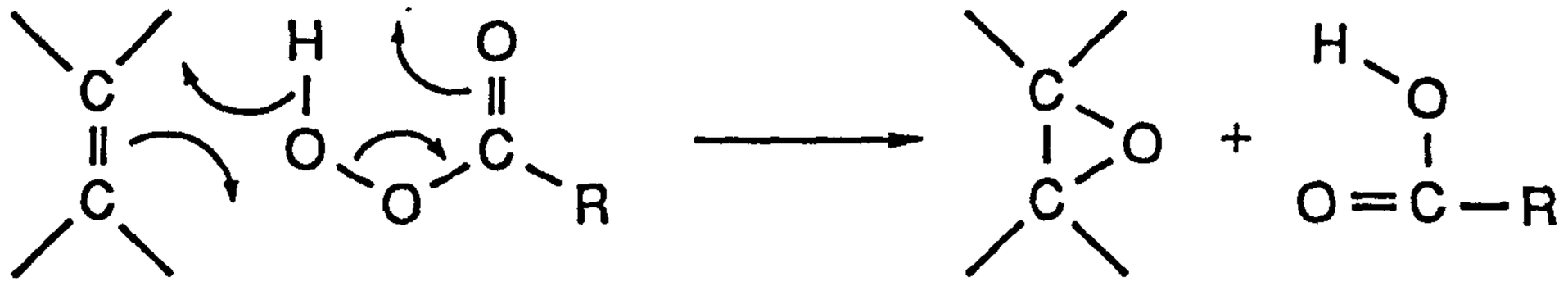
The EI-MS [Table 12] shows a molecular ion at m/z 236 which is 16 mass units higher than for (73) indicating addition of an oxygen atom. The fragment ions produced by cleavage of the C-5/6 bond are at m/z 125 and m/z 111. The A ring fragment at m/z 125 is the same as for (73) and therefore has the same structure. The B ring fragment at m/z 111 is 16 mass units higher than that for both TDN and (73), and indicates that this ring has also been oxygenated.

4. Attempted synthesis of 12,13-epoxyTDN (71).

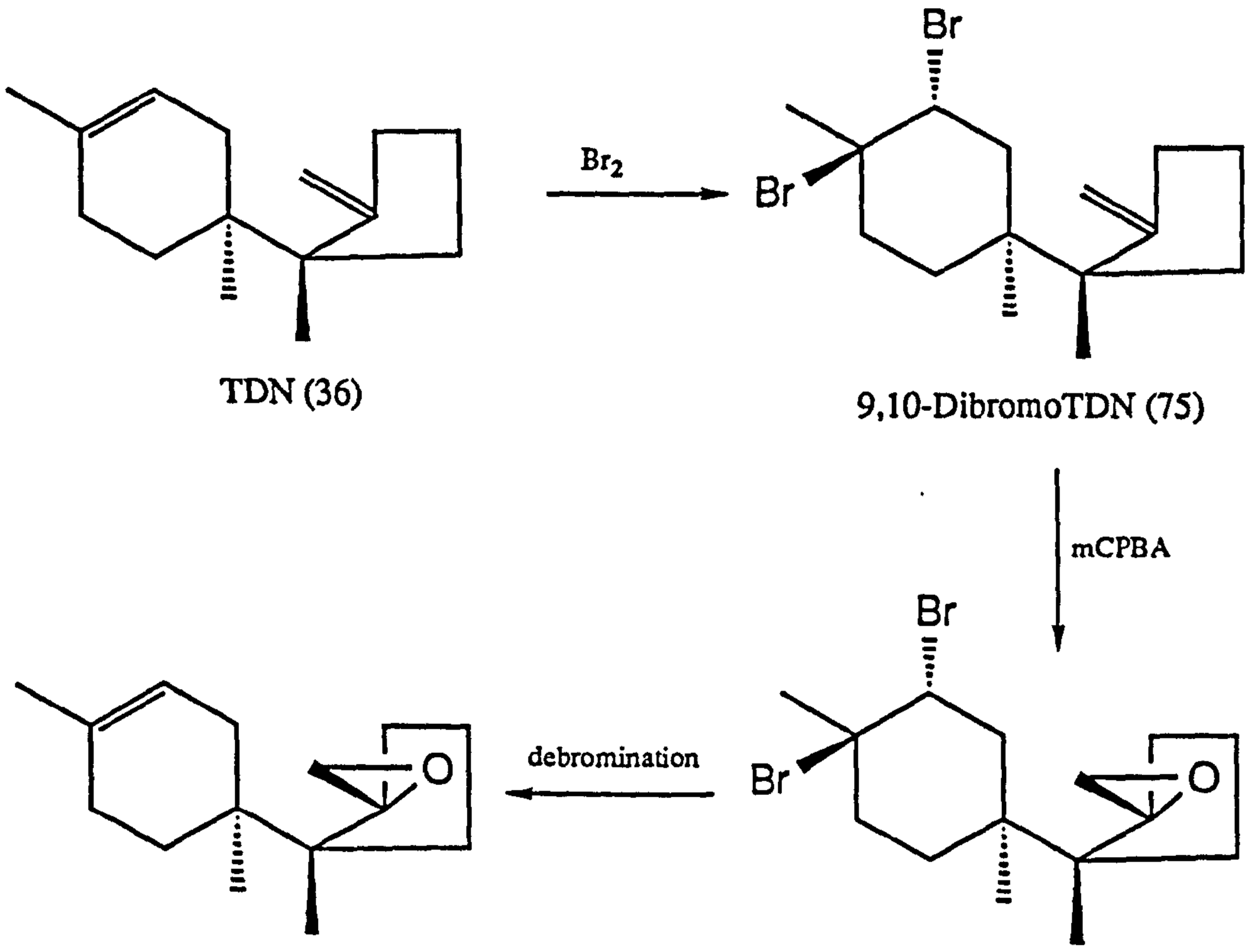
It is clear from the syntheses in 1 and 3 (above) that the C-12,13 double bond of TDN cannot be selectively epoxidated in the presence of the C-9,10 double bond. This is in accordance with the epoxidation mechanism [Fig.36] which dictates that the rate of epoxidation of an olefin is increased by having electron donating substituents attached to it. Thus, the terminal C-12,13 double bond will react more slowly than the C-9,10 double bond which has a 9-methyl substituent. A synthetic scheme involving protection of the C-9,10 double bond by bromination, epoxidation of the C-12,13 double bond, followed by regeneration of the C-9,10 double bond by debromination was therefore attempted [Scheme 2].

A solution of TDN and pyridine (0.5 equivalents) in dichloromethane at 0°C was treated by dropwise addition of a solution of bromine in dichloromethane until one equivalent of bromine had been added. TLC analysis showed that all the

Fig.36: Epoxidation of alkenes using peroxyacids.



Scheme 2: Proposed synthesis of 12,13-epoxyTDN.



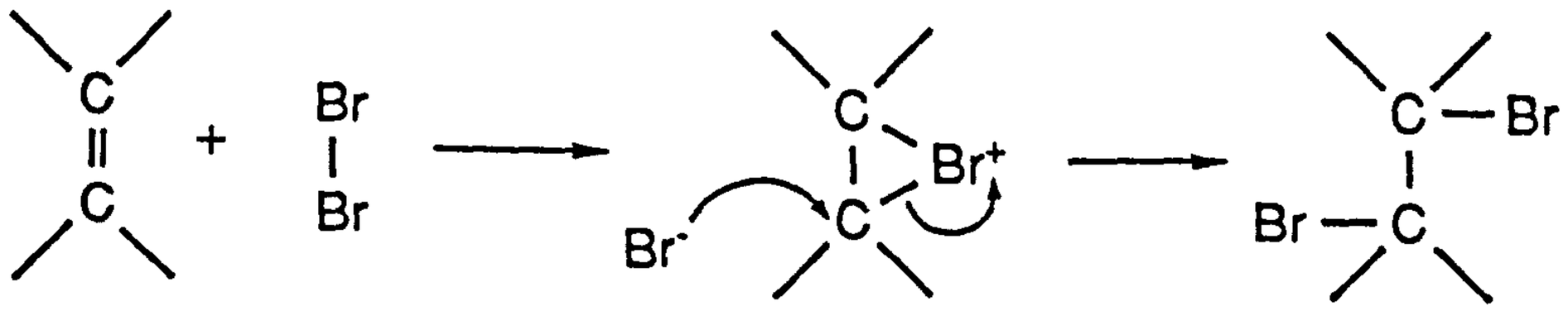
TDN (36)

9,10-DibromoTDN (75)

12,13-EpoxyTDN (71)

9,10-Dibromo-12,13-epoxyTDN (76)

Fig.37: Bromination of alkenes.



Bromonium ion

Trans-dibromide

starting material had reacted, and a single product had been formed. Work-up and purification yielded 9,10-*trans*dibromoTDN (75) (50%) as a viscous colourless oil. Analysis by ^{1}H NMR and ^{13}C NMR [Tables 10 and 11] was used to confirm the identity of the product.

Comparison of the ^{1}H NMR data with that for TDN indicated that the desired product had been formed. The resonance due to H-10 is shifted upfield from δ5.23 to δ4.78 because of the loss of the C-9,10 double bond, and its resultant position on a saturated carbon with a bromine attached. It no longer exhibits coupling to H-16, but couples with both the protons at C-11 ($J_{10,11}$=4.6 and 2.3Hz). The C-16 methyl signal is shifted downfield from δ1.63 to δ2.03 by the deshielding effect of the halogen at C-9, and is now a singlet in the absence of long range coupling to the protons at C-10 and C-11. One of the C-11 protons is also deshielded by the bromine at C-10, and is shifted downfield out of the unresolved methylene region to appear as a double doublet at δ2.75 ($J_{11,10}$=4.3Hz, J_{gem}=15.6Hz). This is similar to the H-11 resonance found in the data for 9β-hydroxy,10α-phenylselenoTDN (78) (see Table 10) and suggests that these compounds have the same stereochemistry i.e. (75) is 9β,10α-dibromoTDN. Resonances due to the C-13 protons appear at δ4.85 and δ5.03 consistent with their position on an unsaturated carbon, and indicating that selective bromination of the C-9,10 double bond has been achieved. A similar comparison of the ^{13}C NMR data shows that only the resonances for C-9, C-10 and C-16 are significantly different from those in the TDN spectrum. C-9 is shifted upfield from δ132.2 to δ72.1, and C-10 from δ120.6 to δ58.6 indicating that these carbons are no longer vinylic but are in a more shielded environment attached to bromine atoms.

FAB-MS [Table12] shows highest mass ions at m/z 361, 363 and 365

corresponding to $[M-H]^-$. This is equivalent to the molecular ion for TDN (m/z 204) plus two bromine atoms. Bromine exists in two isotopic forms with atomic masses of 79 and 81. In nature these are present in approximately equal abundance, which accounts for there being three molecular ions, two mass units apart and in a ratio of 1:2:1. The A ring fragment ions at m/z 267 and 269 produced by cleavage of the C-5/6 bond indicate that the bromines are in the A ring. The B ring fragment at m/z 95 is the same as for TDN.

Reaction of one equivalent of bromine with TDN has therfore yielded the 9,10-dibromide (75) as the major product, with bromination of the terminal olefin at C-12,13 not occurring to any significant extent. This is as expected since the addition of bromine to an alkene is thought to proceed via an intermediate bromonium ion [Fig.37] formed by electrophilic attack on the double bond. In accordance with this mechanism, electron-donating substituents attached to the double bond help to stabilise the cyclic bromonium ion. Consequently addition of bromine to the tri-substituted C-9,10 double bond will occur more readily than to the terminal, di-substituted C-12,13 alkene.

The 9,10-dibromide (75) was epoxidised by treatment with one equivalent of *m*CPBA in the presence of disodium hydrogenphosphate. The progress of the reaction was monitored using TLC analysis, and this showed that all the starting material had reacted after 6 hours, with the formation of a single major product. Work-up and purification yielded 9,10-dibromo-12,13-epoxyTDN (76) (62%) as white crystals.

The ^{1}H NMR spectrum [Table 10] is similar to that for (75) except for that the C-13 protons are shifted upfield from ca. δ4.93 to appear as an AB quartet centred around δ3.04 (J_{AB}=4.0Hz), confirming introduction of the epoxide group at this

position.

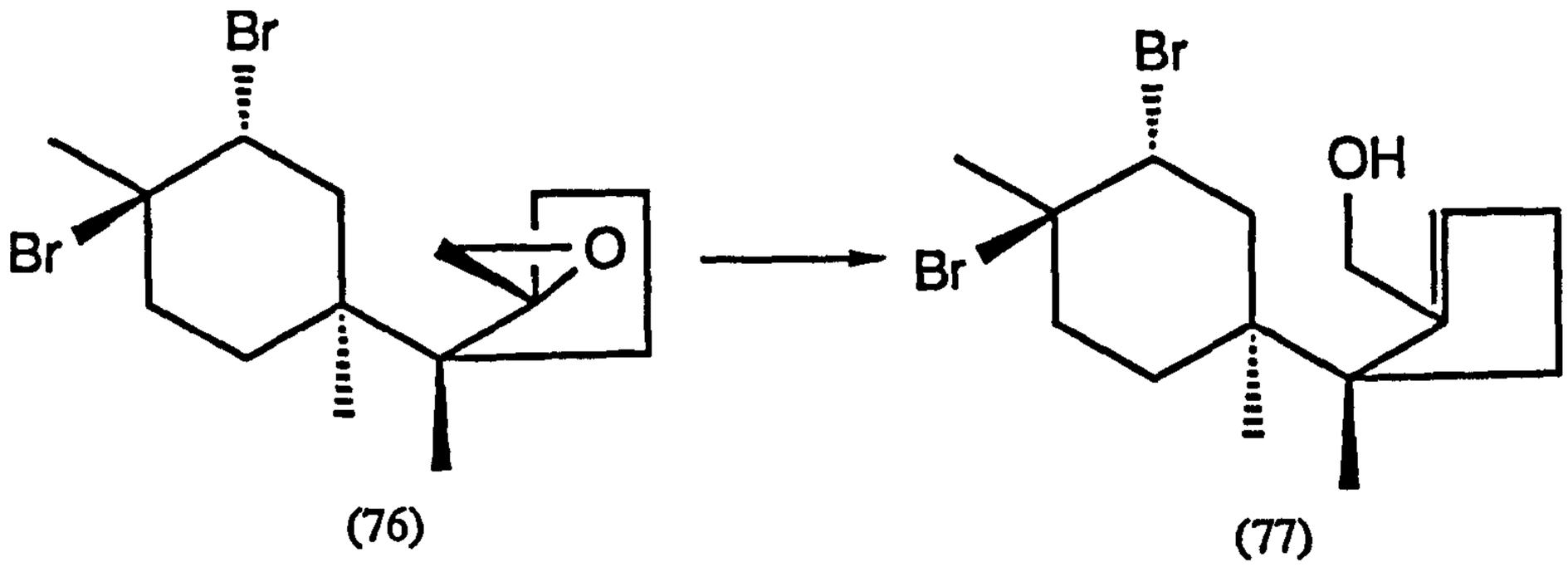
FAB-MS [Table 12] shows molecular ions at m/z 377, 379 and 381 which are 16 mass units heavier than the corresponding ions for (75), consistent with epoxidation having occurred.

A brief survey of the literature indicated that a large number of methods for the dehalogenation of vicinal dihalides were available[101,102,103]. Of these, heating with sodium iodide in acetone appeared to offer the mildest procedure since it is effective at relatively low temperatures.

The bromoepoxide (76) in dry acetone was treated with an excess of sodium iodide, and the mixture stirred at 38°C. GC and TLC analysis indicated the formation of numerous products, none of which predominated. When the reaction was repeated using 9,10-dibromoTDN, analysis showed that after 4 hours all the starting material had reacted, yielding TDN as the major product (95% of the compounds running on GC). Regeneration of the C-9,10 double bond by debromination is therefore possible using these reaction conditions. The failure to produce 12,13-epoxyTDN in the same way from (76) is probably due to the presence of the 12,13-epoxide which may be involved in unwanted rearrangement reactions.

Evidence for the instability of (76) was obtained during ^{13}C NMR analysis with $CDCl_3$ as solvent when a rearrangement was observed [Fig.38]. The spectrum of the product (77) [Table 13] indicates that the A ring structure has remained the same, but in the B ring the 12,13-epoxide has been opened producing a double bond between C-2 and C-12, and hydroxylation at C-13. Interpretation of the spectrum was aided by comparison with the ^{13}C NMR data for 9,10-dibromoTDN (75) and for FS-2[60] [Table 14], which is one of a group of four related TDN

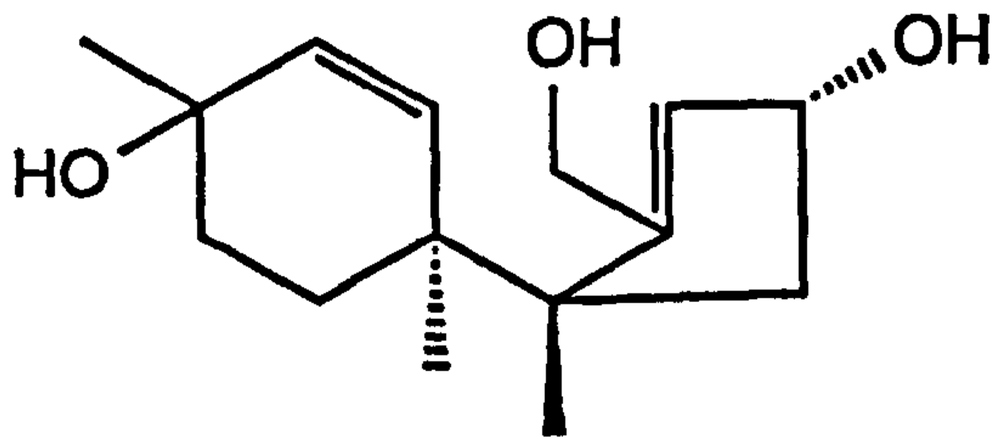
Fig.38: Observed rearrangement of 9,10-dibromo-12,13-epoxyTDN in $CDCl_3$ solution.



(76) (77)

Table 13: ^{1}H and ^{13}C NMR data for (77).

H-2	5.77 (*t*, J=1.8Hz)	C-2	127.2
H-3	1.25-2.26 (9H, *m*)	C-3	30.0
H-4		C-4	35.5
H-7		C-5	57.5
H-8	2.72 (1H, *dd*, J=15.8, 4.7Hz)	C-6	37.9
H-11		C-7	29.1
		C-8	33.3
		C-9	72.1
H-10	4.77 (*ddd*, J=4.6, 2.2, 2.0Hz)	C-10	58.2
H-13	4.23 (*br dd*, J=14.4, 1.8Hz)	C-11	36.9
	4.48 (*br d*, J=14.9Hz)	C-12	150.0
		C-13	61.6
H-14	1.09 (*s*)	C-14	22.4
H-15	1.21 (*s*)	C-15	20.3
H-16	2.04 (*s*)	C-16	35.8



FS-2 (56)

Table 14: ^{1}H and ^{13}C NMR data for FS-2[60].

H-2	5.75 (*dd*, J=3.3, 1.5Hz)	C-2	133.0
H-3	1.3-2.1 (8H, *m*)	C-3	73.4
H-4		C-4	47.4
H-7		C-5	54.6
H-8		C-6	40.0
H-11	5.58 (*d*, J=10.2Hz)	C-7	27.8
		C-8	35.0
H-10	5.67 (*dd*, J=10.2, 1.7Hz)	C-9	65.7
H-13	4.23 (*br d*, J=14.5Hz)	C-10	135.6
	4.35 (*br d*, J=14.5Hz)	C-11	133.2
		C-12	154.0
H-14	1.09 (*s*)	C-13	60.7
H-15	0.95 (*s*)	C-14	21.3
H-16	1.27 (*s*)	C-15	22.0
		C-16	30.9

metabolites produced by *Fusarium sporotrichioides* and *Fusarium sambucinum*[81,83] (see Fig.25). The chemical shifts for all the A ring carbons are almost the same as those determined for (75), indicating an identical A ring structure. In the B ring, the resonance at δ61.6 corresponds to C-13 which is hydroxylated, and those at δ127.2 and δ150.0 are due to C-2 and C-12 respectively. These values are similar to the shifts of the equivalent carbons in FS-2.

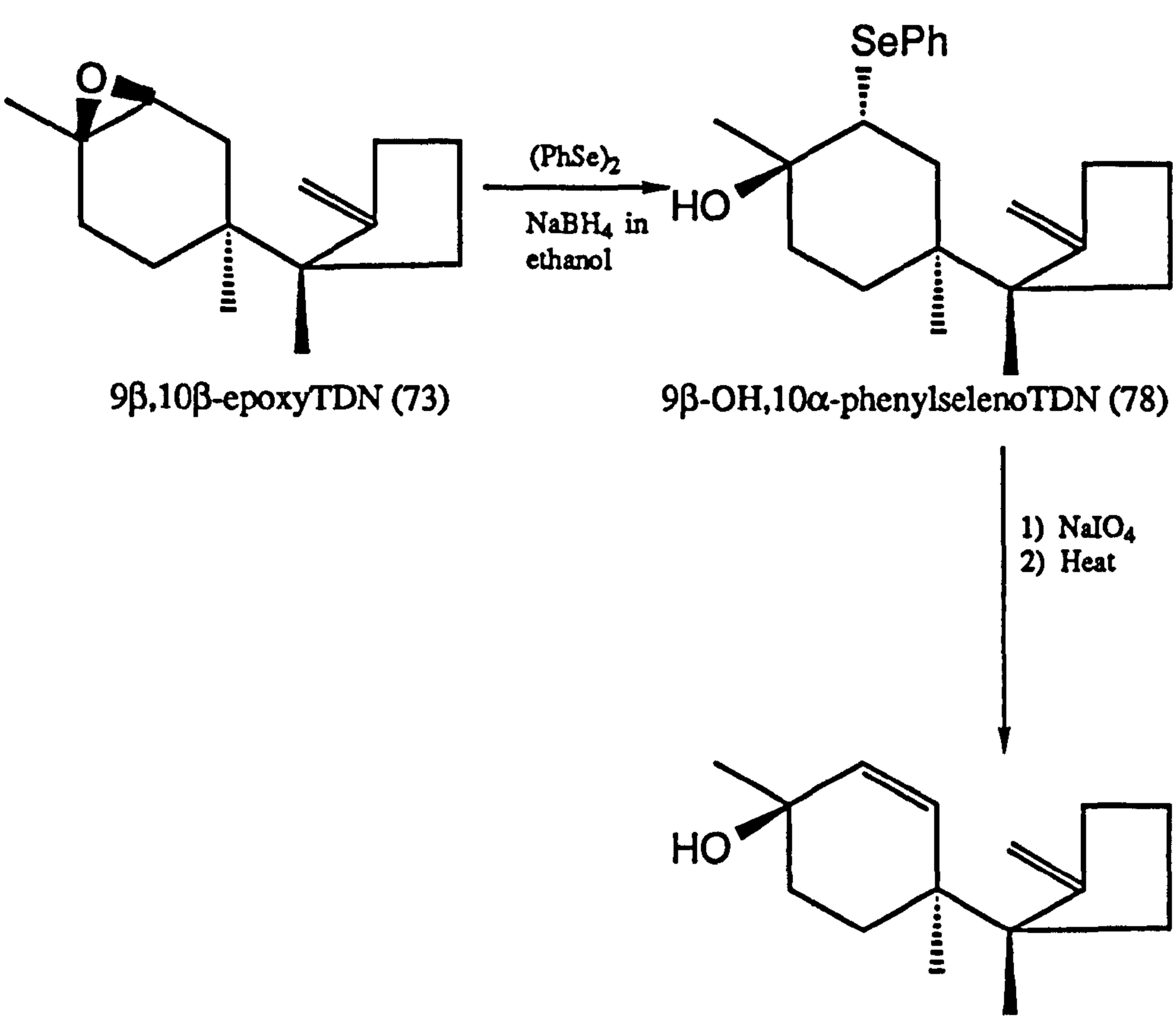
^{1}H NMR analysis [Table 13] provided confirmation of the product's structure. The C-16 methyl resonance at δ2.04, and H-10 at δ4.77 agree closely with the shifts observed in the ^{1}H NMR spectrum of (75) (see Table 10). The C-13 protons are an AB quartet at δ4.23 and δ4.48, and exhibit geminal coupling with J=14.4Hz. This is characteristic of the protons in a -CH_2OH group, as seen in the data for FS-2. H-2 appears at δ5.77 due to its position on an unsaturated carbon, and again this value is similar to that for the C-2 proton in FS-2.

The nature of the rearrangement of (76) to (77) gives rise to the suggestion that the natural metabolites FS-1, FS-2, FS-3 and FS-4 (see Fig.25) could be the products of a similar reaction. They may be derived from parent compounds possessing 12,13-epoxide groups which are unstable and rearrange during purification, or when in $CDCl_3$ solution for NMR analysis.

5. 9β-Hydroxytricho-10,12-diene (70).

The synthesis of 9β-hydroxytricho-10,12-diene from TDN, via the 9β,10β-epoxide (73), had previously been completed as part of a chemical degradation scheme[54] [Figs.39 and 40]. The sequence involves the production of the phenylselenide anion (79) which is an excellent nucleophile and reacts with (73) to produce the trans-hydroxyphenylselenide (78). This is oxidised to the phenylselenoxide (80)

Fig.39: Scheme for the synthesis of 9β-hydroxytricho-10,12-diene[54].

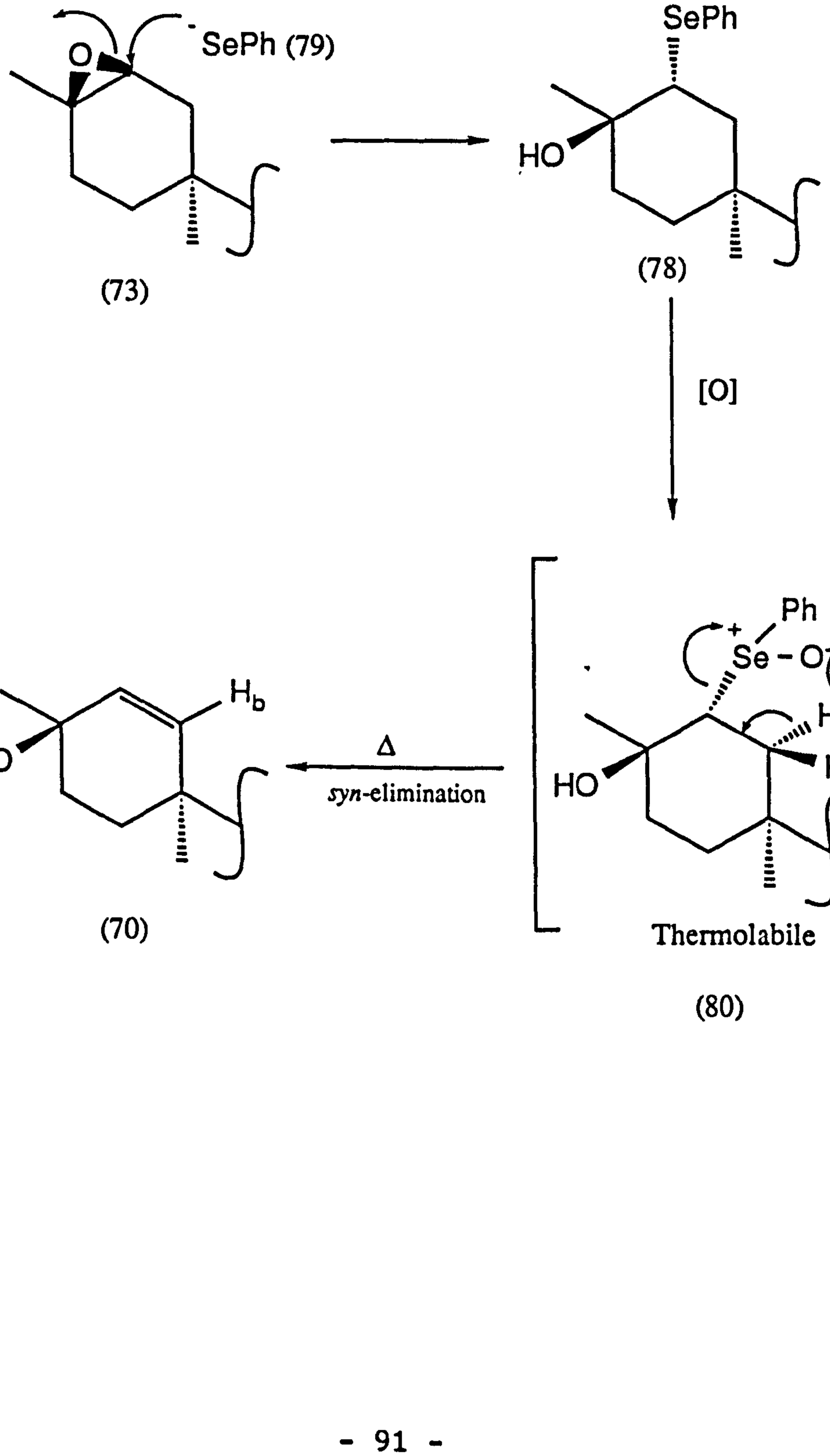


9β,10β-epoxyTDN (73)

9β-OH,10α-phenylselenoTDN (78)

9β-Hydroxytricho-10,12-diene (70)

Fig.40: Mechanisms involved in the synthesis of 9β-hydroxytricho-10,12-diene[104].

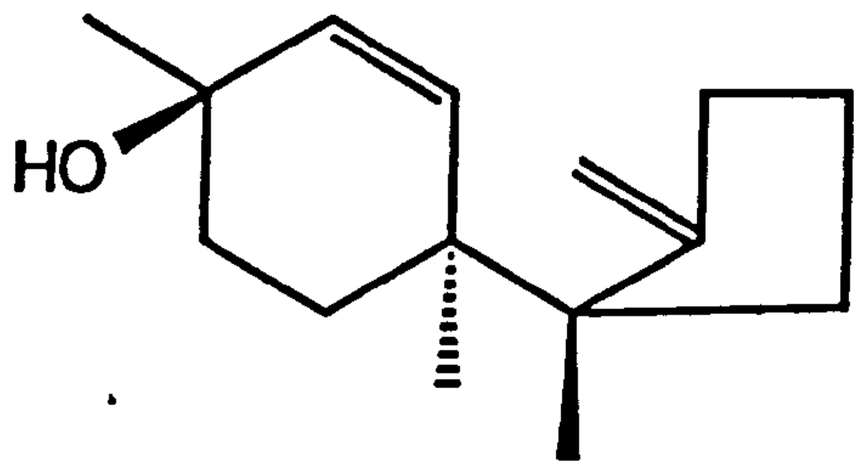


which is unstable , and decomposes to the allylic alcohol (70) on heating. Decomposition is known to occur away from the hydroxyl group and via *syn* elimination[104].

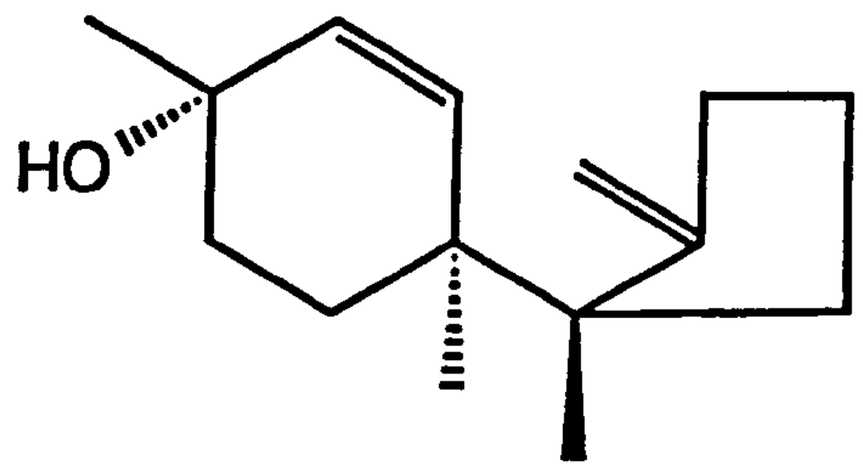
Epoxide (73) was treated with sodium phenylselenide, generated *in situ* by reaction of diphenyldiselenide with sodium borohydride. The reaction was heated and stirred in an inert atmosphere for 18 hours, and then stopped by addition of dilute acid. Work-up and subsequent purification yielded 9β-hydroxy,10α-phenylselenoTDN (78) (68%) as a viscous colourless oil which crystallised on standing and cooling. The ^{1}H NMR data were in agreement with literature values[54].

Compound (78) in tetrahydrofuran was oxidised by reaction at 0°C with a solution of sodium periodate (3 equivalents) in 70% aqueous methanol. After stirring at 0°C for 90 mins, the reaction mixture was heated in a sealed vessel at 75°C. TLC analysis showed that all the starting material had reacted after 3 hours heating, with the formation of three products. Work-up and purification yielded one major product (A:24%), accompanied by two more polar products (B:9%) and (C:5%) [Fig.41].

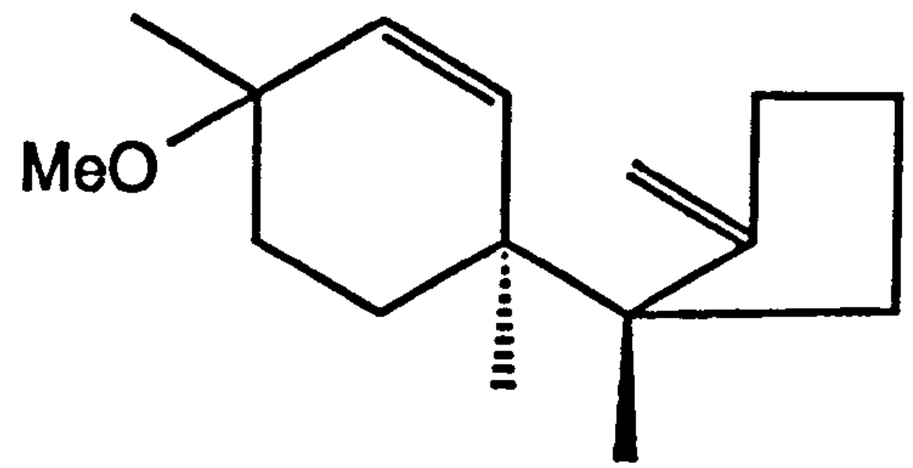
^{1}H and ^{13}C NMR data for compound B [Tables 10 and 11] were in close agreement with literature values for 9β-hydroxytricho-10,12-diene[54] (70). Partial assignment of the ^{1}H NMR spectrum [Fig.42] was achieved by comparison with that for TDN. The resonances for the B ring protons are similar, with those at C-2, C-3 and C-4 being in the unresolved region δ1.3-2.4. The C-13 protons remain non-equivalent and are at δ4.83 and δ4.99. In the A ring a number of differences are evident. The C-16 methyl resonance has shifted upfield from δ1.63 to appear as a singlet at δ1.27, indicating the loss of the C-9,10 double bond. It is still



B: 9β-Hydroxytricho-10,12-diene (70)



C: 9α-Hydroxytricho-10,12-diene (82)

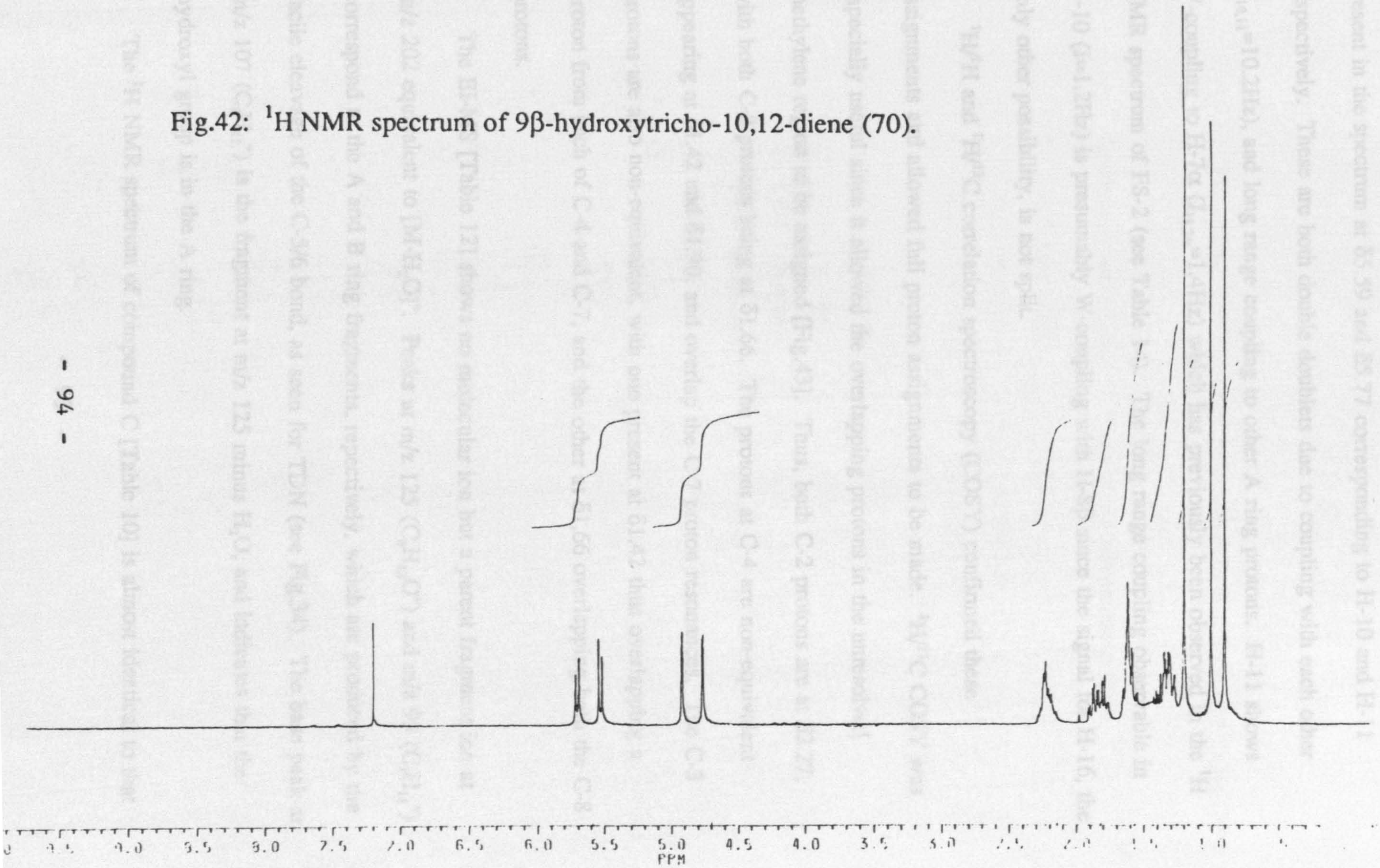


A: 9-methoxytricho-10,12-diene (81)

Mixture of β/α, 85/15.

Fig.41: Products obtained from the thermolysis of 9β-hydroxy,10α-phenylselenoTDN (78) at 75°C.

Fig.42: ^{1}H NMR spectrum of 9β-hydroxytricho-10,12-diene (70).



distinguishable from the methyl groups at C-14 and C-15 because of the deshielding effect of the hydroxyl group at C-9. Two vinylic protons are now present in the spectrum at δ5.59 and δ5.77 corresponding to H-10 and H-11 respectively. These are both double doublets due to coupling with each other ($J_{10,11}$=10.2Hz), and long range coupling to other A ring protons. H-11 shows W-coupling to H-7α ($J_{11,7\alpha}$=1.4Hz) which has previously been observed in the ^1H NMR spectrum of FS-2 (see Table 14). The long range coupling observable in H-10 (J=1.2Hz) is presumably W-coupling with H-8β since the signal for H-16, the only other possibility, is not split.

$^1H/^1H$ and $^1H/^{13}C$ correlation spectroscopy (COSY) confirmed these assignments and allowed full proton assignments to be made. $^1H/^{13}C$ COSY was especially useful since it allowed the overlapping protons in the unresolved methylene region to be assigned [Fig.43]. Thus, both C-2 protons are at δ2.27, with both C-8 protons being at δ1.66. The protons at C-4 are non-equivalent appearing at δ1.42 and δ1.90, and overlap the C-7 proton resonances. The C-3 protons are also non-equivalent, with one present at δ1.42 thus overlapping a proton from each of C-4 and C-7, and the other at δ1.66 overlapping both the C-8 protons.

The EI-MS [Table 12] shows no molecular ion but a parent fragment ion at m/z 202 equivalent to $[M-H_2O]^+$. Peaks at m/z 125 ($C_8H_{13}O^+$) and m/z 95 ($C_7H_{11}^+$) correspond to the A and B ring fragments, repectively, which are produced by the facile cleavage of the C-5/6 bond, as seen for TDN (see Fig.34). The base peak at m/z 107 ($C_8H_{11}^+$) is the fragment at m/z 125 minus H_2O, and indicates that the hydroxyl group is in the A ring.

The ^1H NMR spectrum of compound C [Table 10] is almost identical to that

Fig.43: Data from $^{1}H/^{13}C$ COSY of 9β-Hydroxytricho-10,12-diene (70).

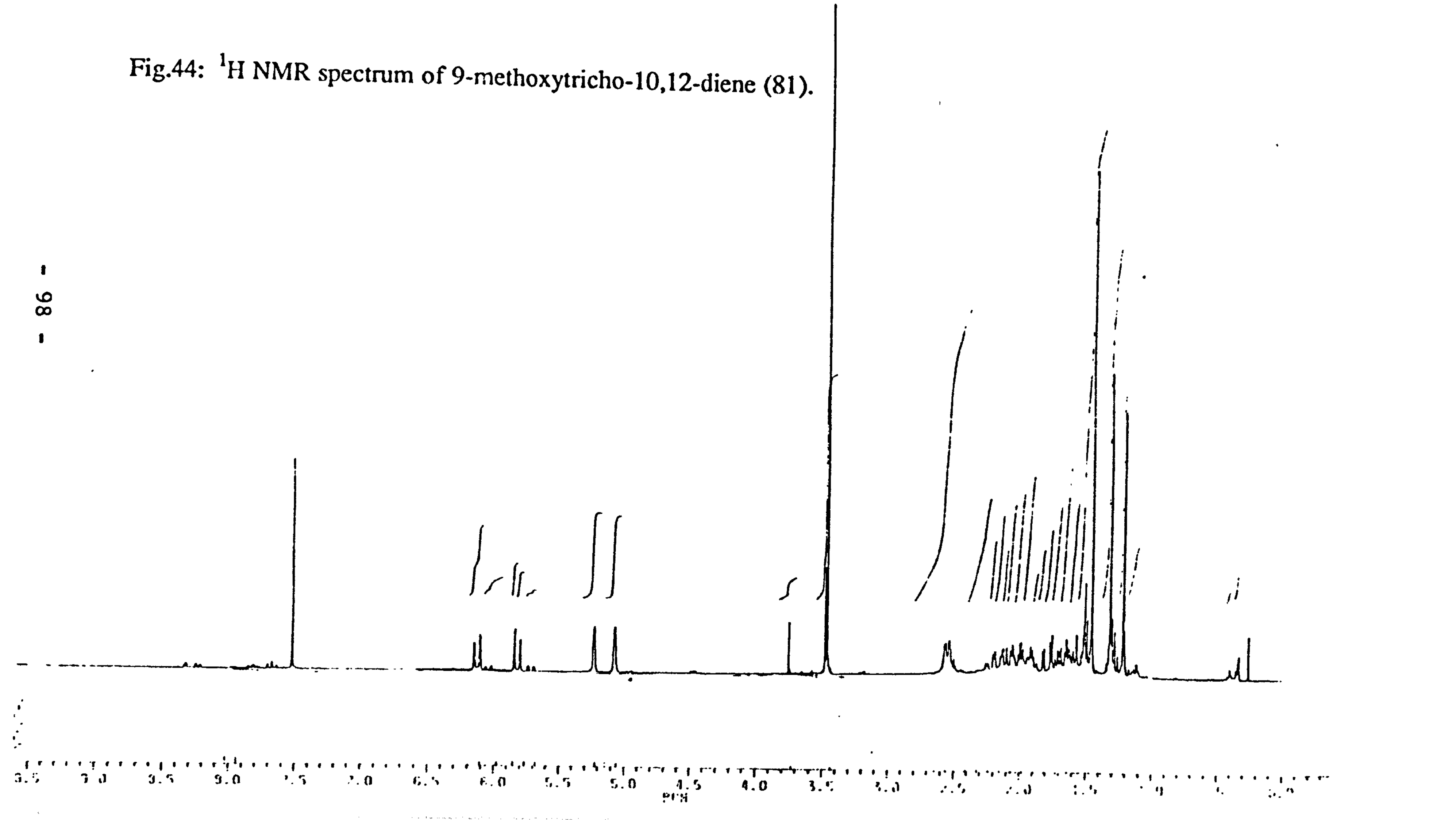
Carbon resonance (δppm)			Cross peaks with protons at δppm
C-2	38.6	(methylene)	2.27
C-3	23.2	(methylene)	1.42 and 1.66
C-4	37.2	(methylene)	1.42 and 1.90
C-7	26.2	(methylene)	1.40 and 1.92
C-8	34.1	(methylene)	1.66
C-10	131.7	(methine)	5.59
C-11	136.5	(methine)	5.77
C-13	106.6	(ethylene)	4.83 and 4.99
C-14	24.3	(methyl)	1.07
C-15	20.2	(methyl)	0.97
C-16	29.7	(methyl)	1.27

for the 9β-alcohol (70). The only differences are the slight upfield shifts of the resonances due to H-10 and H-11, and a change in the separation of the C-14 and C-15 methyl signals. The EI-MS data for the two compounds B and C are in exact agreement, whilst the ^{13}C NMR data [Table 11] show small chemical shift differences (1.6-3.2ppm) for the resonances corresponding to C-7, C-8, C-9, C-10, C-11 and C-16. This is consistent with the compounds being identical apart from the stereochemistry at C-9. Compound C was therefore identified as 9α-hydroxytricho-10,12-diene (82).

Compound A was the major product in the reaction and was identified by comparison of its ^1H NMR spectrum [Table 10] with that for (70). The most striking difference is the resonance at δ3.20, which integration shows to be equivalent to three protons, and is indicative of a methoxy group. Slight differences in the chemical shifts for H-10 and H-11, together with the upfield shift of the C-16 methyl resonance from δ1.27 to δ1.19 indicates that the methoxy group is at C-9. This is confirmed by the EI-MS [Table 12] which shows fragments at m/z 219 $[M-CH_3]^+$ and 202 $[M-CH_3OH]^+$. Peaks at m/z 139 ($C_9H_{13}O^+$) and m/z 95 ($C_7H_{11}^+$) correspond to the A and B ring fragments produced by cleavage of the C-5/6 bond. The base peak at m/z 107 ($C_8H_{11}^+$) is the fragment at m/z 139 minus CH_3OH, and indicates that the methoxy group is in the A ring. Compound A is therefore 9-methoxytricho-10,12-diene (81).

Closer examination of the ^1H NMR spectrum obtained for compound A [Fig.44] indicated that it was in fact a mixture of two compounds. Minor signals are visible for H-10 at δ5.52 (dd, J=10.4, 1.7Hz), H-11 at δ5.83 (dd, J=10.4, 1.7Hz) and the methoxy protons at δ3.22. "Compound A" is therefore a mixture of the two stereoisomers. Comparison of the chemical shifts for H-10 and H-11 with

Fig.44: ^{1}H NMR spectrum of 9-methoxytricho-10,12-diene (81).



the corresponding protons in both the 9α- and 9β-hydroxy compounds (82) and (70) (see Table 10) suggests that the stereochemistry of the major isomer is 9β, with the minor product being 9α-methoxy. Further study showed that the two compounds could be separated by TLC. However, attempts to confirm the stereochemistries by DNOES were unsuccessful. Irradiation of the methoxy protons and the C-16 methyl protons failed to produce observable NOEs on the C-15 methyl protons in either stereoisomer. The ratio of 9β/9α was determined to be approximately 85/15 by measurement of intensities of the methoxy resonances at δ3.20 and δ3.22.

Interpretation of the results.

According to the mechanisms for the reactions involved in the synthesis of 9β-hydroxytricho-10,12-diene (see Fig.40), it is not possible to produce the 9α-hydroxy isomer (82) from 9β,10β-epoxyTDN (73). The isolation of (82) from the reaction mixture was therefore unexpected. One explanation would be that 9α,10α-epoxyTDN was present in the starting material, and (82) was produced from it by a parallel series of reactions via the 9α-hydroxy,10β-phenylselenide. However, GC and ^{1}H NMR analysis of (73) provided no evidence for contamination with the 9α-epoxide.

The identification of 9-methoxytricho-10,12-diene, in both the 9α and 9β stereoisomeric forms, led to a more satisfactory hypothesis to explain these observations [Fig.45]. This assumes that the 9β-alcohol (70) is indeed the only product in the reaction sequence from (73). However, under the reaction conditions employed, it subsequently forms a carbocation (83) by the acid catalysed loss of the 9β-hydroxyl. Nucleophilic attack by methanol then leads to the

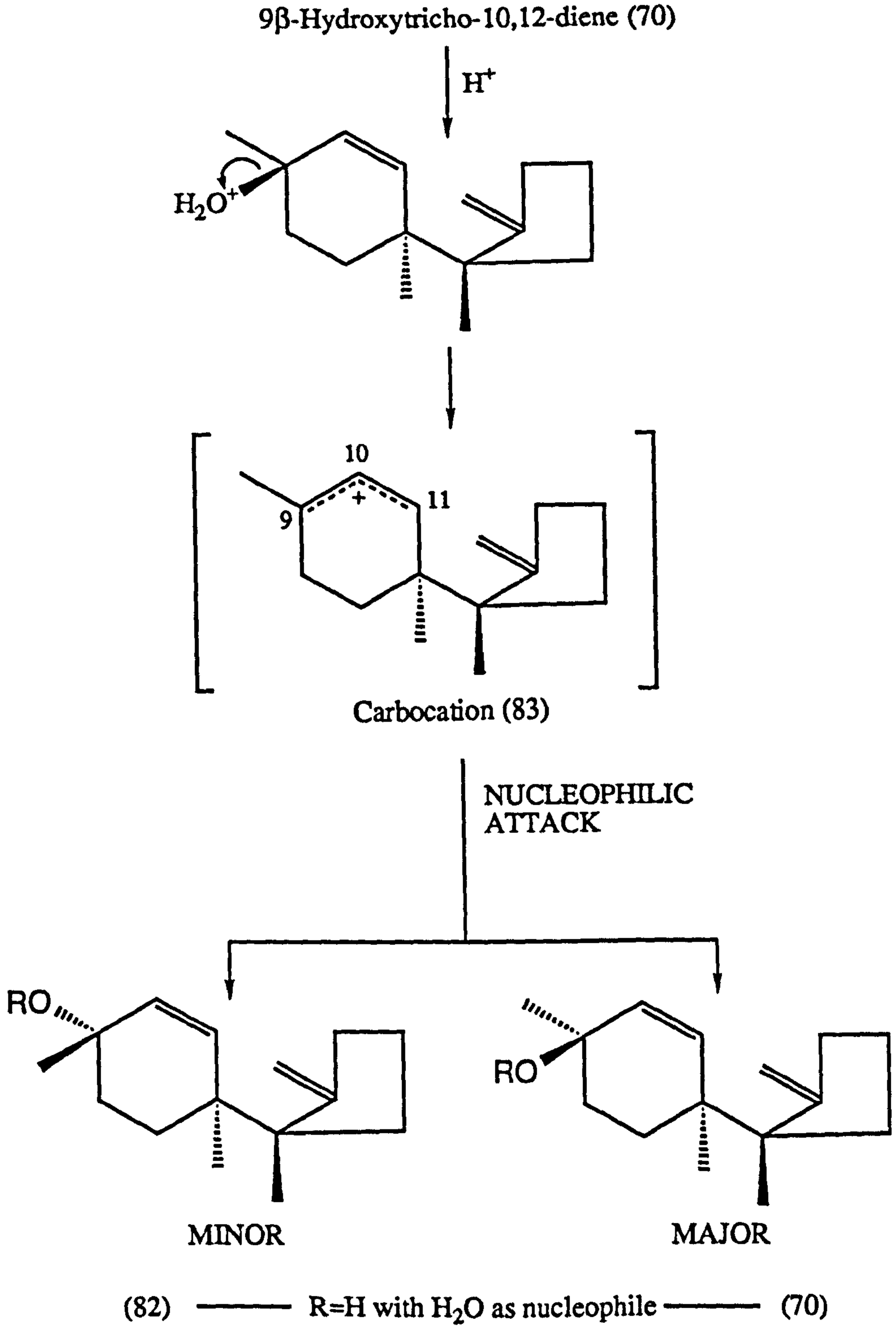


Fig.45: Proposed formation of 9α-hydroxy, and 9α- and 9β-methoxy-tricho-10,12-dienes from 9β-hydroxytricho-10,12-diene (70).

formation of both the 9α- and 9β-methoxy derivatives (81A) and (81B). The 9β isomer is the major product since access to the carbocation from the β-face is less restricted than from the α-face. Similarly, if the attacking nucleophile is water, (70) will be reformed as the major product, whilst the minor product is the 9α-hydroxy isomer (82). Nucleophilic attack at C-11 of the carbocation intermediate forming the 11-hydroxy or 11-methoxy derivatives is theoretically possible [Fig.46]. However, the 9-substituted compounds are the kinetically favoured products since formation of these compounds involves the intermediacy of the more stable tertiary carbocation (83A). In addition, the presence of the bulky adjacent B ring may also prevent nucleophilic attack at C-11 so that only derivatives formed by attack at C-9 are produced, with formation of any 11-substituted compounds in the reaction not occurring.

According to this hypothesis it should therefore be possible to produce these compounds from pure 9β-hydroxytricho-10,12-diene using the appropriate conditions. The 9β-alcohol (70) was dissolved in a 1:1 mixture of tetrahydrofuran and 70% aqueous methanol, and sufficient 1N HCl was added to produce a pH of 3-4. The mixture was heated to 75°C and the reaction monitored using TLC analysis with reference to appropriate standards. After 90 mins over 50% of the starting material had been converted to 9-methoxytricho-10,12-diene. Further TLC analysis revealed this to be a mixture of both the 9β and 9α stereoisomers, present in a ratio of approximately 4:1, respectively. In a similar reaction where no methanol was present, TLC analysis indicated the formation of the 9α-alcohol after 90 mins. The progress of the reaction was monitored for 18 hours, but the 9β-hydroxy isomer was always the major compound present. No 9-methoxytricho-10,12-diene was detected.

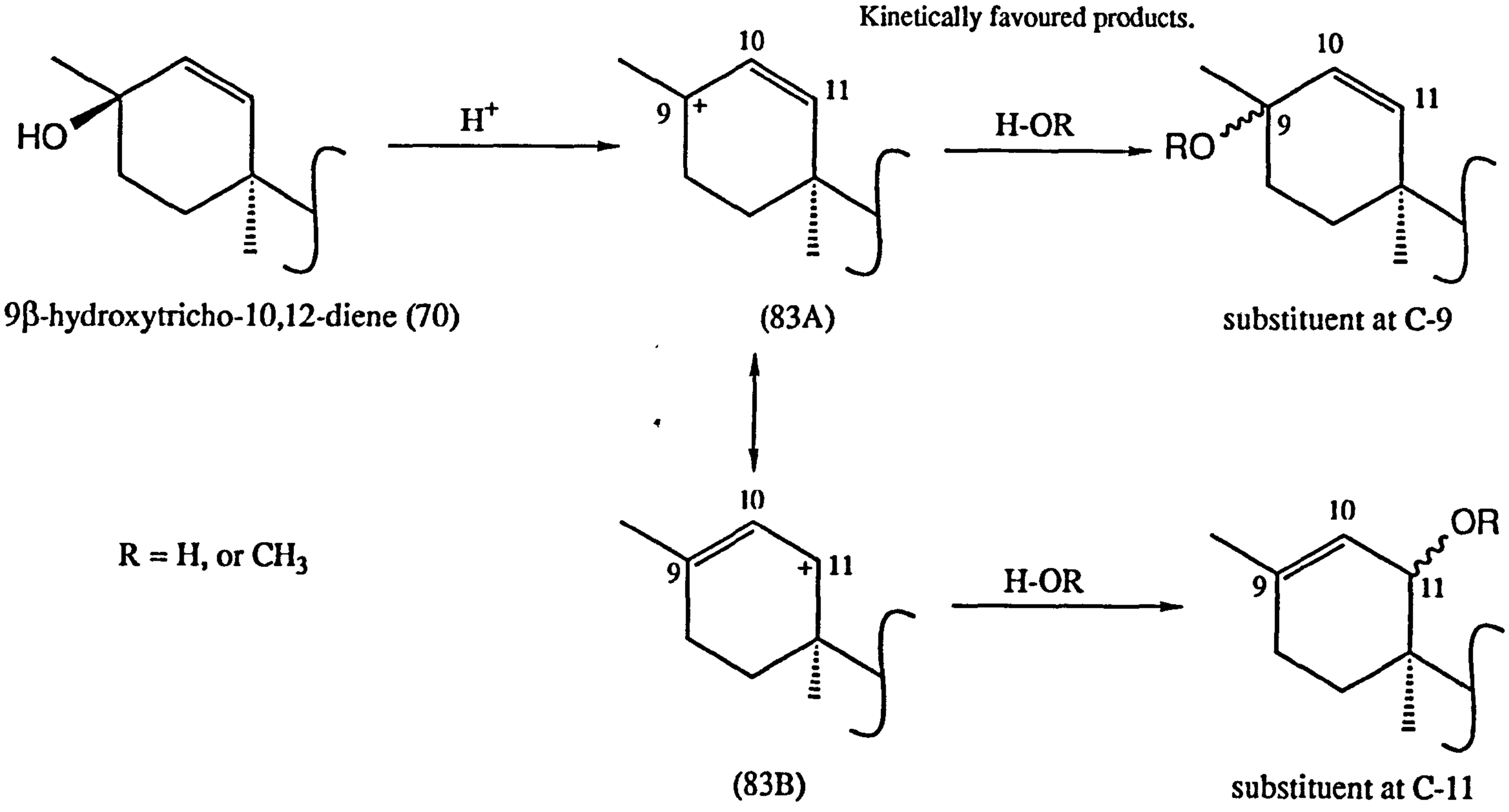


Fig.46: Possible product formation in the reaction of 9β-hydroxytricho-10,12-diene at low pH.

These results therefore support the involvement of a carbocation which is produced from the 9β-hydroxide by heating under acid catalysed conditions.

Revised synthesis of 9β-hydroxytricho-10,12-diene (70).

In the only previously reported synthesis of (70)[54], the formation of these additional compounds was not observed, and the desired product was obtained in 53% yield. The only difference between the two procedures is the temperature at which the phenylselenoxide (80) was heated to cause decomposition. In the literature the reaction mixture was refluxed (60°C), whilst in this laboratory, because of the small scale employed, it was heated in a Reactivial at a slightly higher temperature (75°C). This suggests that carbocation formation is favoured at the higher temperature used. Consequently, the sodium periodate oxidation of 9β-hydroxy,10α-phenylselenoTDN to the phenylselenoxide was repeated, and the final heating was carried out at 60°C, with methanol present as solvent. Work-up yielded the 9β-alcohol (70) (48%) as colourless waxy crystals. No other products were detected.

Synthesis of 9α-hydroxytrich-10,12-diene (82).

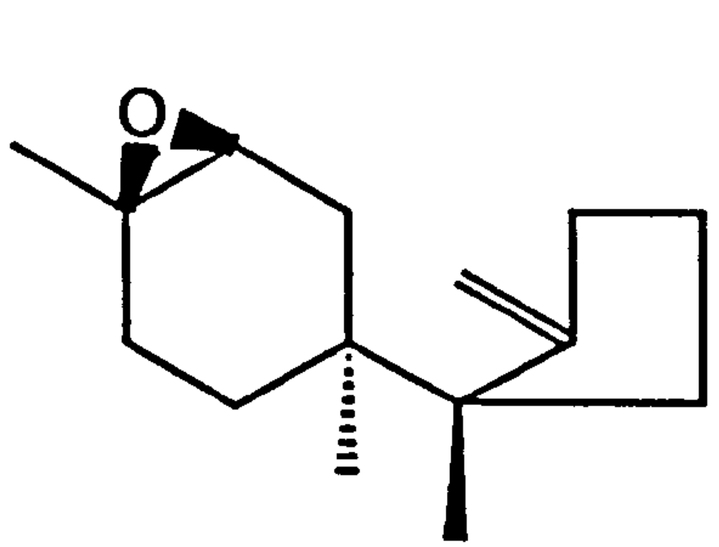
According to the proposed mechanism for the formation of (82) (see Fig.45), the best method for synthesising this compound from the 9β-hydroxyphenylselenide (78) is by heating at 75°C in the absence of any methanol. Work-up and purification yielded (82) (11%) as white crystals. The 9β-alcohol (70) (63%) was also isolated, but the 9-methoxy derivatives were, as expected, not detected.

Inhibition studies.

With a range of semi-synthetic TDN derivatives available [Fig.47] it was necessary to screen them for activity as inhibitors of post-TDN biosynthesis. This was assessed by means of a competition feeding experiment.

A whole-cell system of *Fusarium culmorum* was prepared by filtering a culture of the fungus (30ml; 42hrs old), washing the filtered mycelia thoroughly with water, and then resuspending it in distilled water (6ml) in a conical flask (25ml). A large excess of unlabelled derivative (5mg in 100µl acetone) was fed to the system followed by addition of [^{14}C]TDN (0.5mg in 50µl acetone) 30 mins later. After 6hrs incubation (27°C, 250rpm) the cultures were worked up by filtration, and ethyl acetate extraction. The effect of the analogue on the incorporation of [^{14}C]TDN was observed by separation of the extract using TLC with subsequent visualisation by autoradiography. Any biotransfomations of the analogues were detected by TLC analysis using sulphuric acid and PNBP/TEPA as spray reagents. Feeding experiments to mycelia which had been boiled were used to demonstrate that such transformations were enzymic and not merely chemical. A control in which no analogue was present was used to observe the uninhibited incorporation of [^{14}C]TDN into trichothecene toxins. The inhibitory effects of the analogues were compared to that produced in the presence of unlabelled TDN.

Fig.48 shows the autoradiogram obtained from the competitive feeding experiments with 9β,10β;12,13-diepoxyTDN and 9β,10β-epoxyTDN. Lane 2 demonstrates the uninhibited incorporation of [^{14}C]TDN into a range of trichothecene toxins including 3-AcDON, DHC, CAL, and ITD. By comparing this "normal" radiolabelled toxin profile to those obtained in the presence of the two



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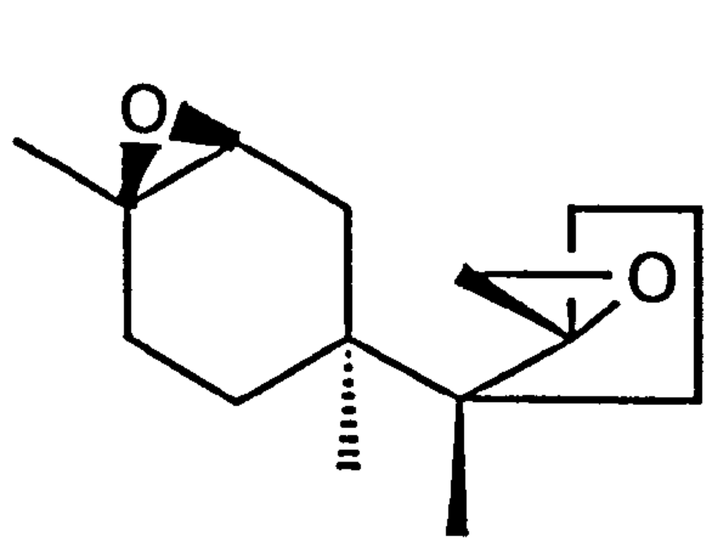

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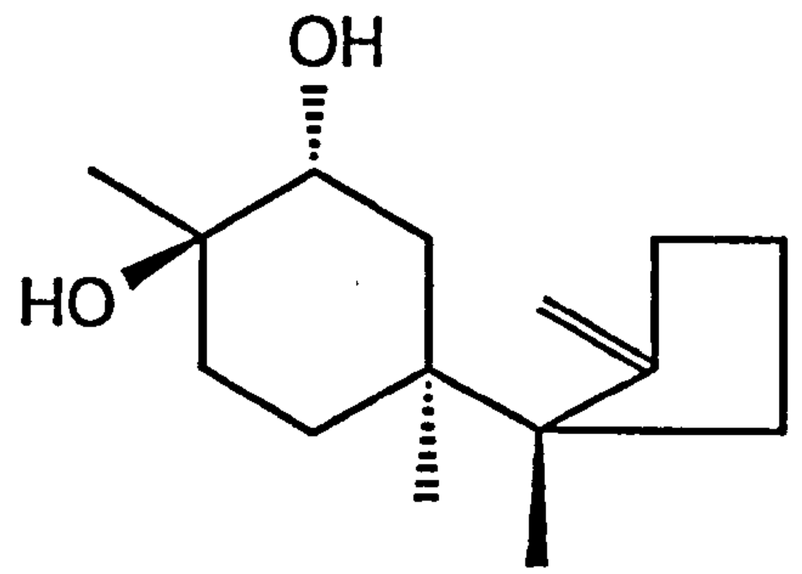

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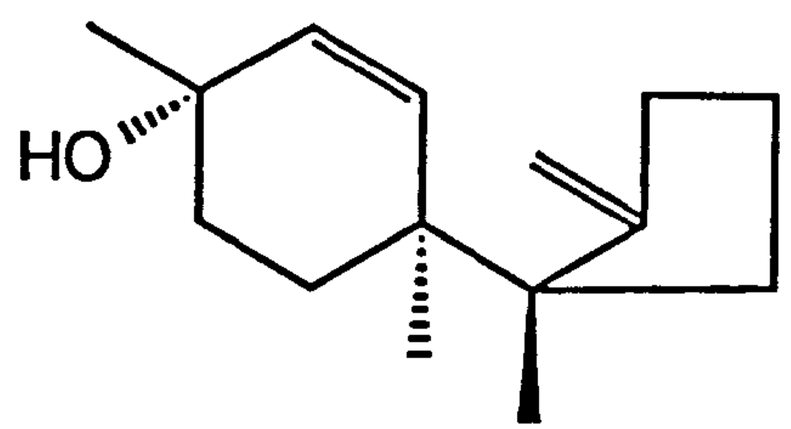

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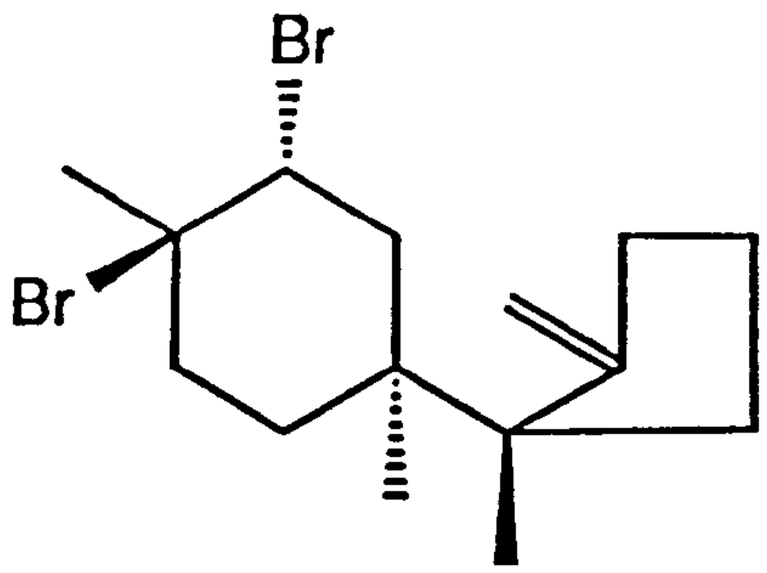

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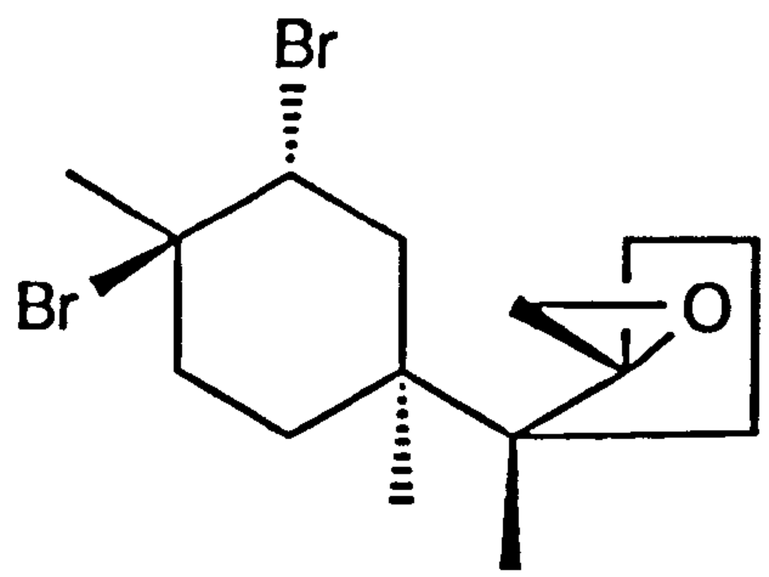
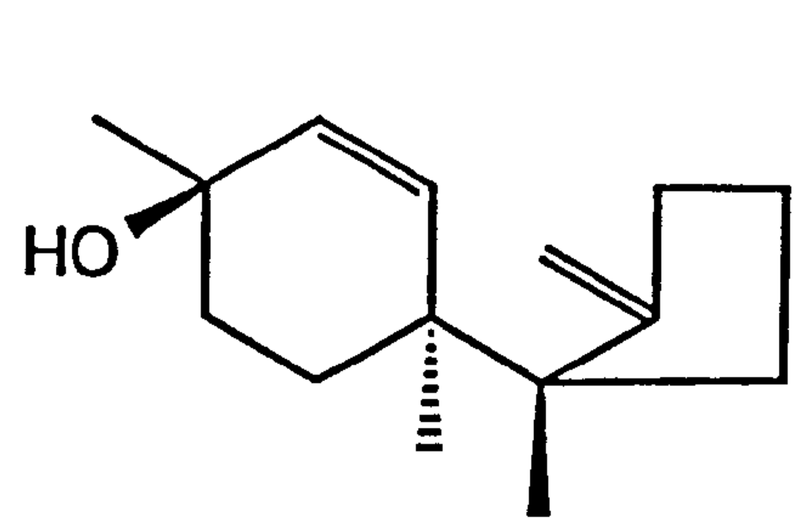


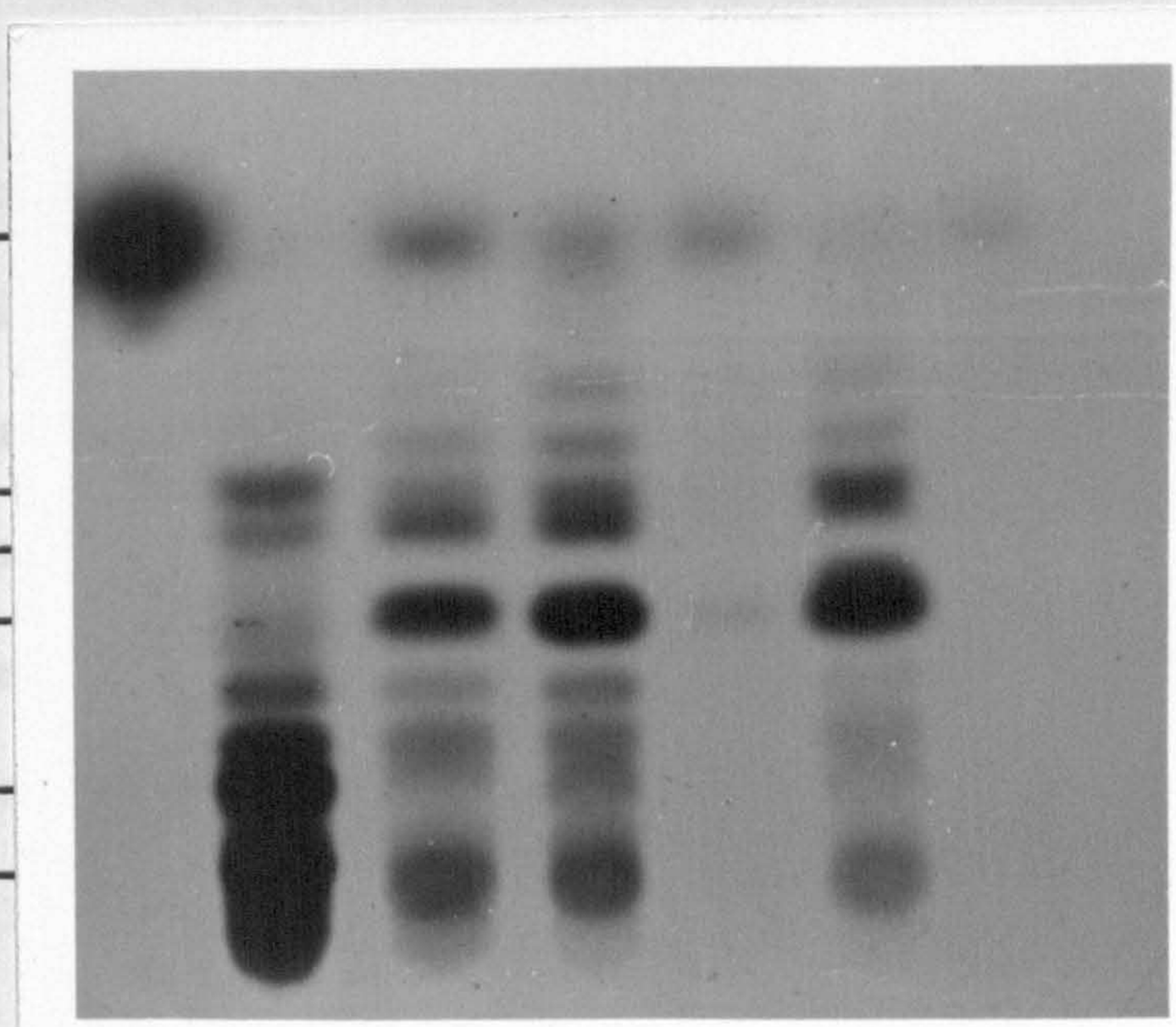
(76)



(70)

Fig.47: TDN analogues screened for activity as inhibitors of post-TDN biosynthesis.

Fig.48: Incorporation of [^{14}C]TDN into the trichothecene toxins of *Fusarium culmorum* in the presence of epoxide derivatives of TDN.



Lane 1: [^{14}C]TDN standard
Lane 2: Fed [^{14}C]TDN (0.5mg)
Lane 3: Fed [^{14}C]TDN (0.5mg) + unlabelled TDN (5mg)
Lane 4: Fed [^{14}C]TDN (0.5mg) + 9β,10β-epoxyTDN (5mg)
Lane 5: Fed [^{14}C]TDN (0.5mg) + 9β,10β;12,13-diepoxyTDN (5mg)

6hr incubation
TLC: Hexane-EtOAc, 1:1
Autoradiogram: 48hr exposure

epoxide analogues (lanes 4 and 5), it is obvious that inhibition has occurred. The intensities of the major trichothecene products 3-AcDON and DHC have been greatly reduced, and a highly active metabolite (X), only present in traces in the "normal" profile, has accumulated. The same effects were observed when unlabelled TDN was used (lane 3) which suggests that X could be a precursor to 3-AcDON and DHC. On additional TLC analysis, X produced a blue colour on reaction with the PNBP/TEPA reagent indicating that it is an epoxide derivative. This analysis also demonstrated that both the epoxide substrates had been biotransformed into at least two major epoxide metabolites.

A series of competitive feeding experiments were then performed to assess the effects of the other TDN analogues on post-TDN biosynthesis. All of the results are summarised in Table 15. All the analogues tested initiated the production of labelled metabolite X, and caused a reduction in the incorporation of [^{14}C]TDN into the major trichothecene toxins. The level of inhibition from 9β-hydroxytricho-10,12-diene (70) is comparable to that caused by TDN and both epoxide derivatives, whilst the dibromide (75) appears to have less of an inhibitory effect. The bromoepoxide (76) and the 9,10-diol (74) are the most active compounds tested since they almost completely inhibit the incorporation of [^{14}C]TDN into 3-AcDON and DHC. In addition, the 9,10-diol is not significantly biotransformed by the fungus suggesting that it may be a non-competitive enzyme inhibitor. Of the other analogues used all apart from the 9,10-dibromide were biotransformed, although the bromoepoxide showed some chemical breakdown in the boiled control and so its observed transformations may not be totally enzymic.

The isolation and identification of the new TDN metabolite (X) was now a priority. In order to obtain a sample of this compound for analysis a large scale

INHIBITOR	INCORPORATION OF [^{14}C]TDN INTO:-			BIOTRANSFORMATION OF INHIBITOR
	METABOLITE (X)	3-AcDON	DHC	
NONE	NO	HIGH	HIGH	----------
TDN (36)	YES	LOW	LOW	YES. TRICHOTHECENE PRODUCTS
9β,10β-EpoxyTDN (73)	YES	LOW	LOW	YES. 2 MAJOR EPOXIDE PRODUCTS
9β,10β;12,13-DiepoxyTDN (72)	YES	LOW	LOW	YES. 1 MAJOR EPOXIDE PRODUCT
9,10-DibromoTDN (75)	YES	MEDIUM	MEDIUM	NO.
9,10-Dibromo-12,13-epoxyTDN (76)	YES	NIL	NIL	YES. 1 MAJOR PRODUCT
9β,10α-DihydroxyTDN (74)	YES	NIL	NIL	NO.
9β-Hydroxytricho-10,12-diene (70)	YES	LOW	LOW	YES. SEVERAL PRODUCTS
9α-Hydroxytricho-10,12-diene (82)	*	*	*	YES. SEVERAL PRODUCTS

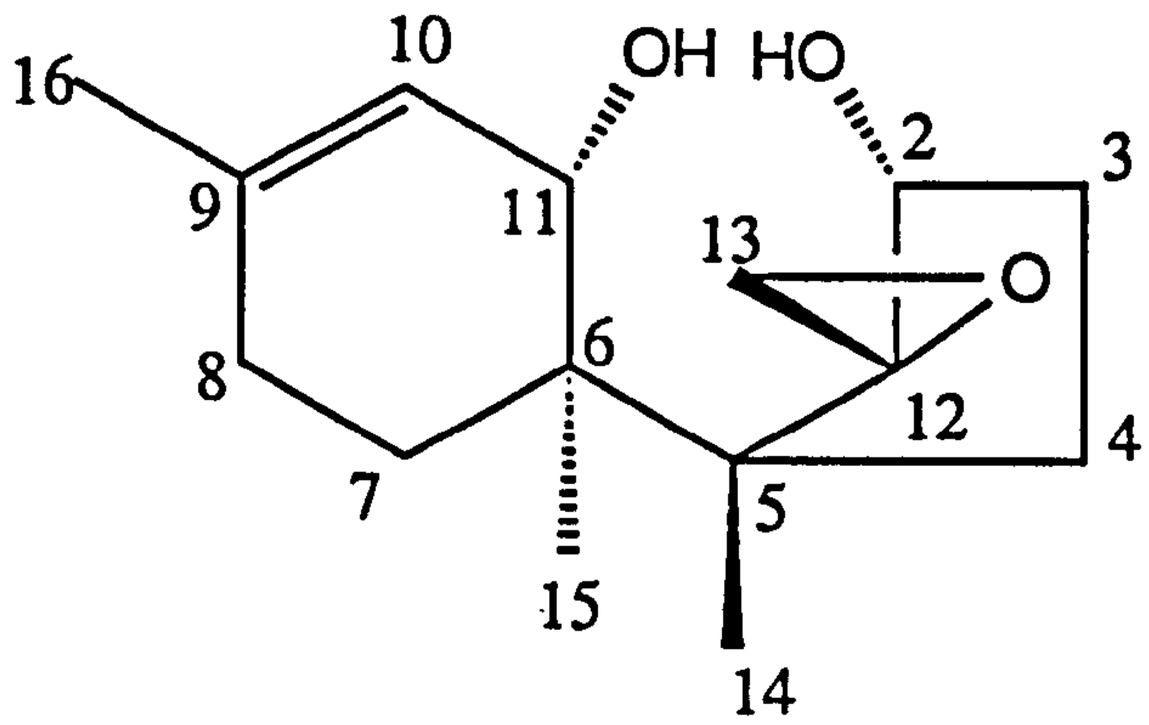
* = NO INFORMATION AVAILABLE.

Table 15: Summary of the results of competitive feeding experiments used to screen TDN analogues for activity as inhibitors of post-TDN biosynthesis.

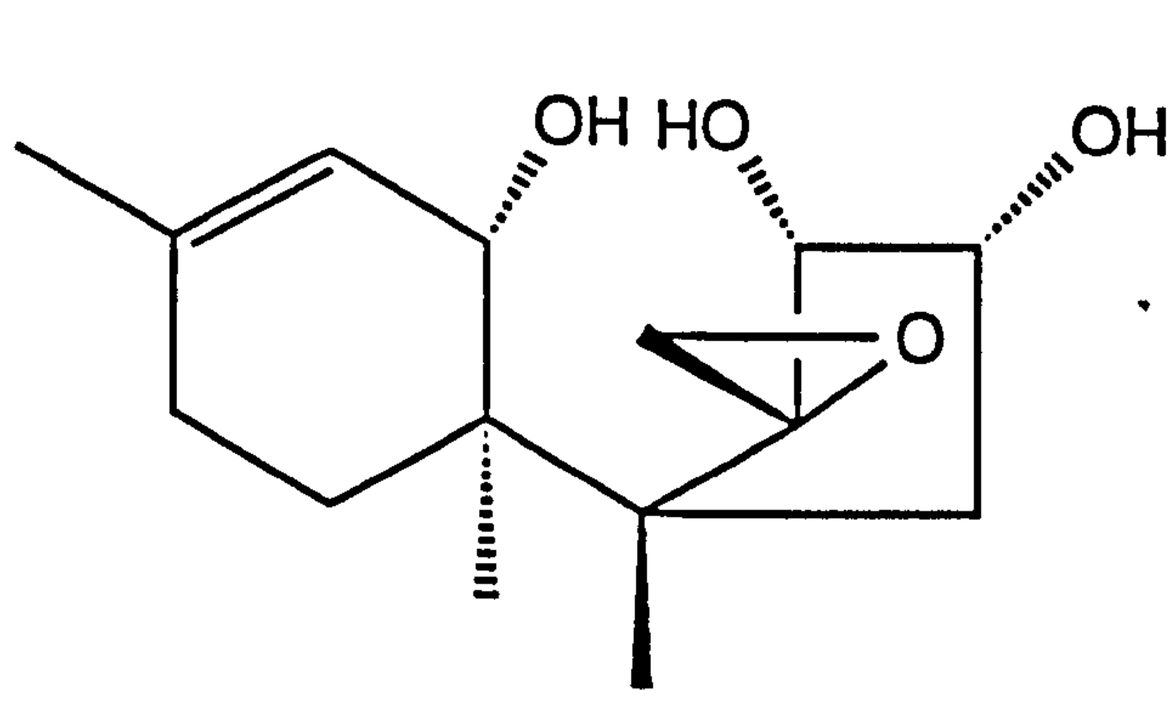
feeding experiment was undertaken. This represented a scaled-up version of the competitive inhibition of [^{14}C]TDN incorporation by TDN, except no radiolabelled precursor was employed.

The mycelia from 0.6L culture of *F. culmorum* (42hrs old) was collected by filtration, washed and then resuspended in distilled water (160ml) in a 1L Erlenmeyer flask. TDN (156mg) as a solution in acetone (0.6ml) was added and the culture incubated for 30 mins (27°C, 250rpm). A further portion of TDN (44mg in 0.2ml acetone) was added and the incubation continued for 5.5hrs. The culture was then harvested by filtration and extraction with ethyl acetate. The combined organic extracts were dried over magnesium sulphate, and evaporated to an oil (120mg). TLC analysis (hexane-ethyl acetate, 1:1) indicated the presence of the metabolite (Rf=0.35), which produced a bright sky blue colour on reaction with PNBP/TEPA reagent. This colour has been reported to be characteristic of uncyclised trichothecenes such as trichodiol (37) and trichotriol (48)[61]. The oil was chromatographed by CTLC (1mm plate) using hexane-ethyl acetate, 1:1, and collecting 1ml fractions. The TDN metabolite (11.8mg) was obtained as white crystals (mp. 138-140°C) from fractions 33-36. It was identified as 12,13-epoxy-2α,11α-dihydroxyTDN (84) by a combination of MS, ^{1}H NMR and ^{13}C NMR methods. Identification was aided by comparison with the spectral data reported for tricho-9-ene-2α,3α,11α-triol (85)[61].

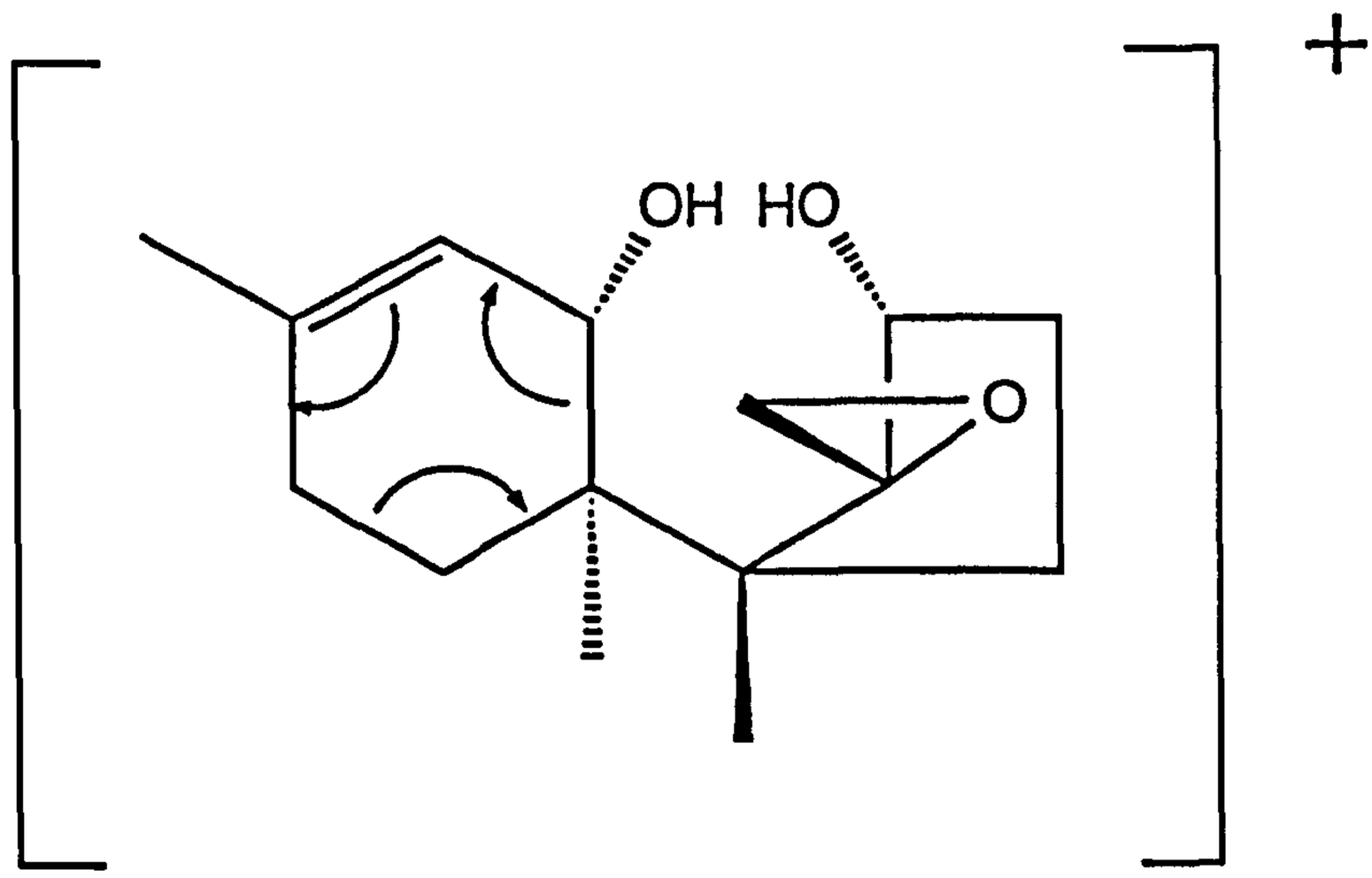
EI-MS showed a molecular ion at m/z 252.173 consistent with a molecular formula of $C_{15}H_{24}O_3$ (requires 252.1741), and four double bond equivalents as expected for an uncyclised trichothecene skeleton. The base peak in the spectrum at m/z 84 (C_5H_8O) is consistent with a retro Diels-Alder (RDA) fragmentation in ring A as seen for (85)[61] and indicates monohydroxylation of this ring [Fig.49].



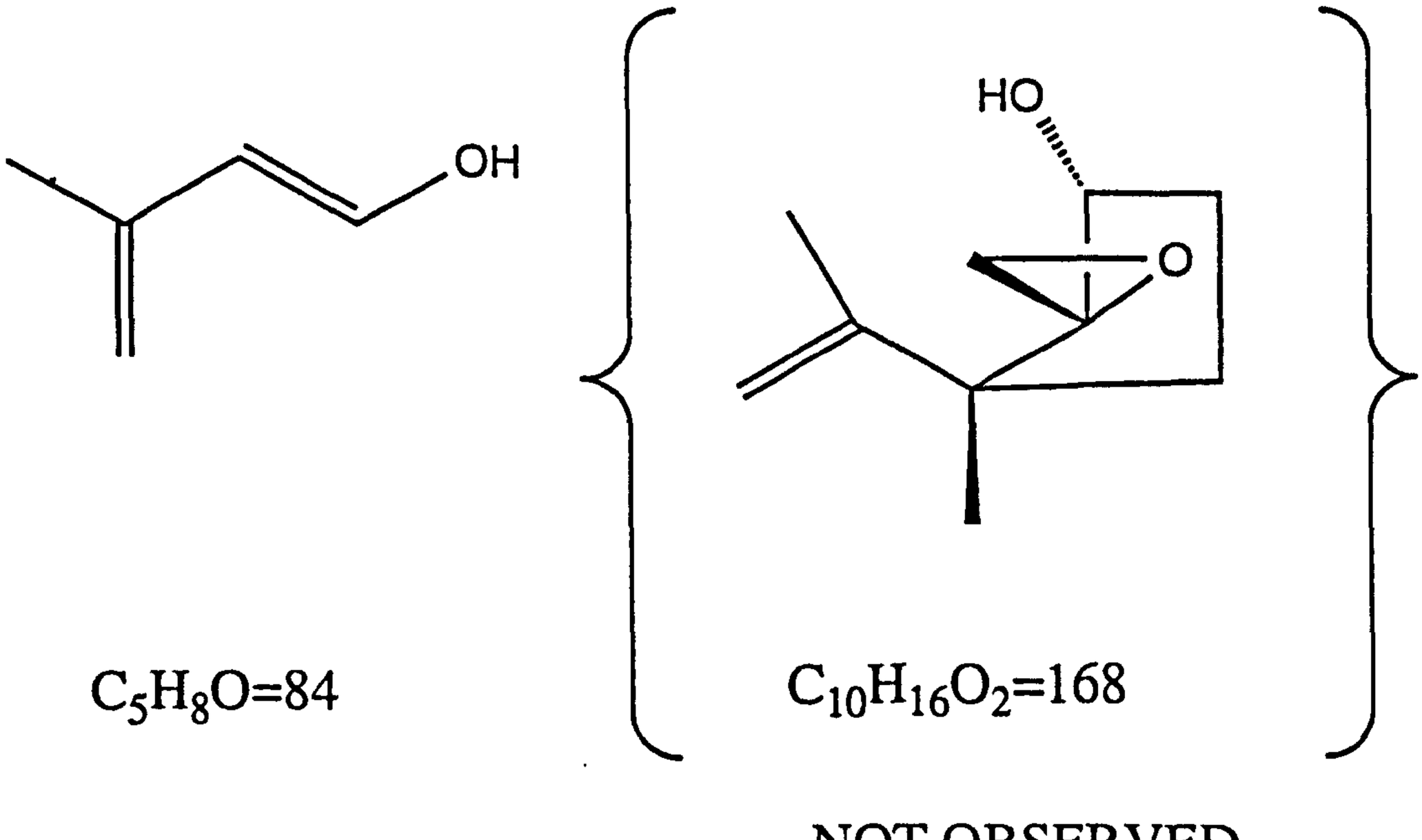
12,13-Epoxy-2α,11α-dihydroxyTDN (84)



Tricho-9-ene-2α,3α,11α-triol (85)



12,13-Epoxy-2α,11α-dihydroxyTDN



C_5H_8O=84

$C_{10}H_{16}O_2$=168

NOT OBSERVED

Fig.49: Retro Diels-Alder fragmentation of 12,13-epoxy-2α,11α-dihydroxyTDN.

Trimethylsilylation was used to determine the total number of hydroxyl groups present. GC-MS of the product after reaction with bis(trimethylsilyl)-trifluoroacetamide (BSTFA) indicated the formation of two products, a di-TMS ether (86) ($[M-CH_3]^+$ at m/z 381) and a mono-TMS ether (87) ($[M]^+$ at m/z 324), thus demonstrating that two hydroxyl groups are in fact present. The EI-MS for the di-TMS ether shows that the RDA fragment ion is shifted from m/z 84 to m/z 156 as expected, whilst in the mono-TMS ether the peak at m/z 156 is insignificant and m/z 84 is retained. This indicates that the second hydroxyl group is in the B ring, and that it is more readily alkylated than the ring A hydroxyl [Fig.50].

The ^1H NMR spectrum [Fig.51, Table 16] indicates the presence of a 12,13-epoxide (trichothecene numbering) by the characteristic AB quartet of signals centred at δ3.19 (J_{AB}=4.2Hz) which correspond to the C-13 methylene protons associated with the epoxide group. Comparison with the ^1H NMR data for (85)[61] allowed the resonances at δ5.14 and δ4.60 to be assigned to H-10 and H-11 respectively, locating the A ring hydroxyl at C-11. The stereochemistry at this position was defined from the size of the coupling constants for H-10 and H-11. Both signals are broad singlets i.e. $J_{10,11}$≈0Hz. This indicates that the dihedral angle between these two protons is about 90° and molecular models predict that this is only possible when H-11 is in the β position, i.e. an 11α-hydroxyl. This contrasts with the trichothecenes where H-11 is α and the C-11 oxygen substituent β, making $J_{10,11}$≈5.5Hz.

The B ring hydroxyl was readily assigned to position 2α from the multiplicity of the resonance at δ3.60. This is a doublet (J=4.1Hz) and can therefore be assigned as H-2β since it is very similar to the signal found in EPT (3) (δ3.72, d,J=4.6Hz) (see Table 18) indicating similar stereochemistries. $J_{2\beta,3\alpha}$≈0Hz in both

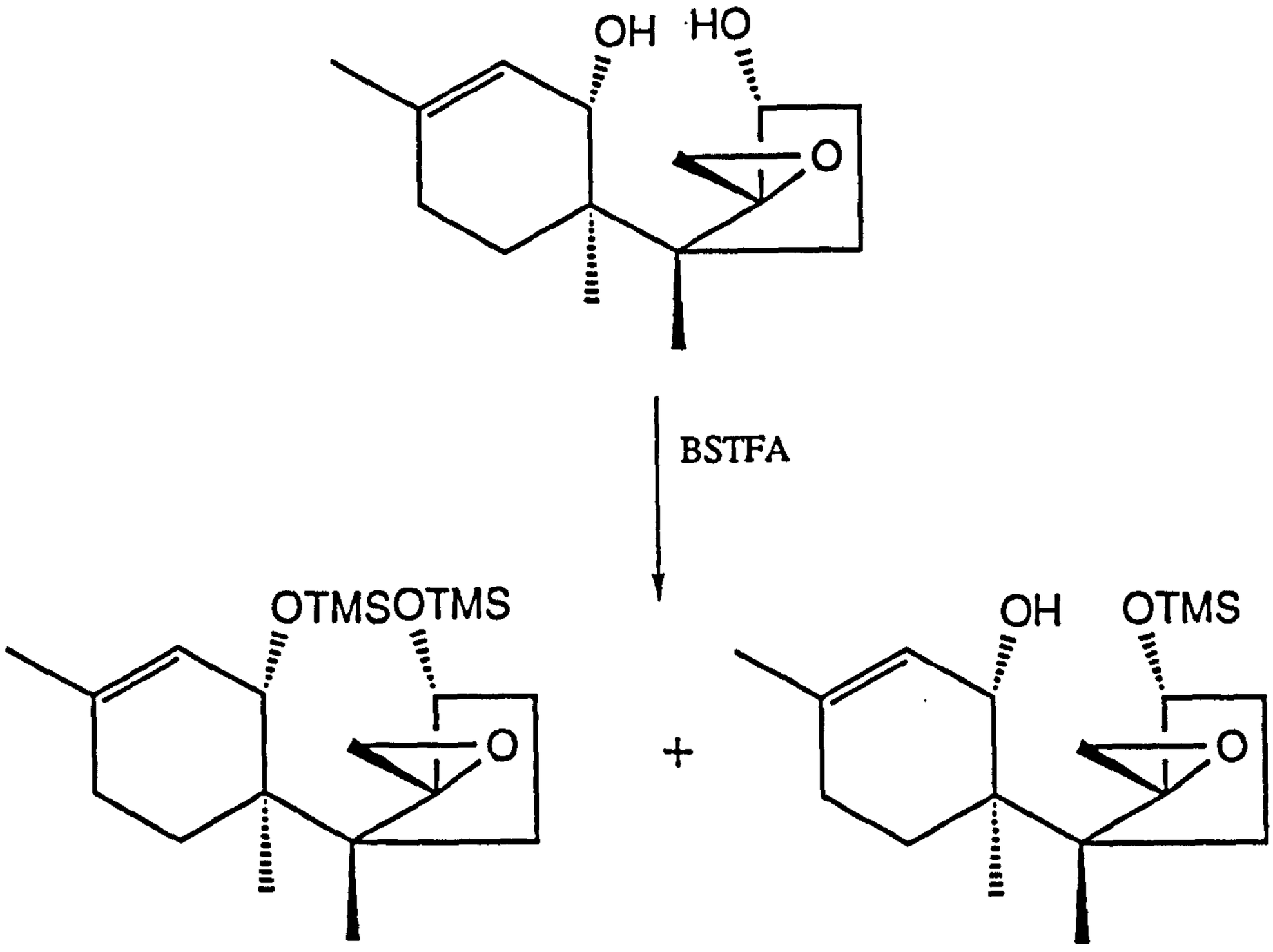


Fig.50: GC-MS of the trimethylsilyl-derivatives of 12,13-epoxy-2α,11α-dihydroxyTDN.

Fig.51: ^{1}H NMR spectrum of isotrichodiol (84).

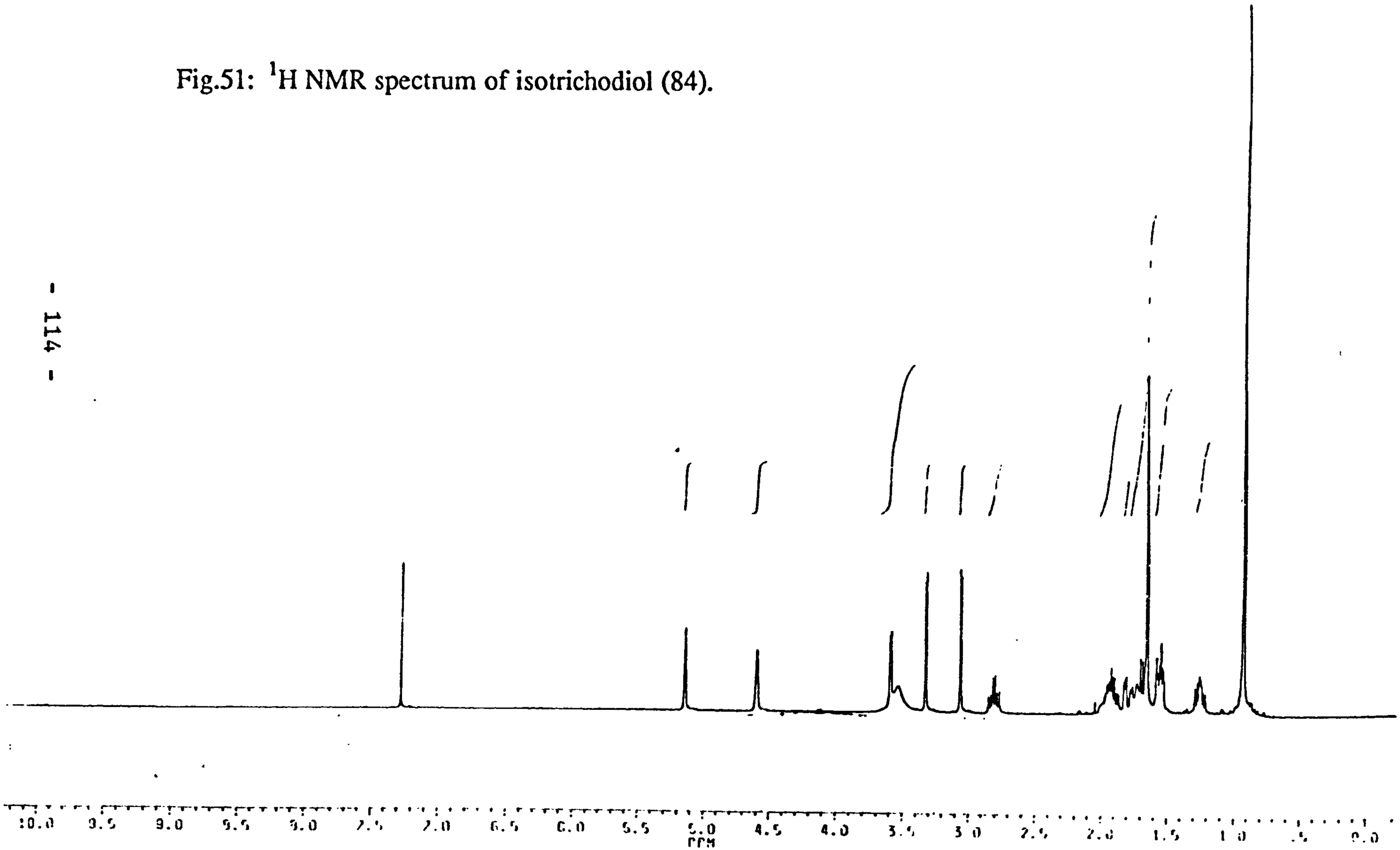


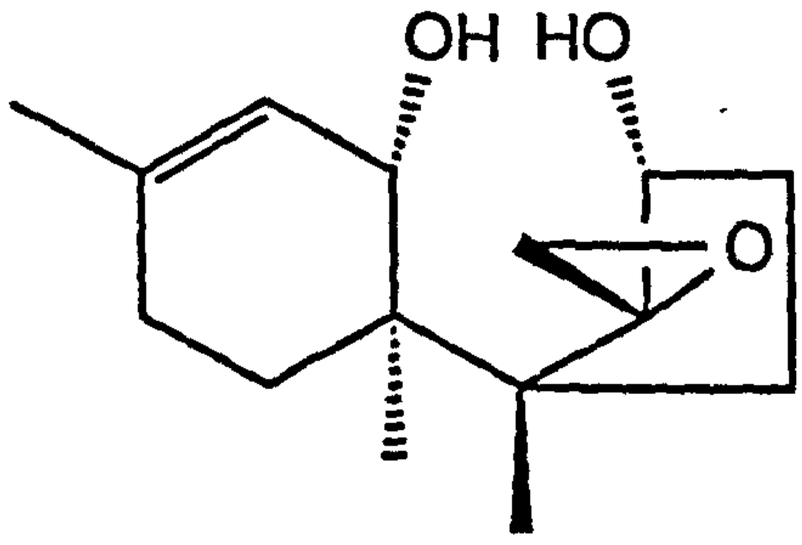
Table 16: NMR data for isotrichodiol (84)

^{1}H NMR		^{13}C NMR	
H-2β	3.60 (*d*, J=4.1Hz)	C-2	81.7
H-3α	1.69 (*dd*, J=13.6, 6.8Hz)	C-3	30.4
β	1.91 (*tdd*, J=13.3, 7.6, 4.3Hz)	C-4	37.3
H-4α	2.82 (*td*, J=13.3, 6.9Hz)	C-5	46.9
β	1.55 (*m*)	C-6	41.2
H-7α	1.55 (*m*)		
β	1.25 (*ddd*, J=14.1, 13.6, 6.2Hz)	C-7	29.2
H-8α	2.00 (*br m*)	C-8	27.9
β	1.79 (*br dd*, J=18.1, 5.8Hz)	C-9	135.1
H-10	5.14 (*br s*)	C-10	125.6
H-11β	4.60 (*br s*)	C-11	72.0
H-13	3.06 (*d*, J=4.2Hz) 3.32 (*d*, J=4.2Hz)	C-12	69.4
H-14	0.94 (*s*)	C-13	49.5
H-15	0.93 (*s*)	C-14	20.4
H-16	1.66 (*br s*)	C-15	12.6
		C-16	22.4

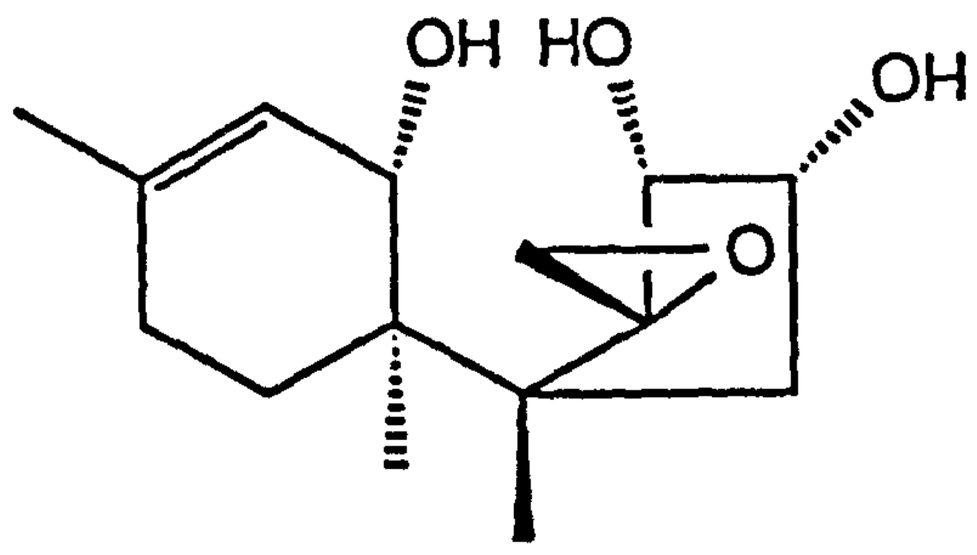
compounds which is as expected since molecular models predict dihedral angles of about 90° for these two protons. Resonances for protons on C-3 and C-4 when these positions are hydroxylated generally appear further downfield at ca. δ4.5 and are more complex multiplets (eg. H-4α in verrucarol (8) is a double doublet at δ4.69, and H-3β in isotrichodermol (5) is a double double doublet at δ4.45[60]). A full assignment of the proton NMR spectrum was facilitated by $^1H/^1H$ COSY [Fig.52] and NOE difference spectra. Significant couplings obtained from COSY and DNOES are shown in Figures 53 and 54 respectively.

The NOE observed between H-2β (δ3.60) and the C-13 proton at δ3.06 confims the stereochmistry of hydroxylation at this position. $^1H/^1H$ COSY located the other B ring protons at δ2.82, δ1.91, δ1.69 and δ1.55 [Fig.52B], and these were then assigned from their characteristic coupling patterns. Thus H-3β at δ1.91 is the most complex multiplet showing coupling to all the other B ring protons ($J_{3\beta,2\beta}$=4.1Hz, $J_{3\beta,3\alpha}$=13.13Hz, $J_{3\beta,4\alpha}$=13.3Hz, $J_{3\beta,4\beta}$=7.6Hz). H-3α at δ1.69 only couples with its geminal partner, and H-4α ($J_{3\alpha,4\alpha}$=6.8Hz), with $J_{3\alpha,2\beta}$ and $J_{3\alpha,4\beta}$ both approximately 0Hz, as expected from molecular models which predict dihedral angles of 90° for these pairs of protons. H-4α at δ2.82 couples with all its neighbours ($J_{4\alpha,4\beta}$=13.3Hz, $J_{4\alpha,3\beta}$=13.3Hz, $J_{4\alpha,3\alpha}$=6.9Hz), and also shows long range W-coupling to the C-14 methyl resonance at δ0.94 thus confirming its stereochemistry as being α.

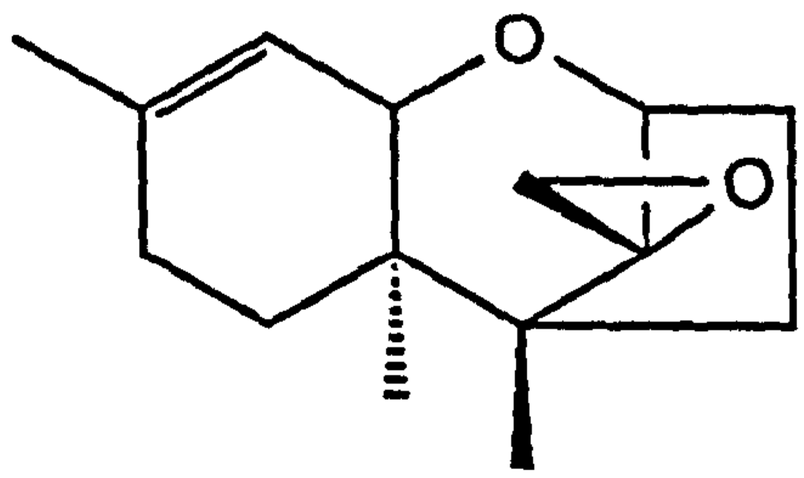
Similarly, $^1H/^1H$ COSY identified the remaining A ring protons as δ1.25, δ1.55, δ1.79 and δ2.00 [Fig.52A]. An observed NOE between the C-13 proton at δ3.32 and the proton at δ1.25 helped assign the latter as H-7β. Long range W-coupling of this resonance with the C-15 methyl protons at δ0.93 confirmed this assignment. COSY 90 also demonstrated long range coupling of H-10 at δ5.14,



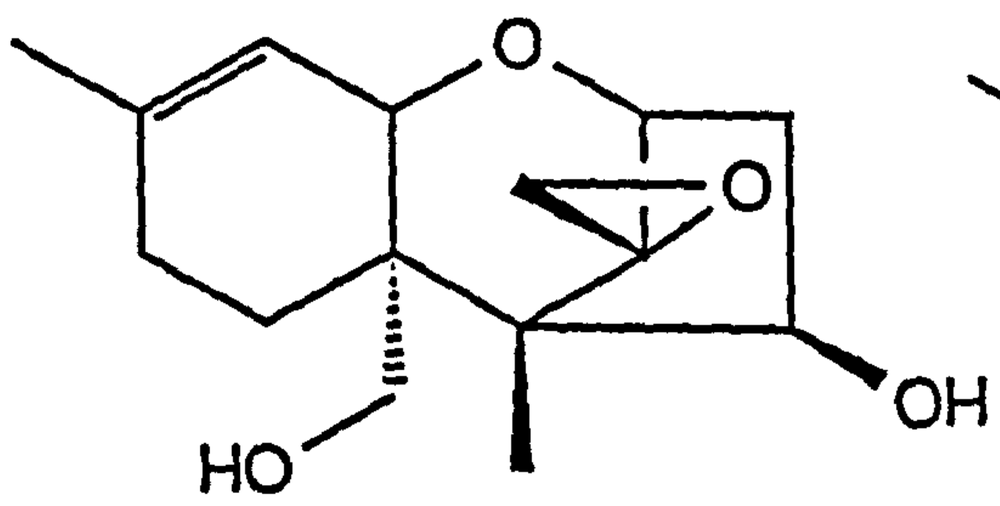
ITdiol (84)



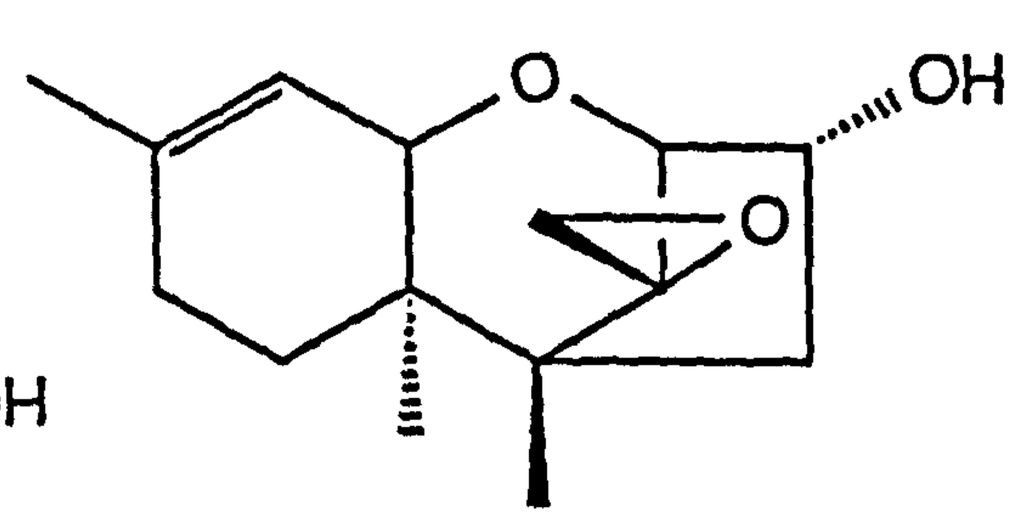
Tricho-9-ene-2α,3α,11α-triol (85)



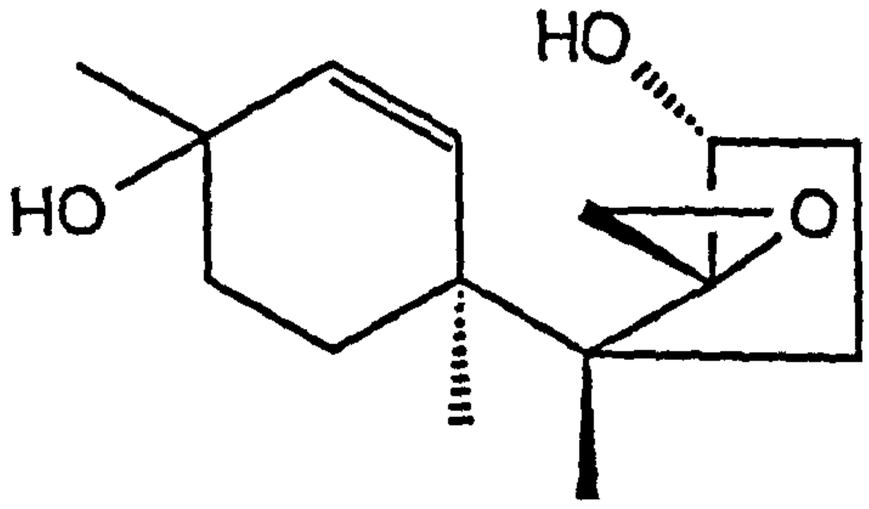
EPT (3)



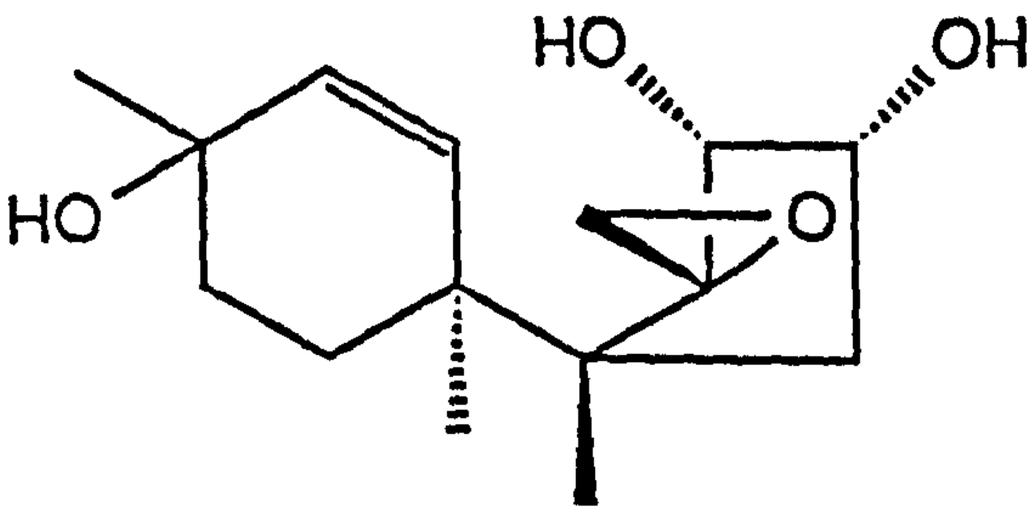
Verrucarol (8)



Isotrichodermol (5)



Trichodiol (37)



Trichotriol (48)

Fig.52A: $^{1}H/^{1}H$ COSY of isotrichodiol showing coupling of the A ring protons.

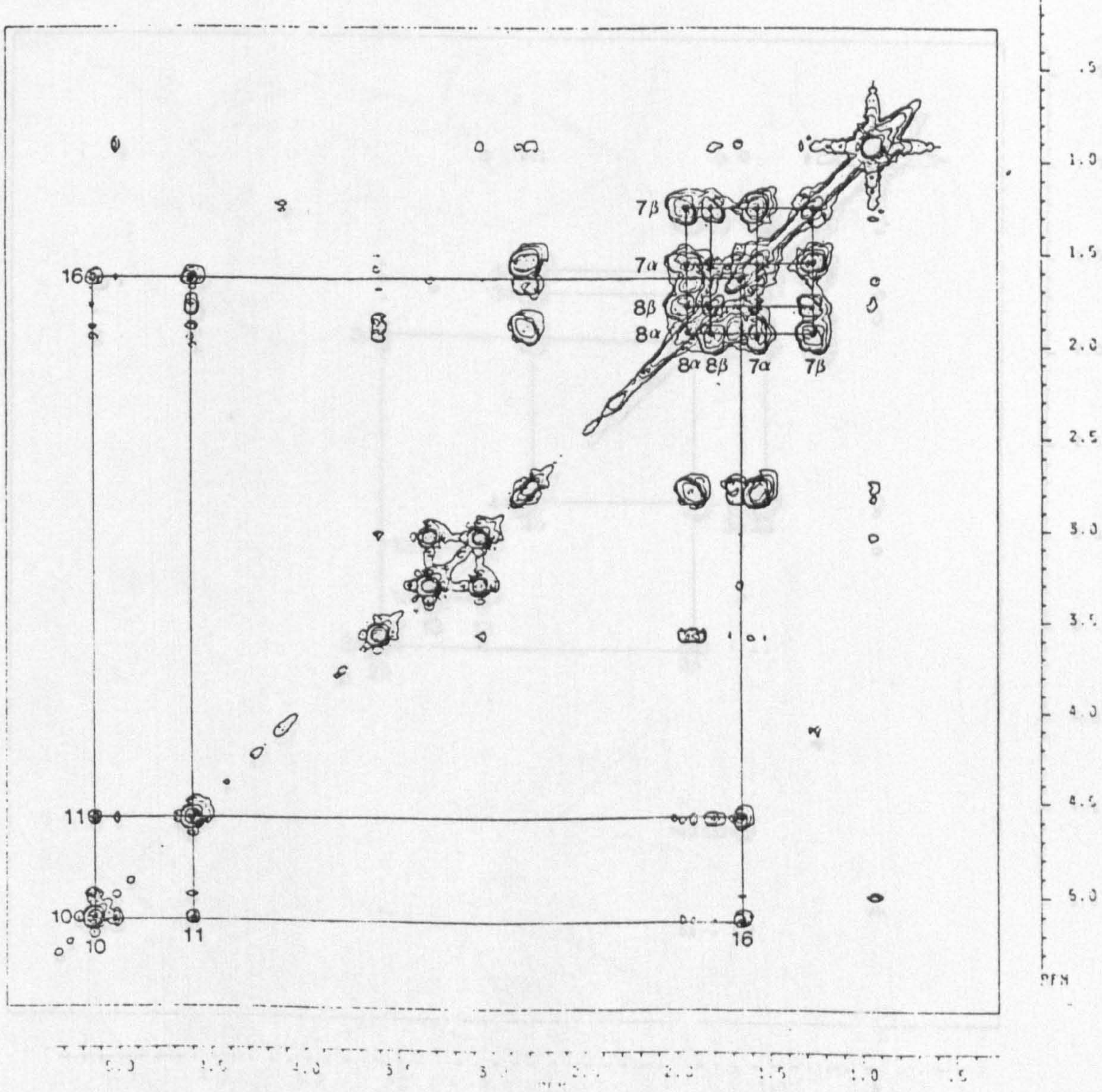


Fig.52B: $^{1}H/^{1}H$ COSY of isotrichodiol showing coupling of the B ring proton

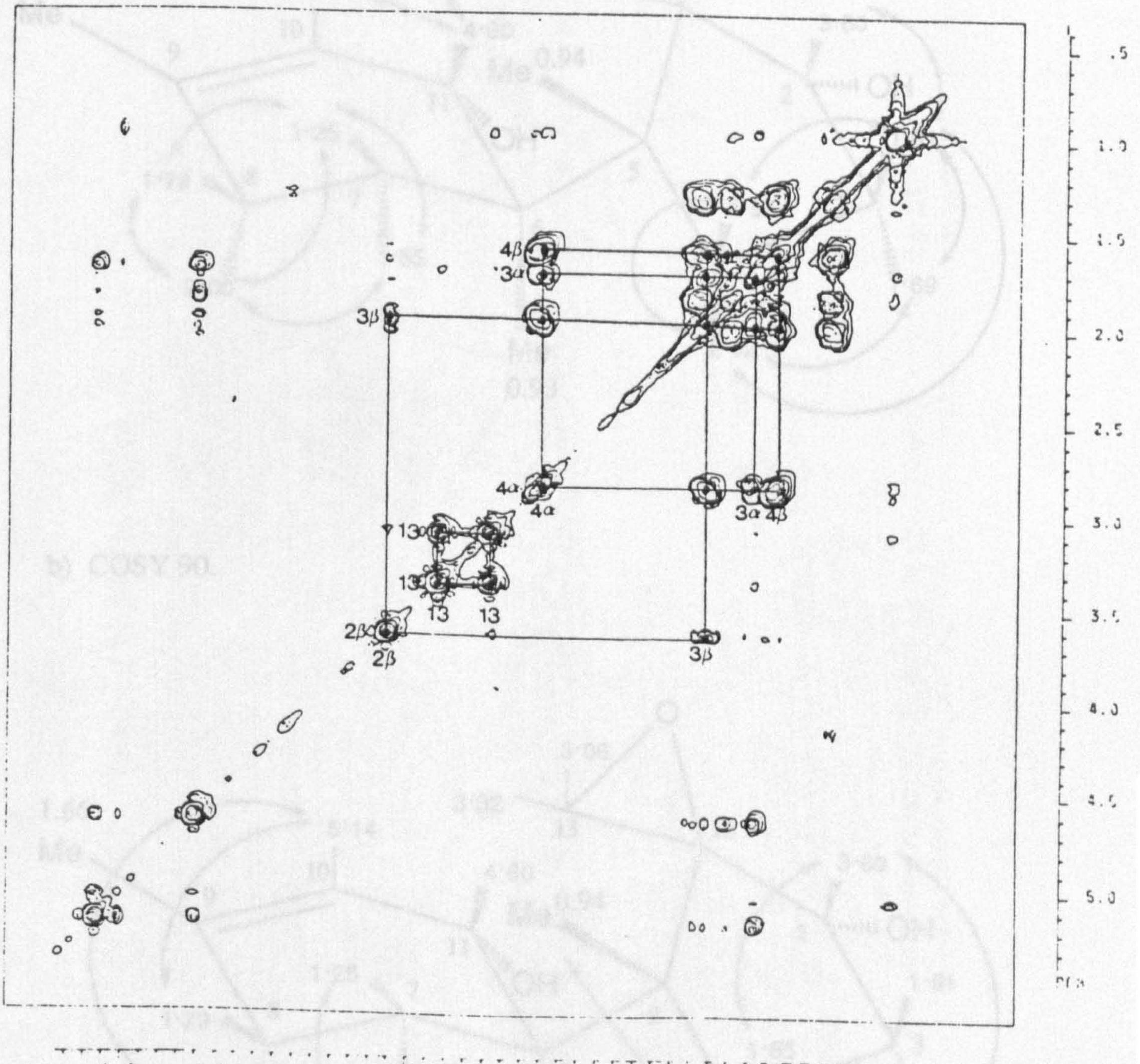
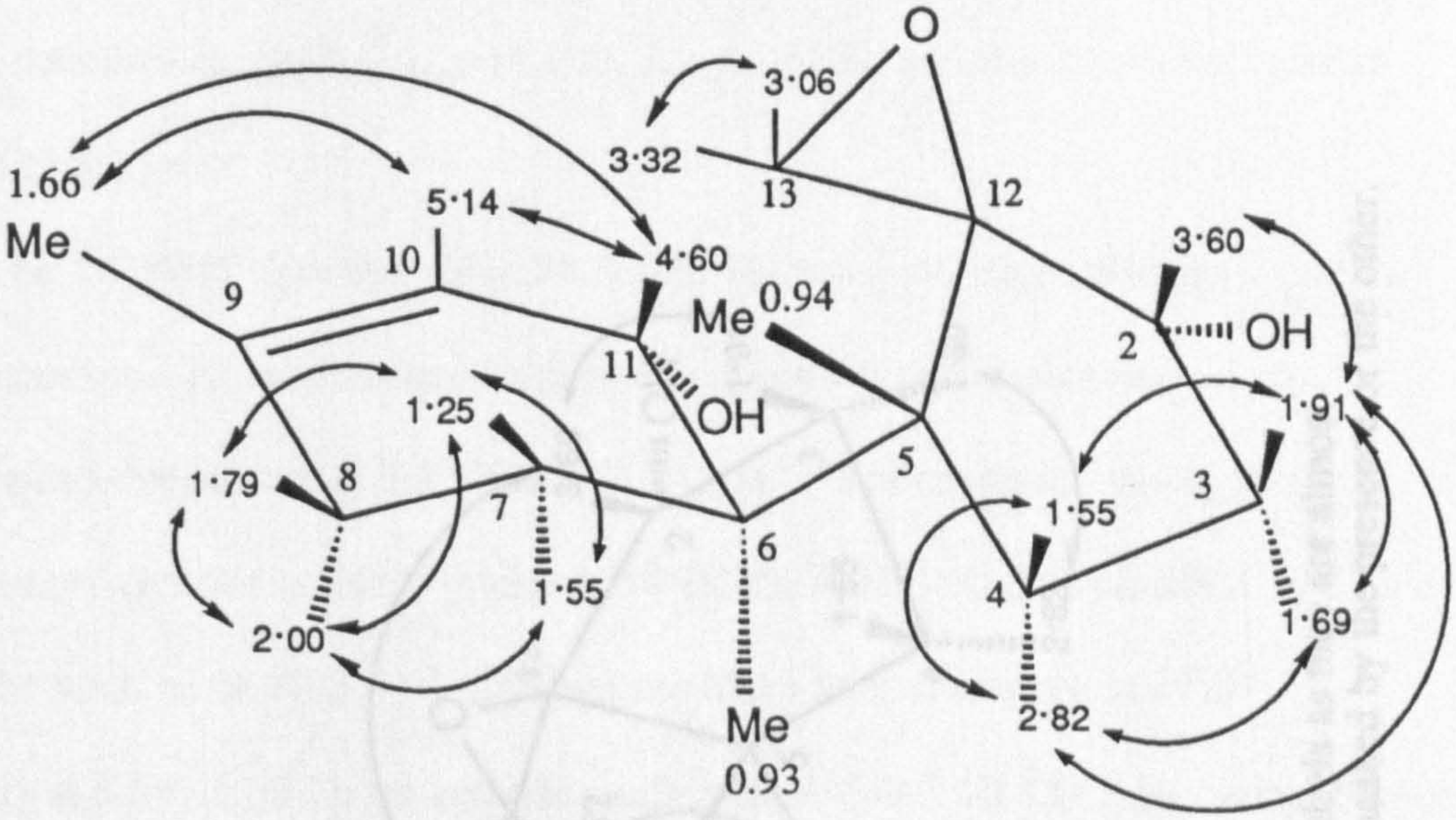


Fig.53: Observed couplings in ITdiol from $^{1}H/^{1}H$ COSY.

a) COSY 45.



b) COSY 90.

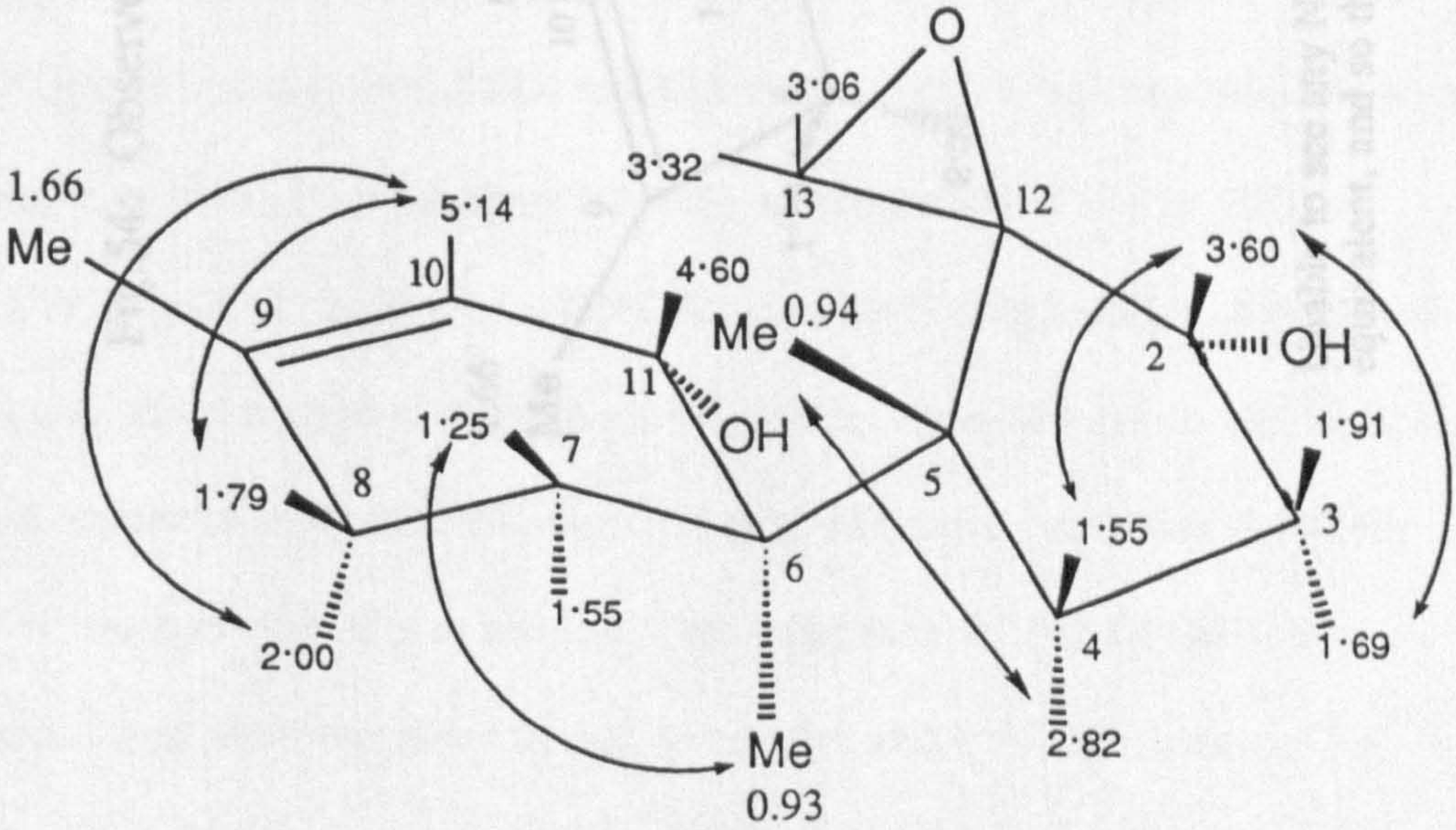
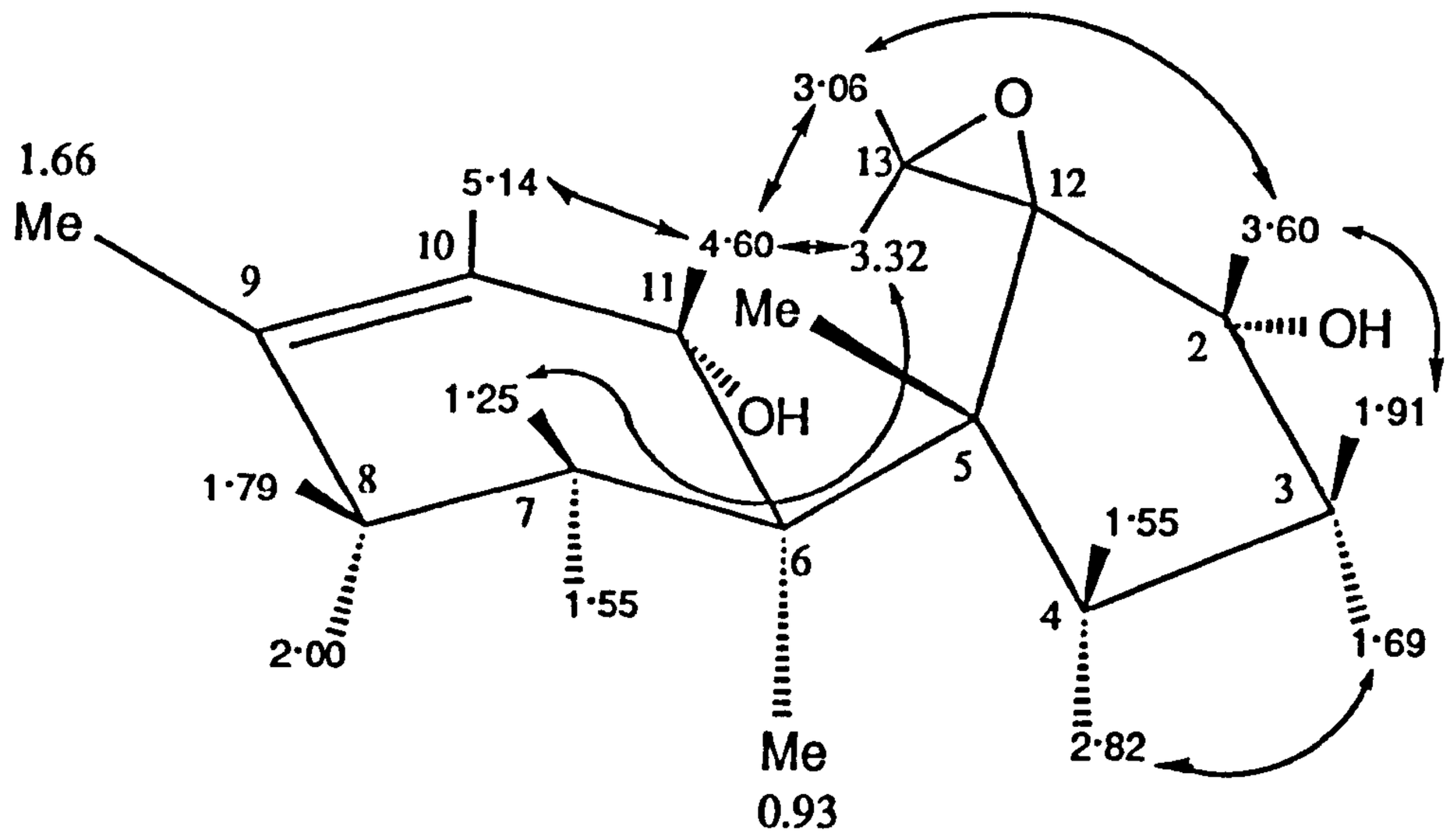


Fig.54: Observed NOE's in ITdiol.



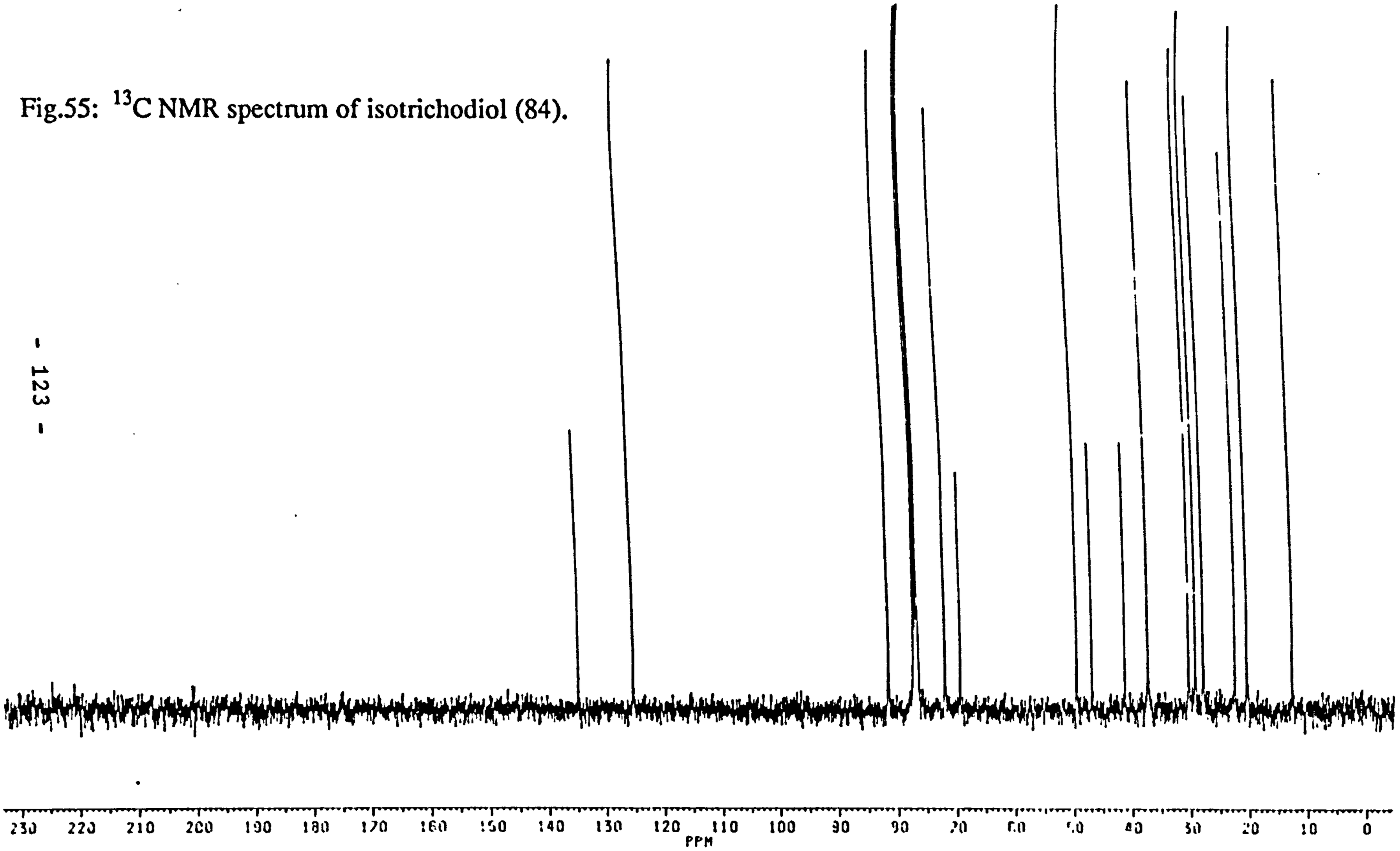
Unable to see any NOE's on 14 and 15 methyls as they are almost equivalent, and so the response of one is masked by the presence of the other.

through the C-9,10 double bond, to the protons at δ1.79 and δ2.00, thus indicating these to be the C-8 protons. The remaining resonance at δ1.55 was therefore assigned to H-7α. Molecular models predict a dihedral angle of about 90° between the H-7α and H-8β protons i.e. $J_{7\alpha,8\beta}$ ≈0Hz. The broad double doublet at δ1.79 must therefore be H-8β ($J_{8\beta,8\alpha}$=18.1Hz, $J_{8\beta,7\beta}$=5.8Hz), and the broad multiplet at δ2.00 H-8α.

The ^{13}C NMR spectrum [Fig.55, Table 16] provided supporting evidence for this structure. Having assigned the proton spectrum, full assignment of the ^{13}C data was achieved using $^{1}H/^{13}C$ COSY [Fig.56]. The spectrum shows four quaternary carbons at δ41.2 (C-6), δ46.9 (C-5), δ135.1 (C-9), and δ69.4 (C-12); a vinylic methine at δ125.6 (C-10); two methines next to oxygen at δ72.0 (C-11) and δ81.7 (C-2); an epoxide methylene at δ49.5 (C-13); three methyl groups at δ12.6 (C-15), δ20.4 (C-14) and δ22.4 (C-16); and four methylene carbons at δ27.9 (C-8), δ29.2 (C-7), δ30.4 (C-3) and δ37.3 (C-4). Subsequent comparison with the ^{13}C NMR data for (85) showed that the spectra are very similar with the only difference being the resonances for C-3 and C-4. These are shifted upfield from δ70.9 to δ30.4 and from δ44.4 to δ37.3 respectively, which is consistent with the compounds being identical apart from the hydroxylation at C-3 in (85).

12,13-Epoxy-2α,11α-dihydroxyTDN (84) is a novel compound, and represents the first uncyclised trichothecene to be isolated from *Fusarium culmorum*. It is a structural isomer of trichodiol (37) with the only difference being that the allylic alcohol function in ring A is at position 11α rather than 9. The trivial name isotrichodiol was therefore given to this compound, and similarly the name isotrichotriol is proposed for tricho-9-ene-2α,3α,11α-triol (85), which is isomeric with trichotriol (48). A compound with the structure of ITdiol has been proposed

Fig.55: ^{13}C NMR spectrum of isotrichodiol (84).



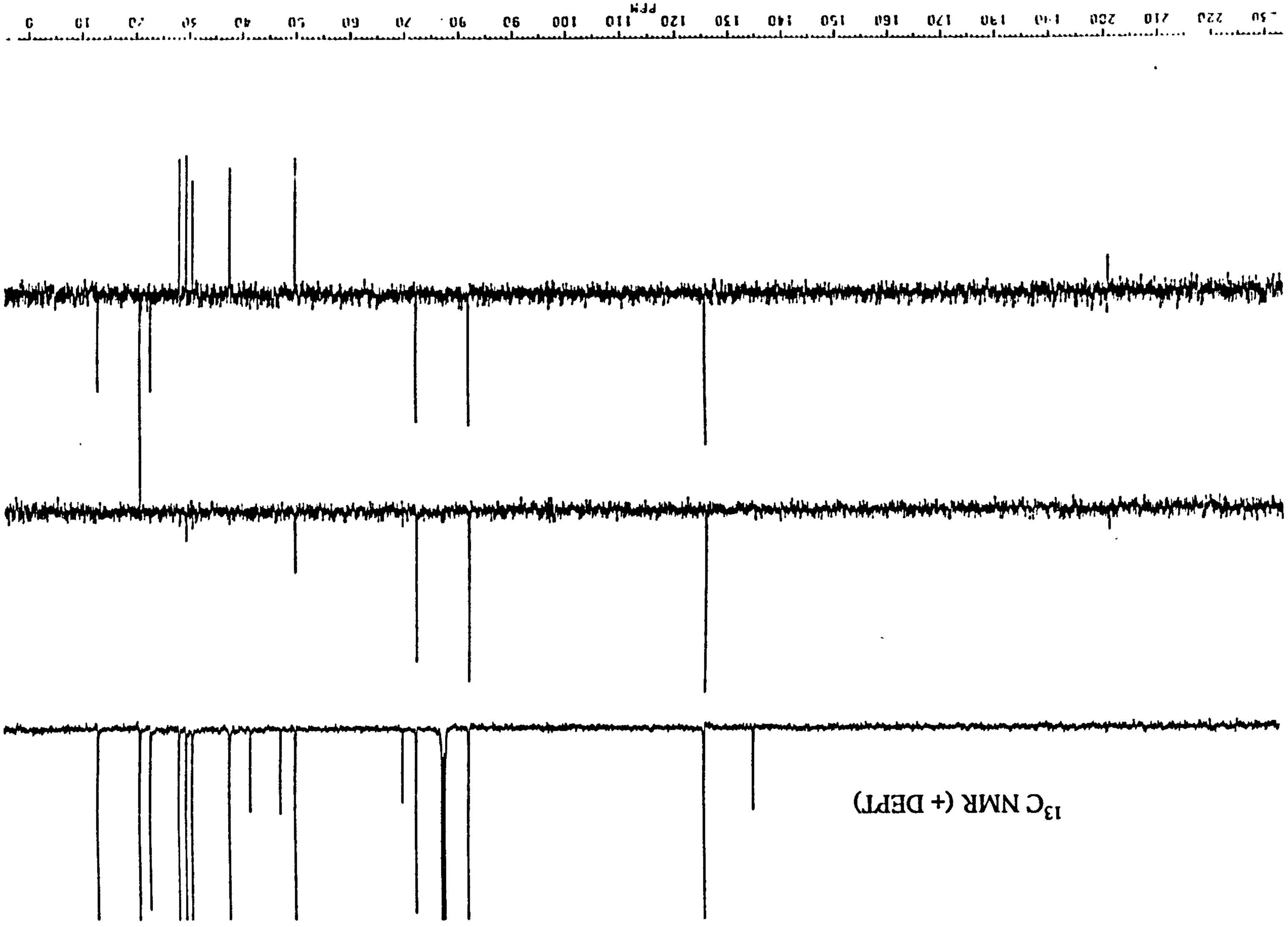
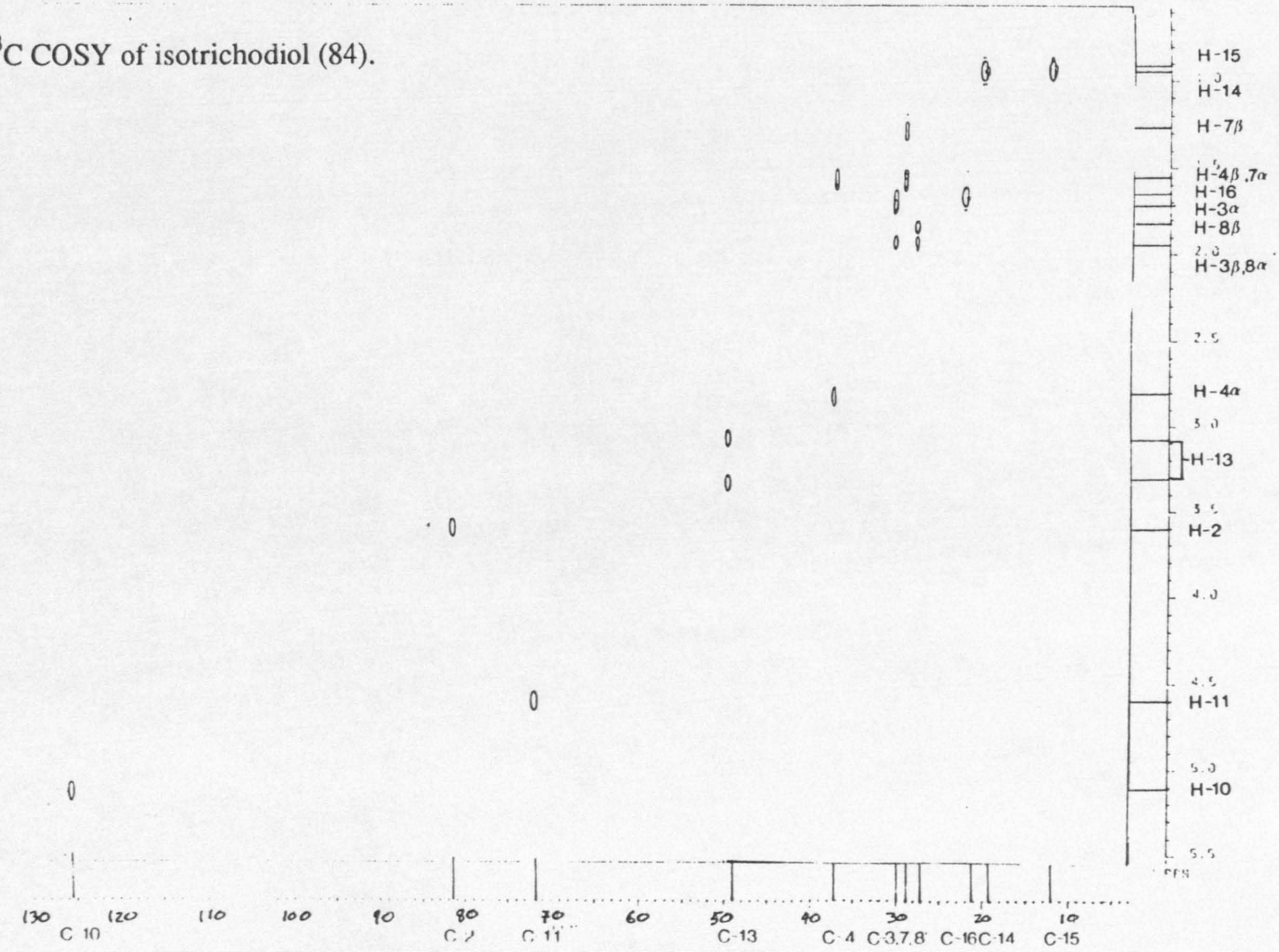

PPM
^{13}C NMR (+ DEPT)

Fig.56: $^{1}H/^{13}C$ COSY of isotrichodiol (84).



as the biosynthetic precursor to sambucoin and sambucinol[77] (see Fig.28), two trichothecene-related metabolites produced as minor metabolites in some *Fusarium* species, including *F. culmorum.*

The accumulation of isotrichodiol (ITdiol) in *F. culmorum* cultures challenged with large amounts of TDN may be due to the exhaustion of cofactors required for transformation of TDN to trichothecenes. The feeding experiments are being performed in distilled water, and this could be expected to deprive the fungus of nutrients essential for its normal metabolic processes. Accordingly, ITdiol could be an intermediate in the biosynthesis of trichothecenes. A similar mechanism may be responsible for the inhibitory action of the TDN derivatives, or alternatively they may be acting as enzyme inhibitors blocking the transformation of ITdiol to the next trichothecene in the sequence. Both 9β,10α-dihydroxyTDN (74) and the bromoepoxide (76) strongly suppress the incorporation of TDN, and the fact that (74) is not biotransformed by the fungus supports their probable role as enzyme inhibitors. However, the 9β-hydroxide (70) and both the epoxide derivatives (72) and (73) are less effective, and, since all are biotransfomed, their activity may be related to cofactor exhaustion.

<u>Transformation of trichodiene to trichothecenes.</u>

Challenging cultures of *Fusarium culmorum* with large quantities of TDN has been shown to cause inhibition of trichothecene biosynthesis (see Fig.48, lane 3). TDN is still however incorporated into the major trichothecene products 3-AcDON and DHC, which indicates that the inhibition is not total and is probably mediated by exhaustion of essential cofactors. The accumulation of ITdiol points to it being an intermediate. Other trichothecene structures involved in the production of

3-AcDON and DHC can therefore also be expected to have accumulated to some extent, and isolation and characterisation of these will provide information about the later stages of trichothecene biosynthesis.

Low activity [^{14}C]TDN (600mg in 0.8ml acetone) was fed to a whole-cell system prepared as before from a liquid culture of *F. culmorum* (1.8L; 42hrs old), and after 6hrs incubation (27°C, 250rpm) the culture was harvested. TLC analysis (hexane-ethyl acetate, 1:1) with reference to appropriate standards indicated that TDN had been incorporated into a range of trichothecene products. These were purified using CTLC (2mm plate) eluting first with hexane-ethyl acetate, 8:2, (60ml) followed by hexane-ethyl acetate, 1:1, collecting 2ml fractions.

The pure trichothecenes isolated were identified by EI-MS and ^{1}H NMR analysis, and are listed in Table 17. Autoradiography showed that all the products were ^{14}C-labelled, demonstrating that they are derived from the exogenous [^{14}C]TDN supplied, and not merely from the biosynthesis of endogenous substrates. Specific incorporations, however, were not determined.

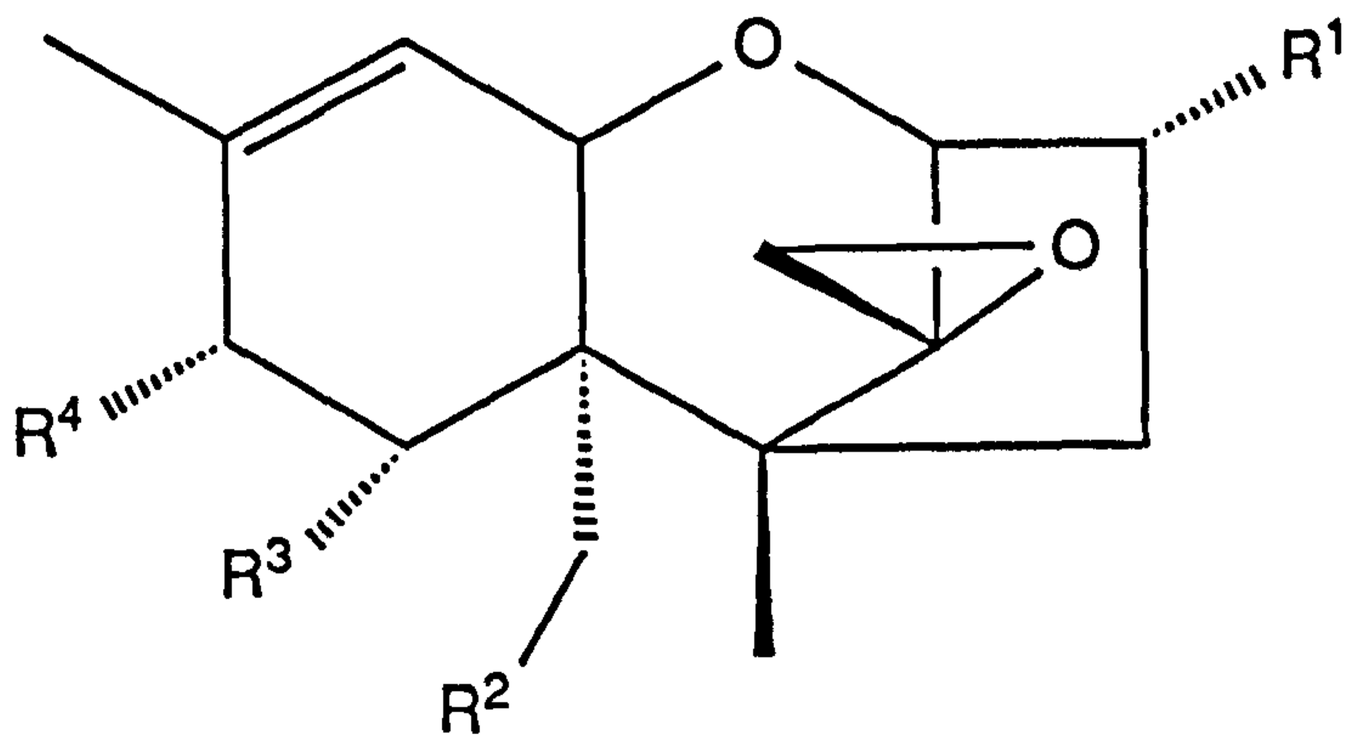
EPT (3), ITD(68), CAL (6), 3-AcDON (14) and DHC (7) have previously been isolated from *F. culmorum* in this laboratory, and yielded spectral data in accordance with published values[59] [Table 18]. The two novel isolates 7α-hydroxycalonectrin (7-hydroxyCAL) (88) and 15-deacetylcalonectrin (15-deacetylCAL) (89) were readily identifiable by comparison of their ^{1}H NMR spectra [Table 18] with those of the series of *F. culmorum* trichothecenes already characterised.

Table 17: Trichothecenes produced from TDN (600mg) in *F.culmorum*, as isolated by CTLC.

Fraction number	Compound	Amount (mg)
18-21	Trichodiene	233*
34-41	12,13-Epoxytrichothecene (EPT)	1.5
47-51	Isotrichodermin (ITD)	1.0
55-59	Calonectrin (CAL)	2.5
74-80	Isotrichodiol (ITdiol)	23.0
87-91	15-Deacetylcalonectrin (15-deacetylCAL)	3.5
97-100	7α-Hydroxycalonectrin (7-OH CAL)	4.0
102-END	3-Acetyldeoxynivalenol (3-AcDON)+	17.0
	7,8-Dihydroxycalonectrin (DHC)+	21.0

* Complete recovery of TDN from the culture is hampered by adsorbtion to mycelia.

+ Required additional purification (CTLC, ether-acetone 9:1).



	R^1	R^2	R^3	R^4
EPT (3)	H	H	H	H
ITD (68)	OAc	H	H	H
15-DeacetylCAL (89)	OAc	OH	H	H
CAL (6)	OAc	OAc	H	H
7-OH CAL (88)	OAc	OAc	OH	H
DHC (7)	OAc	OAc	OH	OH
3-AcDON (14)	OAc	OH	OH	=O

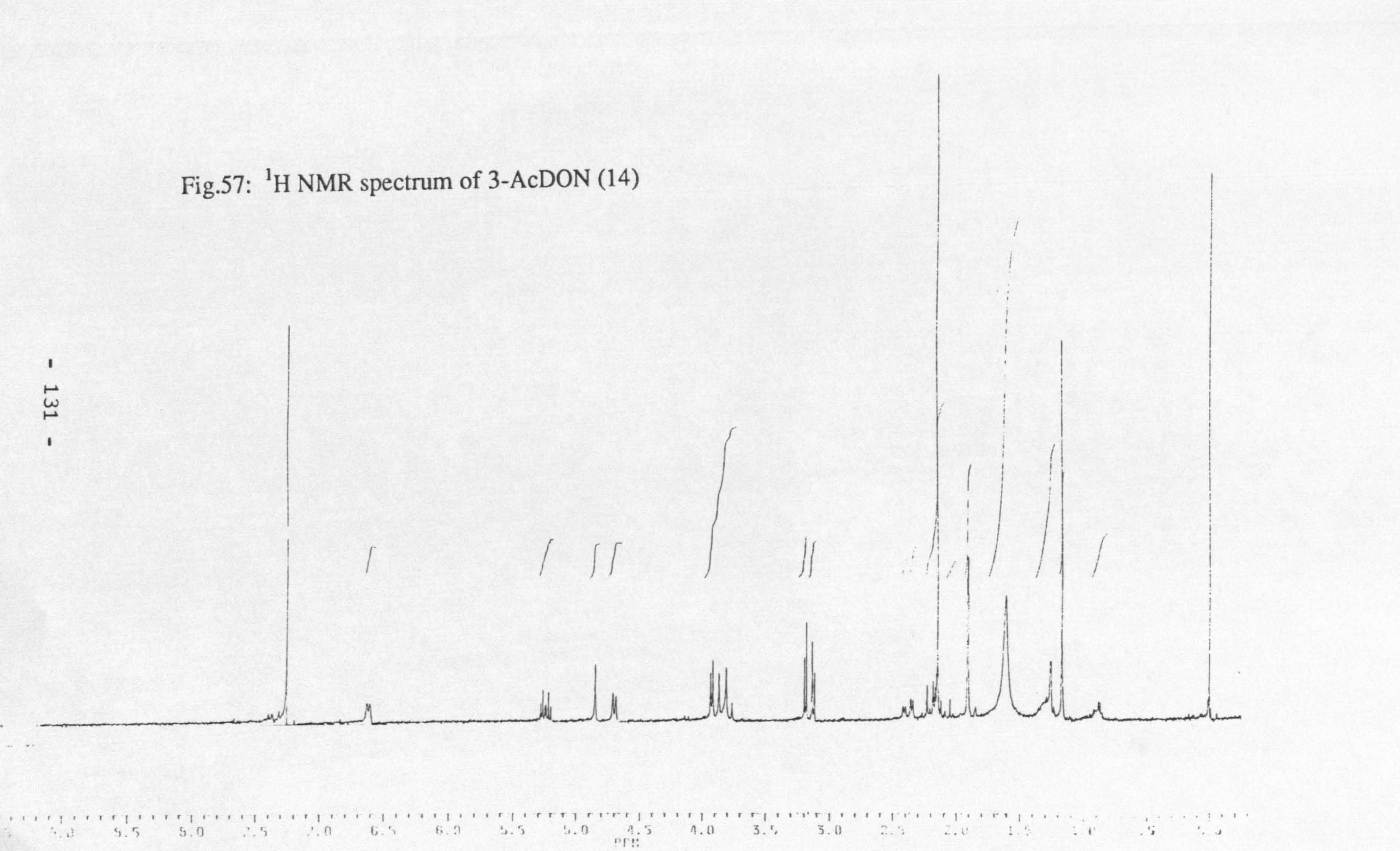
Table 18: ^{1}H NMR chemical shifts (δppm) and coupling constants (Hz) for trichothecenes isolated from *F.culmorum*.

	12,13-Epoxytrichothecene (3)	Isotrichodermin (5)	15-Deacetylcalonectrin (89)	Calonectrin (6)
H-2β	3.72 (*d*, J=4.7)	3.75 (*d*, J=4.7)	3.75 (*d*, J=4.7)	3.76 (*d*, J=4.6)
H-3α	1.8-2.2 (8H, *m*)	5.18 (*ddd*, J=10.1, 5.1, 5.1)	5.17 (*ddd*, J=9.7, 5.0, 4.7)	5.17 (*ddd*, J=9.9, 5.0, 4.9)
β				
H-4		1.8-2.2 (6H, *m*)	1.5-2.2 (6H, *m*)	1.8-2.2 (6H, *m*)
H-7				
H-8				
H-10	5.42 (*dq*, J=5.5,1.4)	5.46 (*dq*, J=5.5, 1.4)	5.48 (*br d*, J=5)	5.50 (*dq*, J=5.4, 1.3)
H-11α	3.70 (*d*, J=5.4)	3.98 (*br d*, J=6.4)	3.98 (*br d*, J=4.9)	4.03 (*d*, J=5.4)
H-13	2.89 (*d*, J=4.3)	2.86 (*d*, J=3.95)	2.87 (*d*, J=4.0)	2.86 (*d*, J=4.0)
	3.16 (*d*, J=4.3)	3.10 (*d*, J=3.95)	3.10 (*d*, J=4.0)	3.11 (*d*, J=4.0)
H-14	0.81 (*s*)	0.82 (*s*)	0.93 (*s*)	0.84 (*s*)
H-15	0.77 (*s*)	0.76 (*s*)	3.50 (*d*, J=11.7)	3.83 (*d*, J=12.2)
			3.69 (*d*, J=11.8)	4.11 (*m*)
H-16	1.71 (*br s*)	1.72 (*br s*)	1.74 (*br s*)	1.73 (*br s*)
Acetates		2.14 (*s*)	2.12 (*s*)	2.05 (*s*)
				2.13 (*s*)

	7α-Hydroxycalonectrin (88)	7α,8α-Dihydroxycalonectrin (7)	3-Acetyldeoxynivalenol (14)
H-2β	3.80 (*d*, J=4.4)	3.79 (*d*, J=4.4)	3.91 (*d*, J=4.4)
H-3β	5.19 (*ddd*, J=8.3, 7.1, 4.6)	5.18 (*ddd*, J=11.2, 4.4, 4.4)	5.23 (*ddd*, J=11.2, 4.5, 4.5)
H-4α	1.5-2.2 (2H, *m*)	2.37 (*dd*, J=15.0, 4.4)	2.38 (*dd*, J=14.8, 4.5)
4β		2.16 (*dd*, J=15.0, 11.2)	2.20 (*dd*, J=15.1, 11.2)
H-7β	4.58 (*br dd*, J=11, 6)	4.54 (*d*, J=5.4*)	4.84 (*s*)
H-8α	1.5-2.2 (1H, *m*)		
8β	2.43 (*dd*, J=17.7, 5.6)	4.02 (*d*, J=5.4*)	
H-10	5.41 (*br d*, J=5.7)	5.61 (*dq*, J=5.7, 1.3)	6.62 (*dq*, J=5.9, 1.5)
H-11α	4.12 (*d*, J=5.7)	4.34 (*d*, J=5.8)	4.69 (*d*, J=5.9)
H-13	3.12 (*d*, J=4.4)	3.11 (*d*, J=4.3)	3.12 (*d*, J=4.3)
	3.26 (*d*, J=4.4)	3.22 (*d*, J=4.3)	3.18 (*d*, J=4.3)
H-14	1.16 (*s*)	1.15 (*s*)	1.17 (*s*)
H-15	4.07 (*d*, J=12.3)	4.16 (*d*, J=12.3)	3.78 (*d*, J=11.7)
	4.32 (*d*, J=12.3)	4.47 (*d*, J=12.3)	3.88 (*d*, J=11.8)
H-16	1.74 (*br s*)	1.89 (*br s*)	1.91 (*dd*, J=1.5, 0.5)
Acetates	2.10 (*s*)	2.04 (*s*)	2.15
	2.11 (*s*)	2.12 (*s*)	
Hydroxyls		2.60 (*br s*)	
		3.00 (*br d*, J=9.5)	

* D_2O shake

Fig.57: ^{1}H NMR spectrum of 3-AcDON (14)



^{1}H NMR analysis of *F. culmorum* toxins.

The trichothecenes show many common structural features, and this leads to characteristic resonances in their ^{1}H NMR spectra. All possess a C-9,10 double bond; methyl groups at C-14 and C-16; a 12,13-epoxide function; and an ether link between C-2 and C-11. Consequently, the signals due to the protons at these positions will be similar for each trichothecene, although chemical shifts may vary according to the nature of any substituents present.

The most characteristic signals are those arising from the C-13 epoxide protons which appear as an AB quartet centred at about δ3.0-3.2 with J≈4.3Hz. The resonances for H-2 and H-11 appear downfield from these because of the deshielding effect of the oxygen atom in the bridge between C-2 and C-11. They are readily distinguishable from each other by the size of their coupling constants, and because H-11 is generally shifted further downfield by the effect of the C-9,10 double bond and any substituents at C-8. H-2 is therefore a doublet at ca. δ3.8 with $J_{2,3\beta}$≈4.7Hz. Coupling with H-3α is not observed since the dihedral angle is 90° ie $J_{2,3\alpha}$=0Hz. H-11 is also a doublet with $J_{11,10}$≈5.7Hz, and appears between ca. δ3.8 and δ4.8.

The vinylic proton, H-10, is the most deshielded resonance, appearing at ca. δ5.6, or ca. δ6.5 if a C-8 ketone is present. The C-14 and C-16 methyl protons resonate upfield at ca. δ0.8-2.0, and are obvious from the integration. They are distinguishable since the H-16 signal is split by long range coupling to H-10 and H-11, and also appears downfield from H-14 due to the adjacent double bond.

1) 3-Acetyldeoxynivalenol (3-AcDON) (14).

The spectrum for 3-AcDON [Table 18, Fig.57] had previously been fully assigned by a combination of COSY and fully-coupled 2D NMR techniques[71]. The strong deshielding effect of the α,β-unsaturated ketone means that H-10 is the most downfield signal at δ6.62. It appears as a double quartet due to coupling to H-11 ($J_{10,11}$=5.9Hz) and long range coupling to the C-16 methyl ($J_{10,16}$=1.5Hz). The 8-ketone substituent also has a deshilding effeect on H-11 which is at δ4.69, whilst H-2 is more shielded and appears upfield at δ3.91.

In addition to the signals for the C-13 protons centred at δ3.15, there is another AB quartet centred downfield from this at δ3.83 (J_{AB}=11.8Hz) corresponding to the C-15 methylene protons. Further downfield is the H-3β resonance at δ5.23 due to deshielding by the 3α-acetoxy substituent. This is a double double doublet as a result of coupling to the protons on adjacent carbons C-2 and C-4 ($J_{3\beta,2}$=4.5Hz, $J_{3\beta,4\alpha}$=4.5Hz, $J_{3\beta,4\beta}$=11.2Hz). H-4α and H-4β are non-equivalent and are located upfield at δ2.38 and δ2.20 respectively. Both are double doublets due to coupling with each other (J_{gem}=14.8Hz) and with H-3β. H-7β couples only with the 7α-hydroxyl and is located at δ4.84. The three methyl resonances are obvious from the integration, and are all present in the upfield region of the spectrum between δ1.1 and δ2.2. The C-16 signal is recognisable as a double doublet at δ1.91, showing long range coupling with H-10 ($J_{16,10}$=1.5Hz) and H-11 ($J_{16,11}$=0.5Hz). The downfield signal at δ2.15 corresponds to the acetate methyl, whilst the C-14 protons are upfield at δ1.17.

2) 7,8-Dihydroxycalonectrin (DHC) (7).

The minor differences between the structures of 3-AcDON and DHC are reflected in corresponding changes in the ^{1}H NMR spectra. The appearance of a second acetate signal at δ2.04, and a downfield shift for the H-15 methylene doublets from ca. δ3.85 to ca. δ4.3 indicate the introduction of a 15-acetyl group. The resonances for H-10 and H-11 are shifted upfield to δ5.61 and δ4.34 respectively, because of the loss of the deshielding effect of the 8-ketone group. Similarly, the H-7β signal is shifted upfield from δ4.84 to δ4.54 and now exhibits coupling to H-8β ($J_{7\beta,8\beta}$=5.4Hz) located at δ4.02.

3) 7α-Hydroxycalonectrin (7-hydroxyCAL) (88).

The ^{1}H NMR spectrum is largely similar to that of DHC, and agrees with published data[105]. The loss of the C-8α hydroxyl has resulted in both the H-8 protons being present in the unresolved methylene region δ1.5-2.2, and in the slight upfield shifts (approximately 0.2ppm) experienced by H-10, H-11 and H-16. H-7β now couples with both protons at C-8 and is therefore a double doublet at δ4.58. The coupling constants, $J_{7,8\alpha}$≈11Hz and $J_{7,8\beta}$≈6HZ, are consistent with dihedral angles of about 180° and 50°, respectively, between H-7 and the C-8 protons, and indicate that the C-7 hydroxyl is in the α position.

7-HydroxyCAL is a new isolation for *F. culmorum* but it has previously been isolated from *F. graminearum*[105].

4) Calonectrin (CAL) (6).

There is only one structural difference between 7-hydroxyCAL and CAL, the loss of the C-7α hydroxyl group. This is reflected in the ^{1}H NMR spectrum by the

disappearance of the H-7β resonance from δ4.54 into the unresolved region at δ1.8-2.2, and in the slight upfield shift in the C-15 methylene doublets from ca. δ4.2 to ca. δ3.95. The C-14 methyl signal is also shifted from δ1.16 to δ0.84, and the separation and chemical shifts for the C-13 epoxide protons are slightly different.

5) 15-Deacetylcalonectrin (15-deacetylCAL) (89).

The ^{1}H NMR data agrees with literature values[106], and differs only slightly to those for CAL. The upfield shift for the H-15 methylene doublets from ca. δ3.9 to ca. δ3.6, and the loss of an acetyl signal from δ2.05 are consistent with the removal of the C-15 acetyl group.

15-DeacetylCAL has not previously been isolated from *F. culmorum* in this laboratory, although it has been reported by other groups from both *F. graminearum*[106] and *F. culmorum*[93].

6) Isotrichodermin (ITD) (68).

ITD is the first compound in the series to lack substitution at C-15. Consequently, when compared to the spectrum for 15-deacetylCAL the major differences are the loss of the H-15 methylene doublets at δ3.50 and δ3.69, and and the appearance of a new methyl singlet at δ0.76, which are consistent with the removal of the C-15 hydroxyl.

7) 12,13-Epoxytrichothec-9-ene (EPT) (3).

EPT is the completely unsubstituted trichothecene, and thus only the resonances for H-2, H-10, H-11, H-13, H-14, H-15 and H-16 are easily identifiable. These are

almost identical to those in the spectrum for ITD, except for H-11 which is shifted slightly upfield from δ3.98 to δ3.71. Other differences evident when comparing the spectra are the loss of both the acetate methyl signal (δ2.14) and the H-3β resonance (δ5.18) which result from the loss of the C-3α acetoxy substituent. The resonances for the protons at C-3, C-4, C-7 and C-8 are all located in the methylene region of the spectrum at δ1.8-2.2, and are indistinguishable from each other since they form a complex overlapping multiplet in this region.

Implication of results.

It has been proposed that in the biosynthesis of trichothecenes from TDN the first cyclised compounds formed are EPT derived from trichodiol, and isotrichodermol produced from trichotriol (see Fig.17). The isolation of both radiolabelled EPT and ITD from a culture of *F. culmorum* which had been fed [^{14}C]TDN supports this hypothesis. The range of other labelled trichothecenes also produced show various levels of oxygenation and esterification, which suggest a linear pathway from EPT and isotrichodermol to 3-AcDON and DHC [Fig.58]. This postulated sequence also involves the possibility of hydroxylation of EPT at C-3 to produce isotrichodermol. Further sequential oxygenations lead to 3,15-dioxygenated (15-deacetylCAL and CAL), 3,7,15-trioxygenated (7-hydroxyCAL) and 3,7,8,15-tetraoxygenated (DHC and 3-AcDON) trichothecenes. The range of trichothecene structures isolated from this strain of *F. culmorum* by previous groups of workers [Table 19] points to the operation of a metabolic grid rather than a unique pathway. However, many of these compounds were isolated only as minor metabolites, and from cultures which were over 7 days old. Some structures could therefore be the

Fig.58: Proposed post-EPT oxygenation sequence in *F. culmorum*.

* = not isolated in this experiment.

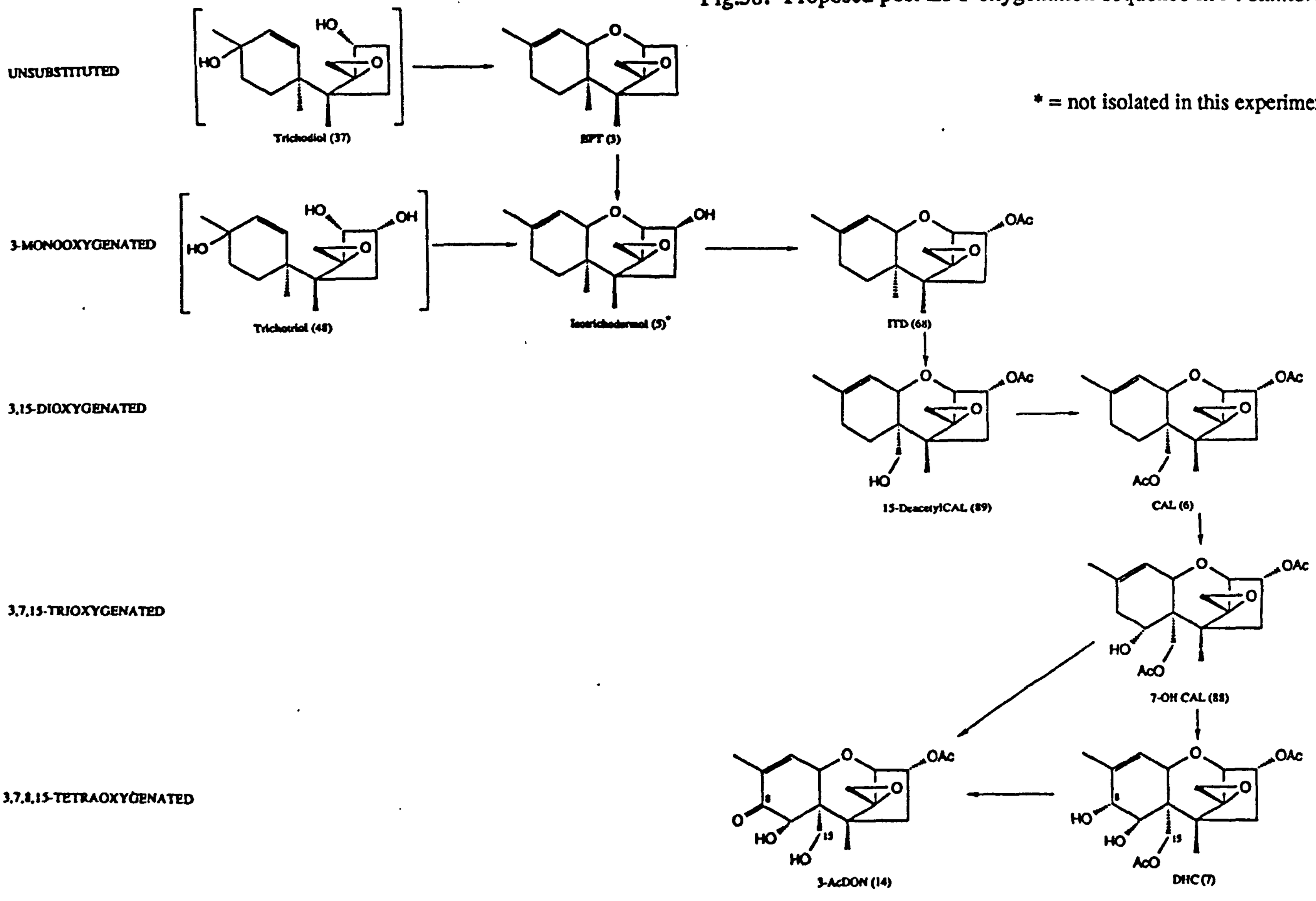


Table 19: Trichothecenes isolated from *F.culmorum* CMI 14764.

	3α	7α	8α	15	Ref.
<u>Unsubstituted</u>					
EPT					59, *
<u>3-Mono-oxygenated</u>					
Isotrichodermol	OH				107
ITD	OAc				59, 93, *
<u>3,8-Dioxygenated</u>					
8-OH ITD	OAc		OH		107
<u>3,15-Dioxygenated</u>					
DideacetylCAL	OH			OH	93
3-DeacetylCAL	OH			OAc	93
15-DeacetylCAL	OAc			OH	93, 108, *
CAL	OAc			OAc	59, 108, *
<u>3,7,15-Trioxygenated</u>					
7-OH CAL	OAc	OH		OAc	*
<u>3,8,15-Trioxygenated</u>					
8-OH CAL	OAc		OH	OAc	93
8-Keto-CAL	OAc		=O	OAc	93
8-Keto-15-deacetylCAL	OAc		=O	OH	93
<u>3,7,8,15-Tetraoxygenated</u>					
DHC	OAc	OH	OH	OAc	59, 93, *
DON	OH	OH	=O	OH	59
3-AcDON	OAc	OH	=O	OH	59, 92, 93, *
3,15-DiAcDON	OAc	OH	=O	OAc	59

* = This work

result of non-specific enzyme reactions on the trichothecenes produced on the main biosynthetic pathway. The trichothecenes reported here were isolated from a TDN feeding experiment with an incubation time of just 6hrs, and are therefore much more likely to be biosynthetic intermediates en route from TDN to the major trichothecene products 3-AcDON and DHC. Similarly, this suggests that ITdiol, also isolated in this experiment, is likewise an intermediate.

Transfomation of [^{14}C]TDN to [^{14}C]ITdiol.

The previous experiment in which labelled ITdiol was obtained by challenging *F. culmorum* cultures with large amounts of low activity TDN proceeded in sufficiently high yields to suggest that higher activity [^{14}C]ITdiol could be produced in a similar way. Radiolabelled ITdiol was required for use in biosynthetic studies, thus allowing any biotransformations of ITdiol by *F. culmorum* to be monitored using scintillation counting procedures and autoradiography.

[^{14}C]TDN (280mg; 127KBqmM^{-1}) was fed to a whole-cell system of *F. culmorum*, and incubated for 6hrs as before. Work-up yielded an oil (154mg) which was then purified by CTLC (hexane-ethyl acetate, gradient elution) to give [^{14}C]ITdiol (8mg; 83.7KBqmM^{-1}). This represents a specific incorporation of [^{14}C]TDN into ITdiol of 67%. In repeat experiments this figure was closer to 50% [Table 20].

The yields of [^{14}C]ITdiol produced by this method were variable and unpredictable, but this is to be expected when using a biological system which may show significant variations between culture batches. The values for specific incorporation range from 45-67%, and indicate that ITdiol is derived directly from

Table 20: Production of [^{14}C]ITdiol from [^{14}C]TDN in *F. culmorum*.

[^{14}C]TDN fed		Recovered TDN	[^{14}C]ITdiol produced		Incorporation	Specific incorporation
mg	KBqmM^{-1}	mg	mg	KBqmM^{-1}	%	%
280	124	NR	8	83.7	1.5	67
400	173	130	8	79.2	0.7	45
700	1237	450	26	633	1.5	51

$$\% \text{ Incorporation} = \frac{\text{Total activity of ITdiol}}{\text{Total activity added to culture}} \times 100$$

NR = Not Recorded

TDN and not via non-specific incorporation of ^{14}C-labels produced from catabolism of [^{14}C]TDN. Figures for total incorporation are low (0.7-1.5%) and consequently this may seem a relatively inefficient method for producing [^{14}C]ITdiol. However, on each occasion 30-65% of the [^{14}C]TDN fed was recovered from the cultures and could therefore be used again.

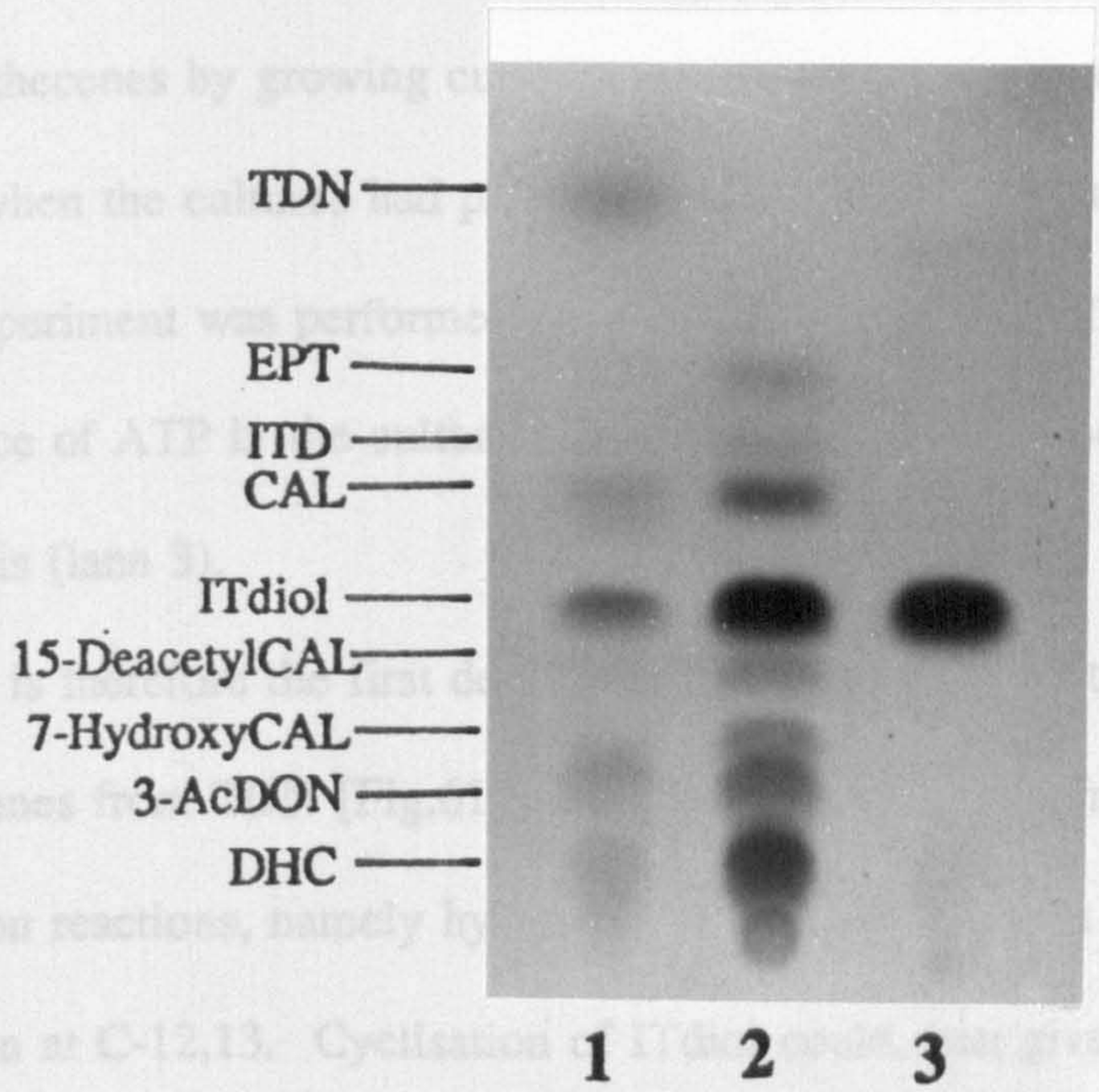
Biotransformations of [^{14}C]ITdiol.

With a good supply of [^{14}C]ITdiol available it was now possible to undertake feeding experiments to *F. culmorum* to establish the role, if any, of ITdiol in the biosynthesis of trichothecenes and trichothecene-related metabolites.

A whole-cell system of *F. culmorum* was prepared from a liquid culture of the fungus (50ml; 42hrs old), and [^{14}C]ITdiol (2.1mg; 0.67KBq in 0.1ml acetone) was fed to it. A similar system, but consisting of mycelia which had been boiled for 10 mins, received [^{14}C]ITdiol as above, and a third received [^{14}C]TDN (2.1mg; 0.67KBq in 0.1ml acetone). The cultures were incubated (27°C, 250rpm) for 6hrs, and then worked up by extracting the filtrates with ethyl acetate. The extracts were analysed by TLC (hexane-ethyl acetate, 1:1) and autoradiography [Fig.59].

The autoradiogram shows that [^{14}C]ITdiol has been transformed into a range of trichothecenes (lane 2) very similar to that obtained for [^{14}C]TDN (lane 1). The boiled control (lane 3) shows no transformation of the substrate indicating that the observed metabolism of [^{14}C]ITdiol in lane 2 is enzymic and not chemical. Further TLC analysis (hexane-ethyl acetate, 1:1, and ether-acetone, 9:1) with comparison to appropriate standards identified the labelled compounds produced from [^{14}C]ITdiol as EPT, ITD, CAL, 7-hydroxyCAL, 15-deacetylCAL, 3-AcDON and DHC. In a larger scale feeding experiment specific incorporations in the range of 31-79%

Fig.59: Incorporation of [^{14}C]ITdiol into the trichothecene toxins of *Fusarium culmorum*.



Lane 1: Fed [^{14}C]TDN
Lane 2: Fed [^{14}C]ITdiol
Lane 3: Fed [^{14}C]ITdiol, boiled mycelia

6hr incubation
TLC: Hexane-EtOAc, 1:1

were recorded for 3-AcDON, DHC and 7-hydroxyCAL [Table 21] indicating ITdiol is indeed a very efficient and direct precursor of these trichothecenes.

In further experiments it was demonstrated that incorporation of [^{14}C]ITdiol into trichothecenes by growing cultures of *F. culmorum* was almost completely inhibited when the cultures had previously been exposed to xanthotoxin, or if the feeding experiment was performed in an N_2 atmosphere [Fig.60]. In the latter case, the presence of ATP in the culture medium did not restore trichothecene biosynthesis (lane 3).

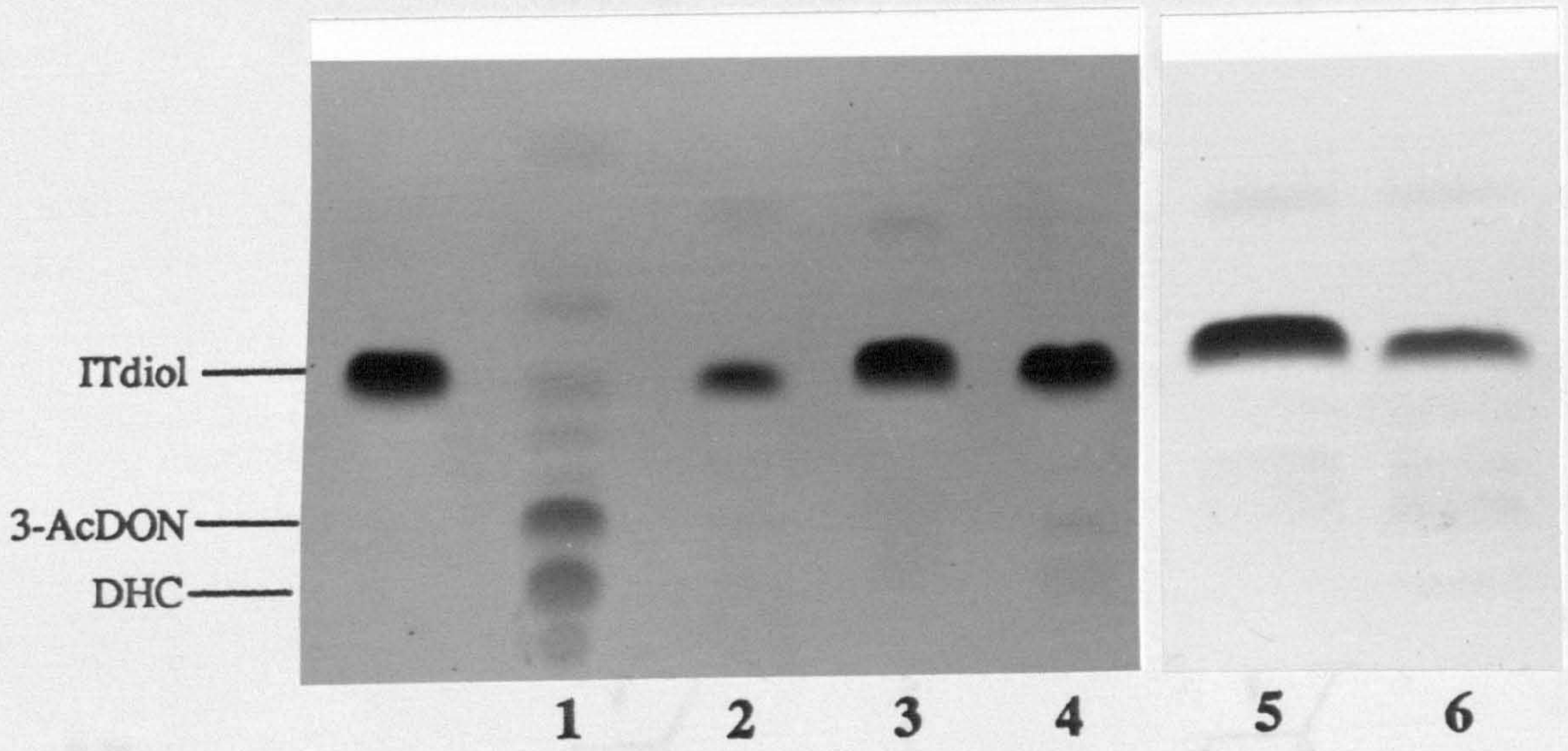
ITdiol is therefore the first demonstrated intermediate in the biosynthesis of trichothecenes from TDN [Fig.61]. It is probably produced from TDN via three oxygenation reactions, namely hydroxylations at positions 2α and 11α, and epoxidation at C-12,13. Cyclisation of ITdiol could then give rise to EPT, or alternatively allylic isomerisation producing trichodiol could be followed by 3α-hydroxylation to trichotriol and cyclisation to isotrichodermol. Sequential oxidation and esterification reactions on either EPT or isotrichodermol could then produce the range of more complex trichothecene structures (see Fig.58). The stereochemistry at position 11 of ITdiol is the opposite to that found in all the trichothecenes, and an inversion of configuration at this centre must therefore take place during the biosynthesis. Several mechanisms for the cyclisation of ITdiol to trichothecenes can be formulated to account for this inversion [Fig.62]. The simplest involves cyclisation to EPT occurring in one step via S_N2 attack of the 2α-hydroxyl onto C-11, accompanied by elimination of the 11α-hydroxyl. Alternatively, cyclisation could involve a carbocation intermediate (90) formed from ITdiol by loss of the C-11 hydroxyl. This carbocation could then cyclise to EPT via an S_N1 mechanism involving attack of the C-2α hydroxyl onto C-11, or

Table 21: Incorporation of [^{14}C]ITdiol (18mg, 52.9KBqmM^{-1}) into trichothecene toxins in *F. culmorum*.

Trichothecene^{+}	mg Isolated	Specific Activity KBqmM^{-1}	Specific Incorporation %
3-AcDON	1.4	41.6	79
DHC	2.6	31.3	59
7-HydroxyCAL	0.6	19.4	31

$^{+}$ Purified to constant specific activity by repeated CTLC and prep.TLC. Radiochemical purity checked by TLC/autoradiography using 3 different solvent systems.

Fig.60: Incorporation of [^{14}C]ITdiol into the trichothecene toxins of *Fusarium culmorum* in the presence of nitrogen and xanthotoxin as inhibitors.



Lane 1: Fed [^{14}C]ITdiol
Lane 2: Fed [^{14}C]ITdiol in the presence of N_2
Lane 3: Fed [^{14}C]ITdiol in the presence of N_2 and ATP
Lane 4: Fed [^{14}C]ITdiol in the presence of xanthotoxin (0.7mM)
Lane 5: Fed [^{14}C]ITdiol in the presence of xanthotoxin (0.4mM)
Lane 6: Fed [^{14}C]ITdiol in the presence of xanthotoxin (0.1mM)

6hr incubation TLC: Hexane-EtOAc, 1:1

Fig.61: ITdiol as an intermediate in the biosynthesis of trichothecenes from TDN.

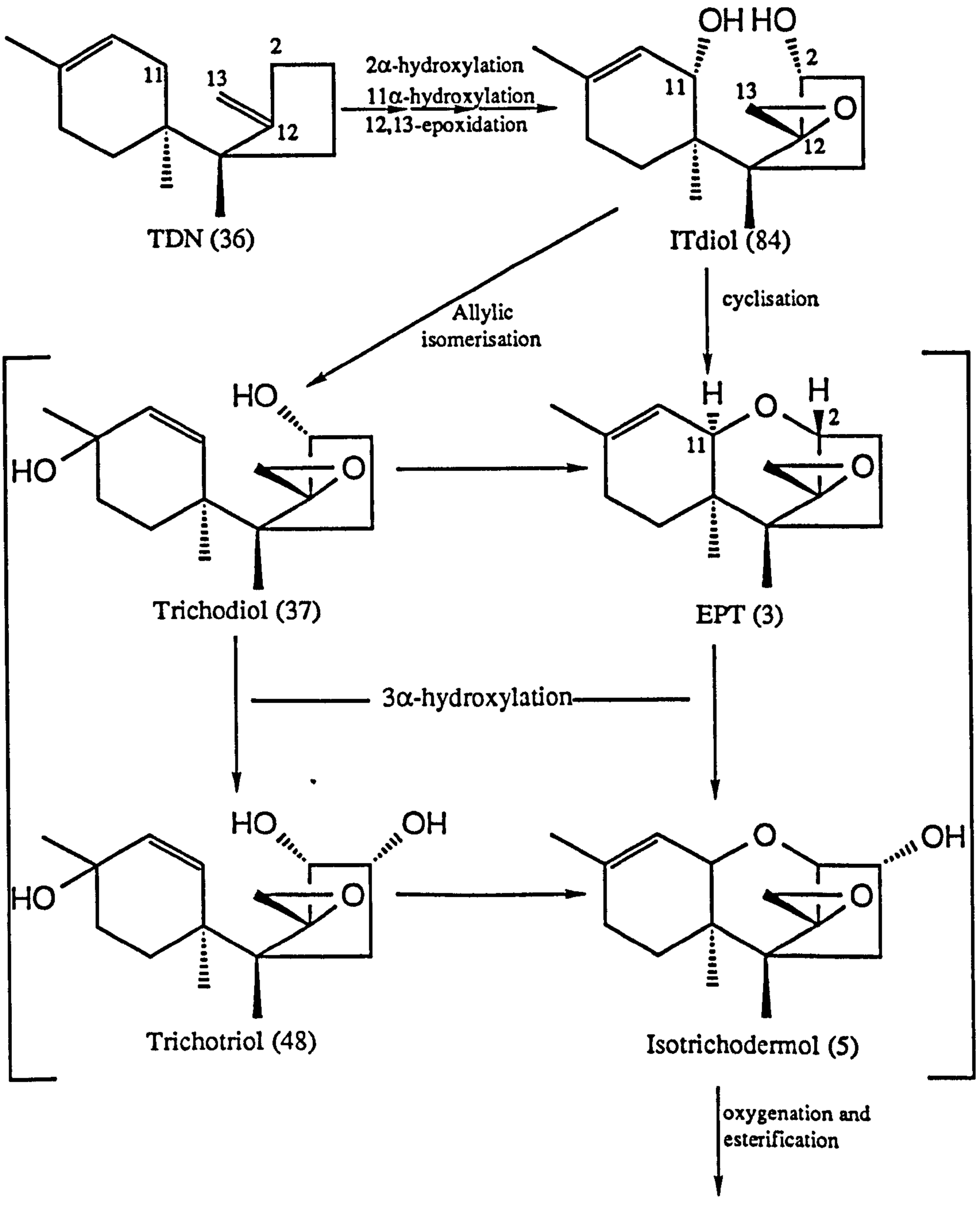
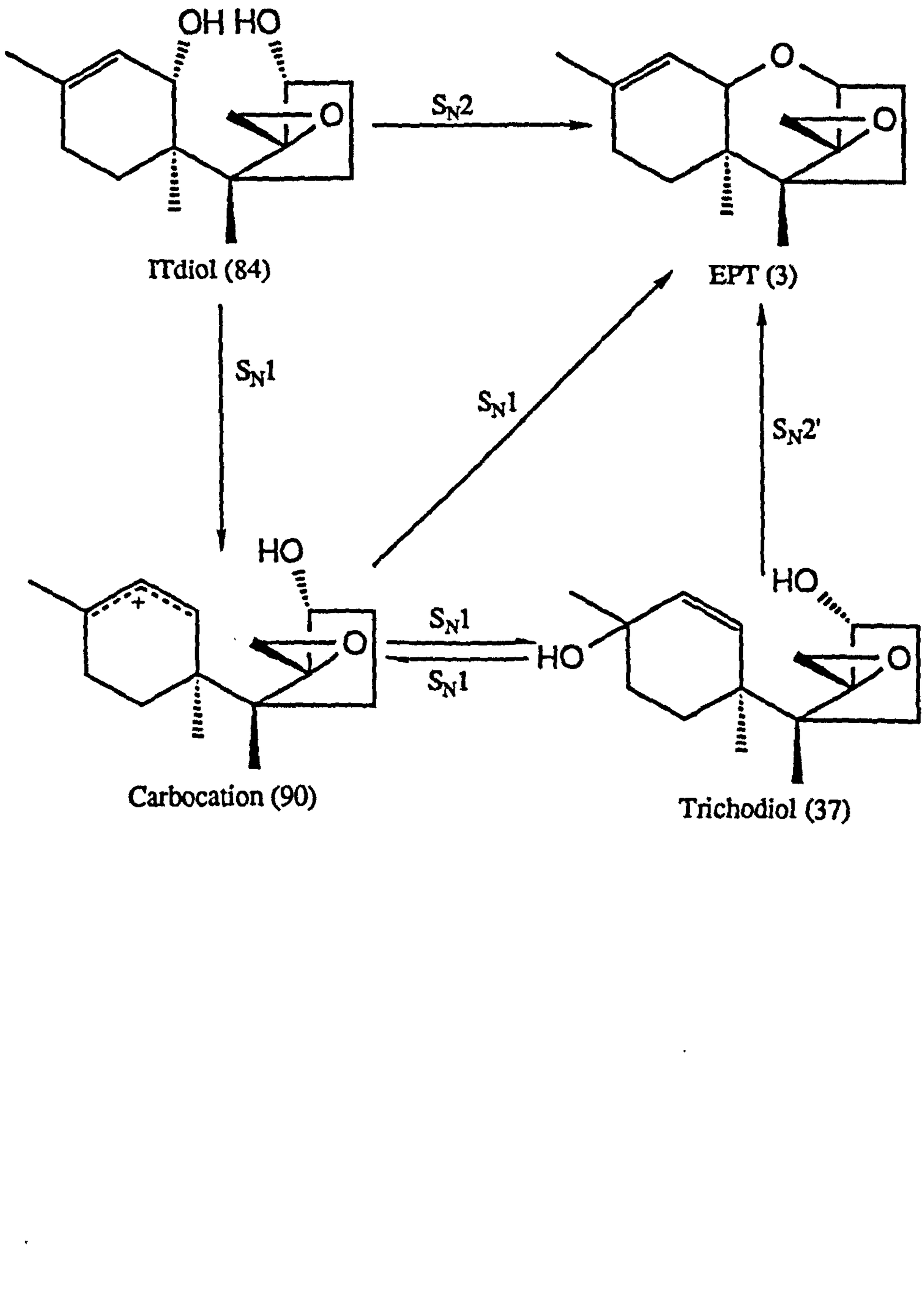


Fig.62: Possible mechanisms for the cyclisation of ITdiol to trichothecenes.

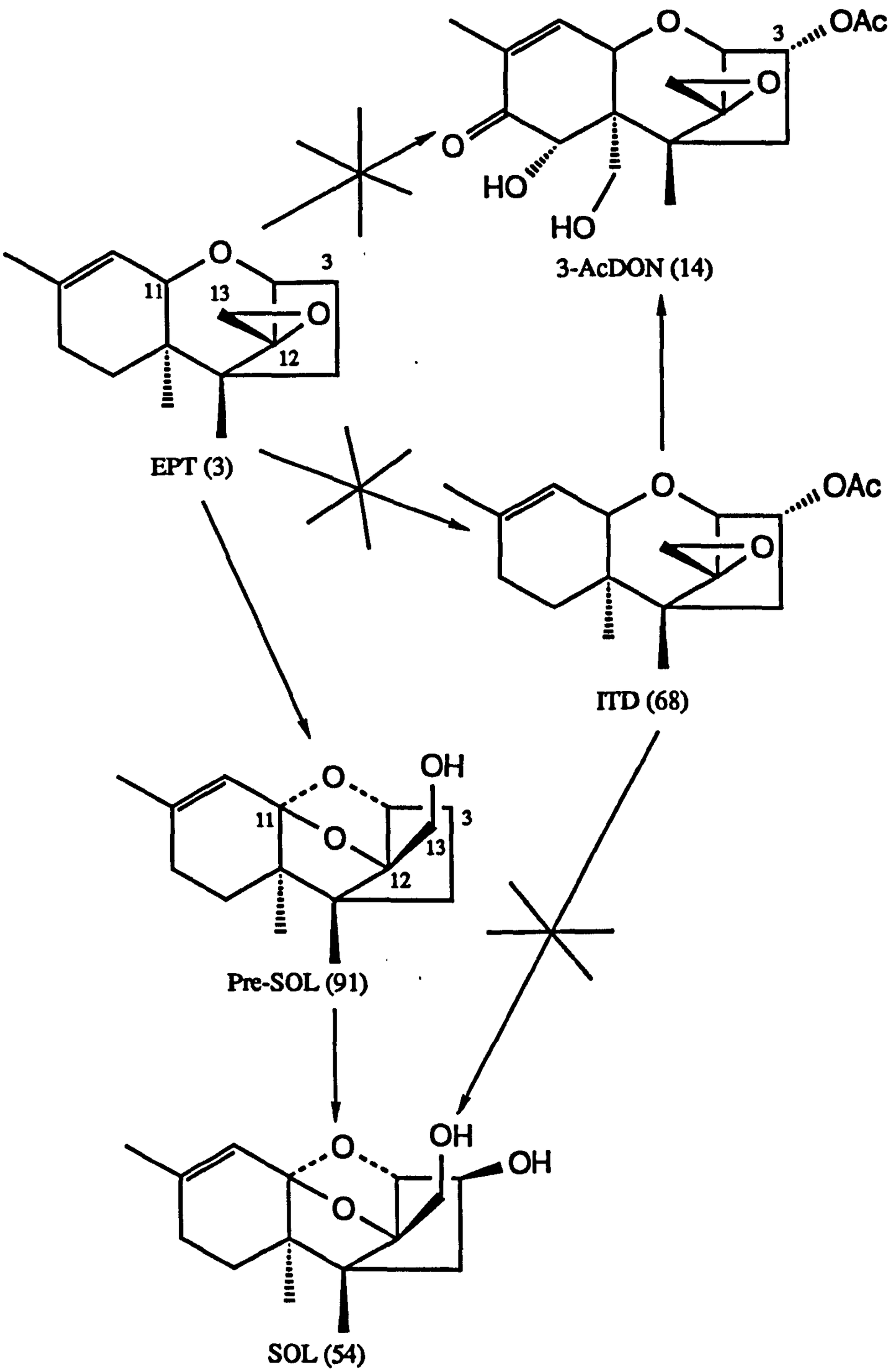


alternatively it could form trichodiol through an S_N1 reaction with H_2O. The trichodiol formed might then cyclise to EPT by a concerted S_N2' type reaction, or via reformation of the carbocation (90). In each mechanism, phosphorylation of the hydroxyl would provide a better leaving group, but there is no evidence to suggest that this is necessary. C-3α hydroxylation of trichodiol would produce trichotriol which could then cyclise to isotrichodermol by mechanisms similar to those proposed for the cyclisation of trichodiol to EPT.

Under conditions inhibiting oxygenation reactions, as produced by using xanthotoxin or N_2, the biotransformation of ITdiol was almost completely blocked. Cyclisation to EPT, either directly or via trichodiol, appears to be non-oxidative and should not therefore be inhibited. Subsequent transformation of EPT does however involve oxygenation, and so would be prevented. A significant conversion of ITdiol to EPT may therefore have been expected, but was not found with either xanthotoxin or N_2 as inhibitors. This suggests that either cyclisation of ITdiol to EPT may not be a major route to the trichothecenes, or, less likely, that it involves an oxygenase enzyme. As expected, biotransformations to trichotriol and isotrichodermol were not observed since these would require 3α-oxygenation reactions to take place.

Zamir *et al*[109] have recently shown that EPT is not in fact a precursor to the 3-oxygenated trichothecenes. Feeding experiments to *Fusarium culmorum* using specifically labelled [^{2}H]- and [^{3}H]-EPT demonstrated that it was not incorporated into either ITD or 3-AcDON but is instead a major precursor to sambucinol (54) (SOL), a trichothecene-related metabolite. An intermediate in the biosynthesis of SOL from EPT was isolated, and identified as 3-deoxysambucinol (91) (named pre-SOL) [Fig.63]. It was proposed that EPT could give rise to pre-SOL by

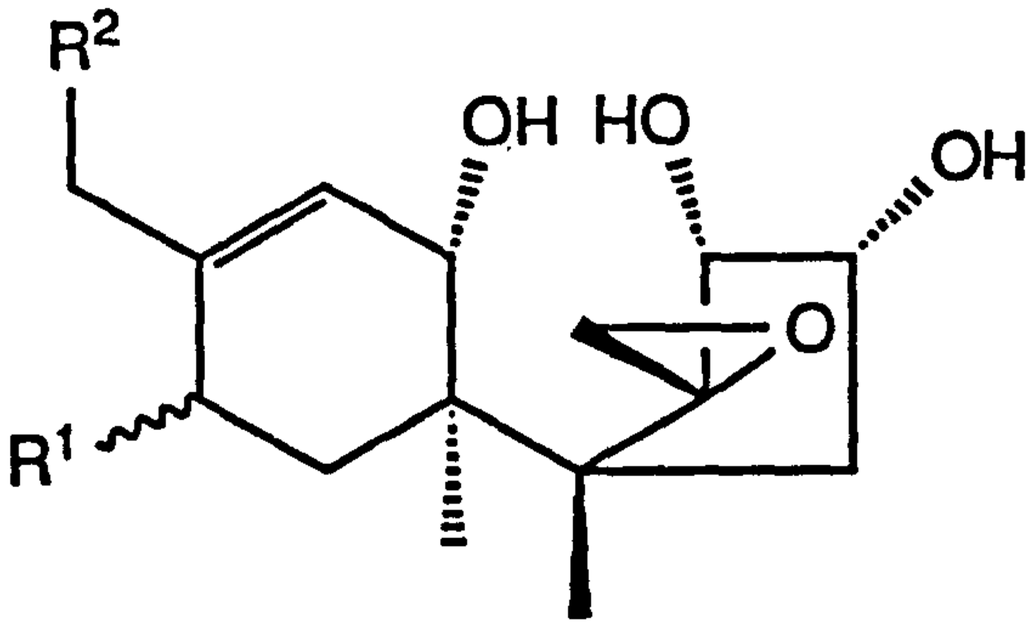
Fig.63: Biosynthetic relationship of EPT, ITD, SOL and 3-AcDON as demonstrated by Zamir *et al*[109].



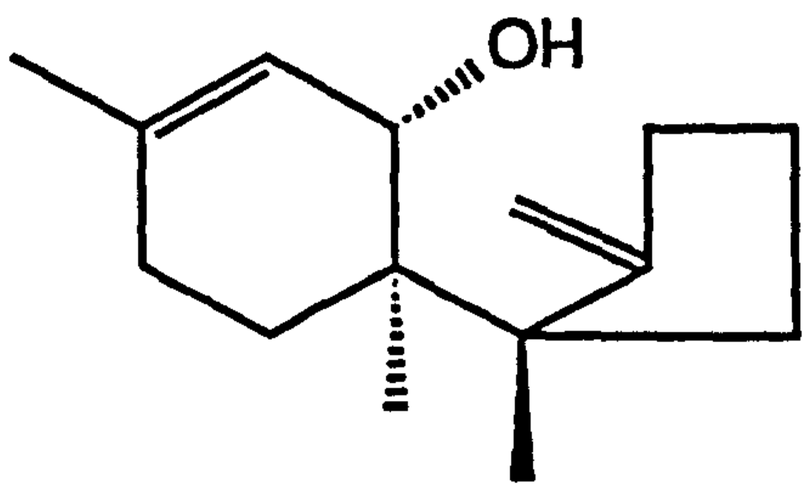
hydroxylation at C-11 with inversion of configuration, and subsequent attack of this hydroxyl onto C-12 of the epoxide group. Hydroxylation of pre-SOL in the C-3β position then gives rise to SOL. The same group in similar work demonstrated that ITD is a precursor to 3-AcDON but not to SOL.

McCormick *et al*[110] have also demonstrated the involvement of ITD in trichothecene biosynthesis. They developed mutant strains of *Fusarium sporotrichioides* all blocked in their ability to synthesise T-2 toxin (the major trichothecene produced by this fungus), but which accumulated a variety of uncyclised trichothecenes with structures closely related to that of ITdiol [Fig.64]. Feeding experiments using unlabelled material showed that one mutant (MB 5493), which accumulates TDN and 11α-hydroxyTDN (92), was able to use ITD to resume biosynthesis of T-2 toxin. Isotrichodermol (5), isotrichotriol (85), and both 9α- and 9β-trichotriol also caused a resumption of T-2 toxin production, although other compounds such as EPT, trichodiol, and the uncyclised trichothecenes with hydroxyls at C-8 or C-16 did not [Table 22].

From all this information a revised biosynthetic scheme can be proposed [Fig.65] in which ITdiol is a common intermediate in the biosynthesis of both SOL, and oxygenated trichothecenes such as 3-AcDON from TDN. This scheme suggests that the first biosynthetic step in the sequence is allylic hydroxylation of TDN at position 11 giving 11α-hydroxyTDN (92). ITdiol is then produced by hydroxylation at C-2α and epoxidation at C-12,13, in an order yet to be determined. Cyclisation of ITdiol, perhaps via allylic isomerisation to trichodiol, can then give rise to EPT which is a precursor to SOL. Alternatively, 3α-hydroxylation of ITdiol yields isotrichotriol which can give rise to the oxygenated trichothecenes by cyclisation to isotrichodermol, perhaps via trichotriol.



R^1	R^2	
H	H	Tricho-9-ene-2α,3α,11α-triol (Isotrichotriol) (85)
αOH	H	Tricho-9-ene–2α,3α,8α,11α-tetraol (93)
βOH	H	Tricho-9-ene-2α,3α,8β,11α-tetraol (94)
H	OH	Tricho-9-ene-2α,3α,11α,16-tetraol (95)



11α-Hydroxytrichodiene (92)

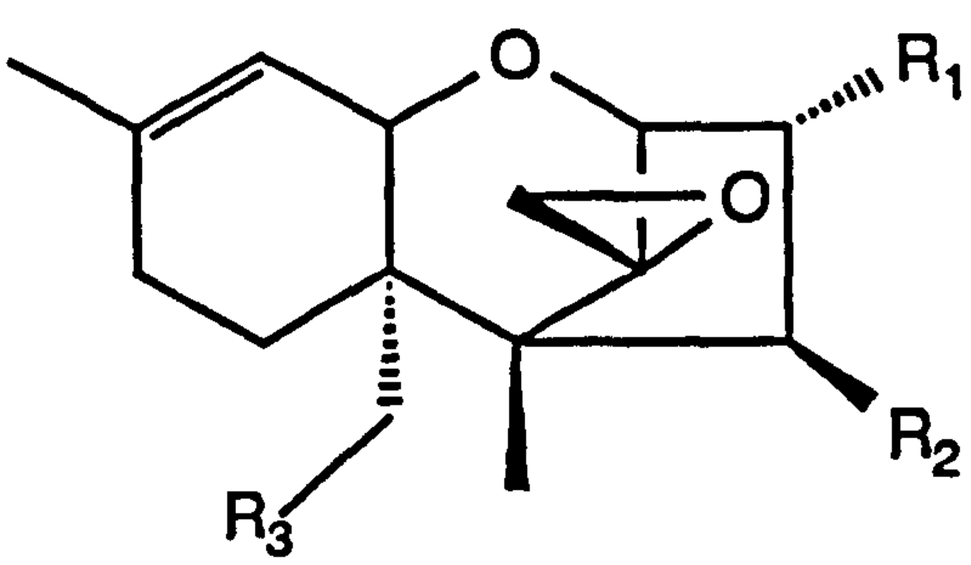
Fig.64: Uncyclised trichothecenes isolated from mutant strains of *Fusarium sporotrichioides*[61].

Table 22: Results from feeding possible T-2 toxin precursors to *F. sporotrichioides* MB 5493[110].

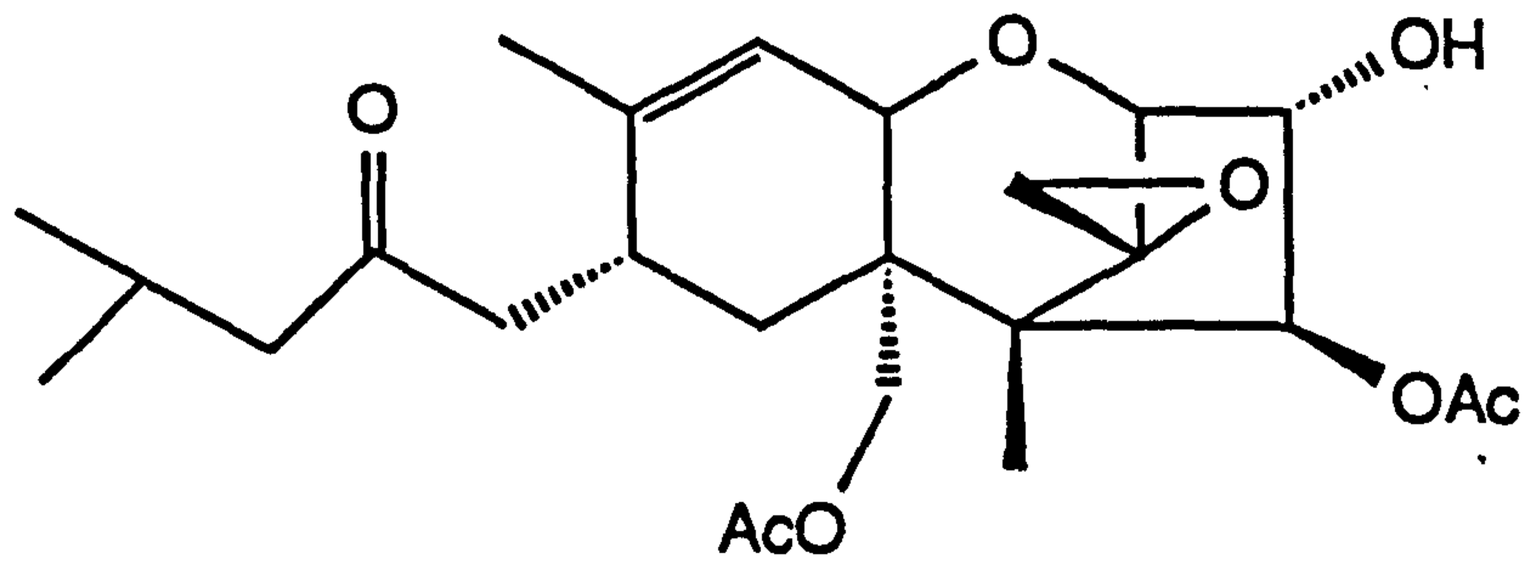
Compound fed	Appearance of T-2 toxin*
EPT	-
Trichodiol	-
ITD	+
Isotrichodermol	+
Trichodermin	-
Trichodermol	-
15-HydroxyEPT	-
15-AcetoxyEPT	-
Isotrichotriol	+
Tricho-9-ene-2α,3α,8α,11α-tetraol	-
Tricho-9-ene-2α,3α,8β,11α-tetraol	-
Tricho-9-ene-2α,3α,11α,16-tetraol	-
9α-Trichotriol	+
9β-Trichotriol	+

*+ = 30-60% conversion to T-2 toxin

- = T-2 toxin not detected



	R_1	R_2	R_3
Isotrichodermol	OH	H	H
ITD	OAc	H	H
Trichodermol	H	OH	H
Trichodermin	H	OAc	H
15-HydroxyEPT	H	H	OH
15-AcetoxyEPT	H	H	OAc



T-2 toxin (11)

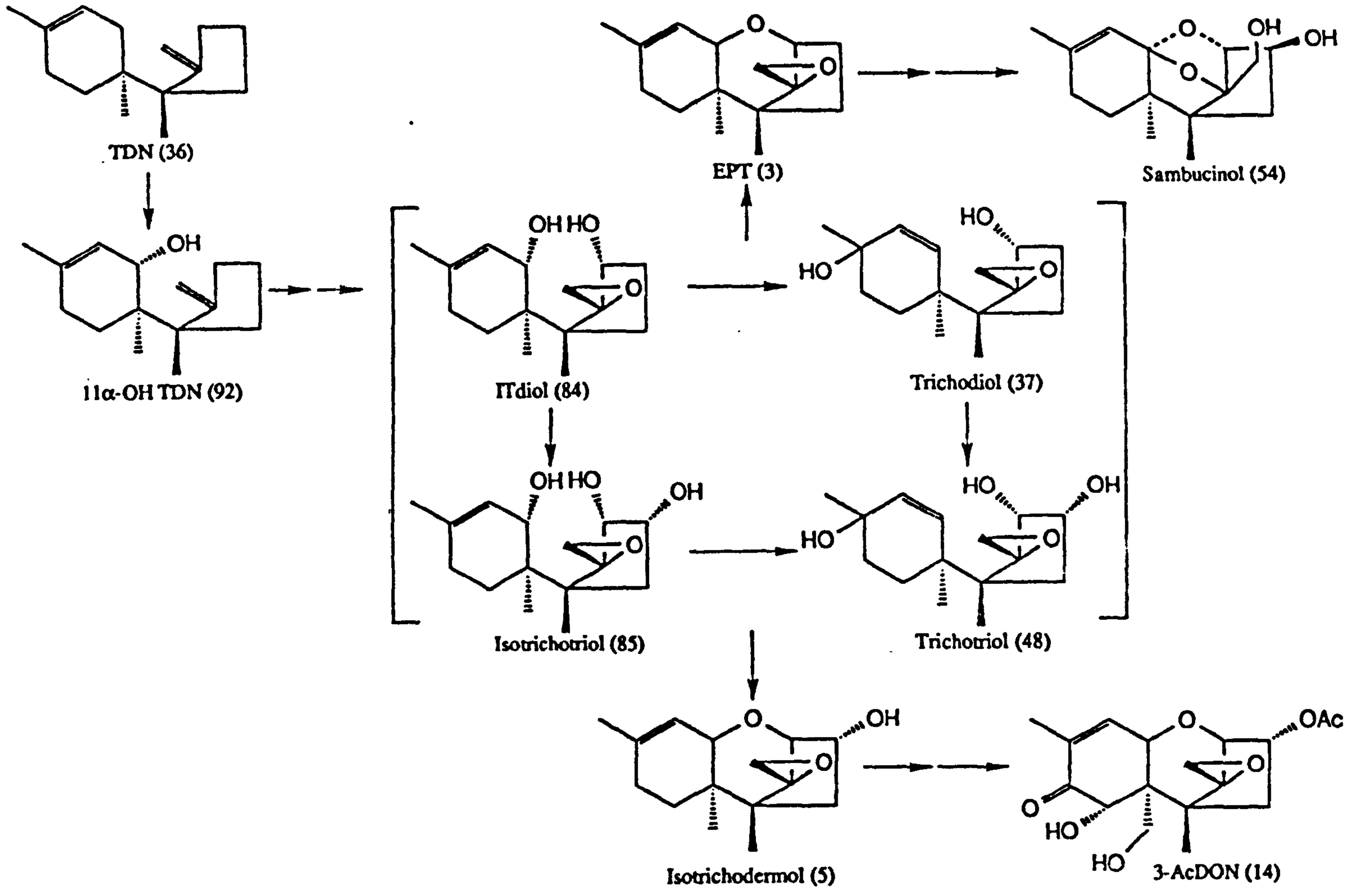


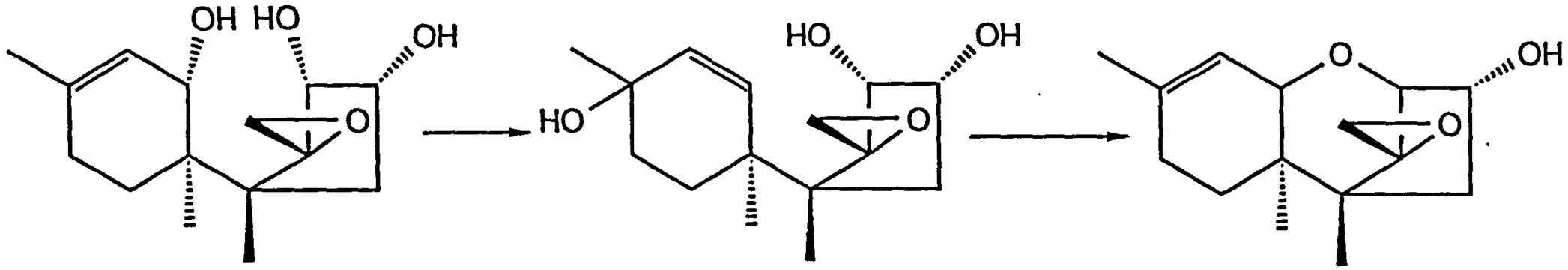
Fig.65: Proposed biosynthesis of trichothecenes and sambucinol from TDN with ITdiol as a common intermediate.

McCormick *et al*[110] have demonstrated that isotrichotriol (85) can be converted to both trichotriol (48) and isotrichodermol (5) under acid catalysed conditions [Fig.66]. At pH 5.7 to 6.5 only trichotriol was produced whilst at lower pH's (4.7 to 5.2) isotrichodermol was also formed. At pH 4.1 all the isotrichotriol was converted to isotrichodermol. These results support the proposed biosynthesis of isotrichodermol from isotrichotriol via trichotriol, and since the pH in fungal cultures typically drops to ≈4.5, it was suggested that this cyclisation may be non-enzymatic. However, the rates of the chemical transformations are slow, taking up to 7 days for complete conversion of isotrichotriol to isotrichodermol, and so in the fungus this part of the pathway is likely to be controlled by appropriate enzymes.

<u>Biotransformations of 9β,10β-epoxyTDN and 9β,10β;12,13-diepoxyTDN.</u>

From the results of the competitive feeding experiments in which TDN analogues were screened for activity as inhibitors of post-TDN biosynthesis (see Table 15), it was evident that both 9β,10β;12,13-diepoxyTDN (72) and 9β,10β-epoxyTDN (73) were being transformed by *Fusarium culmorum* into one or two major metabolites respectively. Identification of these products might provide useful information since the enzymes responsible could be those involved in the biosynthesis of trichothecenes from TDN.

^{14}C-Labelled samples of the two epoxide derivatives (72) and (73) were prepared by semi-synthesis from [^{14}C]TDN (see p. 67 and 80). Samples of these (0.25mg; 3.92MBq) were fed to whole-cell systems of *F. culmorum* prepared from a 46hr old culture. Controls in which the mycelia had been boiled for 10 mins (and then cooled) prior to receiving the labelled substrates were also performed. The cultures were incubated for 6hrs, and then harvested by extraction of the



Isotrichotriol (85) Trichotriol (48) Isotrichodermol (5)

pH 7.3	100%		
pH 5.7	82%	17%	
pH 5.2	62%	18%	20%
pH 4.7	25%	20%	55%
pH 4.1	0%	0%	100%

(Quantities determined by GG-MS of derivatised extracts)

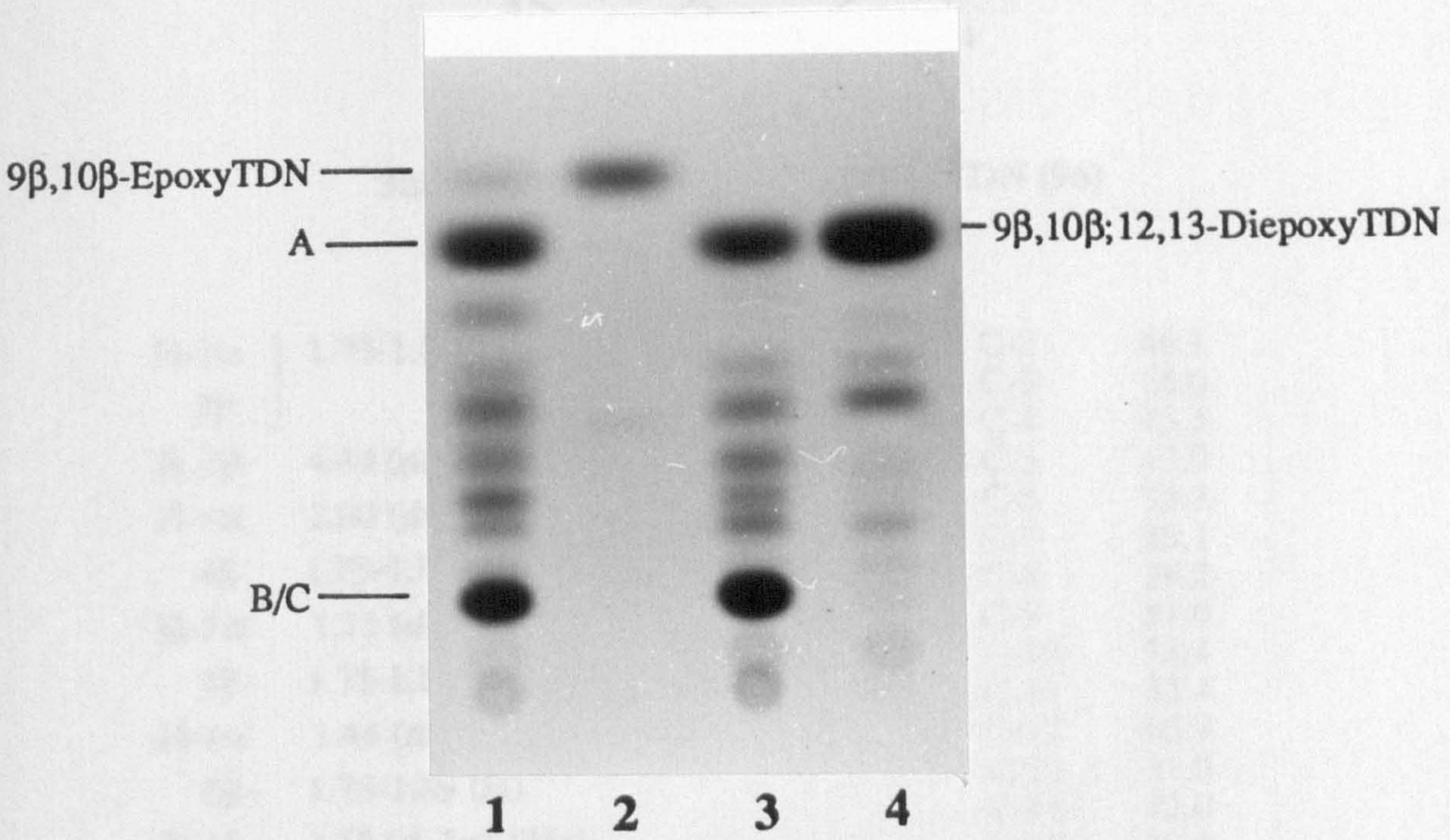
Fig.66: Acid catalysed rearrangement of isotrichotriol at a variety of pH's (7 day incubation)[110].

filtrate with ethyl acetate. Extracts were analysed by TLC (hexane-ethyl acetate, 1:1) and autoradiography [Fig.67], with scintillation counting being used to measure product formation . Lane 1 shows that the 9,10-epoxide (73) has been converted into two more polar, major products (A:7.3% and B:7.9%) plus several minor compounds. No transformations were observed in the boiled control (lane 2) indicating that this process is therefore enzymic and not chemical. Similarly, the diepoxide (72) (lane 3) has been biotransformed to one more polar, major compound (C:11.4%) which was not produced in the boiled control (lane 4). Rf values suggested that A was identical to (72), and that B and C were the same compound. In order to obtain samples of these compounds for analysis these feeding experiments were repeated on a larger scale using unlabelled substrate. Thus, compounds A (2mg; 4.6%) and B (3.2mg; 6.9%) were isolated and purified from a whole-cell *F. culmorum* system which had been fed the 9,10-epoxide (73) (40mg), and compound C (8mg;18.7%) from a similar experiment in which the diepoxide (72) (40mg) was used.

Compound A was identified as the diepoxide (72) by ^{1}H NMR and EI-MS analysis which produced data in agreement with that for the semi-synthetic standard. ^{1}H NMR analysis of compounds B and C indicated that they were indeed the same compound, and were identified as 3α-hydroxy-9β,10β;12,13-diepoxyTDN (96) by a combination of EI-MS, ^{1}H and ^{13}C NMR techniques.

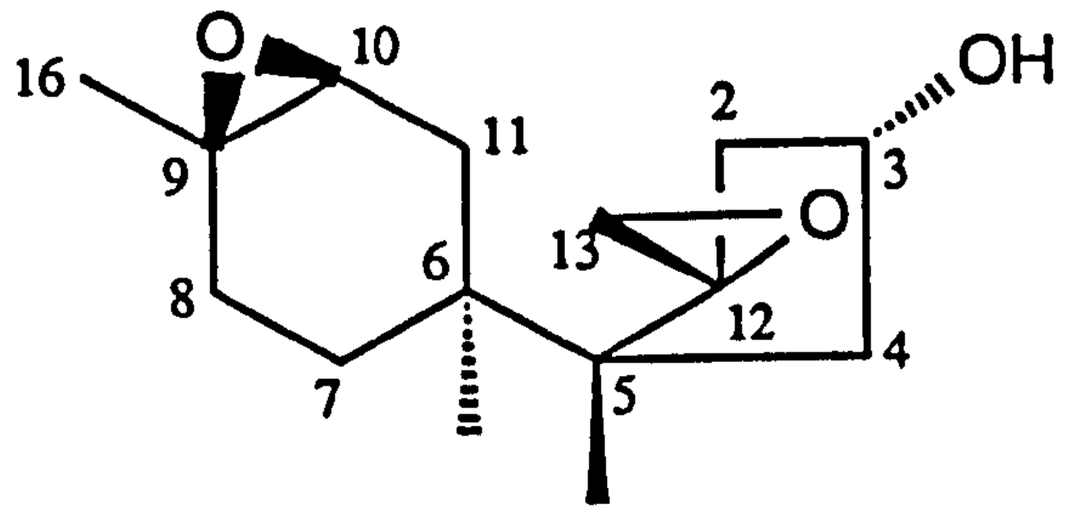
The ^{1}H NMR spectrum [Table 23, Fig.68] is similar to that for (72) (see Table 10, Fig.35) except for the downfield signal at δ4.44. This indicates the presence of a hydroxyl group which can be readily assigned to position 3α by the complexity of the signal. It is a doublet of double double doublets which is only possible for a proton at C-3, and the size of the coupling constants (J=8.9, 8.9, 6.9, 6.9Hz)

Fig.67: Biotransformations of 9β,10β-epoxyTDN and 9β,10β;12,13-diepoxyTDN in *Fusarium culmorum* whole-cells.



Lane 1: Fed [^{14}C]-9β,10β-epoxyTDN
Lane 2: Fed [^{14}C]-9β,10β-epoxyTDN, boiled mycelia
Lane 3: Fed [^{14}C]-9β,10β;12,13-diepoxyTDN
Lane 4: Fed [^{14}C]-9β,10β;12,13-diepoxyTDN, boiled mycelia

6hr incubation
TLC: Hexane-EtOAc, 1:1

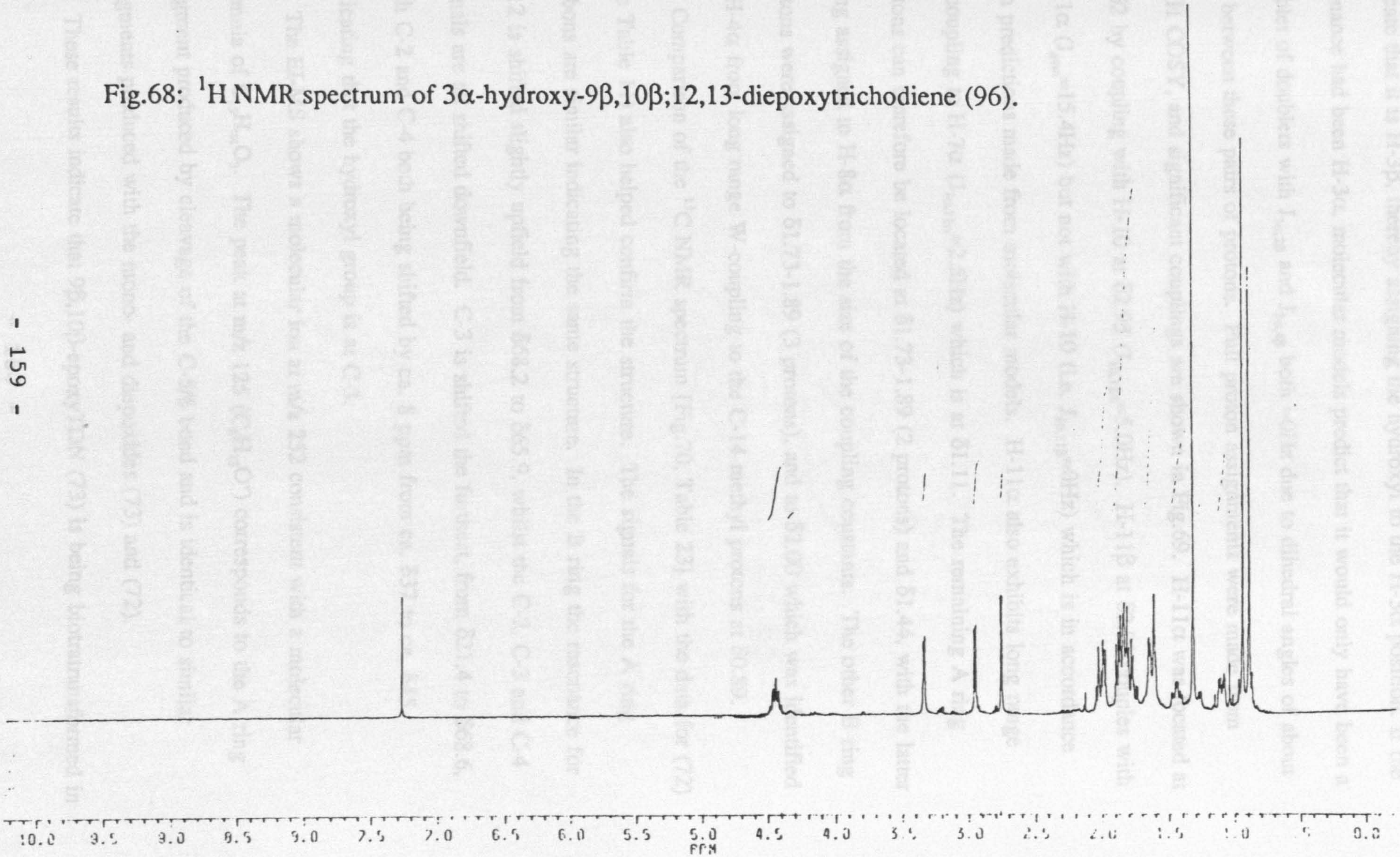


3α-Hydroxy-9β,10β;12,13-diepoxyTDN (96)

H-2α 2β	1.73-1.89 (2H, *m*)	C-2	46.1
		C-3	68.6
H-3β	4.44 (*dddd*, J=8.9, 8.9, 6.9, 6.9Hz)	C-4	45.5
H-4α	2.00 (*dd*, J=13.3, 9.0Hz)	C-5	47.9
		C-6	35.3
4β	1.73-1.89 (*m*)	C-7	25.1
H-7α	1.11 (*ddd*, J=13.2, 5.0, 2.5Hz)	C-8	26.2
		C-9	57.0
7β	1.73-1.89 (*m*)	C-10	58.4
H-8α	1.44 (*td*, 12.5, 5.4Hz)	C-11	32.4
		C-12	65.9
8β	1.73-1.89 (*m*)	C-13	51.0
H-10	2.95 (*d*, J=5.0Hz)	C-14	22.0
		C-15	19.4
H-11α	1.62 (*ddd*, J=15.4, 5.1, 2.5Hz)	C-16	22.9
11β	2.00 (*d*, J=15.4Hz)		
H-13	2.75 (*d*, J=4.2Hz)		
	3.33 (*d*, J=4.2Hz)		
H-14	0.89 (*s*)		
H-15	0.94 (*s*)		
H-16	1.32 (*s*)		

Table 23: NMR data for 3α-Hydroxy-9β,10β;12,13-DiepoxyTDN (96).

Fig.68: ^{1}H NMR spectrum of 3α-hydroxy-9β,10β;12,13-diepoxytrichodiene (96).



indicate that it is H-3β, thereby assigning the hydroxyl to the H-3α position. If the resonance had been H-3α, molecular models predict that it would only have been a doublet of doublets with $J_{3\alpha,2\beta}$ and $J_{3\alpha,4\beta}$ both ≈0Hz due to dihedral angles of about 90° between these pairs of protons. Full proton assignments were made from $^1H/^1H$ COSY, and significant couplings are shown in Fig.69. H-11α was located at δ1.62 by coupling with H-10 at δ2.95 ($J_{10,11\alpha}$=5.0Hz). H-11β at δ2.00 couples with H-11α (J_{gem}=15.4Hz) but not with H-10 (i.e. $J_{10,11\beta}$≈0Hz) which is in accordance with predictions made from molecular models. H-11α also exhibits long range W-coupling to H-7α ($J_{7\alpha,11\alpha}$=2.5Hz) which is at δ1.11. The remaining A ring protons can therefore be located at δ1.73-1.89 (2 protons) and δ1.44, with the latter being assigned to H-8α from the size of the coupling constants. The other B ring protons were assigned to δ1.73-1.89 (3 protons), and to δ2.00 which was identified as H-4α from long range W-coupling to the C-14 methyl protons at δ0.89.

Comparison of the ^{13}C NMR spectrum [Fig.70, Table 23] with the data for (72) (see Table 11) also helped confirm the structure. The signals for the A ring carbons are similar indicating the same structure. In the B ring the resonance for C-12 is shifted slightly upfield from δ68.2 to δ65.9, whilst the C-2, C-3 and C-4 signals are all shifted downfield. C-3 is shifted the furthest, from δ21.4 to δ68.6, with C-2 and C-4 both being shifted by ca. 8 ppm from ca. δ37 to ca. δ45 indicating that the hydroxyl group is at C-3.

The EI-MS shows a molecular ion at m/z 252 consistent with a molecular formula of $C_{15}H_{24}O_3$. The peak at m/z 125 ($C_8H_{13}O^+$) corresponds to the A ring fragment produced by cleavage of the C-5/6 bond and is identical to similar fragments produced with the mono- and diepoxides (73) and (72).

These results indicate that 9β,10β-epoxyTDN (73) is being biotratransformed in

Fig.69: Significant couplings observed in (96) from $^{1}H/^{1}H$ COSY.

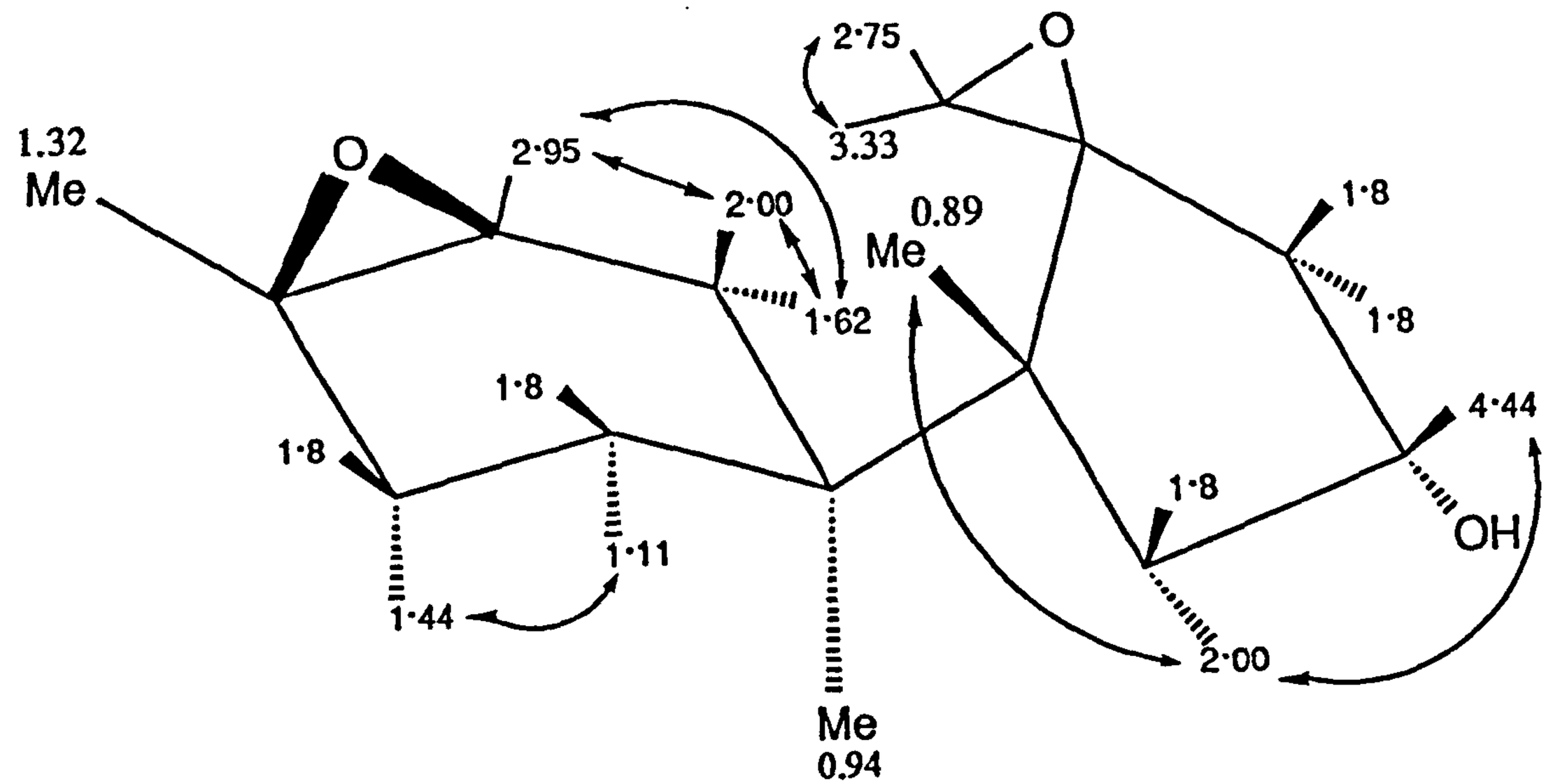
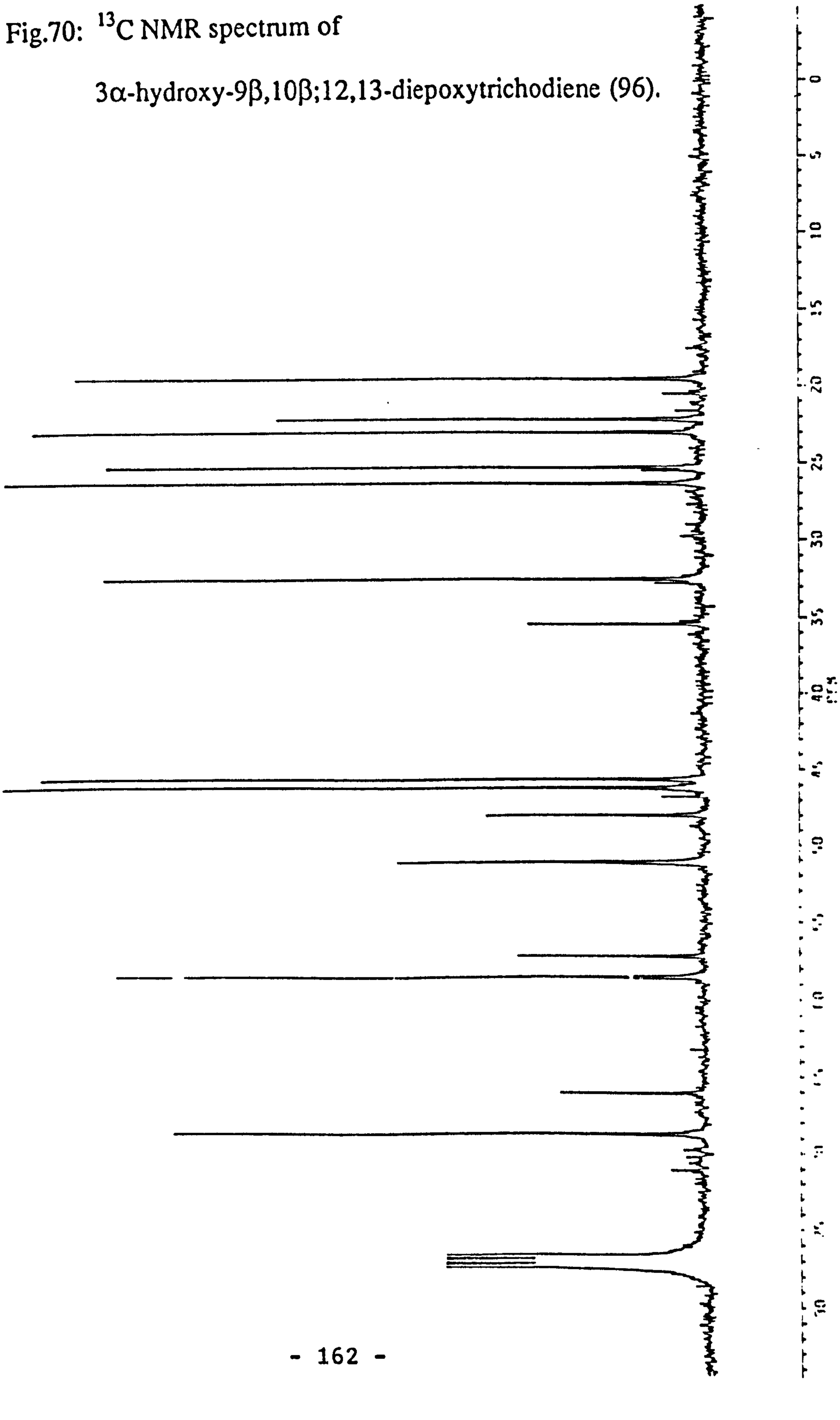


Fig.70: ^{13}C NMR spectrum of

3α-hydroxy-9β,10β;12,13-diepoxytrichodiene (96).

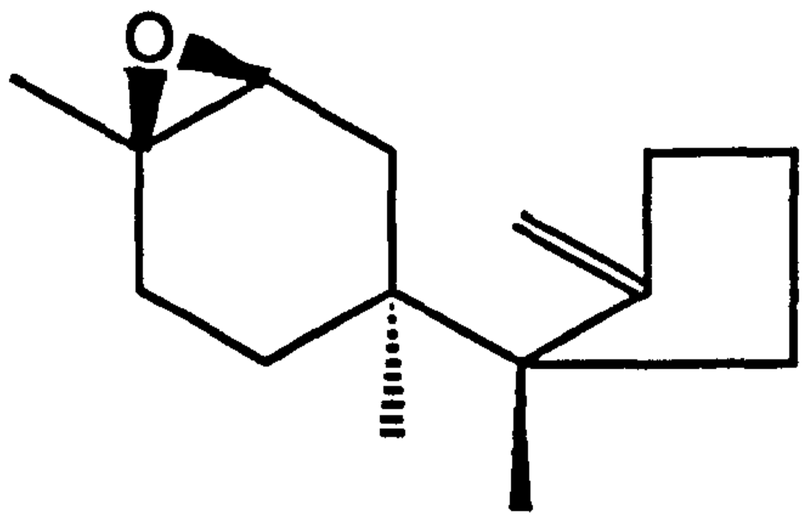


F. culmorum by two sequential oxygenation reactions on the B ring [Fig.71]. It is first converted to the diepoxide (72), demonstrating for the first time the single-step enzymic epoxidation at C-12,13, and then it undergoes hydroxylation at C-3α producing (96). The epoxidase activity is likely to be that involved in the biosynthesis of trichothecenes from TDN, and this is important since it is the 12,13-epoxide group which is responsible for the cytotoxicity of the trichothecenes. A cell-free system from *F. culmorum* capable of transforming (73) to (72) has subsequently been prepared, and it has been demonstrated that the epoxidase requires NADPH and molecular oxygen, and that it is inhibited by carbon monoxide[111]. It thus appears to be a cytochrome P-450 mono-oxygenase system. Significantly, TDN itselfwas not epoxidised. A project aimed at isolating this enzyme is underway.

In the biosynthesis of trichothecenes (see Fig.65) epoxidation at C-12,13 must occur prior to the formation of ITdiol, and the natural substrate is therefore likely to be either 11α-hydroxyTDN (92) or 2α,11α-dihydroxyTDN (97) [Fig.72]. The unnatural substrate, 9β,10β-epoxyTDN, has substitution in ring A but not in ring B, and so 11α-hydroxyTDN is likely to be the natural substrate. This suggests that in the biosynthesis of ITdiol from TDN the oxygenation sequence is 11α-hydroxylation, epoxidation at C-12,13, then 2α-hydroxylation.

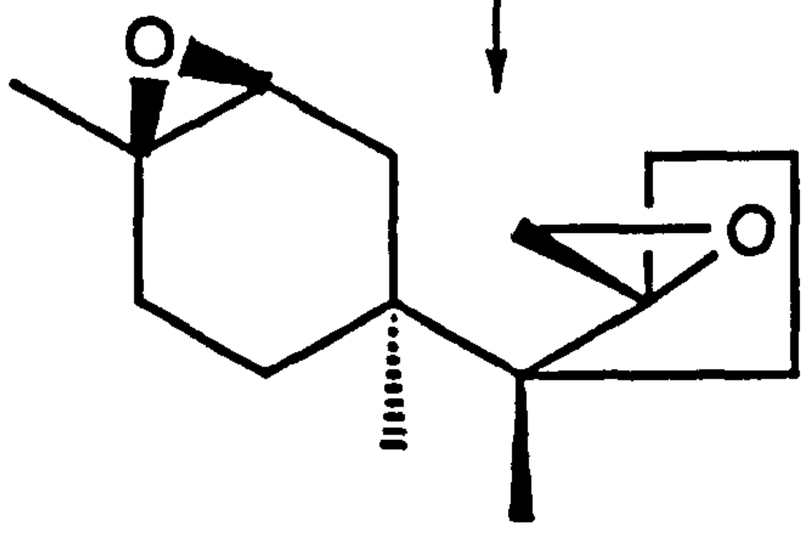
2α-Hydroxylation of 9β,10β-epoxyTDN was not observed which may be because the unnatural substitution in ring A does not allow the 2-hydroxylase to function. 3α-Hydroxylation, however, does occur (producing 3α-hydroxy-9β,10β;12,13-diepoxyTDN) indicating that the 3α-hydroxylase is less restricted. This 3α-hydroxylation reaction is also important because it is proposed that in the biosynthesis of trichothecenes ITdiol/trichodiol are converted to

Fig.71: Biotransformations of 9β,10β-epoxyTDN and 9β,10β;12,13-diepoxyTDN in *F. culmorum*.



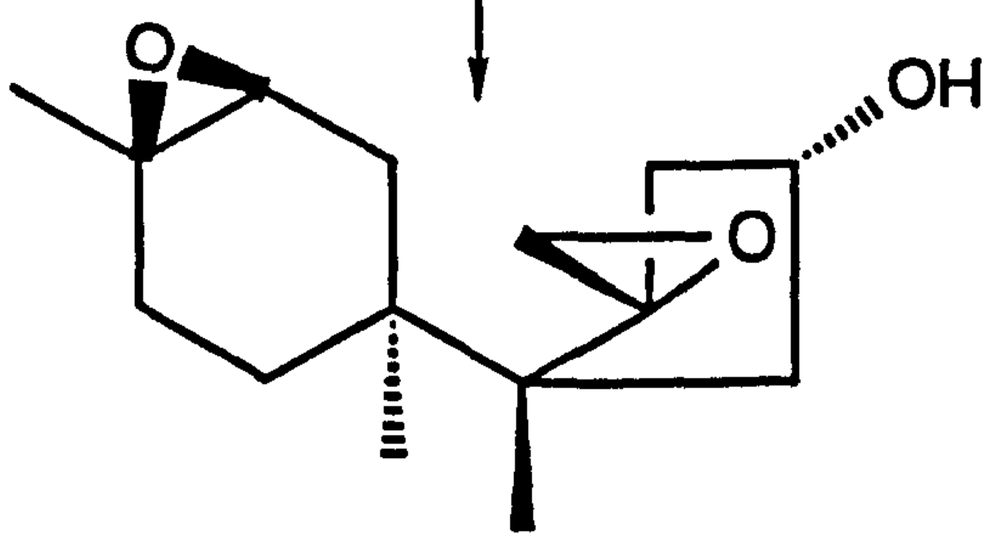
9β,10β-EpoxyTDN (73)

A



9β,10β;12,13-DiepoxyTDN (72)

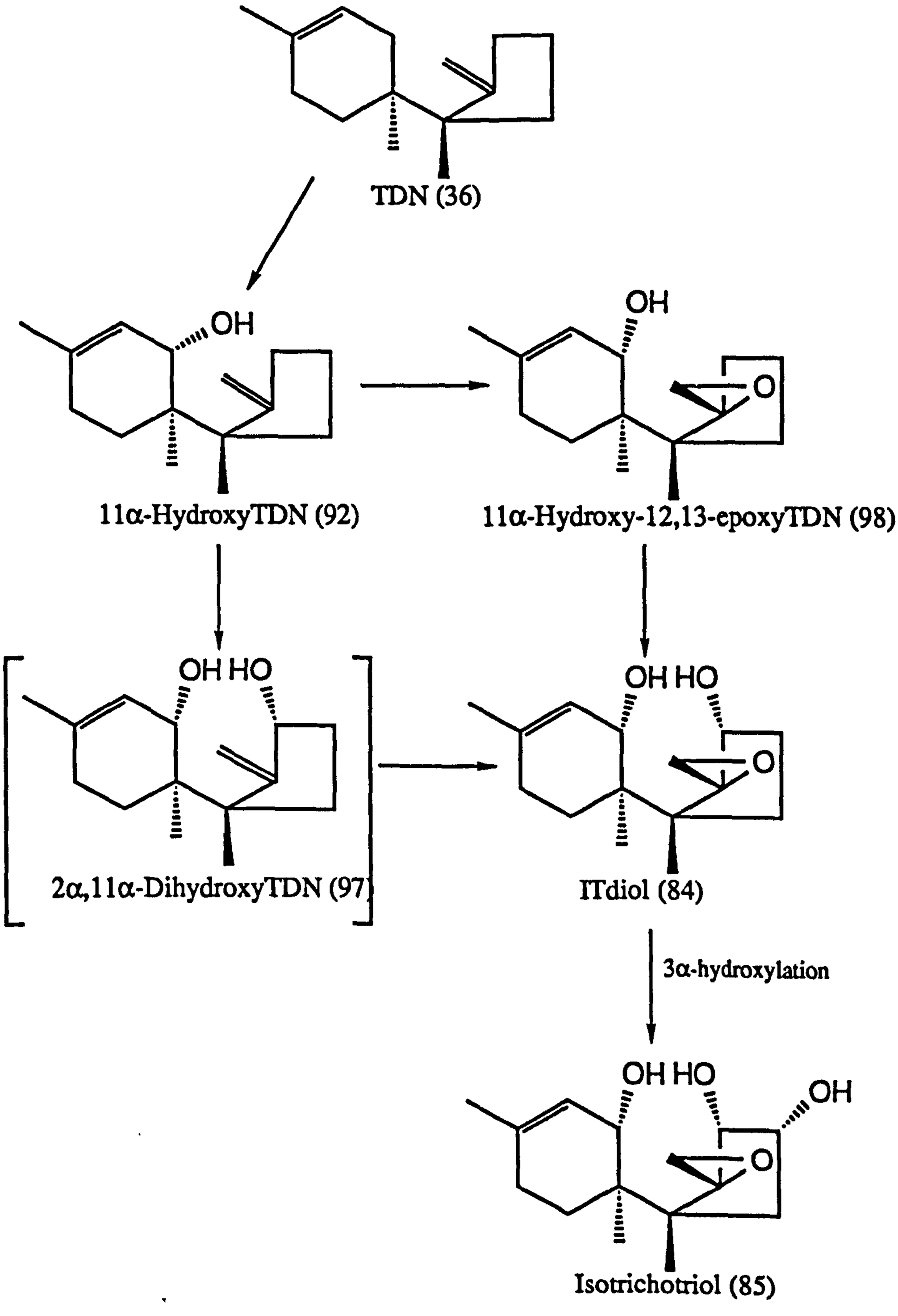
B/C



3α-Hydroxy-9β,10β;12,13-diepoxyTDN (96)

Substrate	Product (% yield)	
	A: Diepoxide (72)	B/C: 3α-OH-Diepoxide (96)
9β,10β-EpoxyTDN	4.6	6.9
9β,10β;12,13-DiepoxyTDN	-	18.7

Fig.72: Proposed biosynthesis of ITdiol from 11α-hydroxyTDN and its subsequent conversion to isotrichotriol.

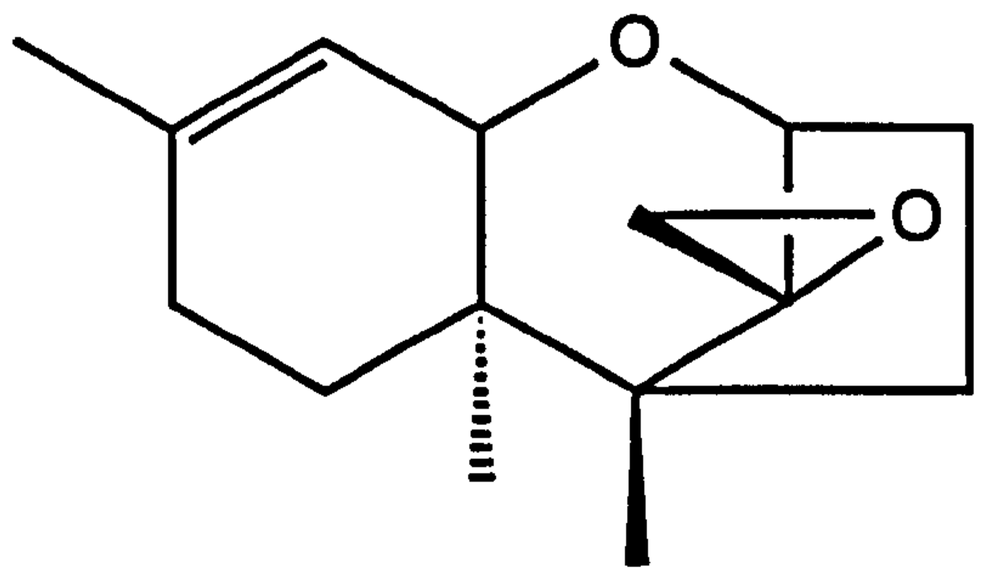


isotrichotriol/trichotriol by hydroxylation at this position. If cyclisation occurs before 3α-hydroxylation, then EPT (3) is produced which is a precursor to SOL (54) and not to the oxygenated trichothecenes (see Fig.65).

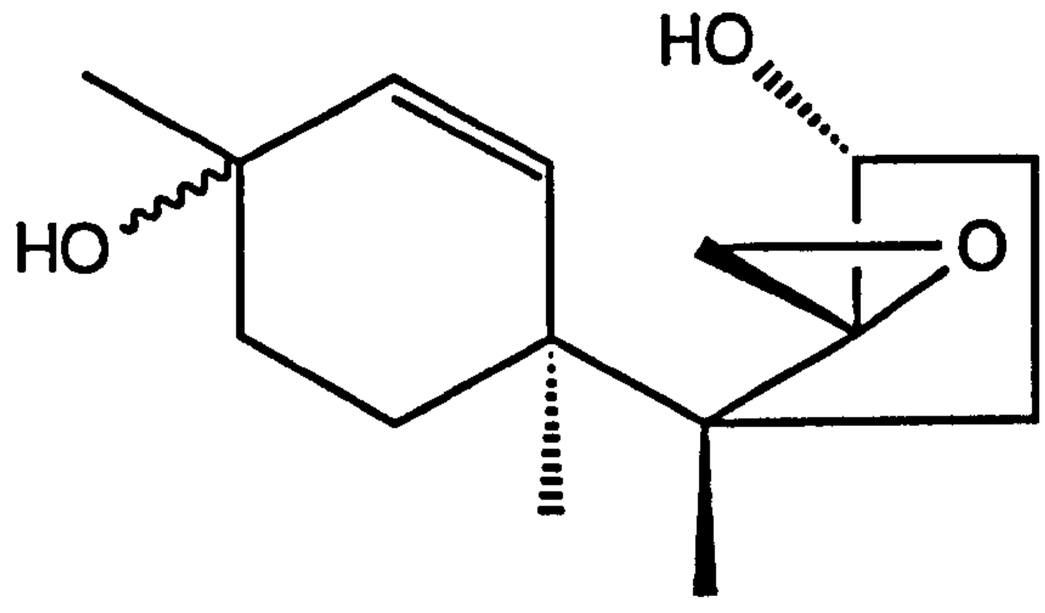
Acid catalysed rearrangements of ITdiol.

McCormick *et al*[110] have demonstrated that in acidic conditions isotrichotriol undergoes isomerisation to trichotriol, and cyclisation to isotrichodermol (see Fig.66). Under similar conditions, ITdiol might therefore rearrange to produce trichodiol and EPT.

Accordingly, ITdiol (7.7mg) was dissolved in sterile culture medium (YEPD-5G) as used by McCormick, adjusted to pH 4.5 with dilute HCl (aq), and, after incubating (28°C, 250rpm) for 3 days, the solution was extracted with ethyl acetate. TLC analysis showed that all the starting material had been consumed, and that three products had been formed. Purification by preparative TLC afforded EPT (3) (2.5mg;35%) in the form of a viscous colourless oil spectrally identical to the natural material (see Table 18). The non-enzymic cyclisation of ITdiol to EPT has therefore been demonstrated, but the process is slow and other products are formed. In an attempt to identify these products, and in particular to establish the presence of trichodiol, the extract from a 6 day incubation of ITdiol (2.0mg) in phosphate buffer at pH 5.8 was analysed using GC-MS following trimethylsilylation. The results demonstrated the presence of EPT (Rt=3.41 mins), the di-TMS ether of residual ITdiol (Rt=5.18 mins), and the di-TMS ethers of three other compounds. One of these with Rt=4.65 mins was identified as 9β-trichodiol (37B) by comparison to the GC-MS of a derivatised standard of 9β-trichodiol obtained from *T. roseum* (see later, p. 178). Another, smaller peak at Rt=5.05 mins produced



EPT (3)



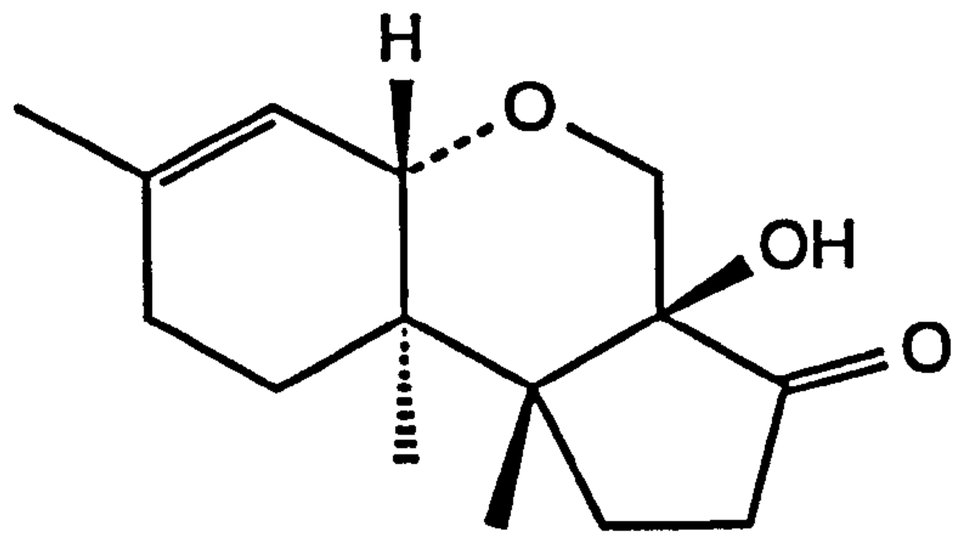
αOH = 9α-Trichodiol (37A)
βOH = 9β-Trichodiol (37B)

spectral data identical to that for 9β-trichodiol, and was therefore identified as the isomeric 9α-trichodiol (37A). The ratio of β to α was calculated as approximately 4:1 from the areas of the respective peaks.

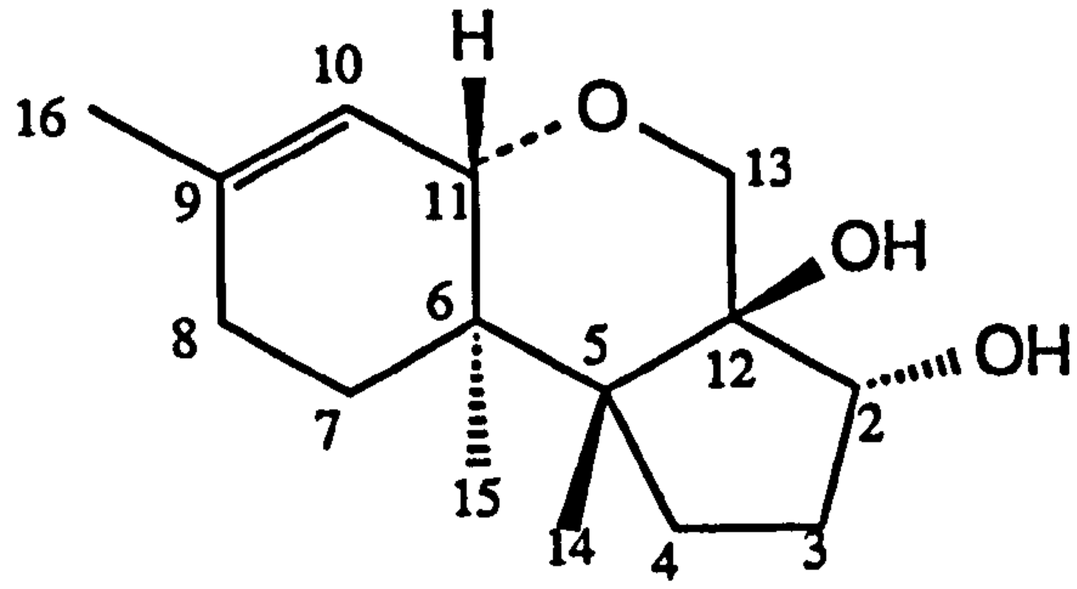
The remaining unknown compound could not be identified using GC-MS analysis alone, and was subsequently isolated in 17% yield from a repeat experiment in which ITdiol was incubated in phosphate buffer at pH 5.9. EI-MS, ^{1}H NMR and ^{13}C NMR analysis determined its structure as (99), and it was later given the trivial name of pre-sambucoin because of its structural and biosynthetic relationship to sambucoin (53) (see Fig.78).

The EI-MS shows a molecular ion at m/z 252 consistent with a molecular formula of $C_{15}H_{24}O_3$, and indicating that, unlike EPT, it had been formed from ITdiol without loss of H_2O. The base peak in the spectrum is at m/z 124, and there is no peak at m/z 84, the base peak in ITdiol produced by retro Diels-Alder fragmentation in ring A.

Comparison of the ^{1}H NMR spectrum [Fig.73, Table 24] with that of ITdiol (see Fig.51, Table 16) indicated that they have similar A ring structures. The C-16 methyl (trichothecene numbering) is a broad singlet at δ1.68 indicating that the C-9,10 double bond is still present, and H-10 and H-11 are broad singlets at δ5.25 and δ4.15 respectively, demonstrating retention of α-oxygenation at C-11. H-11 is shifted upfield from δ4.60 in ITdiol and this, together with the absence of a peak at m/z 84 in the EI-MS (see above), indicates that the C-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 ITdiol. The epoxide protons in ITdiol at δ3.06 andδ3.32 are no longer present, and the C-13 protons now appear as an AB quartet upfield at δ3.75 and δ4.11 (J_{AB}=12.5Hz) indicating that the 12,13-epoxide has been opened, and is



Sambucoin (53)

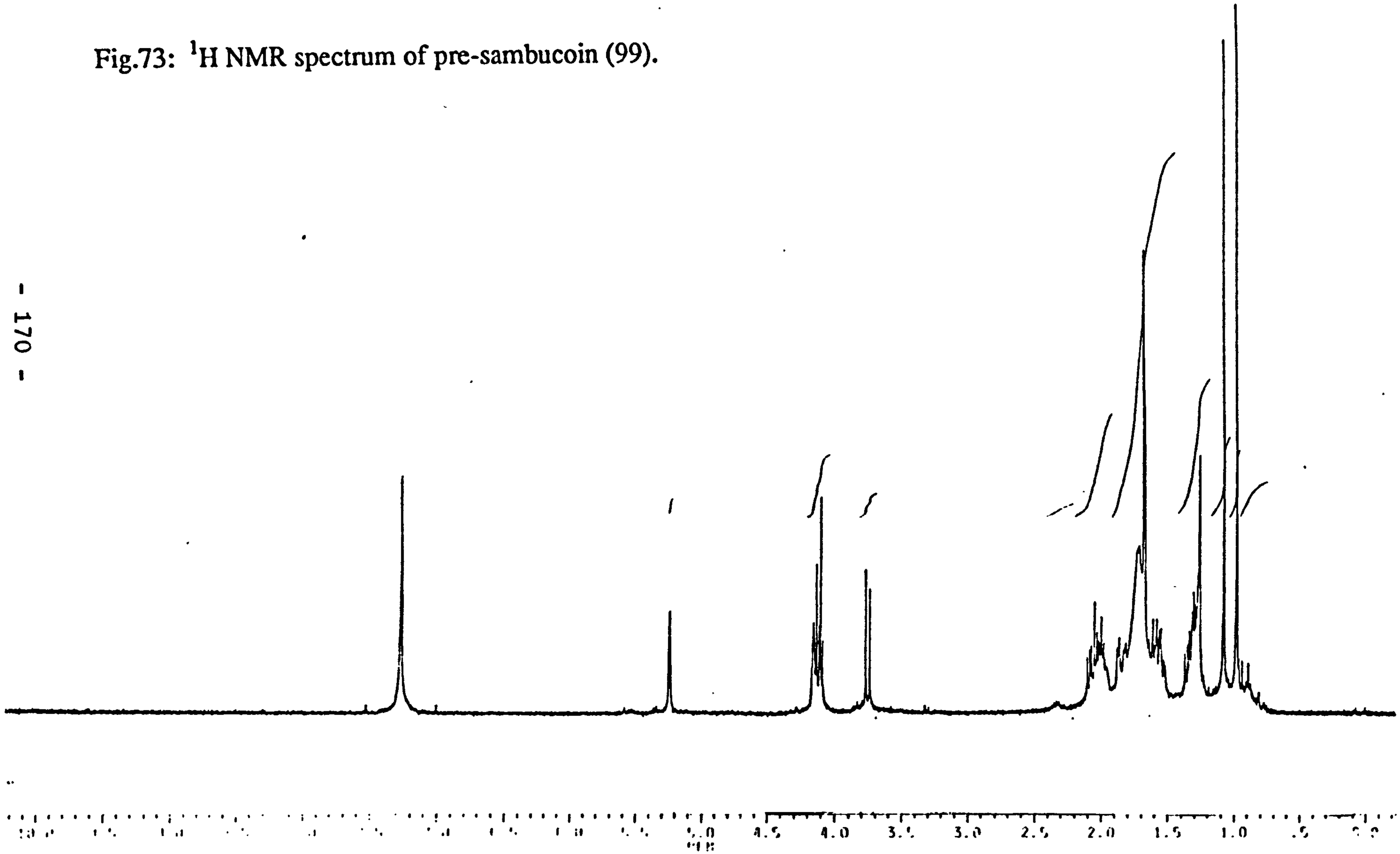


Pre-sambucoin (99)

H-2β	4.09 (*dd*, J=10.7, 6.7Hz)	C-2	80.7	(methine)
H-3α	1.55 (*m*)	C-5	46.1	(quaternary)
3β	1.90 (*ddd*, J=13.6, 7.0, 6.6Hz)	C-6	37	(quaternary)
H-4α	1.98 (*dd*, J=14.0, 8.5Hz)	C-9	135	(quaternary)
4β	1.25 (*ddd*, J=13.8, 12.3, 8.0Hz)	C-10	122.9	(methine)
H-7α	1.25 (*m*)	C-11	71.6	(methine)
7β	1.55 (*m*)	C-12	---	
H-8α	1.99 (*br m*)	C-13	67.8	(methylene)
8β	1.84 (*br dd*, J=18.0, 6.0Hz)	C-3	27.7	(methylene)
H-10	5.25 (*br s*)	C-4	29.5	(methylene)
H-11β	4.15 (*br s*)	C-7	30.9	(methylene)
H-13	3.75 (*d*, J=12.5Hz)	C-8	31.1	(methylene)
	4.11 (*d*, J=12.5Hz)	C-14	16.4	(methyl)
H-14	1.07 (*s*)	C-15	22.3	(methyl)
H-15	0.98 (*s*)	C-16	22.7	(methyl)
H-16	1.68 (*br s*)			

Table 24: NMR data for pre-sambucoin (99).

Fig.73: ^{1}H NMR spectrum of pre-sambucoin (99).



consistent with the formation of an oxygen bridge between C-11α and C-13. The resonance at δ4.09 is due to H-2β and indicates that the C-2α hydroxyl group has been retained.

Full assignment of the proton NMR data was achieved using $^{1}H/^{1}H$ COSY [Fig.74], and significant couplings are shown in Fig.75. Thus, the protons at C-3 and C-4 were located at δ1.55/1.90, and δ1.25/1.98, respectively, and these could then be assigned from their coupling patterns. H-2β is at δ4.09 and is a doublet of doublets coupling with H-3β ($J_{2\beta,3\beta}$=6.7Hz) and H-3α ($J_{2\beta3\alpha}$=10.7Hz). From molecular models, this indicates the dihedral angles for H-2/3 as shown in Fig.76A, which in turn dictates that the dihedral angles between the C-3 and C-4 protons are as shown in Fig.76B. Therefore, in the ^{1}H NMR spectrum the double doublet at δ1.98 is H-4α, and the double double doublets at δ1.25 and δ1.90 are H-4β and H-3β respectively, and H-3α is the multiplet at δ1.55. The protons at C-7 and C-8 were located at δ1.25/1.55 , and δ1.84/2.00 respectively, but could not be fully assigned.

The ^{13}C NMR data [Table 24], although incomplete due to the small sample size, are in full agreement with the structure determined from the ^{1}H NMR analysis. It shows two methines next to oxygen at δ80.7 (C-2) and δ71.6 (C-11), a vinylic methine at δ122.9 (C-10), and a methylene next to oxygen at δ67.8 (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.

Final confirmation of this structure was obtained when a sample was oxidised using dimethysulphoxide-trifluoroacetic anhydride[112] yielding sambucoin (53) (40%) spectrally (^{1}H NMR and MS) identical to the natural product isolated from

Fig.74: $^{1}H/^{1}H$ COSY spectrum of pre-sambucoin (99).

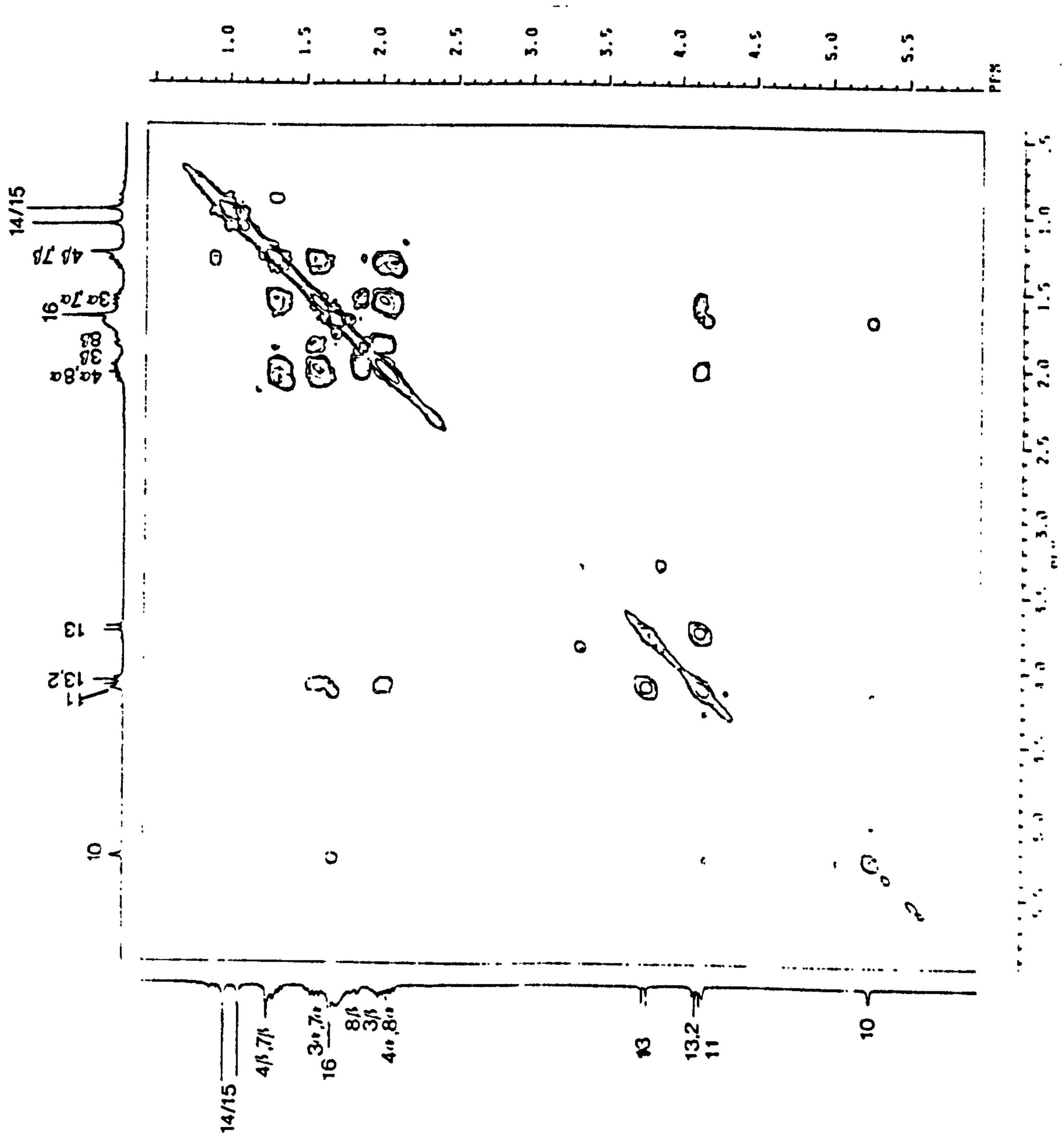


Fig.75: Significant couplings observed in pre-sambucoin (99) using $^{1}H/^{1}H$ COSY.

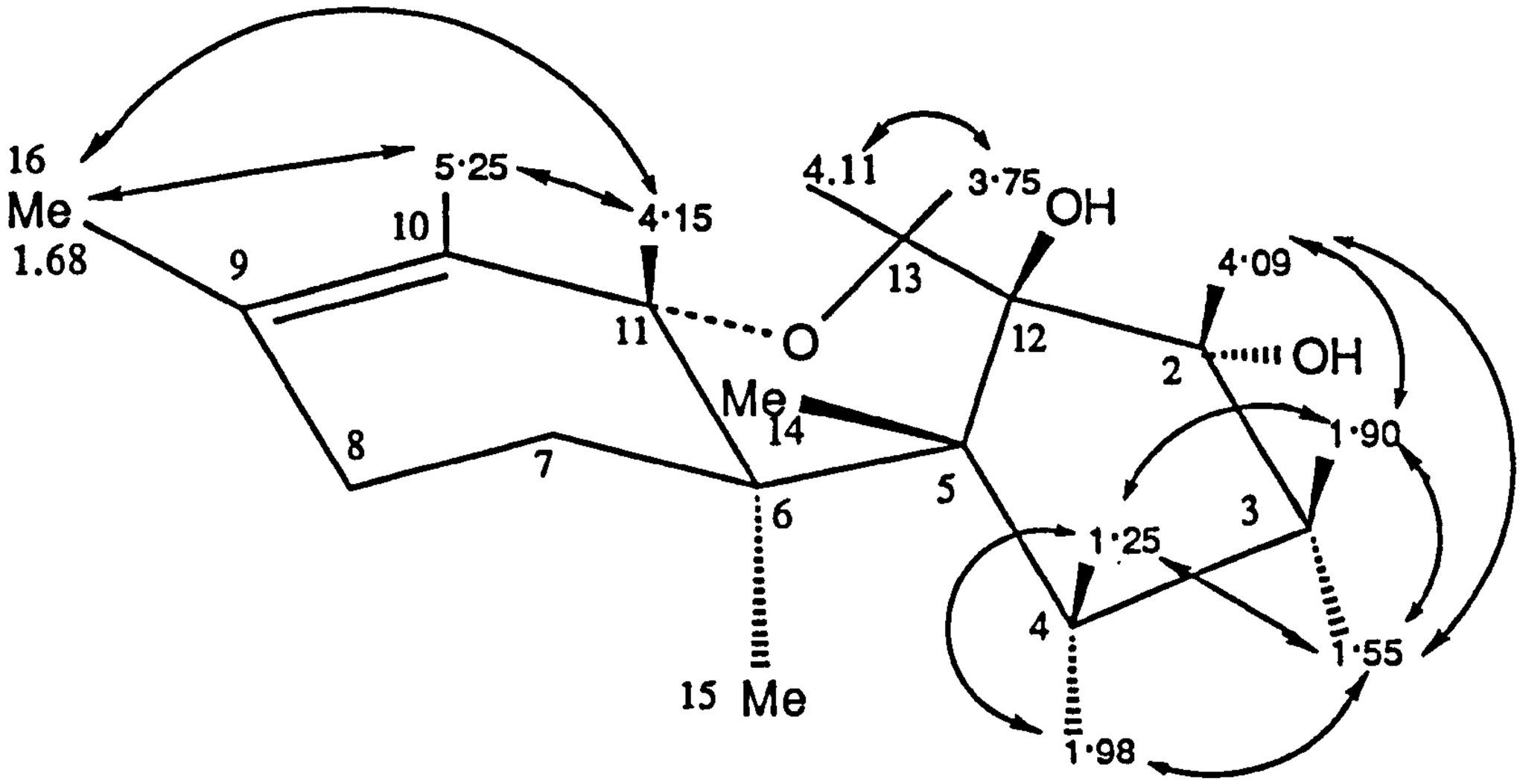
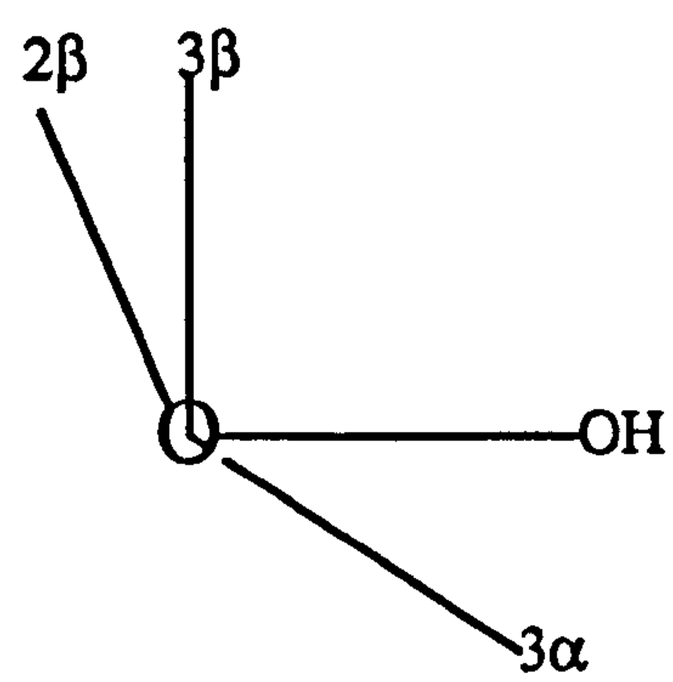
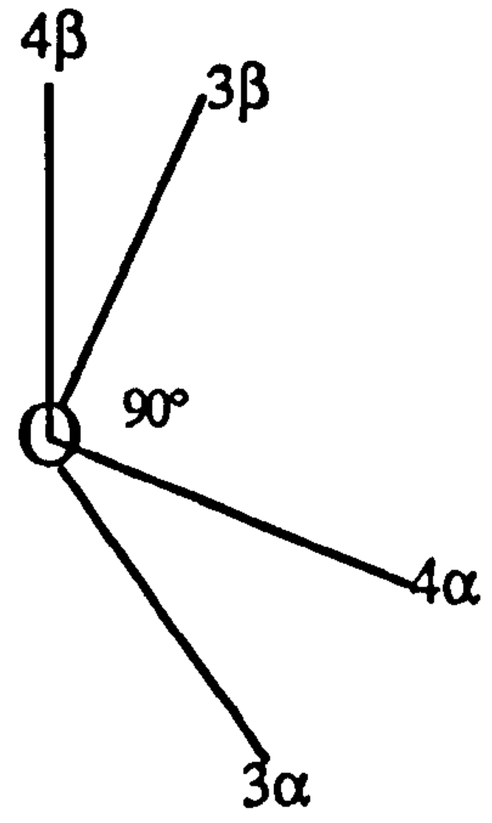


Fig.76: Coupling predictions (Hz) for the C-ring protons of pre-sambucoin from dihedral angles in molecular models.

A)



B)



	3β	3α	4β	4α	2β	Multiplicity
2β	7	11	-	-	-	*dd*
3β	-	GEM	7	0	7	*ddd*
3α	GEM	-	12	8	11	*dddd*
4β	7	12	-	GEM	-	*ddd*
4α	0	8	GEM	-	-	*dd*

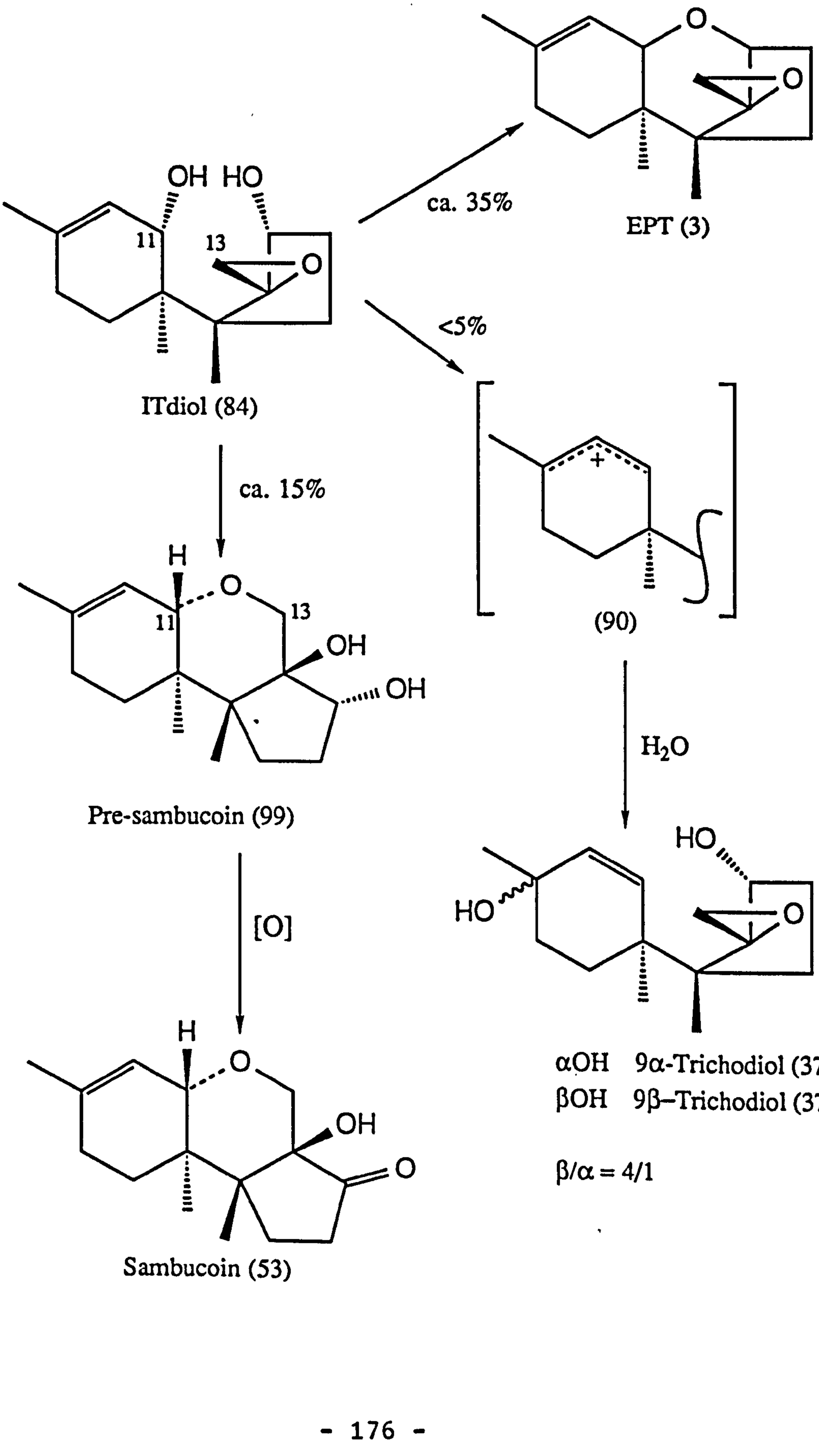
Fusarium sambucinum[77].

Therefore in acid catalysed conditions, ITdiol undergoes a series of changes similar to those reported for isotrichotriol, namely allylic isomerisation to trichodiol and cyclisation to EPT [Fig.77]. Several mechanisms have been formulated for the cyclisation of ITdiol (see Fig.62), which may occur by a direct S_N2 reaction, or via trichodiol or a carbocation intermediate. The trichodiol produced is in both the 9α- and 9β-hydroxy stereoisomeric forms, with the 9β predominating. This suggests that the allylic isomerisation occurs via S_N1 reactions involving a carbocation intermediate similar to that proposed for the formation of 9α- and 9β-methoxytricho-10,12-diene from 9β-hydroxytricho-10,12-diene (see Fig.45). Thus, acid catalysed loss of the C-11 hydroxyl produces the carbocation (90) which then undergoes nucleophilic attack by H_2O to give 9α- and 9β-trichodiol. The 9β isomer is the major product since access from the β-face is less restricted than from the α-face.

In addition to these reactions, ITdiol also forms pre-sambucoin as a major product, and this can be seen as the result of cyclisation by intramolecular attack of the C-11α hydroxyl onto C-13 of the epoxide.

These results support the proposed biosynthesis of EPT from ITdiol, and also suggest that ITdiol is a precursor of sambucoin, a minor trichothecene-related metabolite produced in *Fusarium* species. Therefore ITdiol appears to be a common biosynthetic precursor to sambucinol via cyclisation to EPT, to sambucoin via cyclisation to pre-sambucoin, and to the trichothecenes via 3α-hydroxylation with subsequent cyclisation to isotrichodermol [Fig.78]. It has also been proposed as a precursor to another group of trichothecene-related metabolites, the 11-epi-apotrichothecenes[77] (see Fig.28), but no evidence for this has been found.

Fig.77: Acid catalysed rearrangements of ITdiol.



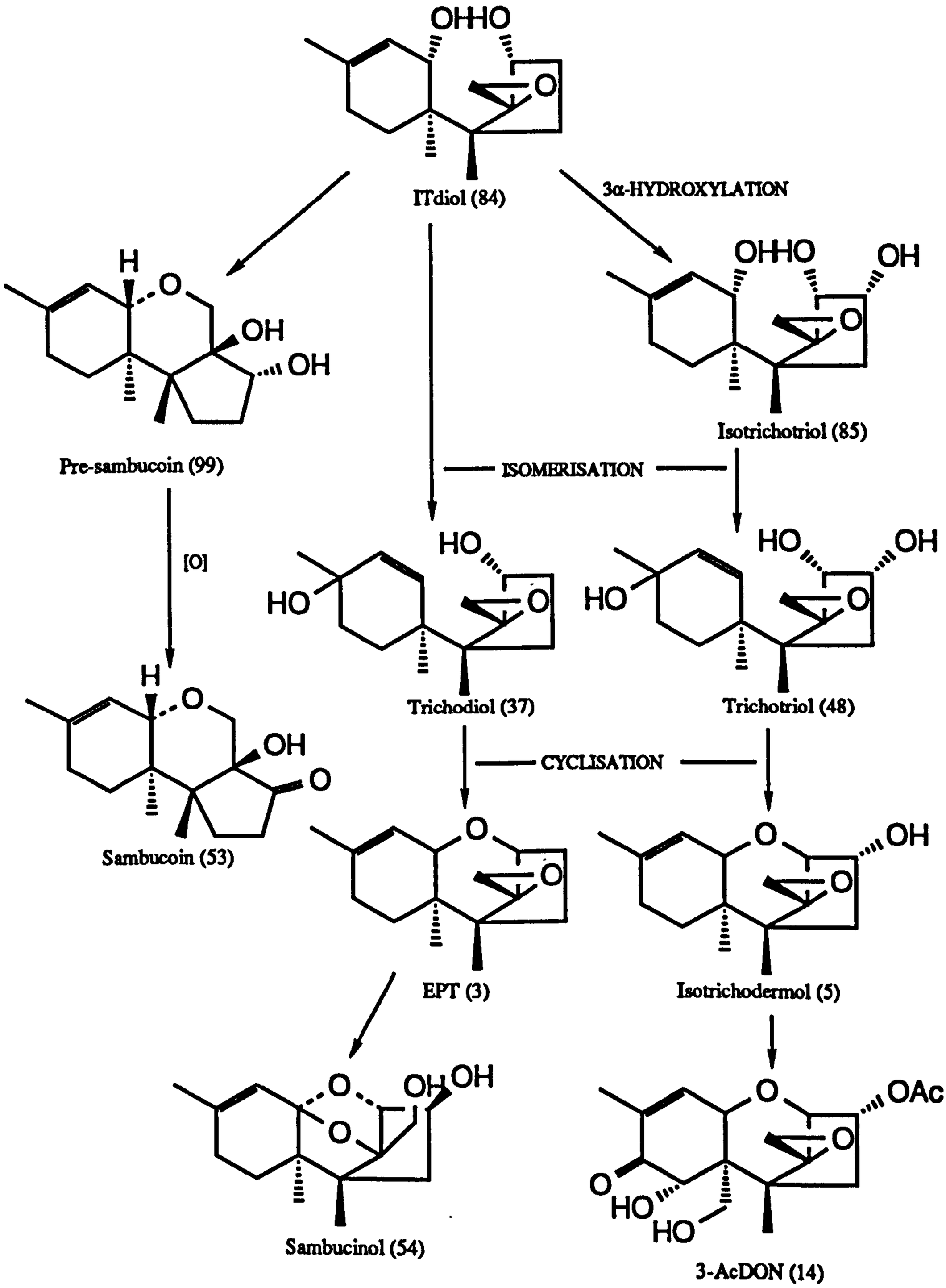


Fig.78: ITdiol proposed as a common precursor to sambucoin, sambucinol and the trichothecenes.

Biotransformation of pre-sambucoin (99).

In order to investigate the biosynthesis of sambucoin further, a ^{14}C-labelled sample of (99) was prepared from [^{14}C]ITdiol by cyclisation at pH 5.0. This material (1.0mg; 0.41MBqmM^{-1}) was fed to a growing culture of *Fusarium culmorum* (72 hours after subculture) and incubated for 18 hours. The culture was then harvested by extraction of the filtrate with ethyl acetate, and the extract spiked by addition of unlabelled sambucoin which facilitated the isolation of any [^{14}C]sambucoin present. Incorporation of [^{14}C]pre-sambucoin into [^{14}C]sambucoin was measured as 7.1% by scintillation counting. This indicated that pre-sambucoin is a biosynthetic precursor to sambucoin (although the incorporation measured was relatively low) and supports the proposed biosynthesis from ITdiol via cyclisation to pre-sambucoin.

Production and identification of uncyclised trichothecenes from cultures of *Fusarium culmorum* 14764 and *Trichothecium roseum* 50661.

It was clear that further investigations into the biosynthesis of trichothecenes from TDN and ITdiol were dependent upon obtaining samples, preferably radiolabelled, of trichodiol, trichotriol and isotrichotriol. The fungus *Trichothecium roseum* was selected for initial studies, as this was the source from which trichodiol was first isolated[78].

Production of trichodiol from *T. roseum* 50661.

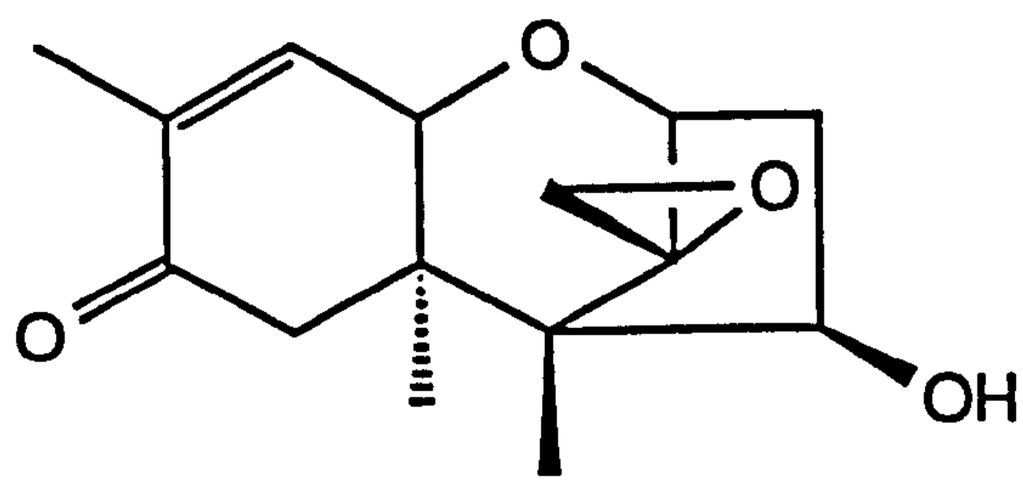
The production of trichothecenes from *T. roseum* was achieved by a two stage shake-culture method which had already been used previously to produce acceptable yields of mycotoxins[94]. In the first stage, seed medium was inoculated

with a spore suspension obtained from mature agar plates of *T. roseum*, and incubated on an orbital shaker at 27°C and 250rpm. After two days incubation the seed culture (50ml) was used to inoculate 2L Erlenmeyer flasks containing production medium (400ml), and the incubation continued. After a further 21 days the cultures were harvested and the toxins extracted. This was achieved by filtration of the cultures, then saturating the filtrate with salt, and extracting repeatedly with ethyl acetate. The filtered mycelium was also extracted as this is known to contain a significant proportion of the trichothecenes produced[94]. It was frozen in liquid nitrogen , then ruptured by the addition of hot water. The slurry was then extracted with a suitable organic solvent (e.g. ethyl acetate). All the organic extracts were combined and analysed by TLC. This indicated the presence of trichothecolone (12) as the major trichothecene product, and a minor, more polar metabolite which reacted with PNBP/TEPA reagent producing the bright sky blue colour characteristic of uncyclised trichothecenes[61]. These were purified by repeated CTLC which yielded trichothecolone (25mgL^{-1}), and 9β-trichodiol (2.5mgL^{-1}). The yield of trichodiol is therefore too low for this to be a practicable method for producing ^{14}C-labelled material.

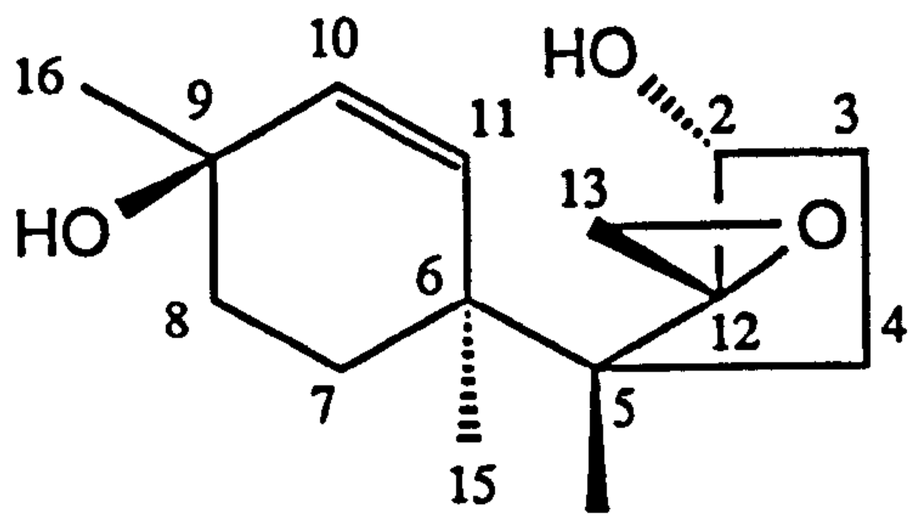
^{1}H NMR and EI-MS analysis of trichothecolone produced data in agreement with literature values[94].

9β-Trichodiol was identified by a combination of MS, ^{1}H NMR and ^{13}C NMR techniques, with identification being aided by comparison with the spectral data for 9β-hydroxytricho-10,12-diene (70) (see Table 10) and ITdiol (see Table 16).

The ^{1}H NMR spectrum [Fig.79, Table 25] shows the presence of a 12,13-epoxide function as indicated by the characteritic AB quartet of signals centred at δ3.24 (J_{AB}=4.0Hz) which correspond to the C-13 methylene protons



Trichothecolone (12)



9β-Trichodiol (37B)

Fig.79: ^{1}H NMR spectrum of 9β-trichodiol (37B).

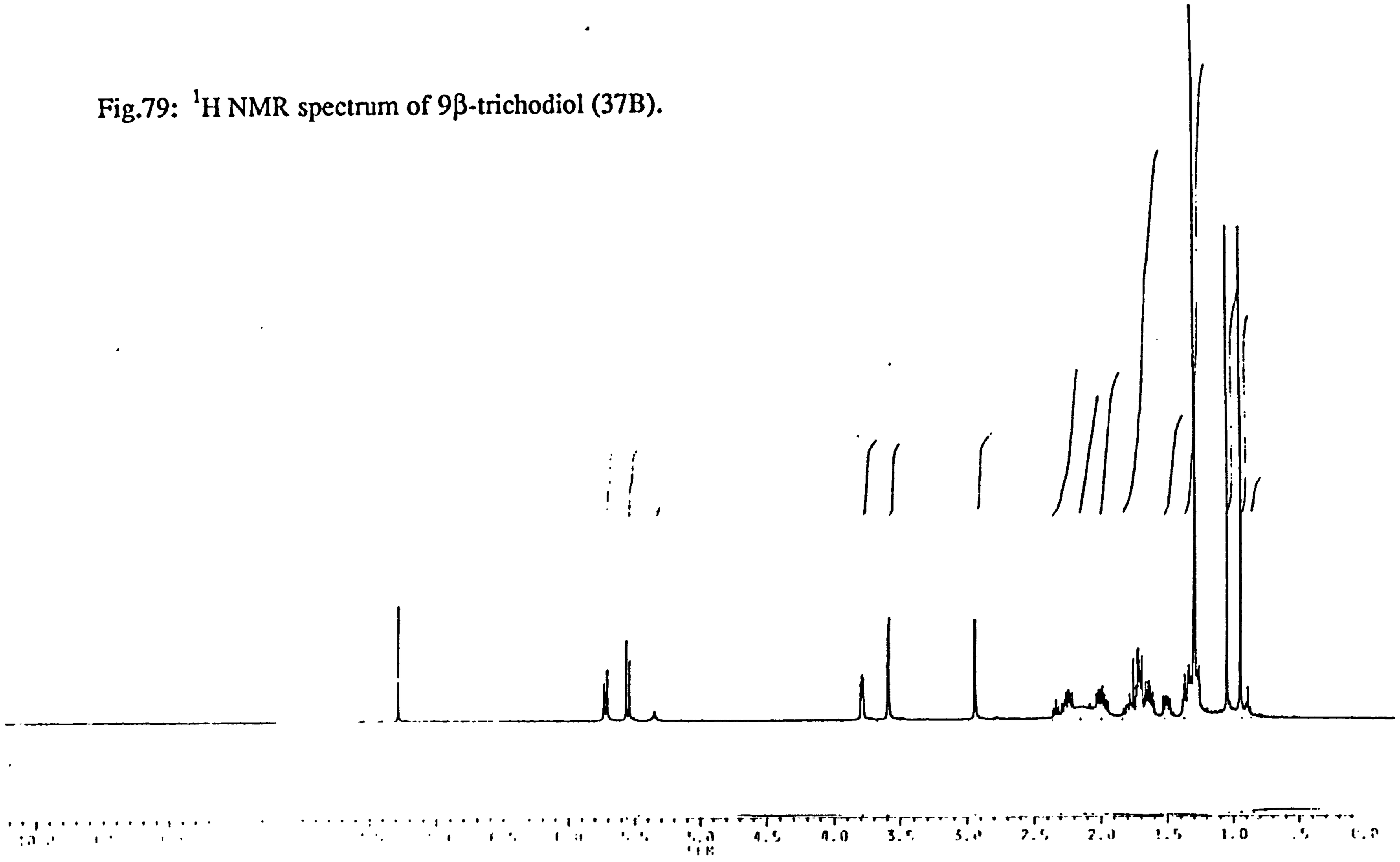


Table 25: ^{1}H NMR chemical shifts (δppm) and coupling constants (Hz) for 9β-trichodiol and 9α–/9β-trichotriol.

	9β-Trichodiol (37B)	9β-Trichotriol (48B)	9α-Trichotriol (48A)
H-2β	3.78 (*dd*, J=5.1, 3.0)	3.68 (*d*, J=4.6)	3.73 (*d*, J=4.7)*
H-3α	1.62 (*dddd*, J=13.3, 7.2, 3.1, 3.0)		
3β	1.97 (*dddd*, J=13.2, 11.2, 7.2, 5.2)	4.25 (*ddd*, J=8.2, 6.4, 4.6)*	4.31 (*m*)
H-4α	2.24 (*ddd*, J=12.0, 12.0, 7.3)	2.12 (*dd*, J=13.2, 8.0)	2.05 (*dd*, J=13.2, 8.1)
4β	1.48 (*ddd*, J=12.9, 7.2, 2.9)	1.75 (*dd*, J=13.2, 6.3)	
H-7α	1.32 (*m*)	1.32 (*m*)	
7β	1.68-1.81 (*m*)	1.8 (*m*)	1.4-1.8 (5H, *m*)
H-8α	1.68-1.81 (*m*)	1.67-1.73 (*m*)	
8β	1.68-1.81 (*m*)	1.67-1.73 (*m*)	
H-10	5.55 (*d*, J=10.2)	5.54 (*d*, J=10.2)	5.5 (*m*)
H-11	5.71 (*br d*, J=10.2)	5.71 (*br d*, J=10.2)	5.5 (*m*)
H-13	2.92 (*d*, J=4.0)	2.99 (*d*, J=4.0)	3.06 (*d*, J=4.3)
	3.57 (*d*, J=4.0)	3.53 (*d*, J=4.0)	3.29 (*d*, J=4.3)
H-14	0.94 (*s*)	1.08 (*s*)	1.07 (*s*)
H-15	1.04 (*s*)	0.89 (*s*)	1.01 (*s*)
H-16	1.29 (*s*)	1.28 (*s*)	1.28 (*s*)

*from CH_3OD shake

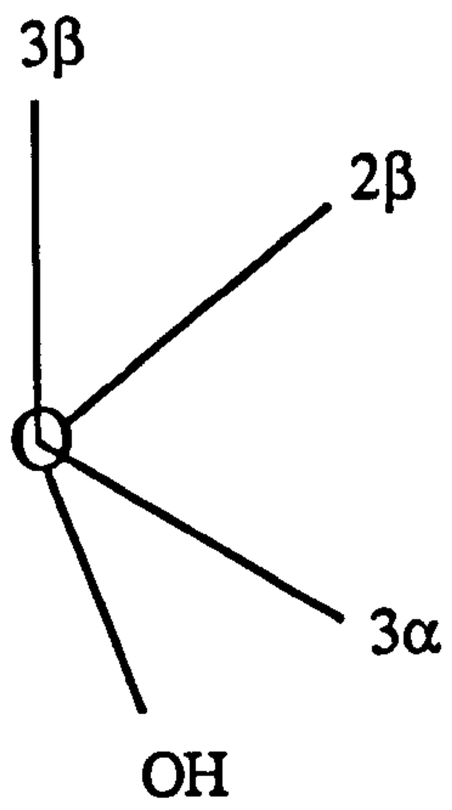
Table 26: ^{13}C NMR chemical shifts (δppm) for 9β-trichodiol and 9β-trichotriol.

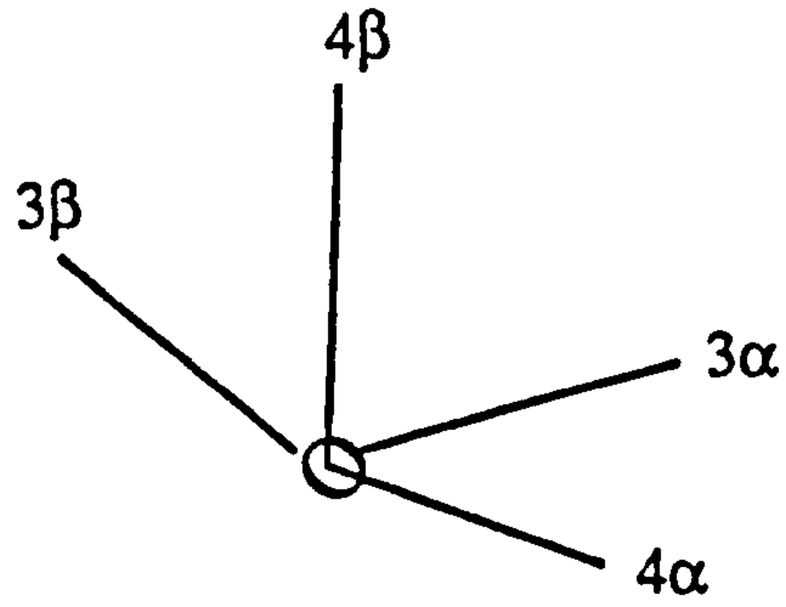
	9β-Trichodiol (37B)	9β-Trichotriol (48B)
C-2	78.9	77.7
C-3	30.0	69.1
C-4	34.1	41.7
C-5	45.6	43.8
C-6	39.3	39.5
C-7	27.1	26.9
C-8	34.0	33.9
C-9	66.6	66.6
C-10	131.1	131.2
C-11	136.4	136.3
C-12	71.5	69.4
C-13	50.1	50.0
C-14	21.2	20.4
C-15	18.9	21.2
C-16	30.7	30.7

associated with the epoxide group. Further downfield, the vinylic protons at δ5.55 (d, J=10.2Hz) and δ5.71 (brd, J=10.2Hz) indicate the presence of a C-10,11 double bond, as seen in the data for (70). Hence, the doublet at δ5.55 corresponds to H-10 ($J_{10,11}$=10.2Hz), and that at δ5.71, broadened by W-coupling to H-7α, is H-11. The C-16 methyl resonance is a singlet at δ1.29, and is similar to the corresponding signal in (70) indicating the presence of a hydroxyl at C-9. The second hydroxyl is in the B ring, and this was assigned to C-2 from the chemical shift of the adjacent proton which, at δ3.78, is similar to H-2β in both ITdiol (δ3.60) and EPT (δ3.72) (see Table 18). The sterochemistry at this position was defined from the size of the coupling constants of H-2 with H-3α and H-3β. Thus, H-2 is a double doublet with J=5.1, 3.0Hz indicating dihedral angles of approximately 45° and 70°, respectively, between this proton and the C-3 protons [Fig.80]. Molecular models show that this is only possible with H-2 in the β position, thereby assigning the hydroxyl α stereochemistry. This is the same as for ITdiol, but the latter has a different coupling pattern for H-2β which is a doublet (J=4.1Hz) rather than a doublet of doublets (J=5.1, 3.0Hz). An explanation for this is the presence of the C-11α hydroxyl in ITdiol which can form an intramolecular hydrogen bond with the hydroxyl at C-2α thereby fixing the conformation of the B ring. In trichodiol this hydrogen bonding is not possible due to lack of hydroxylation at C-11, and therefore the B ring comformation is altered slightly. The occurrence of intramolecular hydrogen bonding in ITdiol but not in trichodiol also helps explain the observation that ITdiol is much less polar, having an Rf≈0.35 on TLC in hexane-ethyl acetate, 1:1, whilst trichodiol remains near the baseline.

A full assignment of the proton NMR was facilitated by $^1H/^1H$ COSY [Fig.81],

Fig.80: Coupling predictions (Hz) for the B-ring protons in 9β-trichodiol from dihedral angles in molecular models.





	3β	3α	4β	4α	2β	Multiplicity
2β	5	3	-	-	-	*dd*
3β	-	GEM	7	11	5	*dddd*
3α	GEM	-	3	7	3	*dddd*
4β	7	3	-	GEM	-	*ddd*
4α	11	7	GEM	-	-	*ddd*

Fig.81: $^{1}H/^{1}H$ COSY spectrum of 9β-trichodiol (37B).

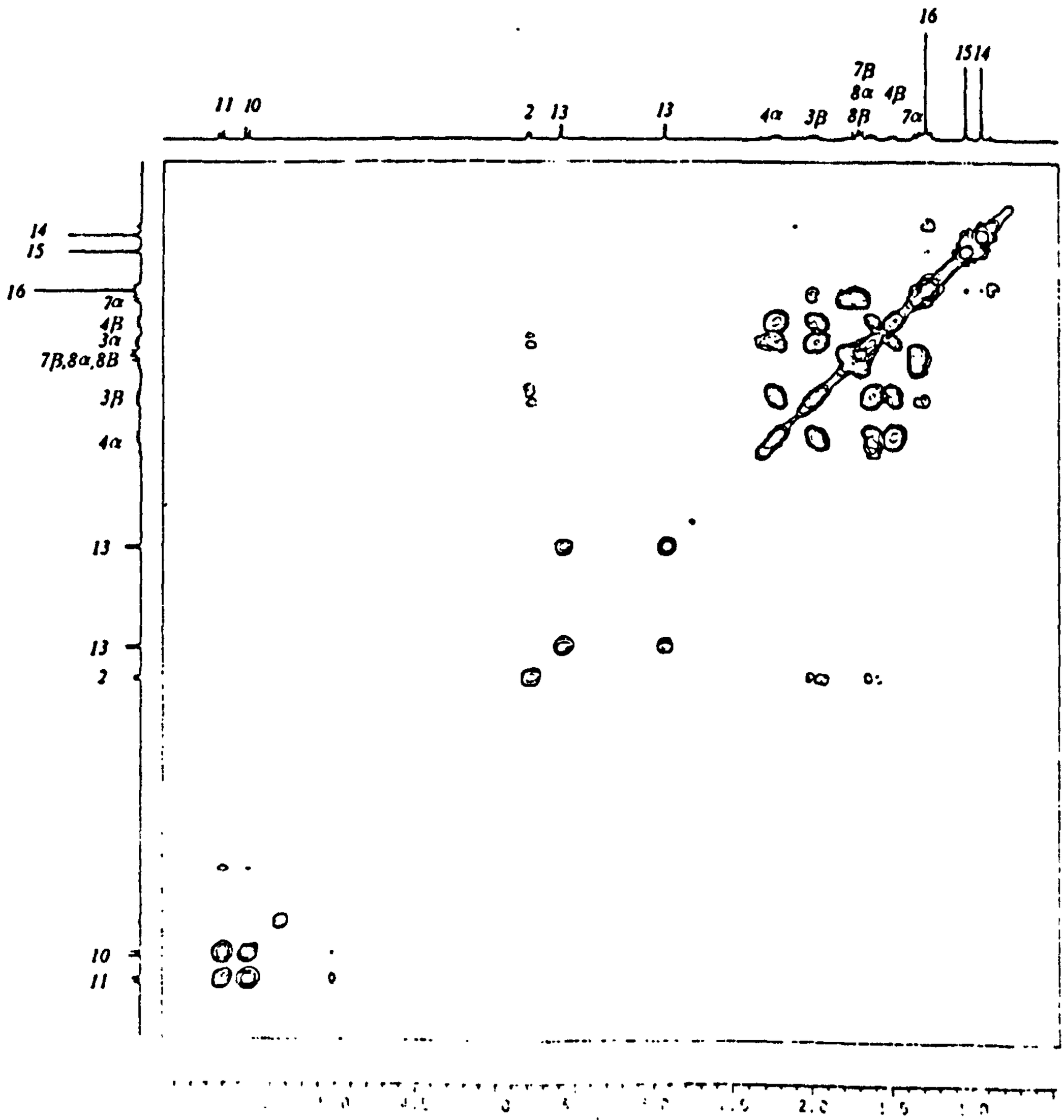
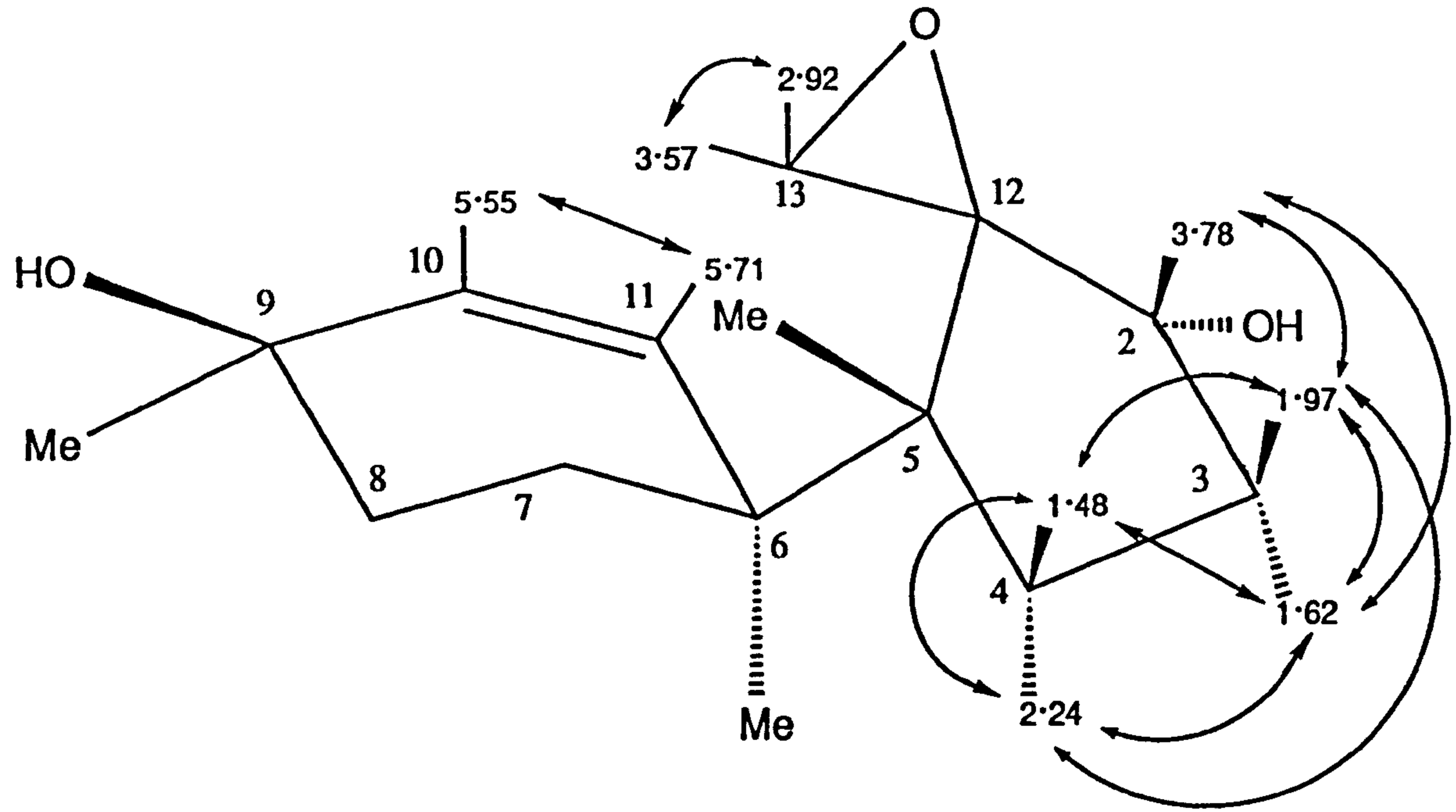


Fig.82: Significant couplings obtained in the $^1H/^1H$ COSY of 9β-trichodiol.



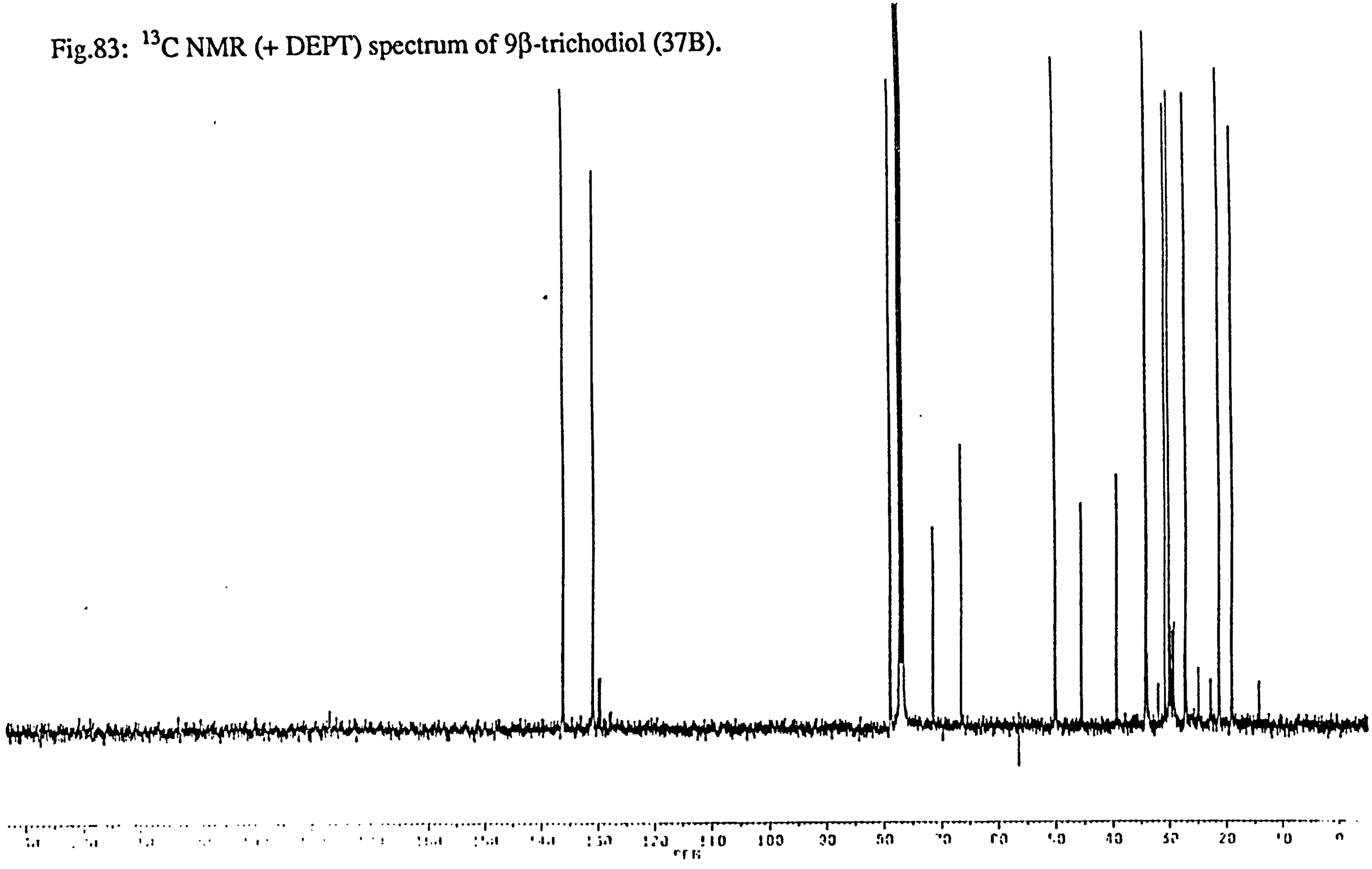
and significant couplings obtained from this are shown in Fig.82. The C-3 protons were therefore located at δ1.62 and δ1.97 from observed coupling with H-2β at δ3.78, and the C-4 protons located at δ2.24 and δ1.48 from coupling with those at C-3. These were then assigned from their characteristic coupling constants. Thus, H-3β is at δ1.97 since it couples with H-2β with $J_{2\beta,3\beta}$=5.2Hz, and H-3α is at δ1.62 ($J_{2\beta,3\alpha}$=3.0Hz). Molecular models predict that H-3α has dihedral angles of approximately 70° and 40° with H-4β and H-4α respectively (see Fig.80), and thus the resonance at δ1.48 can be assigned to H-4β ($J_{3\alpha,4\beta}$=3.0Hz) and that at δ2.24 to H-4α ($J_{3\alpha,4\alpha}$=7.2Hz). The coupling of H-3β with these C-4 protons ($J_{3\beta,4\beta}$=7.2Hz, $J_{3\beta,4\alpha}$=11-12Hz) is also in agreement with dihedral angle predictions.

In ring A, $^1H/^1H$ COSY shows H-10 at δ5.55 coupling only with H-11 at δ5.71, and locates the C-7 and C-8 protons at δ1.32 (one proton) and δ1.68-1.81 (three protons). The former is obscured by the C-16 methyl resonance at δ1.29, and the latter form a complex overlapping multiplet, and so assignment of these protons by determination of their coupling constants was not possible.

The ^{13}C NMR spectrum [Fig.83, Table 26] was fully assigned by using DEPT and $^1H/^{13}C$ COSY [Figures 84 and 85], and is in full agreement with the structure determined from the ^1H NMR data. The spectrum shows two vinylic methine resonances at δ131.1 (C-10) and δ136.4 (C-11), a methine next to oxygen at δ78.9 (C-2), and an epoxide methylene at δ50.1. In addition to the two quaternary carbons at δ45.6 (C-5) and δ39.3 (C-6), there are two others attached to an oxygen substituent appearing downfield at δ71.5 (C-12) and δ66.6 (C-9). Comparison with the ^{13}C NMR data for ITdiol (see Table 16) shows that the resonances for the B ring carbons are very similar, indicating an identical structure.

Similarly, comparison with the ^{13}C NMR data for 9β-hydroxytricho-10,12-diene

Fig.83: ^{13}C NMR (+ DEPT) spectrum of 9β-trichodiol (37B).



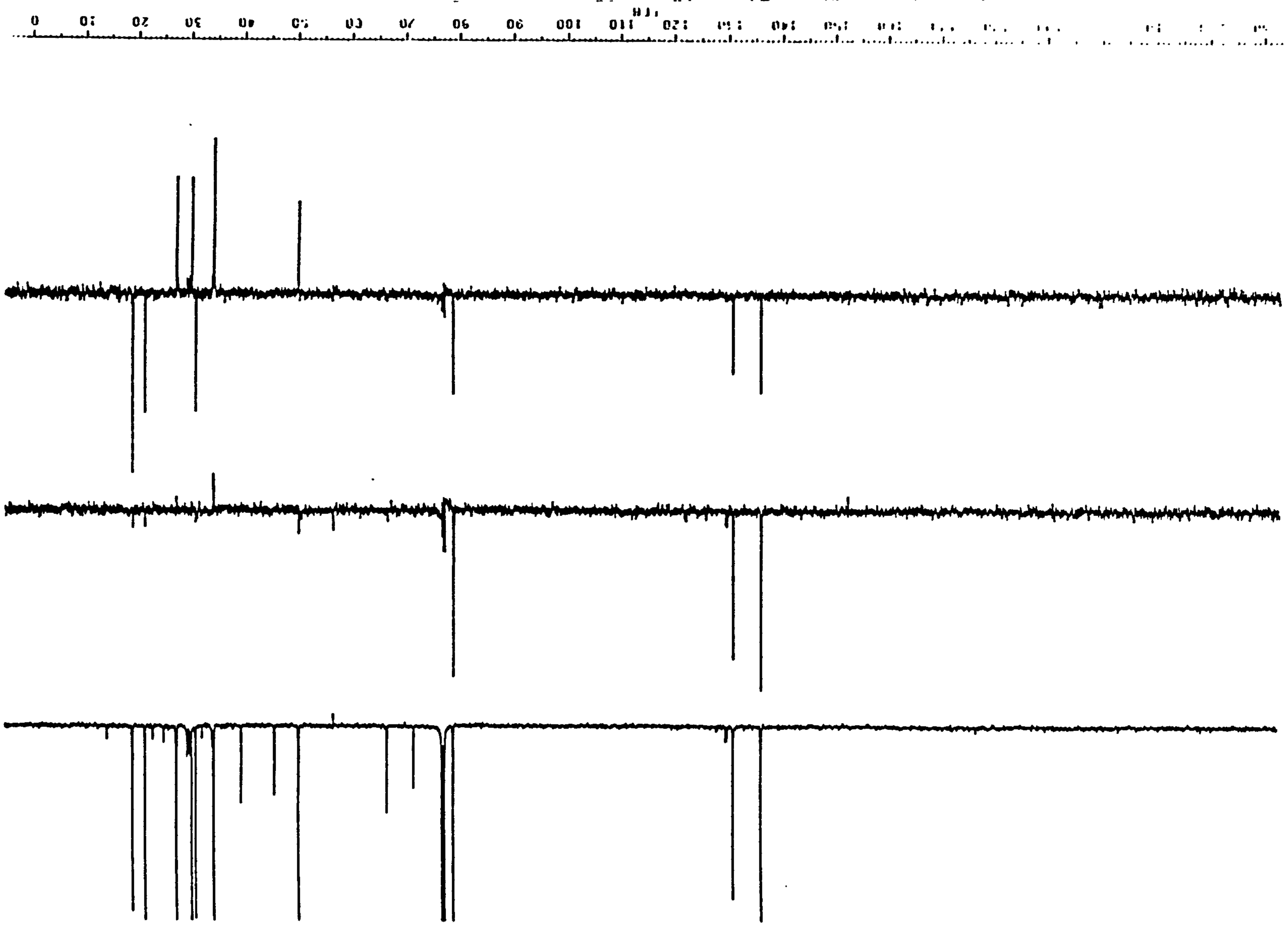


Fig.84: $^{1}H/^{13}C$ COSY spectrum of 9β-trichodiol (37B).

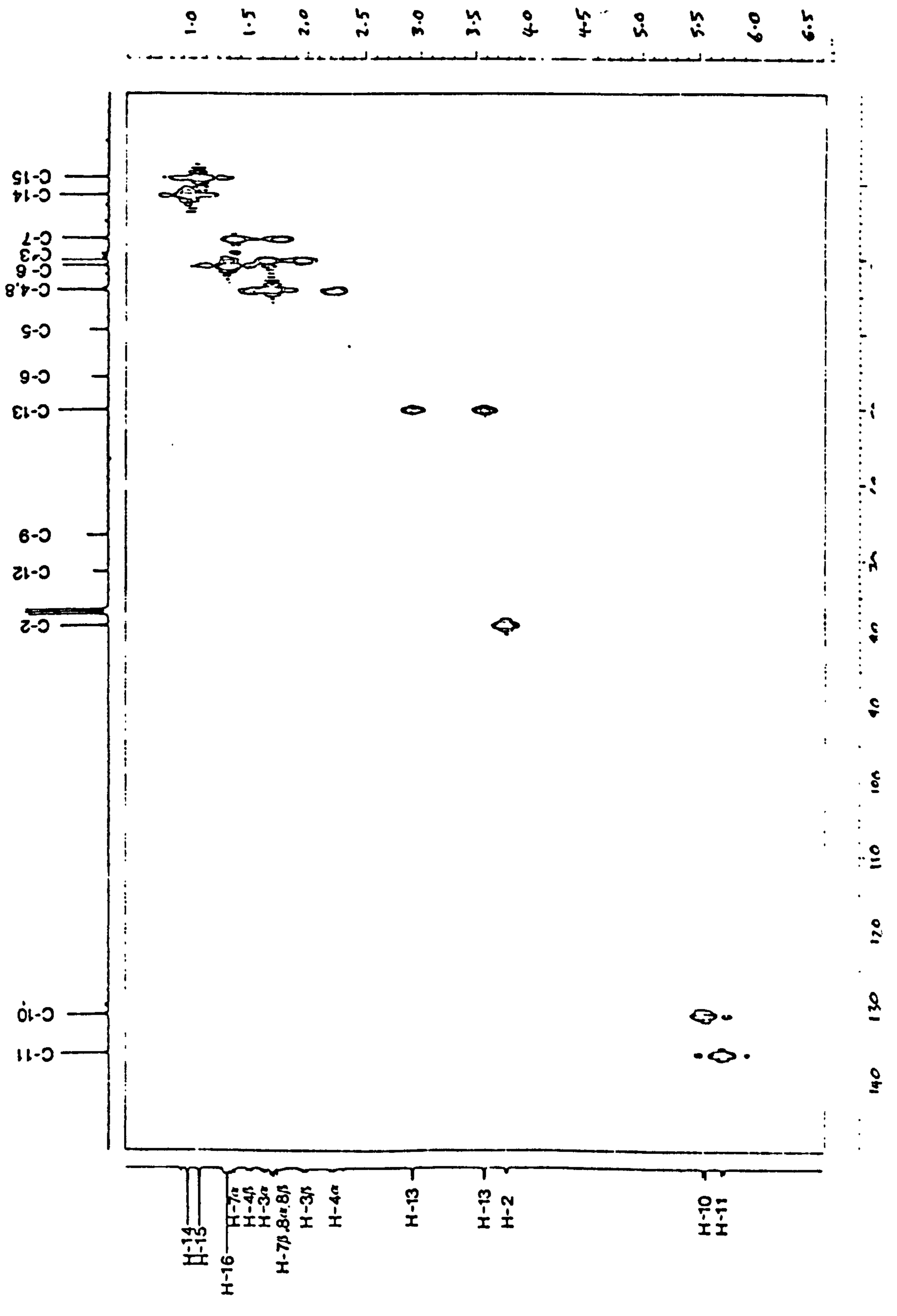


Fig.85: Couplings obtained in the $^{1}H/^{13}C$ COSY of 9β-trichodiol.

	Proton resonance (δppm)	Cross peak with carbon at δppm
H-2	3.78	78.9 (methine)
H-3	1.62 and 1.97	30.0 (methylene)
H-4	1.48 and 2.24	34.1 (methylene)
H-7	1.32 and 1.75	27.1 (methylene)
H-8	1.75	34.0 (methylene)
H-10	5.55	131.1 (methine)
H-11	5.71	136.5 (methine)
H-13	2.92 and 3.57	50.1 (ethylene)
H-14	0.94	21.2 (methyl)
H-15	1.04	18.9 (methyl)
H-16	1.28	30.7 (methyl)

(70) shows that they have identical A ring structures, and indicates that the stereochemistry of the C-9 hydroxyl is β. The stereochemistry of (70) is well defined by virtue of its mechanism of formation from 9β,10β-epoxyTDN (see Fig.40), and it is therefore a reliable standard to use to assign the orientation of the C-9 hydroxyl groups in compounds with similar A ring structures. This is further aided by comparison with 9α-hydroxytricho-10,12-diene (82) which was obtained by acid catalysed isomerisation from the 9β-alcohol (70). These two compounds show characteristic differences in their ^{13}C NMR data for the A ring carbons [Fig.86]. Thus, in the 9β-hydroxy isomer (70) C-9 is at δ66.5, C-10 at δ131.7, C-11 at δ136.5, and C-16 at δ29.7, whilst in the 9α-hydroxy isomer (82) these are slightly different with C-9 appearing upfield at δ69.7, C-10 and C-11 are more equivalent at δ134.0 and δ133.3 respectively, and C-16 is slightly downfield at δ27.7. It is the chemical shift of C-9, and the shift separation between the C-10 and C-11 resonances which seem particularly characteristic.

Thus, comparison of the A ring resonances in the ^{13}C NMR spectrum of 9β-trichodiol with those for (70) and (82) [Fig.86], clearly confirms the β stereochemistry of the C-9 hydroxyl. However, when analysing the data published for 9α- and 9β-trichotriol[60,110] it is evident that the wrong stereochemical assignments have been made. The resonances for C-9, 10 and 11 of "9α-trichotriol" are similar to those for (70) (and 9β-trichodiol) indicating that it is in fact the 9β-hydroxy isomer. Similarly, those for "9β-trichotriol" agree with data for (82), and indicates that it must be the 9α-hydroxy compound. Future references to these compounds will use these revised stereochemical assignments and not the published ones (unless otherwise stated).

The EI-MS for the isolated trichodiol is in agreement with literature data[48], and

Fig.86: Chemical shift values for selected A ring carbons in a range of 9-hydroxytricho-10-enes.

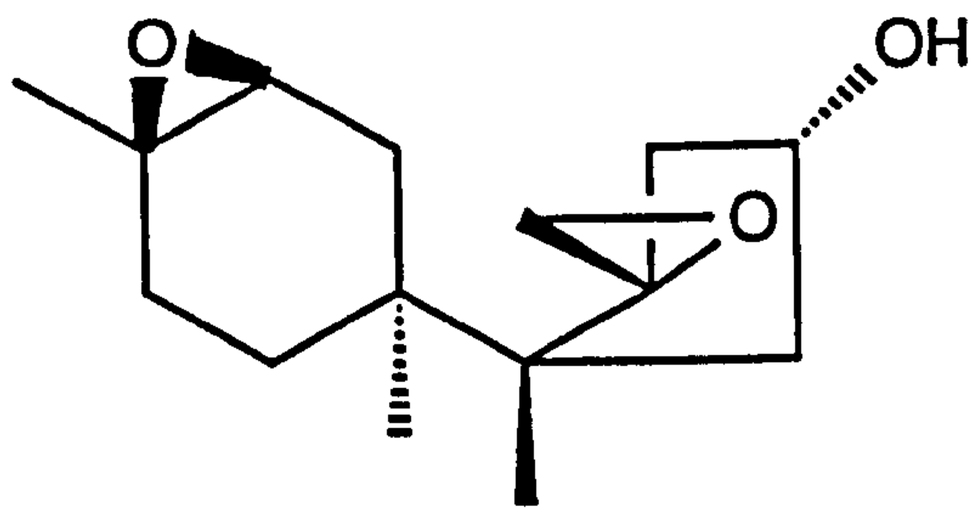
	9β-OH-Tricho-10,12-diene*	9α-OH-Tricho-10,12-diene*	9β-Trichodiol*	9α-Trichotriol[60]	9β-Trichotriol[110]	"Trichodiol"[60]
C-7	26.2	28.8	27.1	27.8	29.5	27.7
C-8	34.1	35.7	34.0	34.8	34.9	35.3
C-9	66.5	69.7	66.6	65.8	69.0	---
C-10	131.7	134.0	131.1	136.0+	133.8	133.8
C-11	136.5	133.3	136.4	132.3+	133.3	133.5
C-16	29.7	27.7	30.7	31.3	27.3	29.6

*This work

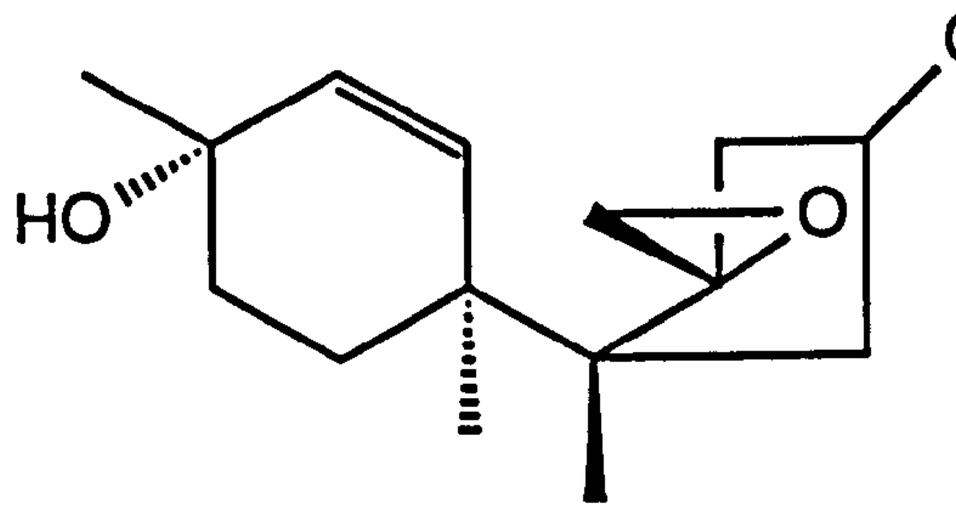
+Wrongly assigned - are interchanged

shows a molecular ion at m/z 252 consistent with a formula of $C_{15}H_{24}O_3$. The peak at m/z 125 ($C_8H_{13}O^+$) corresponds to the A ring fragment produced by cleavage of the C-5/6 bond, and the ion at m/z 107 is equivalent to $[A\text{-}H_2O]^+$ indicating hydroxylation in the A ring. GC-MS of the product after trimethylsilylation showed the formation of a di-TMS ether with a molecular ion at m/z 396, indicating the presence of two hydroxyl groups. The spectrum also shows that the A ring fragment ion has shifted from m/z 125 to m/z 197 indicating the presence of only one hydroxyl group in this ring, with the other being in ring B.

Trichodiol was first isolated from *T. roseum* in 1972[48] but no NMR data were reported, and the stereochemistry of hydroxylation at C-9 was not defined. In 1987, Corley *et al*[60] reported the isolation of trichodiol from *Fusarium sporotrichioides*, listing ^1H NMR and incomplete ^{13}C NMR data for this compound, and suggested that hydroxylation at C-9 in both trichodiol and trichotriol was in the α position. These literature data do not agree with the present NMR assignments, and it is now proposed that the "trichodiol" isolated from *F. sporotrichioides* is in fact a 3-hydroxy isomer, tricho-3,9α-diol (100). The NMR data [Table 27] show that the hydroxylated carbon assigned as C-2 resonates at δ68.6, with the adjacent proton (H-2) resonating at δ4.49. This is in contrast with the values for C-2 at ca. δ80 and H-2 at ca. δ3.7 in ITdiol, isotrichotriol[61], trichotriol[62] and EPT, and agrees more closely with values for C-3 at ca. δ70 and H-3 at ca. δ4.3 in trichotriol and isotrichotriol. Hydroxylation can therefore be reassigned to C-3 with the resonance at δ46.2 reassigned as C-2, and C-4 remaining as δ45.8. These values are in agreement with the resonances for C-2, 3 and 4 in 3α-hydroxy-9β,10β;12,13-diepoxyTDN (96) (see Table 23), confirming the revised B ring structure. The stereochemistry of the hydroxylation at C-9 was confirmed as being α by



3α-Hydroxy–9β,10β;12,13-diepoxyTDN (96)



Tricho-3,9α-diol (100)

Table 27: NMR data published for "trichodiol"[60].

H-2β	4.49 (*m*)	C-2	68.6
H-3α	2.06 (*dd*, J=14, 5Hz)	C-3	46.2
3β	1.85 (*m*)	C-4	45.8
H-4	1.45-1.85 (2H, *m*)	C-5	--
H-7	1.45-1.85 (2H, *m*)	C-6	--
H-8	-----	C-7	27.7
H-10	5.53 (*d*, J=10Hz)	C-8	35.3
H-11	5.47 (*dd*, J=10, 1.5Hz)	C-9	--
H-13	2.76 (*d*, J=4.0Hz)	C-10	133.8
	3.25 (*d*, J=4.0Hz)	C-11	133.3
H-14	1.04 (*s*)	C-12	--
H-15	0.98 (*s*)	C-13	50.7
H-16	1.28 (*s*)	C-14	19.6
		C-15	22.0
		C-16	29.6

comparison with the data for 9α- and 9β-hydroxytricho-10,12-diene (see Fig.86).

Acid catalysed cyclisation of 9β-trichodiol to EPT.

The non-enzymic cyclisation of trichotriol to isotrichodermol has previously been demonstrated[60], and it has long been proposed that trichodiol can undergo a similar cyclisation to produce EPT. In order to determine whether this rearrangement can occur, a sample of 9β-trichodiol was incubated in phosphate buffer at pH 5.0 at 28°C for 5 days. TLC analysis indicated the formation of a single, less polar product which was purified by preparative TLC and identified as EPT (64% yield). EI-MS and ^{1}H NMR spectra agreed with the data for the natural material (see Table 18).

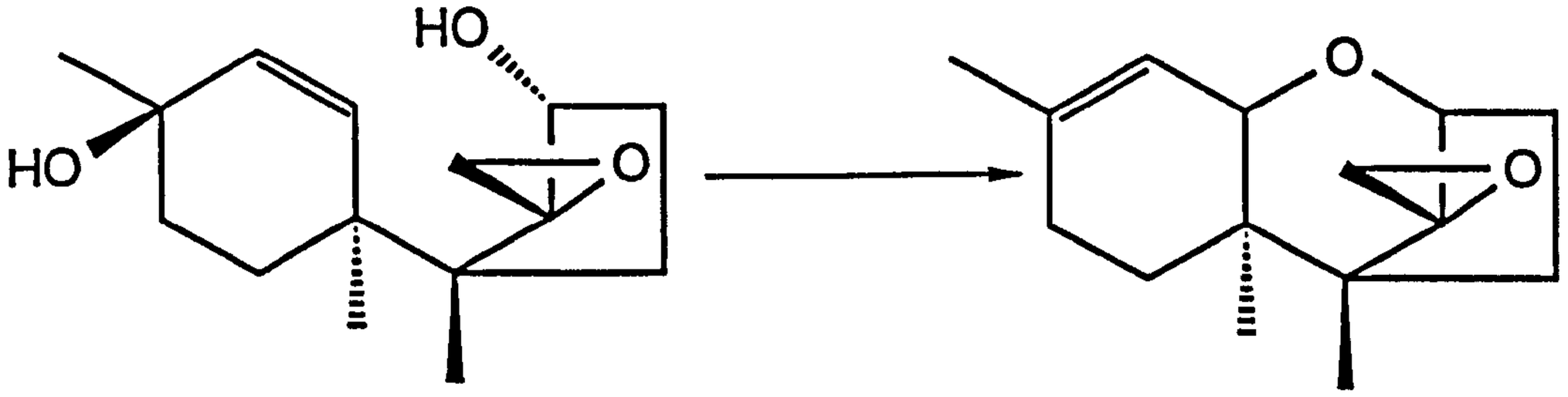
These results demonstrate the cyclisation of 9β-trichodiol to EPT for the first time [Fig.87], and conclusively prove that the B ring hydroxyl is indeed located at C-2α. Cyclisation could occur via an S_N2' mechanism, or via S_N1 reactions involving a carbocation intermediate (see Fig.62).

Production of trichotriol and isotrichotriol from *F. culmorum* CMI 14764.

Although trichotriol and isotrichotriol had not previously been isolated from this fungus, *F. culmorum* was selected as a potential source since it produces high yields of 3α-oxygenated trichothecenes. Also, in this laboratory the high polarity metabolites produced by *F. culmorum* had largely been ignored in favour of the major, less polar compounds (e.g. 3-AcDON, DHC), and so it was possible that these compounds had been overlooked in the past.

A culture of *F. culmorum* (1.6L; 7 days old) was harvested by extraction of the filtrate with ethyl acetate. TLC analysis indicated that in addition to

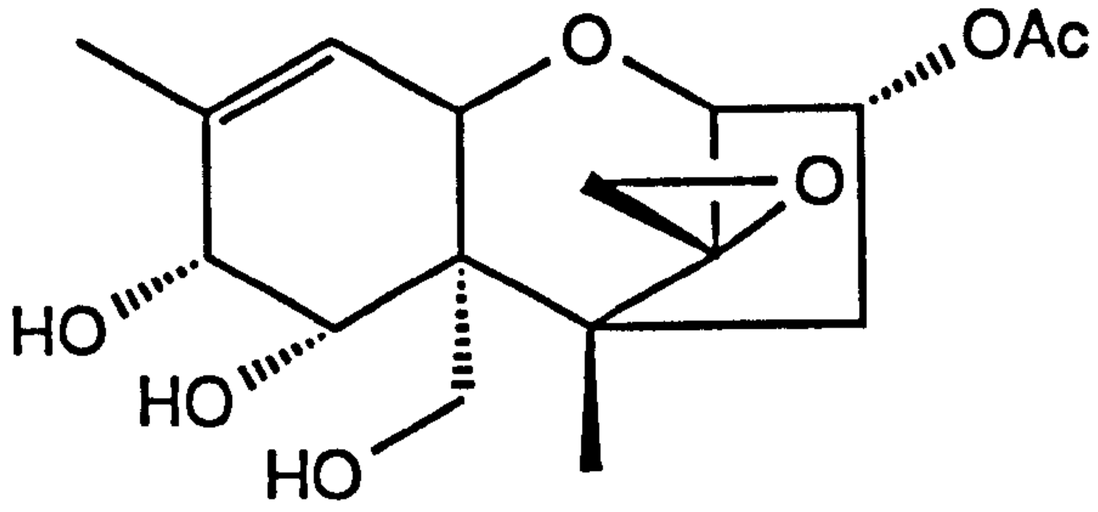
Fig.87: Acid catalysed cyclisation of 9β-trichodiol to EPT.



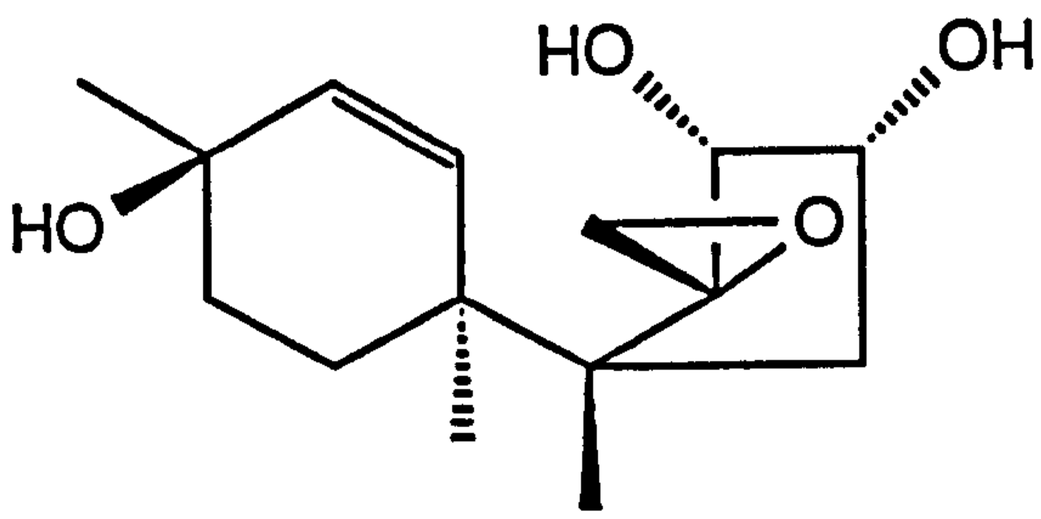
9β–Trichodiol (37B) EPT (3)

3-AcDON and DHC, two minor, more polar metabolites were present both of which reacted with PNBP/TEPA producing a sky blue colour. These were purified by a combination of repeated CTLC and preparative TLC methods. Analysis using a combination of EI-MS, ^{1}H NMR and ^{13}C NMR techniques identified these as 9β-trichotriol (48B) (11.3mgL^{-1}), and 15-deacetyl-7,8-dihydroxycalonectrin (15-deacetylDHC) (101) (16mgL^{-1}).

Identification of 9β-trichotriol was aided by comparison with the NMR data for 9β-trichodiol, and with literature data for the compound reported as "9α-trichotriol"[60] which has now been shown to be 9β-trichotriol (see p. 193). The ^{1}H NMR spectrum [Table 25] is similar to that for 9β-trichodiol (see Table 25), except for the appearance of a multiplet at δ4.25, and the change in multiplicity of the H-2β proton at ca. δ3.7 from a doublet of doublets to a doublet. This is consistent with the introduction of a hydroxyl group at C-3. The size of the coupling constant between H-2β and H-3 ($J_{2\beta,3}$=4.6Hz) indicates that H-3 is β since molecular models predict a dihedral about 90° for H-2β and H-3α i.e. $J_{2\beta,3\alpha}$ would be ≈0Hz. The hydroxyl group at C-3 can therefore be assigned as α. $^{1}H/^{1}H$ COSY [Fig.88] located the remaining B ring protons at δ2.12 and δ1.75, and these were assigned as H-4α and H-4β, respectively, from their coupling constants ($J_{4\alpha,3\beta}$=8.0Hz and $J_{4\beta,3\beta}$=6.3Hz). The vinylic resonance at δ5.71 can be assigned as H-11 since the signal is broadened by W-coupling to H-7α, as reported previously for this compound[60], and this couples with H-10 at δ5.54 ($J_{10,11}$=10.2Hz). $^{1}H/^{1}H$ COSY facilitated full ^{1}H NMR assignments which are in agreement with data published for"9α-trichotriol"[60]. The ^{13}C NMR data is also in agreement except for the assignments for C-10 and C-11 which have been interchanged. The assignments reported here were made from $^{1}H/^{13}C$ COSY which shows that H-11 at



15-DeacetylDHC (101)



9β–Trichotriol (48B)

Fig.88: Significant couplings observed in $^{1}H/^{1}H$ COSY of 9β-trichotriol.

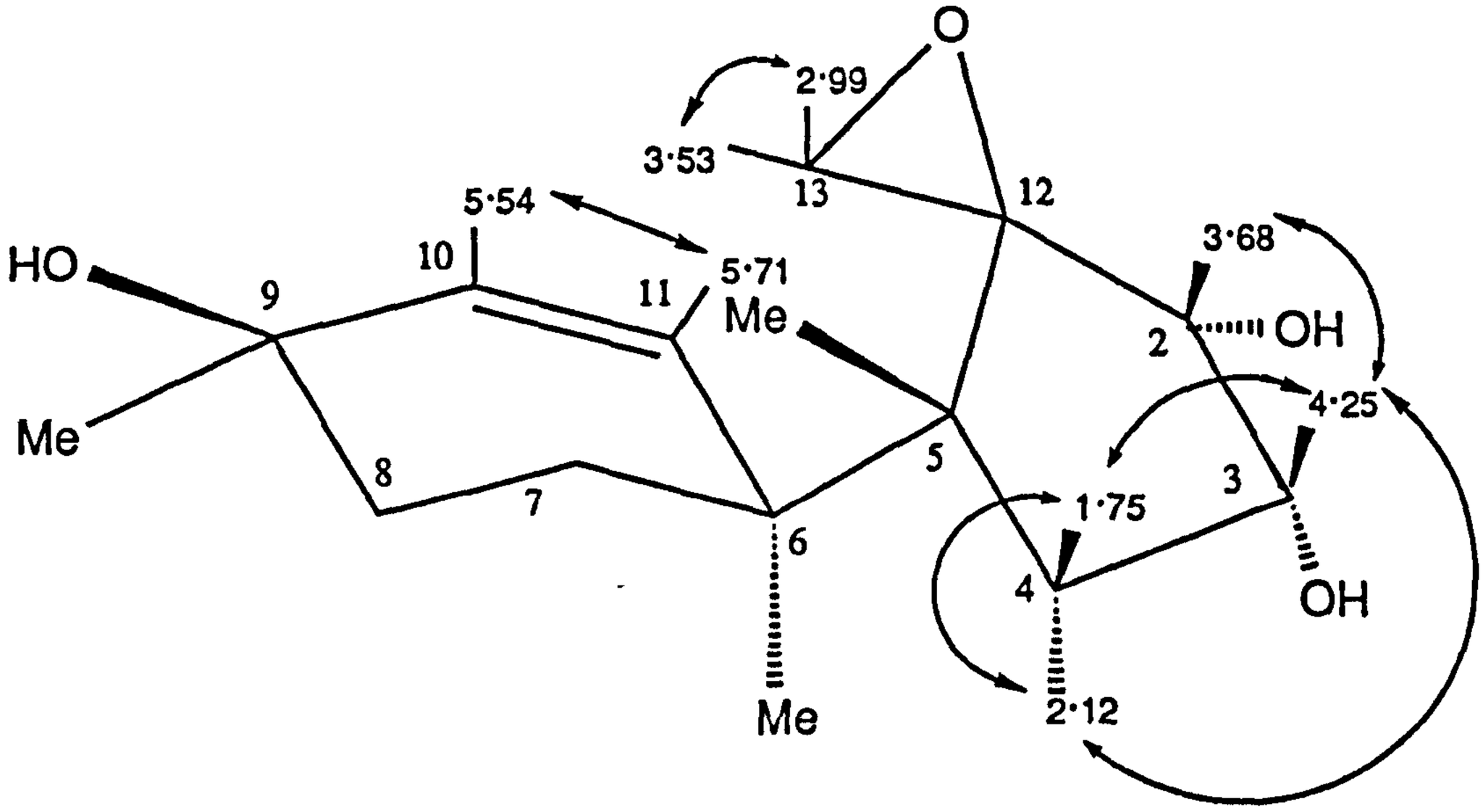


Fig.89: Couplings obtained from $^{1}H/^{13}C$ COSY of 9β-trichotriol.

Carbon resonance (δppm)		Cross peaks with protons at δppm
C-2	77.7	3.68
C-3	69.1	4.25
C-4	41.7	1.75 and 2.12
C-7	26.9	1.32 and 1.8
C-8	33.9	1.7
C-10	131.2	5.54
C-11	136.3	5.71
C-13	50.0	2.99 and 3.53
C-14	20.4	1.04
C-15	21.2	0.89
C-16	30.7	1.28

δ5.71 couples with the carbon at δ136.4 whilst H-10 at δ5.54 couples with the carbon at δ131.1 [Fig.89]. Comparison with the ^{13}C NMR data for 9β-trichodiol (see Table 26) helped to confirm this structure. A ring resonances are very similar indicating the same structure and stereochemistry, whilst in the B ring the main differences are that C-3 is shifted downfield from δ30.0 to δ69.1 and C-4 is shifted to a lesser extent from δ34.1 to δ41.7. This is consistent with the B ring structures being similar apart from hydroxylation at C-3.

The EI-MS shows a molecular ion at m/z 268 ($[C_{15}H_{24}O_4]^+$), and a fragment at m/z 250 corresponding to $[M-H_2O]^+$. The base peak at m/z 125 ($C_8H_{13}O^+$) corresponds to the A ring fragment produced by cleavage of the C-5/6 bond, with the fragment at m/z 107 ($C_8H_{11}^+$) being equivalent to $[A-H_2O]^+$. This is in agreement with the data published for "9α-trichotriol".

The 15-DeacetylDHC (101) produced was readily identified by comparison of its ^1H NMR spectrum [Table 28] with that for DHC (see Table 18). The spectra are similar with the only difference being that the C-15 methylene doublets centred at ca. δ4.31 in DHC are shifted upfield to ca. δ3.77, and one acetate signal has been lost from δ2.04. This is consistent with the removal of the C-15 acetyl group. A D_2O shake caused the signals at δ4.04 and δ4.50 to collapse to doublets, sharpened up the signals for the C-15 methylene protons at δ3.08 and δ3.22, and caused three signals at δ2.89, δ3.35 and δ3.47 to disappear. This is consistent with three hydroxyl groups being present, one each at positions C-7, C-8 and C-15, and confirms the structure as being 15-deacetylDHC. This is a novel compound, and its presence as a minor metabolite in *F. culmorum* cultures suggests that it could be a post-cyclisation intermediate in the biosynthesis of the major trichothecene products 3-AcDON and DHC. Feeding experiments subsequently demonstrated

Table 28: ^{1}H NMR chemical shifts (δppm) and coupling constants (Hz) for 15-deacetyl-7,8-dihydroxycalonectrin (101).

H-2β	3.76 (*d*, J=4.3)	
H-3β	5.17 (*ddd*, J=10.0, 5.1, 4.8)	
H-4α H-4β	2-2.2 (2H, *m*)	
H-7β	4.50 (*br t*)	(*d*, J=5.2)*
H-8β	4.04 (*br s*)	(*d*, J=5.3)*
H-10	5.61 (*d*, J=5.4)	
H-11	4.11 (*d*, J=5.9)	
H-13	3.08 (*d*, J=4.3)	
	3.22 (*d*, J=4.3)	
H-14	1.17 (*s*)	
H-15	3.69 (*br d*)	(*d*, J=12.5)*
	3.86 (*br d*)	(*d*, J=12.5)*
H-16	1.88 (*br s*)	
Acetate	2.09 (*s*)	
Hydroxyls	2.89 (*br s*)	
	3.35 (*br d*, J=8.8)	
	3.47 (*br s*)	

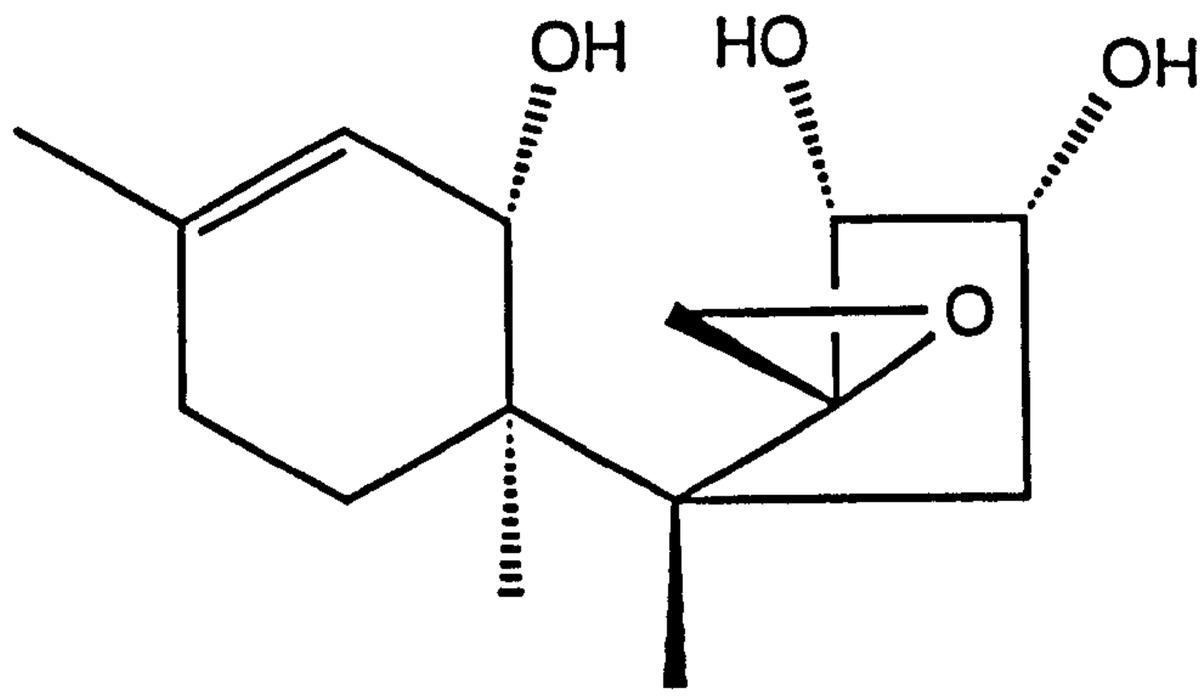
*with D_2O shake

that it is a precursor to 3-AcDON (see p. 227).

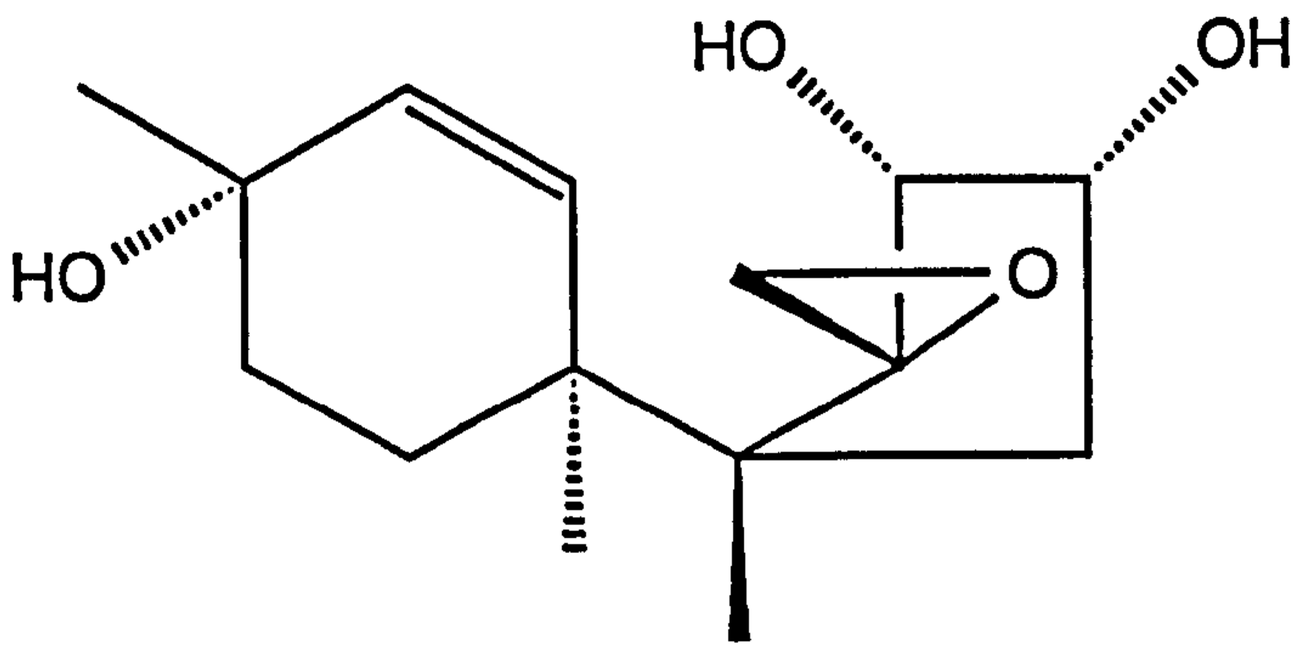
9β-Trichotriol is a novel isolation from *F. culmorum* although it has previously been isolated from *F. sporotrichioides*[60,110]. The yields obtained were sufficiently high (11mgL^{-1}) for this to be a feasible method for obtaining ^{14}C-labelled material. By feeding sodium [2-^{14}C]acetate (6.28MBq; 1.98GbqmM^{-1}) in a multiple batch addition to a culture of *F. culmorum* (0.8L), [^{14}C]-9β-trichotriol (7.0mg) was obtained with a specific activity of 0.30MBqmM^{-1}. [^{14}C]-15-DeacetylDHC (6.5mg; 0.42MBqmM^{-1}) was also isolated.

The presence of other uncyclised trichothecene products such as isotrichotriol were not detected in these *F. culmorum* cultures. It was decided to try xanthotoxin inhibition as a possible method for producing a sample of isotrichotriol. Xanthotoxin has been shown to inhibit the biosynthesis of trichothecenes from TDN (see p. 56), although the inhibition at a concentration of 0.1mM over a 6 day incubation period is not total, and so accumulation of intermediates is likely to occur.

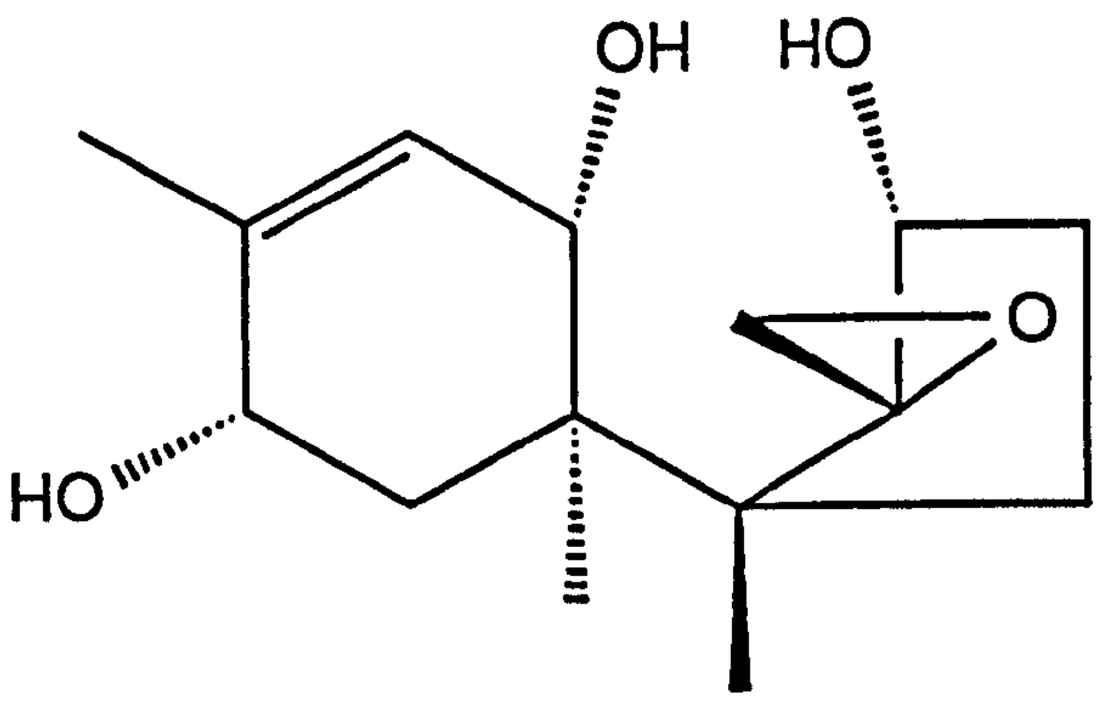
A liquid culture of *F. culmorum* (8.0L) was treated with 0.1mM xanthotoxin 2 days after subculture, and incubation was continued for a further 6 days. The culture was then harvested by extraction of the filtrate with ethyl acetate. Preliminary purification by column chromatography yielded TDN (255mgL^{-1}), and a fraction containing the most polar metabolites (413mg). TLC analysis of this fraction indicated the presence of three compounds which produced a sky blue colour with PNBP/TEPA reagent, characteristic of uncyclised trichothecenes. Purification by repeated CTLC, and preparative TLC methods yielded isotrichotriol (85) (4.6mg), 9α-trichotriol (48A) (1.3mg) and 8α-hydroxyisotrichodiol(102) (2.9mg) which were identified by EI-MS and ^{1}H NMR analysis.



Isotrichotriol (85)



9α-Trichotriol (48A)



8α-Hydroxyisotrichodiol (102)

The data for isotrichotriol agreed with literature values[61]. The ^{1}H NMR spectrum [Table 29] shows H-10 at δ5.15, and H-11β at δ4.64 with $J_{10,11\beta}$≈0Hz indicating a dihedral angle of about 90°, and confirming that the hydroxyl at C-11 is in the α position. The spectrum is therefore similar to ITdiol (see Table 16) except for the appearance of a multiplet at δ4.27, and the decrease in multiplicity for the H-4α resonance at ca. δ2.8 to a doublet of doublets. This is consistent with the presence of a hydroxyl group at C-3α. The EI-MS shows a molecular ion at m/z 268 ($C_{15}H_{24}O_4^+$), and a fragment ion at m/z 84 ($C_5H_8O^+$) due to retro Diels-Alder fragmentation of ring A, as seen for ITdiol (see Fig.49).

Identification of 9α-trichotriol was aided by comparison of its ^{1}H NMR data [Table 29] with that for 9β-trichotriol. The spectra are similar except for the chemical shifts of H-10, H-11 and the C-13 protons. H-10 and H-11, distinct from each other in the 9β-hydroxy isomer (48B) at δ5.54 and δ5.71 respectively, form an overlapping multiplet centred at δ5.5, and the C-13 protons are separated by only 0.23ppm as compared to 0.54ppm for (48B). The shifts and coupling constants for the H-2, H-3 and H-4 signals are essentially the same for both compounds, which suggests that the only difference between the two is the stereochemistry of the C-9 hydroxyl, which is reversed. The ^{1}H NMR and EI-MS data agree with the values published for the compound reported as 9β-trichotriol[110], which work in this laboratory has now shown to be 9α-trichotriol (see p. 193). Insufficient material was available to produce ^{13}C NMR data, and so definitive evidence for the stereochemistry at C-9 is therefore lacking.

8α-Hydroxyisotrichodiol (8α-hydroxyITdiol) is a novel compound, and was identified by ^{1}H NMR and EI-MS analysis, comparing the results with the data for ITdiol (see Table 16), and the 8α- and 8β-hydroxyisotrichotriols isolated from a

Table 29: ^{1}H NMR chemical shifts (δppm) and coupling constants (Hz) for isotrichotriol and 8α-hydroxyITdiol.

	Isotrichotriol (85)	8α-HydroxyITdiol (102)
H-2β	3.63 (*d*, J=4.0)	3.65 (*d*, J=4.0)
H-3α		1.0-2.0 (2H, *m*)
3β	4.27 (*ddd*, J=9.2, 6.6, 4.0)	
H-4α	2.72 (*dd*, J=13.6, 9.3)	2.79 (*ddd*, J=13, 13, 7)
4β	1.83 (*dd*, J=13.5, 6.5)	
H-7α	1.43 (*dd*, J=14.3, 8.4)	1.0-2.0 (3H, *m*)
7β	1.0-1.5 (*m*)	
H-8α	1.8-2.0 (*m*)	
8β	1.8-2.0 (*m*)	3.98 (*br d*, J=5.1)*
H-10	5.15 (*br s*)	5.33 (*br s*)
H-11	4.64 (*br s*)	4.56 (*br s*)
H-13	3.13 (*d*, J=4.3)	3.06 (*d*, J=4.1)
	3.31 (*d*, J=4.3)	3.32 (*d*, J=4.1)
H-14	1.03 (*s*)	1.13 (*s*)
H-15	0.91 (*s*)	0.98 (*s*)
H-16	1.66 (*br s*)	1.81 (*br s*)

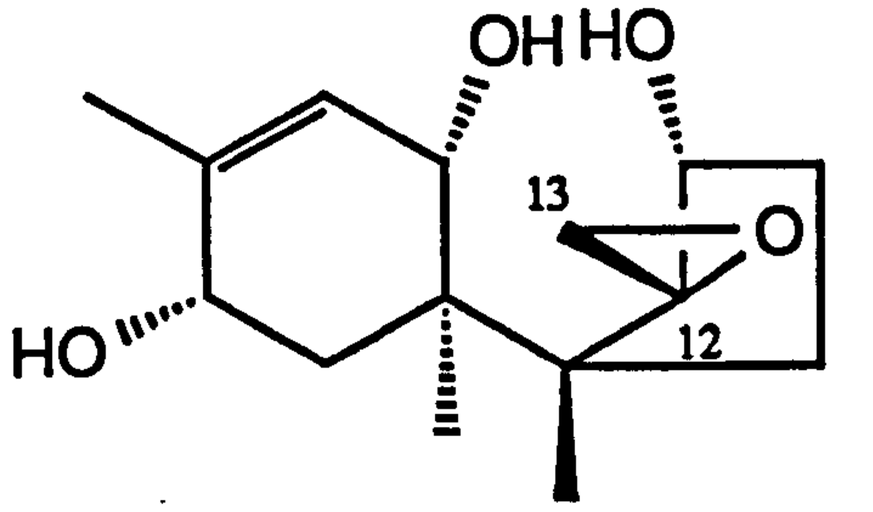
*with CH_3OD shake

mutant strain *F. sporotrichioides*[61].

The EI-MS shows a molecular ion at m/z 268 consistent with a molecular formula of $C_{15}H_{24}O_4$, 16 mass units heavier than ITdiol. The base peak at m/z 100 is consistent with a retro Diels-Alder fragment ion of $C_5H_8O_2$ as seen for 8α-hydroxyisotrichotriol, and indicates the presence of two hydroxyl groups in the A ring. The ^1H NMR spectrum is similar to that for ITdiol with H-2β as a doublet ($J_{2\beta,3\beta}$=4.0Hz) at δ3.65, and H-4α a doublet of triplets at δ2.72 ($J_{4\alpha,4\beta}$≈13Hz, $J_{4\alpha,3\alpha}$≈7Hz and $J_{4\alpha,3\beta}$≈13Hz) consistent with one hydroxyl in the B ring at C-2α. The only differences are the appearance of a resonance at δ3.98, and downfield shifts for the H-10 signal from δ5.14 to δ5.33 and the C-16 methyl resonance from δ1.66 to δ1.81, 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 $J_{8\beta,7\beta}$=5.1Hz, and $J_{8\beta,7\alpha}$≈0Hz, similar to the coupling found in 8α-hydroxyisotrichotriol. With 8β-hydroxyisotrichotriol the H-8α resonance is a broadened double doublet with $J_{8\alpha,7\alpha}$=5.9Hz, and $J_{8\alpha,7\beta}$=9.3Hz[110], as expected from molecular models which predict dihedral angles of ca.50°and 170°, respectively, for these pairs of protons.

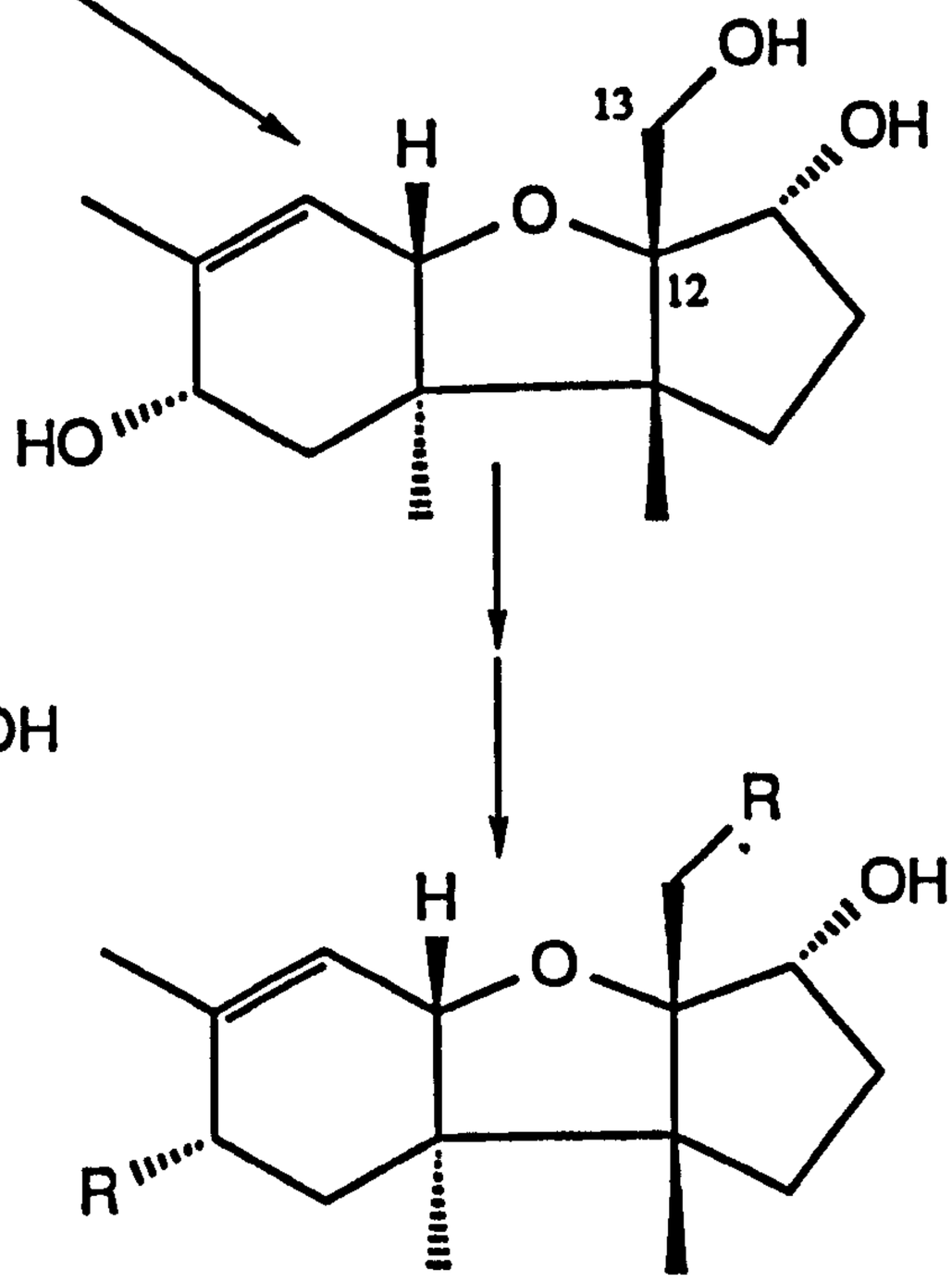
Isotrichotriol and 9α-trichotriol are novel isolations from *F. culmorum* although they have previously been isolated from mutant strains of *F. sporotrichioides*[61,110]. 8α-HydroxyITdiol is a new compound but is not believed to be involved in trichothecene biosynthesis. The related compounds 8α- and 8β-hydroxyisotrichotriol did not restore T-2 toxin production when fed to a mutant of *F. sporotrichioides*[110] (see Table 27) indicating that they are not significant biosynthetic precursors. 8α-HydroxyITdiol may however be involved in the

Fig.90: 8α-HydroxyITdiol proposed as a precursor to 8α-OH sambucoin, and 8α-oxygenated 11-epi-apotrichothecenes.



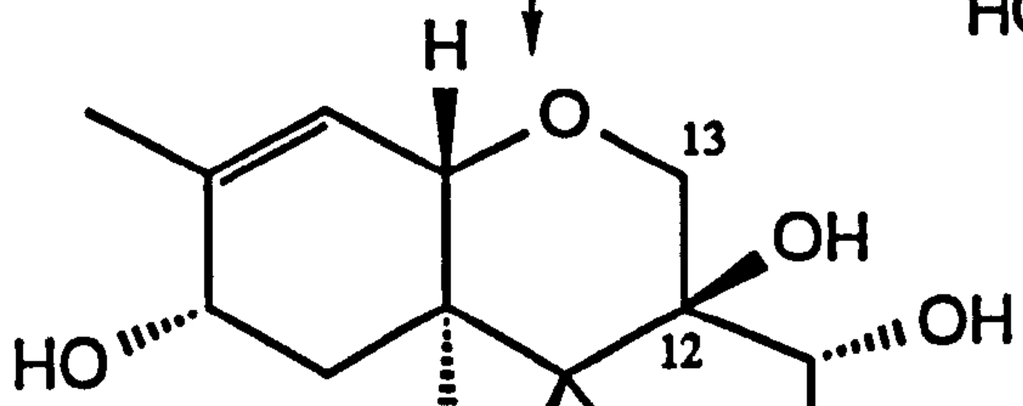
8α-HydroxyITdiol (102)

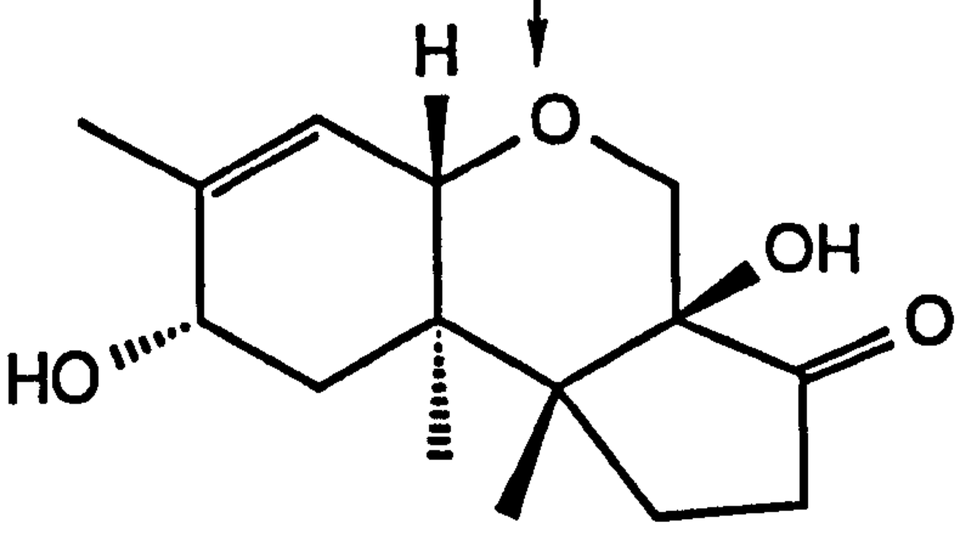
cyclisation to C-12



11-epi-apotrichothecenes
eg.R = OH or OAc

cyclisation to C-13





8α-OH sambucoin (62)

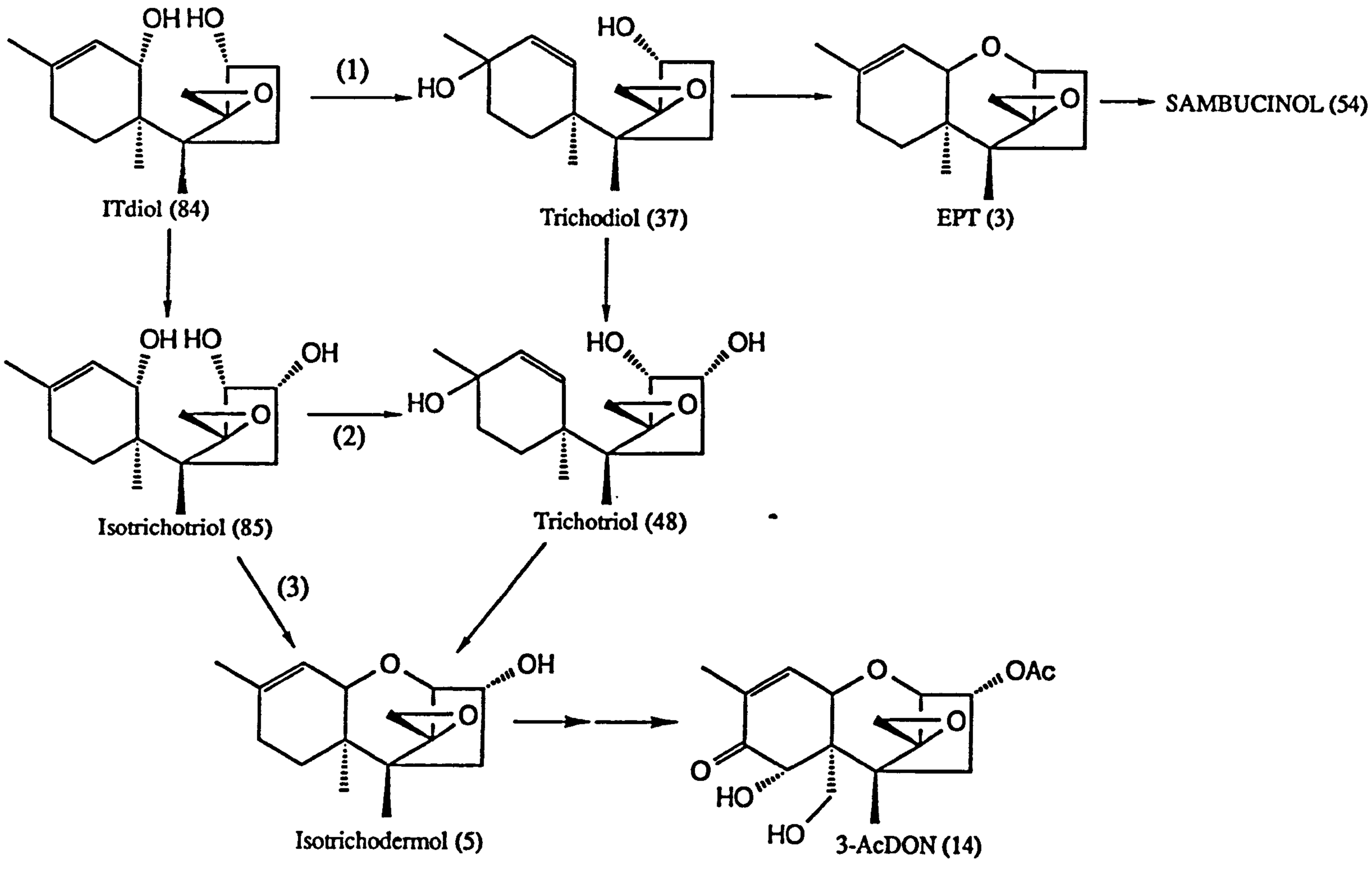
biosynthesis of the minor, trichothecene-related metabolites such as 8α-hydroxysambucoin[82], or the range of 2α,8α-dihydroxy-11-epi-apotrichothecenes isolated from *Fusarium* species[76] [Fig.90]. Alternatively, it may be a dead end metabolite produced by hydroxylation of ITdiol.

9β-trichotriol, previously produced in good yields from an uninhibited *F. culmorum* culture, was not isolated when xanthotoxin was used as an inhibitor. This is probably the result of the practical procedures used for the extraction and purification, and does not necessarily indicate that 9β-trichotriol was not produced in the inhibited culture. Further investigation is needed to ascertain whether both or just single isomers of trichotriol are produced under the various culture conditions.

Studies on the post-ITdiol biosynthesis of trichothecenes.

In the proposed scheme for the production of oxygenated trichothecenes from ITdiol [Fig.91] there are a number of possible biosynthetic pathways. One involves isomerisation to trichodiol followed by 3α-hydroxylation to trichotriol and cyclisation to isotrichodermol. Alternatively, the other two possibilities require 3α-hydroxylation to isotrichotriol which can then give rise to isotrichodermol either by direct cyclisation or by isomerisation-cyclisation via trichotriol. In this laboratory, the successful isolation of ITdiol, 9β-trichodiol, 9β-trichotriol and isotrichotriol meant that it was now possible to further investigate this area of the biosynthesis in *F. culmorum* by means of appropriate feeding experiments.

Fig.91: Three possible biosynthetic pathways to the oxygenated trichothecenes from ITdiol.



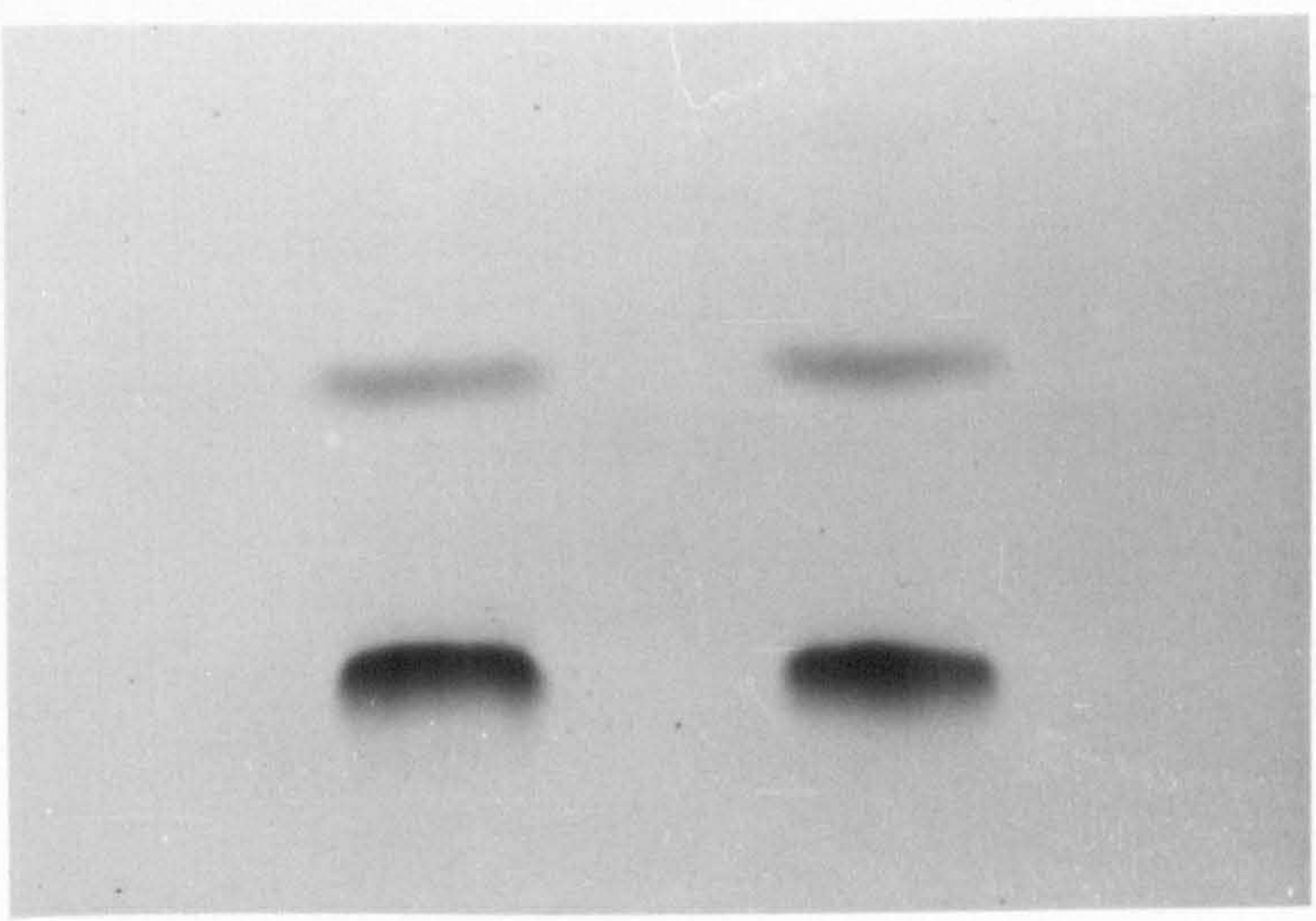
Feeding experiments using [^{14}C]-9β-trichotriol.

Trichotriol has been proposed as the immediate precursor to the first oxygenated trichothecene formed, isotrichodermol. Indeed the non-enzymatic cyclisation of 9β-trichotriol to isotrichodermol has been demonstrated[60,110], and both the 9β- and 9α-hydroxy isomers have been shown to restore T-2 toxin biosynthesis in a blocked mutant[110]. Now that a sample of [^{14}C]-9β-trichotriol had been produced it was possible to study the biosynthesis of trichothecenes from 9β-trichotriol, and in particular the cyclisation to isotrichodermol. The ultimate aim would be to demonstrate the presence of a key cyclase enzyme responsible for the formation of the tetracyclic trichothecene skeleton.

A cell-free system of *Fusarium culmorum* capable of mediating the transformation of 9β,10β-epoxyTDN to 9β,10β;12,13-diepoxyTDN (see Fig.71) had been developed[111], and this system was used to observe the functioning of any cyclase enzyme. The mycelium from a 3 day old culture of *F. culmorum* was liquidised in a blender at 0°C, and then ruptured in a microfluidiser precooled with ice. Removal of cell debris from the homogenate by centrifugation at 4°C yielded the cell-free preparation. [^{14}C]-9β-Trichotriol (0.2mg; 0.30MBqmM^{-1}) was fed to a portion of this preparation in phosphate buffer (100mM; pH 7.15) and incubated (27°C, 250rpm) for 2 hours. A similar experiment using cell-free extract which had been boiled for 10 mins was also performed. The reactions were extracted with ethyl acetate, and the extracts analysed using TLC and autoradiography. Product formation was measured using scintillation counting procedures.

The autoradiogram [Fig.92, lane 1) shows that some cyclisation to isotrichodermol has occurred (5.0%). However, in the boiled control (lane 2) a

Fig.92: Autoradiogram showing the attempted enzymic cyclisation of 9β-trichotriol by a cell-free extract of *F.culmorum*.



Lane 1: Fed [^{14}C]-9β-trichotriol
Lane 2: Fed [^{14}C]-9β-trichotriol, boiled extract

2hr incubation
TLC: Ether-acetone, 85:15

similar level of product formation (5.2%) was detected indicating that the transformation observed in lane 1 is likely to be chemical rather than enzymic.

Cyclase activity responsible for the formation of isotrichodermol has not therefore been observed. It is possible that the enzyme requires co-factors not present in this experiment, e.g. for phosphorylating the leaving group. However, the reaction occurs readily under acid catalysed conditions[60,110] and a phosphorylation step seems unlikely. An alternative explanation is that 9β-trichotriol is not the natural substrate for the cyclase enzyme, and so is not a biosynthetic precursor to the trichothecenes. This is supported by the suprisingly high yields of 9β-trichotriol obtained from the unhibited *F. culmorum* culture (see p. 199), despite the fact that this metabolite had not previously been observed in earlier cultures. Its appearance was accounted for by the fact that the K_2HPO_4 used in the culture medium had just been replaced by a different batch which produced medium with a slightly higher pH (≈6.5 instead of ≈5.5). Trichotriol is known to cyclise non-enzymatically to isotrichotriol at below ca. pH 6[110], and so in previous cultures it may not have been present having been converted instead to isotrichodermol. However, isotrichodermol has never been isolated from *F. culmorum* in this laboratory, probably because it is rapidly transformed to the more oxygenated products such as 3-AcDON. If 9β-trichotriol is likewise a biosynthetic precursor to trichothecenes it should similarly be transformed to later compounds but instead it appears to accumulate at the higher pH. The reported incorporation of both 9α- and 9β-trichotriol into T-2 toxin occurred in a feeding experiment lasting 4 days, and could be explained by slow non-enzymatic cyclisation to isotrichodermol which is a biosynthetic precursor.

In order to study this aspect further, short term feeding experiments using

^{14}C-labelled trichotriol in *F. culmorum* whole-cells were performed.

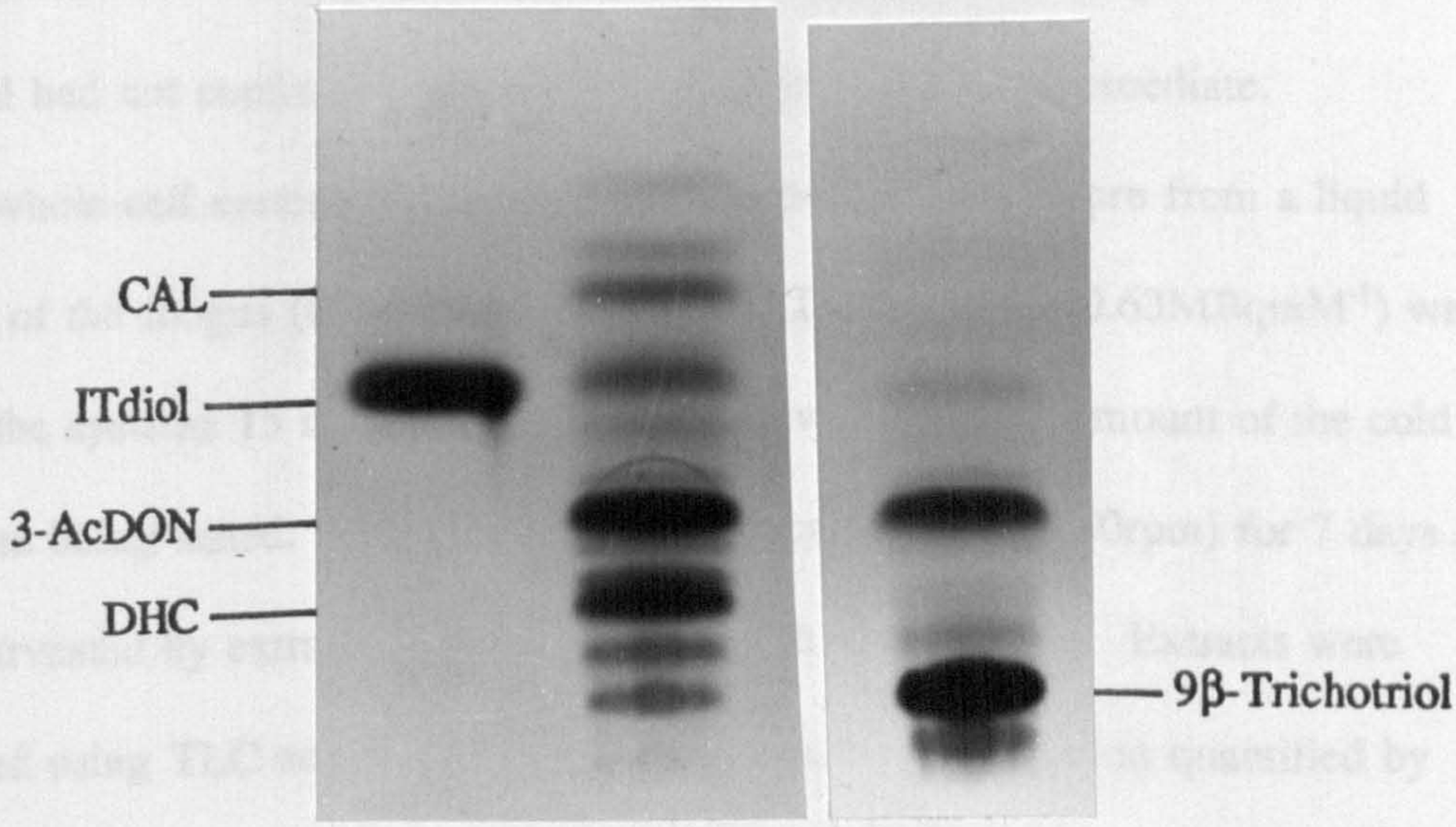
A whole-cell system of *F. culmorum* was prepared from a 4 day old culture of the fungus by resuspending the filtered mycelia in distilled water. [^{14}C]-9β-Trichotriol (0.5mg; 0.30MBqmM^{-1}) was fed to this, and a similar culture received [^{14}C]ITdiol (0.5mg; 0.63MBqmM^{-1}). After incubating (27°C,250rpm) for 7 hours, the cultures were harvested by extraction of the filtrate with ethyl acetate. Extracts were analysed using TLC and autoradiography, with scintillation counting being used to quantify the formation of any products. The autoradiograms [Fig.93] show that virtually all the ITdiol has been transformed to trichothecene products (lane 2), with 37.3% of the radioactivity fed being associated with 3-AcDON and DHC, the major products formed. Lane 3 shows that a large proportion of the 9β-trichotriol substrate remains unaltered although some transformation to trichothecenes has occurred. The activity associated with 3-AcDON and DHC is only 6.9%, which is about one-fifth the incorporation found with ITdiol.

Thesė results indicate that 9β-trichotriol is incorporated into trichothecenes in *F. culmorum*, but the production of 3-AcDON and DHC from it occurs approximately five-times more slowly than from ITdiol. This supports the hypothesis that it is not a natural biosynthetic precursor like ITdiol, but that it can become incorporated into trichothecenes via non-enzymatic cyclisation to isotrichodermol.

<u>Competitive feeding experiments using [^{14}C]ITdiol.</u>

It had not been possible to produce 9β-trichodiol and isotrichotriol in radiolabelled form, and only small quantities of unlabelled material were available for use in feeding experiments. It was therefore decided that the best method for studying

Fig.93: Incorporation of [^{14}C]ITdiol and [^{14}C]-9β-trichotriol into trichothecene toxins in *Fusarium culmorum*.



Lane 1: [^{14}C]ITdiol standard
Lane 2: Fed [^{14}C]ITdiol
Lane 3: Fed [^{14}C]-9β-trichotriol

7hr incubation
TLC: Hexane-EtOAc, 1:1

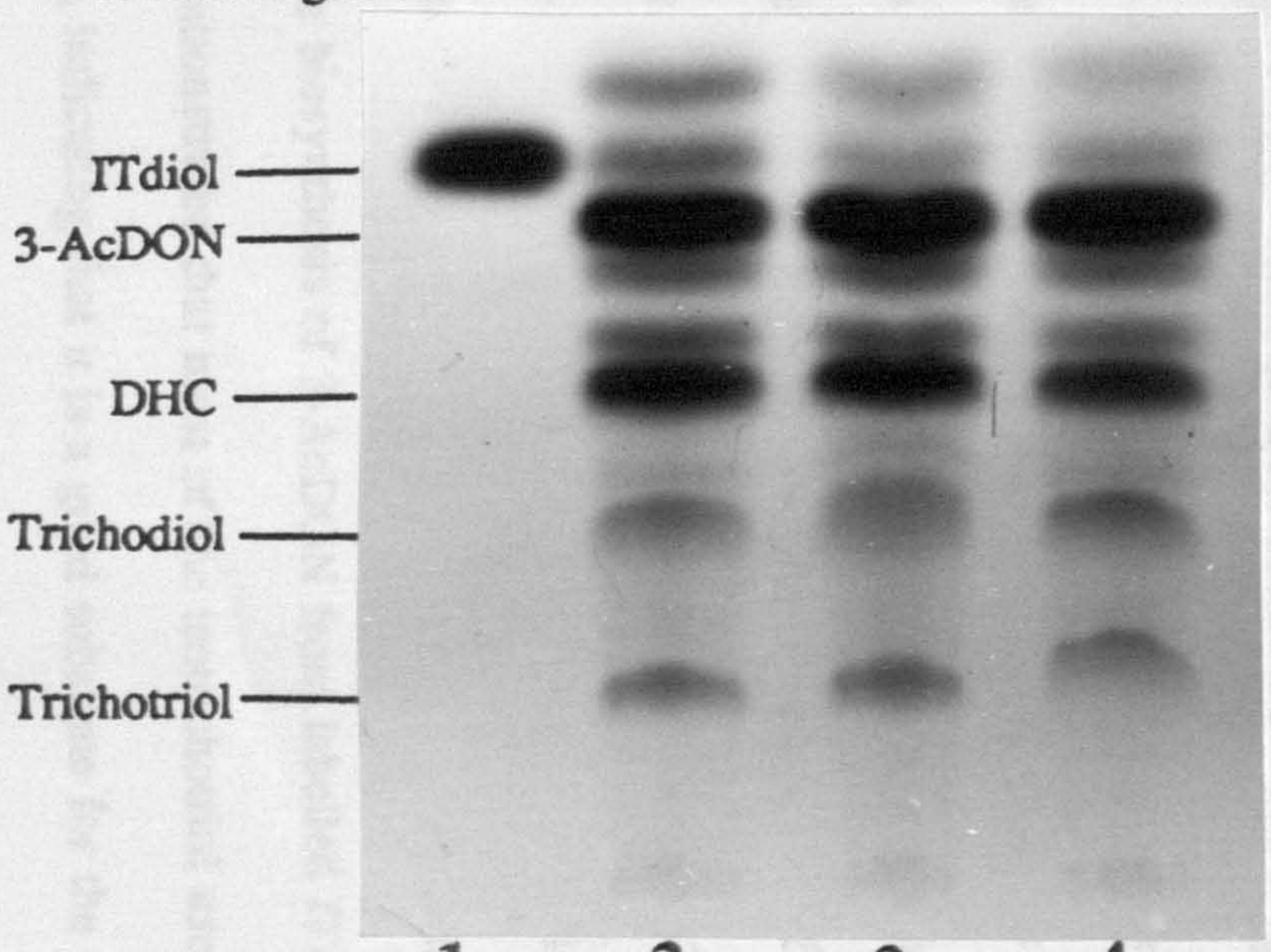
their involvement in trichothecene biosynthesis would be to feed them to *F. culmorum* in competition with [^{14}C]ITdiol. By observing their effects on ITdiol incorporation it should be possible to determine whether or not they are natural intermediates in the biosynthesis of trichothecenes from ITdiol. 9β-Trichotriol was also included in the study because the feeding experiments using ^{14}C-labelled material had not confirmed it to be a natural biosynthetic intermediate.

A whole-cell system of *F. culmorum* was prepared as before from a liquid culture of the fungus (60-96 hours old). [^{14}C]ITdiol (0.5mg; 0.63MBqmM^{-1}) was fed to the systems 15 mins after they had received a similar amount of the cold substrate being tested. The cultures were incubated (27°C, 250rpm) for 7 days and then harvested by extraction of the filtrates with ethyl acetate. Extracts were analysed using TLC and autoradiography, and product formation quantified by scintillation counting.

Autoradiogram A [Fig.94] shows the results from one batch of feeding experiments using 9β-trichotriol and 9β-trichodiol as inhibitors, and autoradiogram B [Fig.94] the results from another using isotrichotriol. In A, lane 2 shows the uninhibited incorporation of [^{14}C]ITdiol into a range of trichothecenes with 3-AcDON and DHC being the major products formed. Some incorporation into 9β-trichotriol and 9β-trichodiol is also evident. Lanes 3 and 4 show the incorporation of [^{14}C]ITdiol into trichothecenes in the presence of 9β-trichodiol and 9β-trichotriol, respectively. The profile of metabolites produced in both cases is identical to that for the uninhibited culture (lane 2), and no accumulation of radioactivity associated with the cold compound added is observed. This indicated that neither 9β-trichodiol nor 9β-trichotriol have inhibited the biosynthesis of trichothecenes from ITdiol. Measurements for the incorporation of radioactivity

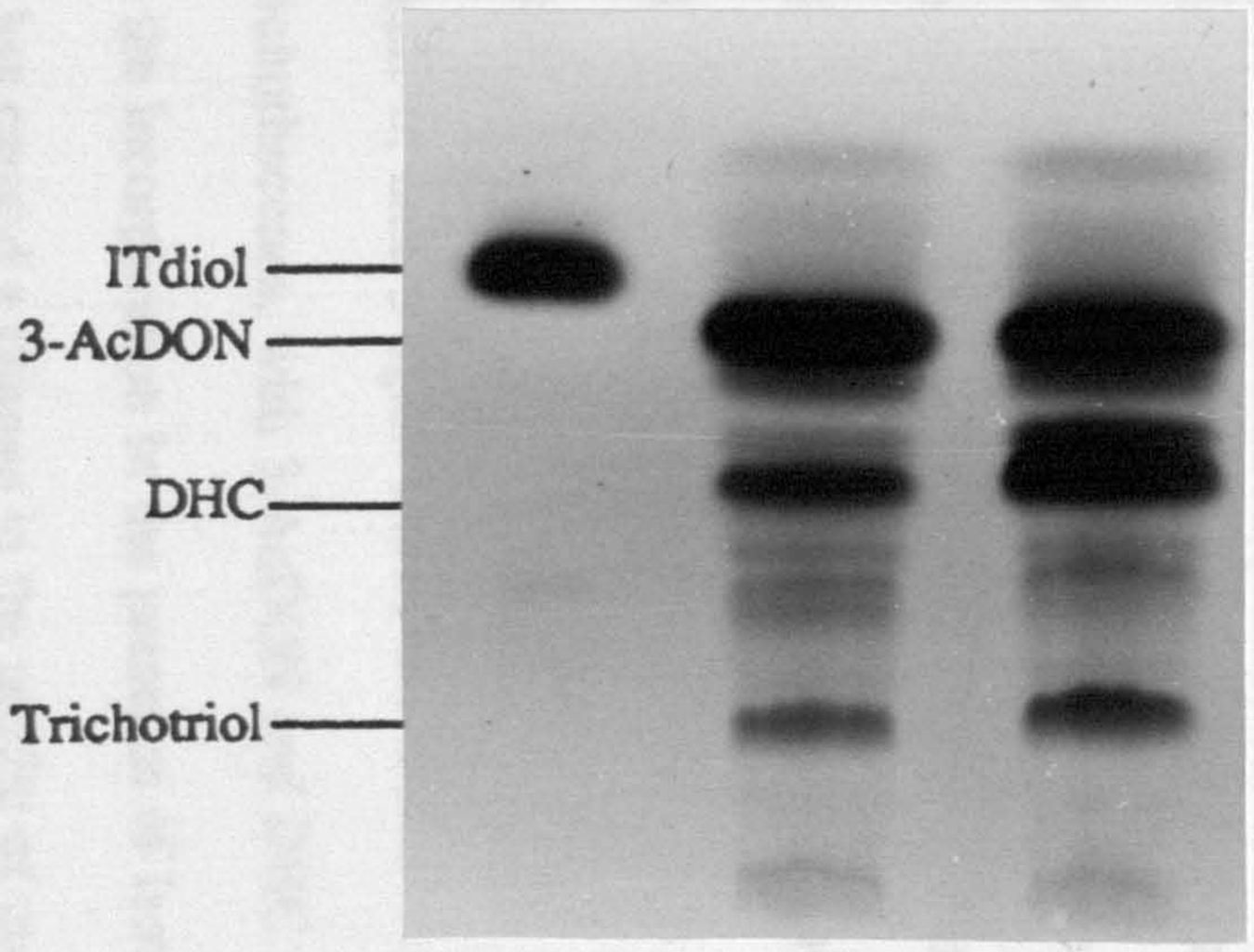
Fig.94: Autoradiograms showing the effect of isotrichotriol, 9β-trichodiol and 9β-trichotriol on the incorporation of [^{14}C]ITdiol into trichothecenes in *F. culmorum*.

Autoradiogram A



Lane 1: [^{14}C]ITdiol standard
Lane 2: Fed [^{14}C]ITdiol (0.5mg)
Lane 3: Fed [^{14}C]ITdiol (0.5mg) + 9β-trichodiol (0.5mg)

Autoradiogram B



Lane 4: Fed [^{14}C]ITdiol (0.5mg) + 9β-trichotriol (0.5mg)
Lane 5: Fed [^{14}C]ITdiol (0.5mg)
Lane 6: Fed [^{14}C]ITdiol (0.5mg) + isotrichotriol (0.5mg)

7hr incubation TLC: Ether-acetone, 9:1

into 3-AcDON and DHC for lanes 2, 3 and 4 [Table 30] also demonstrate that no inhibition has occurred. The values obtained for the formation of both 3-AcDON and DHC in experiments 3 and 4 are at least as high as those determined for the uninhibited system (lane 2). Further TLC analysis showed that significant amounts of both 9β-trichodiol and 9β-trichotriol were recovered unchanged from experiments 3 and 4 respectively, indicating that they had not been metabolised by the fungus as quickly and completely as ITdiol.

In autoradiogram B, lane 5 again shows the uninhibited incorporation of [^{14}C]ITdiol into trichothecenes, with 3-AcDON and DHC as the major products, and lane 6 shows the incorporation in the presence of isotrichotriol. These indicate that isotrichotriol has caused a change in the profile of metabolites produced from [^{14}C]ITdiol, with the amount of labelled DHC formed being significantly increased, and 3-AcDON production being slightly decreased. The results from scintillation counting [Table 31] confirmed these observations, demonstrating an increase in incorporation into DHC from 7.2% to 17.2%, and a decrease into 3-AcDON from 21.2% to 18.5%. These results were also expressed as a ratio of DHC to 3-AcDON, which eliminates any errors due to the amount of extract analysed by autoradiography. In the uninhibited system this value is 0.32, but in the presence of isotrichotriol it increases three-fold to 0.95. Since DHC is a demonstrated precursor to 3-AcDON (see p. 230), these results indicate that isotrichotriol must be inhibiting the biosynthesis of 3-AcDON from labelled ITdiol. Additional TLC analysis also demonstrated that most of the isotrichotriol used had been biotransformed, indicating that it is a good substrate for the fungus.

The effect of isotrichotriol-inhibition on the biosynthesis of trichothecenes from ITdiol is being observed a long way down the pathway, and consequently is not as

Tables 30 & 31: Effect of isotrichotriol, 9β-trichotriol and 9β-trichodiol on the incorporation of ITdiol into 3-AcDON and DHC in *Fusarium culmorum*.

Table 30.

Lane No.	Inhibitor	% Incorporation of [^{14}C]ITdiol into:- 3-AcDON	DHC
2	None	23.0	14.3
3	9β-Trichodiol	29.4	16.2
4	9β-Trichotriol	23.9	12.3

Table 31.

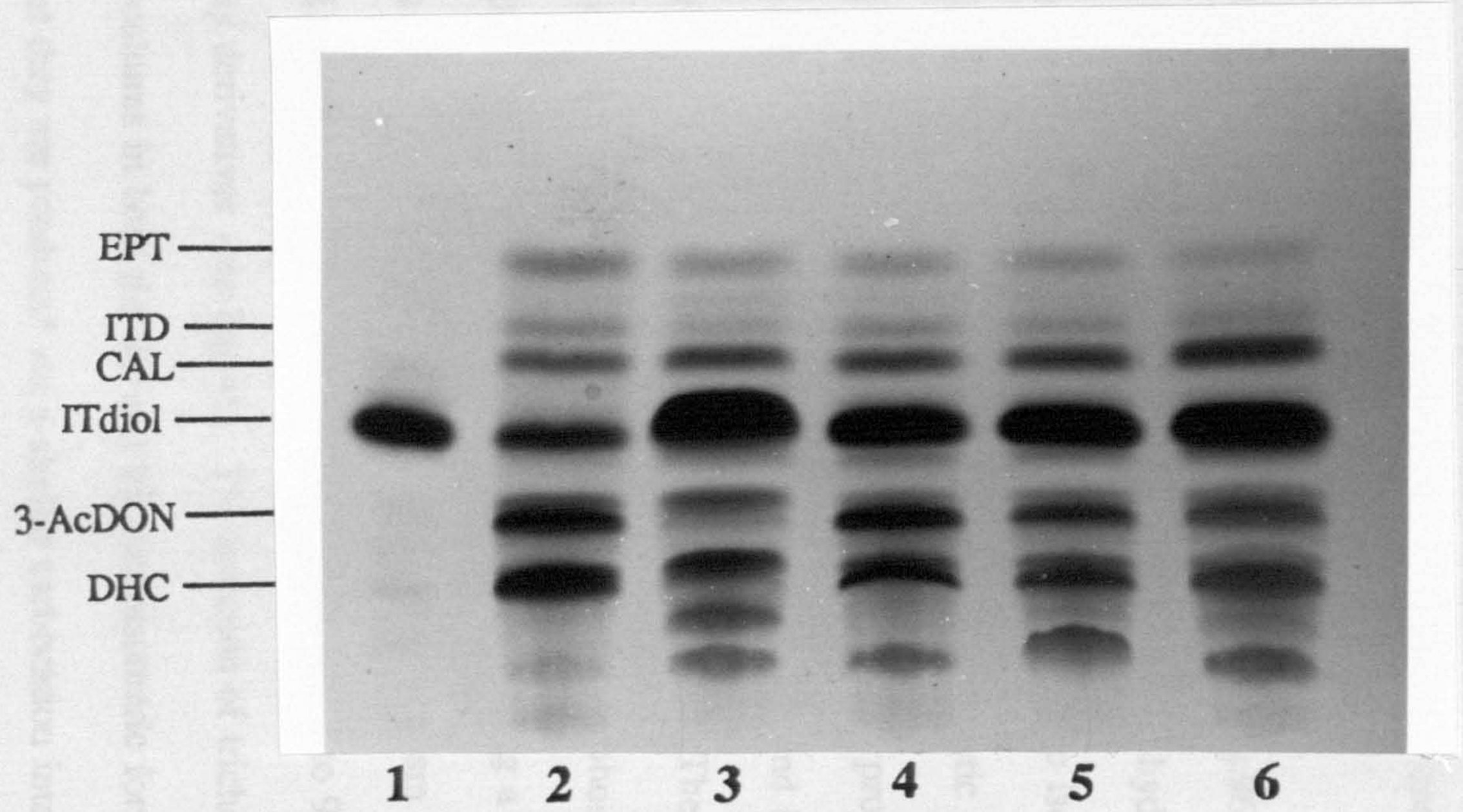
Lane No.	Inhibitor	% Incorporation of [^{14}C]ITdiol into:- 3-AcDON	DHC	Ratio of DHC/ 3-AcDON
5	None	21.8	7.2	0.32
6	Isotrichotriol	18.5	17.7	0.95

$$\% \text{ Incorporation into a compound} = \frac{\text{Activity recovered as that compound}}{\text{Total activity fed in}} \times 100$$

obvious as was hoped. In order to demonstrate this inhibition more clearly, the experiment was repeated using six times more isotrichotriol than [^{14}C]ITdiol. Experiments using a similar excess of 9β-trichotriol, 9β-trichodiol and cold ITdiol were also performed. In the autoradiogram produced [Fig.95] lane 2 shows the uninhibited incorporation of [^{14}C]ITdiol into a range of trichothecenes with DHC and 3-AcDON being the major products formed. By comparing this "normal" metabolite profile with that obtained in the presence of isotrichotriol (lane 6), it is obvious that inhibition has occurred. The intensities of both 3-AcDON and DHC are reduced and there is an increase in the activity remaining as ITdiol, which is similar to the profile obtained in the presence of cold ITdiol (lane 3). Lane 4 shows that 9β-trichodiol has had little effect, with the amount of label associated with 3-AcDON being similar to that found in the uninhibited system (lane 2). The incorporation of label into 3-AcDON and DHC in the presence of 9β-trichotriol (lane 5) is lower than in lane 1 indicating that some inhibition has occurred. This inhibition is not however as great as that produced by isotrichotriol (lane 6), and could be due to the formation of isotrichodermol by acid catalysed cyclisation of trichotriol.

In summary, the competitive feeding experiments demonstrated that the biosynthesis of 3-AcDON from ITdiol is inhibited by isotrichotriol, and, to a lesser extent, by 9β-trichotriol, but not by 9β-trichodiol. The latter two compounds are also poorly utilised by *F. culmorum* whilst isotrichotriol is metabolised as efficiently as ITdiol. These results suggest that isotrichotriol is an intermediate in the post-ITdiol biosynthesis of trichothecenes whilst 9β-trichotriol and 9β-trichodiol are not involved. It is possible that the latter may function as intermediates in a metabolic grid but with biosynthesis via these compounds being only a minor

Fig.95: Repeat experiment investigating the effect of isotrichotriol, 9β-trichodiol and 9β-trichotriol on the incorporation of [^{14}C]ITdiol into trichothecenes in *F.culmorum*.



Lane 1: [^{14}C]ITdiol standard
Lane 2: Fed [^{14}C]ITdiol (0.1mg)
Lane 3: Fed [^{14}C]ITdiol (0.1mg) + unlabelled ITdiol (0.6mg)
Lane 4: Fed [^{14}C]ITdiol (0.1mg) + 9β-trichodiol (0.6mg)
Lane 5: Fed [^{14}C]ITdiol (0.1mg) + 9β-trichotriol (0.6mg)
Lane 6: Fed [^{14}C]ITdiol (0.1mg) + isotrichotriol (0.6mg)

6hr incubation TLC: Hexane-EtOAc, 1:1

pathway, with the majority of production occurring via isotrichotriol. This is supported by the observed incorporation of [^{14}C]-9β-trichotriol into trichothecenes which was found to be only about 20% of that determined for ITdiol. However, this low incorporation can be more satisfactorally explained by the occurrence of acid catalysed cyclisation to isotrichodermol, with trichotriol otherwise not being an enzymic precursor as suggested by the results of the cell-free feeding experiment.

Thus, a new biosynthetic sequence is now proposed [Fig.96] in which the oxygenated trichothecenes are produced from ITdiol via 3α-hydroxylation to isotrichotriol which then undergoes S_N2 or S_N1 cyclisation to isotrichodermol. Trichodiol and trichotriol are not considered to be biosynthetic intermediates in this sequence, and it is proposed that they are shunt metabolites produced non-enzymatically by acid catalysed isomerisation from ITdiol and isotrichotriol, respectively, via carbocation intermediates (90) and (103). The isomerisation of ITdiol to 9β- and 9α-trichodiol (in a ratio of about 4:1) in phosphate buffer at pH 5.8 has been demonstrated, and an S_N1 mechanism involving a carbocation intermediate was proposed (see Fig.77). A similar mechanism is postulated for the acid catalysed conversion of 9β-hydroxytricho-10,12-diene to 9α-hydroxy- and 9β/9α-methoxy derivatives (see Fig.45). The isolation of trichotriol from *F. culmorum* cultures in both the 9α and 9β stereoisomeric forms supports the hypothesis that they are produced via a similar carbocation intermediate derived from isotrichotriol.

In its simplest form, the biosynthetic cyclisation may be an S_N2 process. If an S_N1 mechanism occurs, the carbocations (103) and (90) may be formed from isotrichotriol and ITdiol as enzyme-bound intermediates in the biosynthesis of

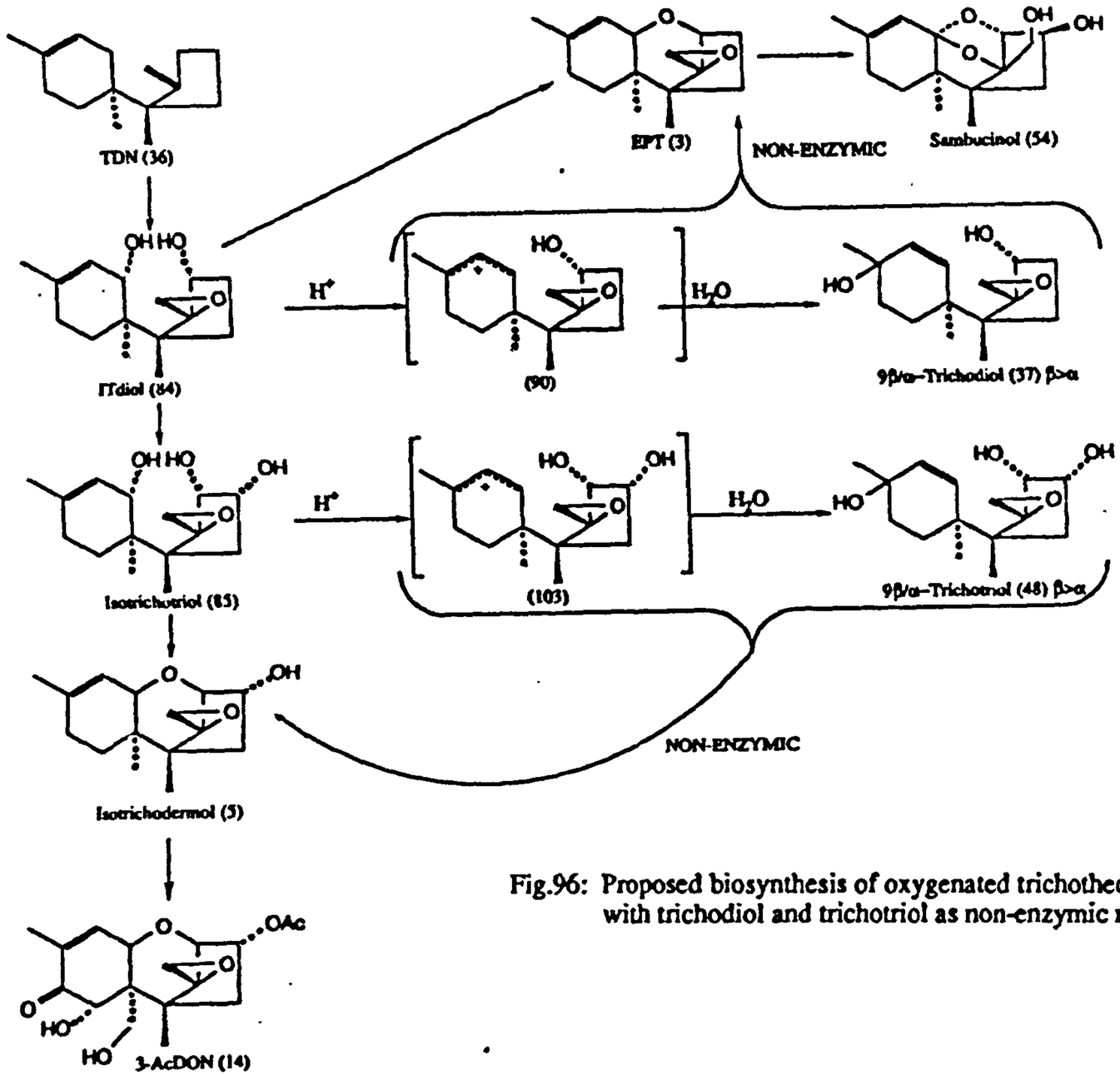


Fig.96: Proposed biosynthesis of oxygenated trichothecenes via ITdiol and isotrichotriol, with trichodiol and trichotriol as non-enzymic metabolites.

isotrichodermol and EPT, respectively [Fig.97]. Then, enzymatic cyclisation can be achieved by intramolecular attack of the C-2α hydroxyl onto C-11 with concomittant reformation of the C-9,10 double bond. Perhaps non-enzymic reaction at low pH breaks the enzyme-carbocation association, allowing 9-hydroxylation and liberation of trichotriol and trichodiol.

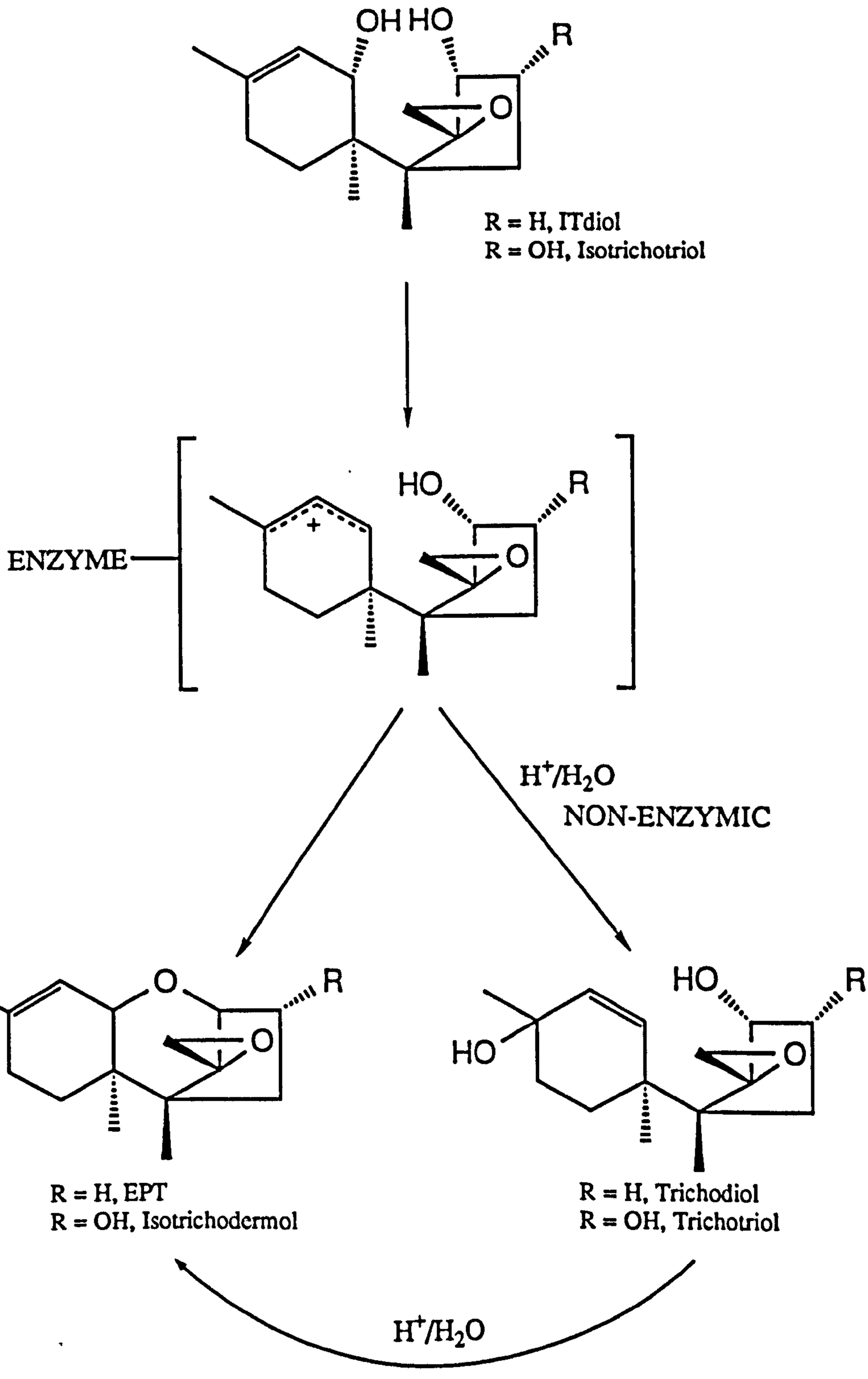
Once formed, it is theoretically possible for trichotriol and trichodiol to isomerise back to isotrichotriol and ITdiol, respectively, via the same carbocation intermediates. This would however require nucleophilic attack at C-11 in the carbocations which is not favoured kinetically, and moreover appears to be prevented by the adjacent B ring (see p. 101). In addition, these compounds in acidic conditions undergo cyclisation rather than isomerisation[60] (see Fig.87), and it is by this process that they are likely to re-enter the biosynthetic pathways. Trichotriol can therefore give rise to oxygenated trichothecenes via cyclisation to isotrichodermol, and trichodiol could similarly be incorporated into sambucinol via cyclisation to EPT, although these acid catalysed cyclisations are relatively slow.

Further work in this area is required, with a particular need for a sample of ^{14}C-labelled isotrichotriol. This could be used in feeding experiments to cell-free systems in an attempt to confirm the hypothesis that it, rather than trichotriol, is a substrate for the cyclase enzyme. Work directed at isolating this important enzyme could then be undertaken.

Further studies in the post-cyclisation biosynthesis of trichothecenes in *F. culmorum.*

In a previous feeding experiment (see p. 126) *F. culmorum* whole-cells were challenged with a large quantity of TDN, and a range of trichothecene products

Fig.97: Proposed cyclisation of ITdiol/isotrichotriol to EPT/ isotrichodermol via an enzyme bound carbocation.



were isolated and identified. Their structures suggested a linear pathway to 3-AcDON and DHC involving sequential oxidation and esterification reactions (see Fig.58). However, the biosynthetic relationship of 3-AcDON and DHC was not clear, and a new trichothecene, 15-deacetylDHC (101), has since been isolated from *F. culmorum* (see p. 199). 15-DeacetylDHC could be a precursor of 3-AcDON by oxidation at C-8, or of DHC by acetylation at C-15 [Fig.98]. In order to clarify the relationship, a feeding experiment employing ^{14}C-labelled substrate was undertaken. A whole-cell system of *F. culmorum* was prepared from a 60 hour old culture by resuspending the filtered mycelia in distilled water. [^{14}C]-15-DeacetylDHC (1mg; 0.42MBqmM^{-1}) was fed to this system, and a control system consisting of mycelia which had been boiled for 10 mins received the same. The cultures were incubated for 4 hours and then harvested by extraction of the filtrates with ethyl acetate. The extracts were analysed by TLC and autoradiography, and product formation measured by scintillation counting.

The autoradiogram [Fig.99, lane 2] shows that 15-deacetylDHC has been biotransformed to a single, less polar product (17% incorporation) which was identified as 3-AcDON by TLC analysis using reference standard materials. No DHC was detected. In the boiled control (lane 1) no changes to the starting material were observed.

15-DeacetylDHC therefore appears to be a direct precursor to 3-AcDON but not to DHC. The transformation to 3-AcDON is a single step involving oxidation of the C-8α hydroxyl to an 8-keto group, and is presumably effected by a single enzyme. Further work leading to the isolation of this enzyme is envisaged.

In order to determine whether DHC is a dead-end metabolite or an intermediate in the production of 3-AcDON, a similar feeding experiment using [^{14}C]DHC

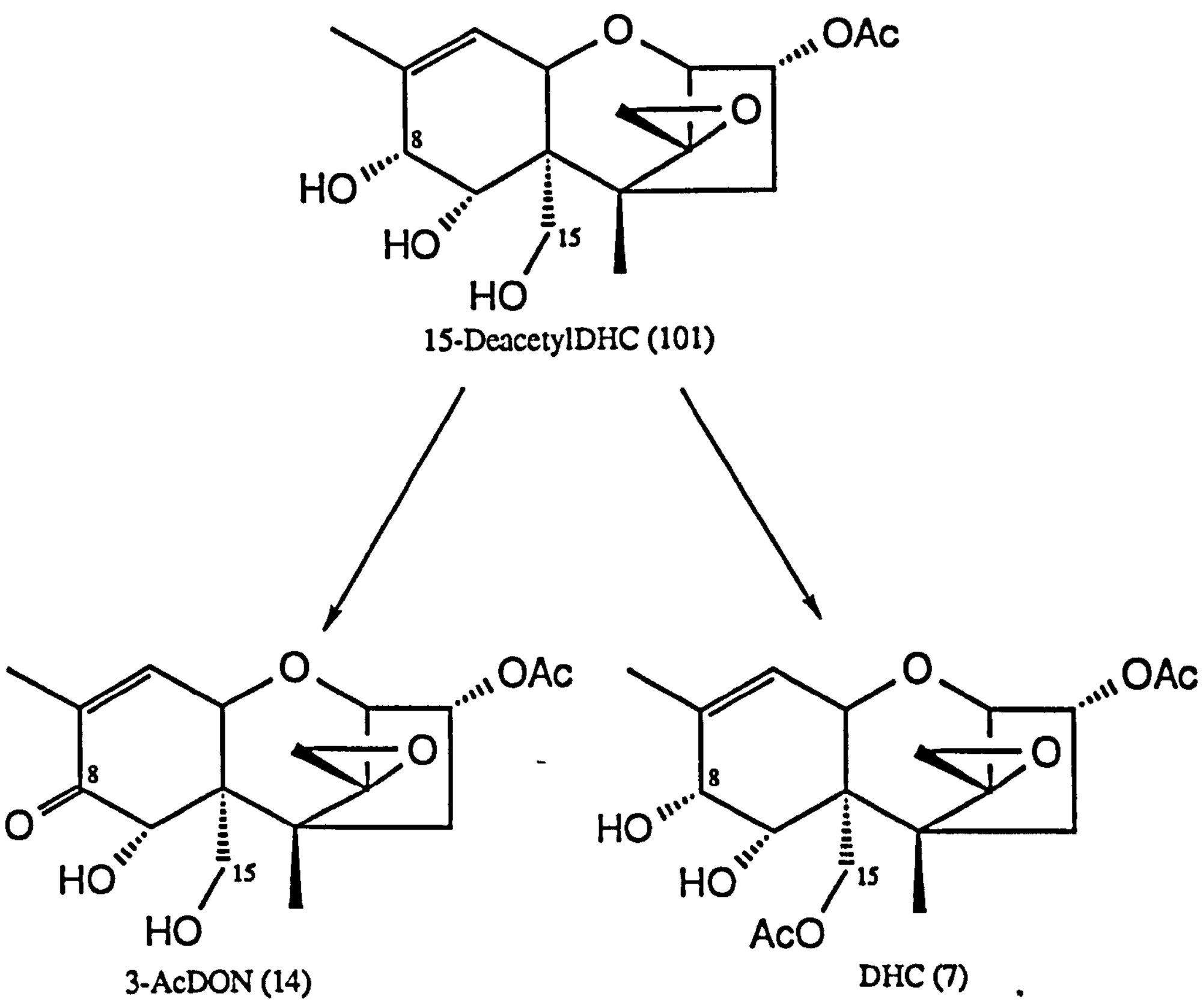
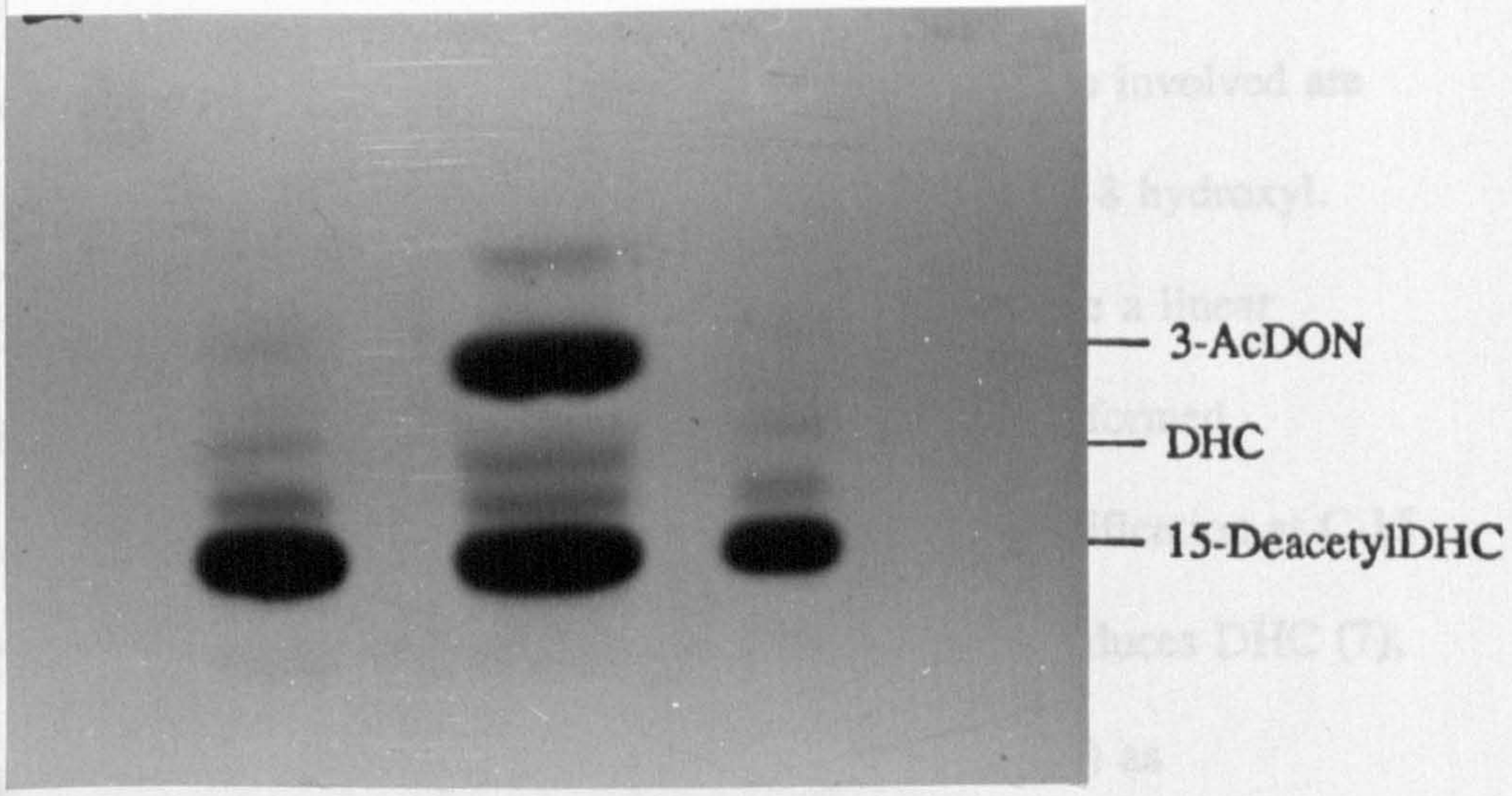


Fig.98: 15-DeacetylDHC as a possible precursor to both DHC and 3-AcDON.

Fig.99: Autoradiogram showing the biotransformation of [^{14}C]-15-deacetylDHC to 3-AcDON in *F.culmorum*.



Lane 1: Fed [^{14}C]-15-DeacetylDHC, boiled mycelia
Lane 2: Fed [^{14}C]-15-DeacetylDHC
Lane 3: [^{14}C]-15-DeacetylDHC standard

4hr incubation
TLC: Ether-acetone, 9:1

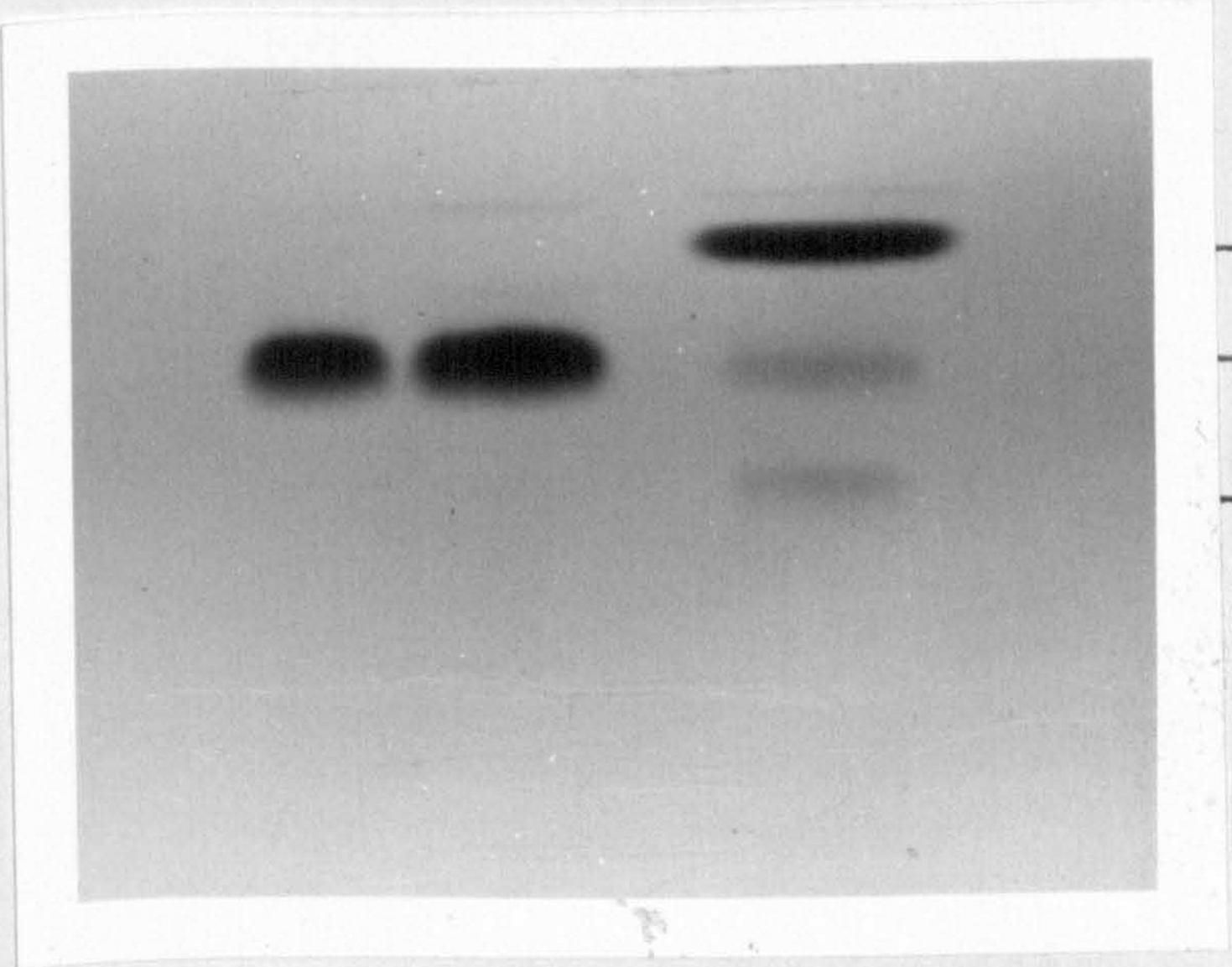
(1mg; 31.0KBqmM^{-1}) was performed. The resultant autoradiogram [Fig.100, lane 3] shows that DHC has been converted to 3-AcDON (26% incorporation). The presence of 15-deacetylDHC was also detected. In the boiled control (lane 2) no changes to the starting material were observed. These results indicate that DHC is in fact a precursor to 3-AcDON, and that 15-deacetylDHC is an intermediate in the biosynthesis of 3-AcDON from DHC [Fig.101]. The enzymic steps involved are therefore 15-deacetylation of DHC, followed by oxidation of the C-8 hydroxyl.

The biosynthesis of 3-AcDON [Fig.102] therefore appears to be a linear sequence from isotrichodermol (5), the first cyclised trichothecene formed. 3-Esterification to ITD (68) is followed by hydroxylation and esterification at C-15 to produce CAL (6). Sequential hydroxylation at C-7 and C-8 produces DHC (7), which then gives rise to 15-deacetylDHC (102) and 3-AcDON (14) as demonstrated above. In this sequence, acetylation at C-15 is later followed by deacetylation. 3-AcDON could theoretically be formed from 15-deacetylCAL (89) by sequential hydroxylations at C-7 and C-8. The 15-acetylation step therefore appears necessary for these hydroxylations to occur. Once this has been achieved, the acetyl group is removed prior to oxidation at C-8.

Inhibition of *Fusarium culmorum* cultures using semi-synthetic derivatives of TDN.

The results of the competitive feeding experiments in which [^{14}C]TDN was fed to *F. culmorum* whole-cells in the presence of an excess of unlabelled TDN derivatives (see Table 15) indicated that all the derivatives tested inhibited to some extent the biosynthesis of trichothecenes from TDN, and stimulated the production of ITdiol. The feeding experiments were however performed under abnormal conditions, with the fungus being suspended in distilled water rather than

Fig.100: Autoradiogram showing the biotransformation of [^{14}C]DHC to 3-AcDON in *F.culmorum*.



Lane 1: [^{14}C]DHC standard
Lane 2: Fed [^{14}C]DHC, boiled mycelia
Lane 3: Fed [^{14}C]DHC

6hr incubation
TLC: Ether-acetone, 9:1

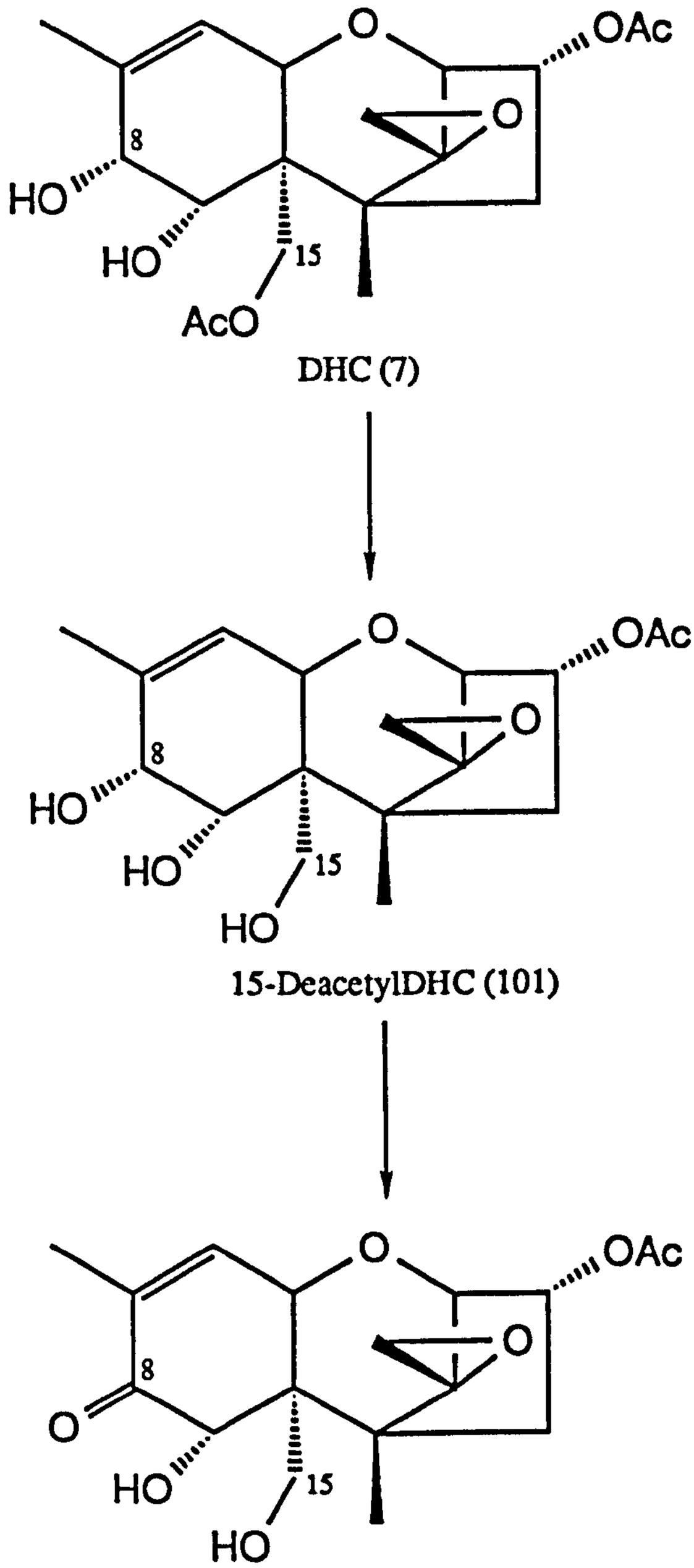


Fig.101: Biosynthesis of 3-AcDON from DHC via 15-deacetylDHC.

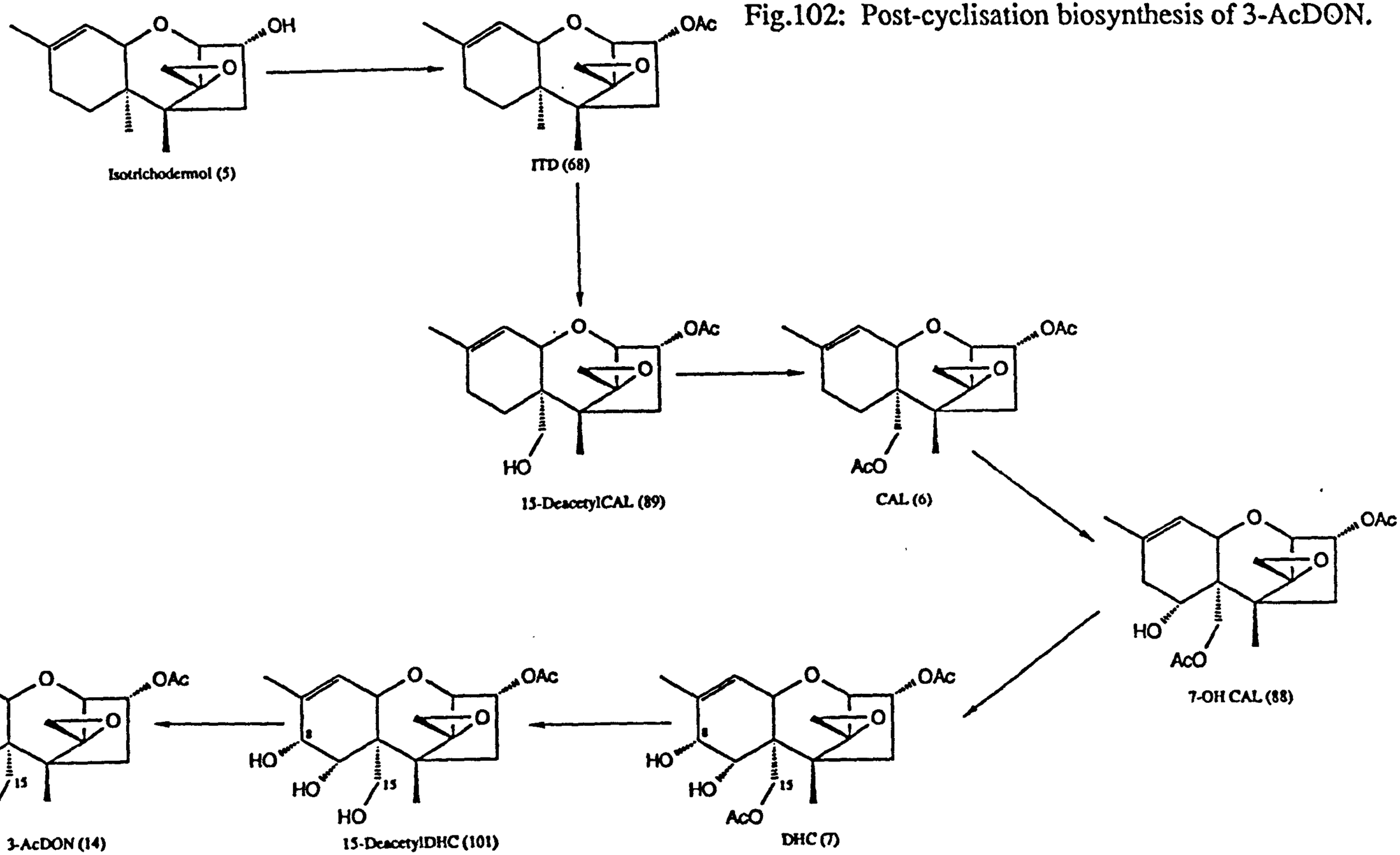


Fig.102: Post-cyclisation biosynthesis of 3-AcDON.

production medium, and under these stressful conditions the effects of the derivatives may have been accentuated. Also, the experiments involved supplying the fungus with exogenous [^{14}C]TDN, which is not naturally found to any significant extent in *F. culmorum* culture broth. In order to obtain a more realistic assessment of the inhibitory activities of the TDN derivatives it was therefore decided to investigate their effects on trichothecene biosynthesis in growing *F. culmorum* cultures by observing the fate of sodium [2-^{14}]acetate supplied to them.

Cultures of *F. culmorum* (25ml) received sufficient amounts of the derivatives to produce a concentration between 2-3mM, 43-60 hours after subculture [Table 32]. Incubation was continued and sodium [2-^{14}C]acetate (740-925KBq) was added after 30 mins. Culture samples (1ml) were taken 5, 24, 72 and 120 hours after addition of the label, and extracted with ethyl acetate. Extracts were then analysed by TLC and autoradiography, with scintillation counting being used to measure total incorporation into the products formed [Table 33].

Table 33 shows the effect of the derivatives on the incorporation with time of [2-^{14}C]acetate into trichothecenes and related minor metabolites. From this it is clear that all the derivatives tested have inhibited the production of trichothecenes from [2-^{14}C]acetate, with 9β,10β-epoxyTDN having the greatest effect and 9,10-dibromoTDN the least. Taking the incorporation in the respective controls after 120 hours as being 100%, Table 34 shows that the 9,10-epoxide (73) in fact causes a 90% reduction in incorporation of label, and the 9,10-dibromide (75) only a 40% reduction. The other derivatives produce effects between these values, with TDN, the only natural biosynthetic intermediate used, causing a 50% inhibition. The mechanisms for the inhibition are unknown, but those derivatives causing

Table 32: TDN derivatives used in the inhibition of growing *Fusarium culmorum* cultures.

Flask	Derivative	Concentration (mM)	Age of culture (hours)	[^{14}C]acetate added (KBq)
1	None	---	43	925
2	9β,10α-DihydroxyTDN (74)	2.01	43	925
3	9,10-Dibromo-12,13-epoxyTDN (76)	2.52	43	925
4	None	---	60	740
5	TDN (36)	1.96	60	740
6	9β,10β-EpoxyTDN (73)	2.00	60	740
7	9β,10β;12,13-DiepoxyTDN (72)	2.03	60	740
8	9,10-DibromoTDN (75)	1.97	60	740
9	9β-OH-Tricho-10,12-diene (70)	2.00	60	740

Table 33: Incorporation of [^{14}C]acetate into trichothecene products.

Flask	Total % incorporation at times 5hr	24hr	72hr	120hr
1	6.6	6.2	6.6	7.6
2	1.2	1.8	1.9	1.8
3	2.4	1.7	3.3	3.7
4	5.3	6.0	5.8	5.5
5	1.3	2.7	2.7	2.7
6	1.7	1.4	0.8	0.5
7	1.5	1.8	1.7	1.7
8	1.8	3.7	3.9	3.3
9	2.2	2.7	2.8	2.9

Table 34: Inhibition of trichothecene production by TDN analogues.

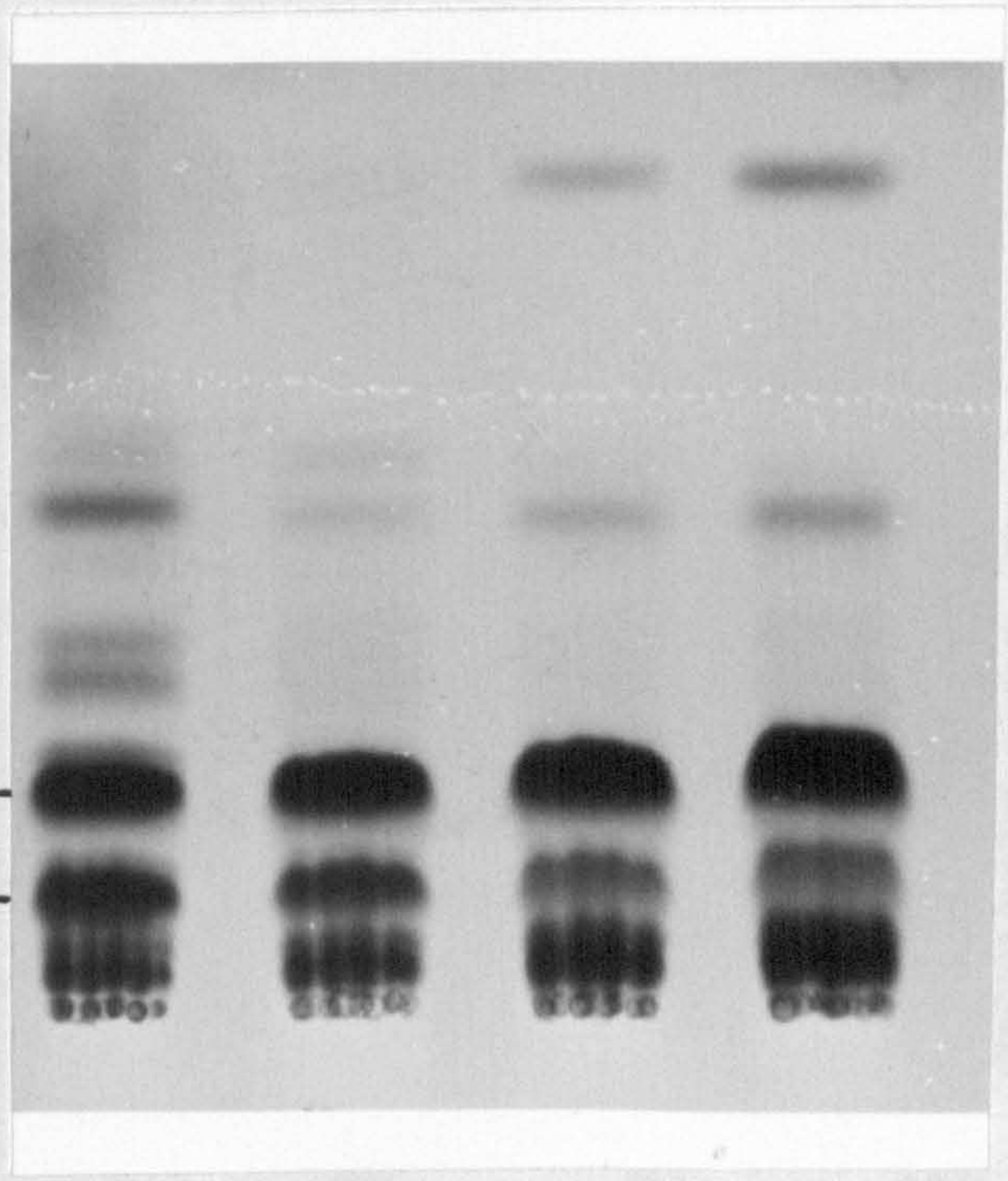
Flask	Derivative	Trichothecene production after 120 hours (% of controls)
1	None	100
2	9β,10α-DihydroxyTDN (74)	24
3	9,10-Dibromo-12,13-epoxyTDN (76)	49
4	None	100
5	TDN (36)	49
6	9β,10β-EpoxyTDN (73)	9
7	9β,10β;12,13-DiepoxyTDN (72)	30
8	9,10-DibromoTDN (75)	60
9	9β-OH-Tricho-10,12-diene (70)	52

inhibition similar to TDN (i.e. (70), (75) and (76)) may be acting as competitive inhibitors, with those producing a greater effect (i.e. (72), (73) and (74)) having a different activity. The autoradiograms [Fig.103] show the effects of the derivatives on the various types of trichothecenes produced. It was hoped that one or more derivatives would cause accumulation of ITdiol, and could therefore be used in future for the large scale production of [^{14}C]ITdiol direct from inhibited cultures, similar to producing [^{14}C]TDN by xanthotoxin-inhibition. However, from the autoradiograms it is evident that although ITdiol accumulates by using the 9,10-dibromide (75), the bromoepoxide (76) and 9β-hydroxytricho-10,12-diene, the effects are transient with little ITdiol being present after 3 days. Interestingly, (75) and (76) also cause a slight accumulation of TDN after 5 hours but this again disappears by 1-3 days.

The use of TDN derivatives for the production of ITdiol by inhibition of *F. culmorum* cultures does not appear to be practicable. ITdiol is present after 5 hours to 1 day in some of the cultures, but yields will be too low considering the amount of TDN and time required to produce the inhibitors. However, the derivatives also cause other interesting changes in the metabolite profiles produced, and further work in this area may provide useful information on trichothecene biosynthesis, and perhaps lead to the identification of novel intermediates. At this stage, it is difficult to identify the compounds present with any certainty from TLC in only one system. However, the profiles after 5 hours and 1 day for (70) and (75) show the appearance of potentially interesting metabolites, less polar than ITdiol, and further work with these inhibitors is envisaged.

Fig.103: Autoradiograms showing the inhibitory effects of a range of TDN-derivatives on trichothecene production in *Fusarium culmorum*.

Series 1: Flask 1.



Flask 2.

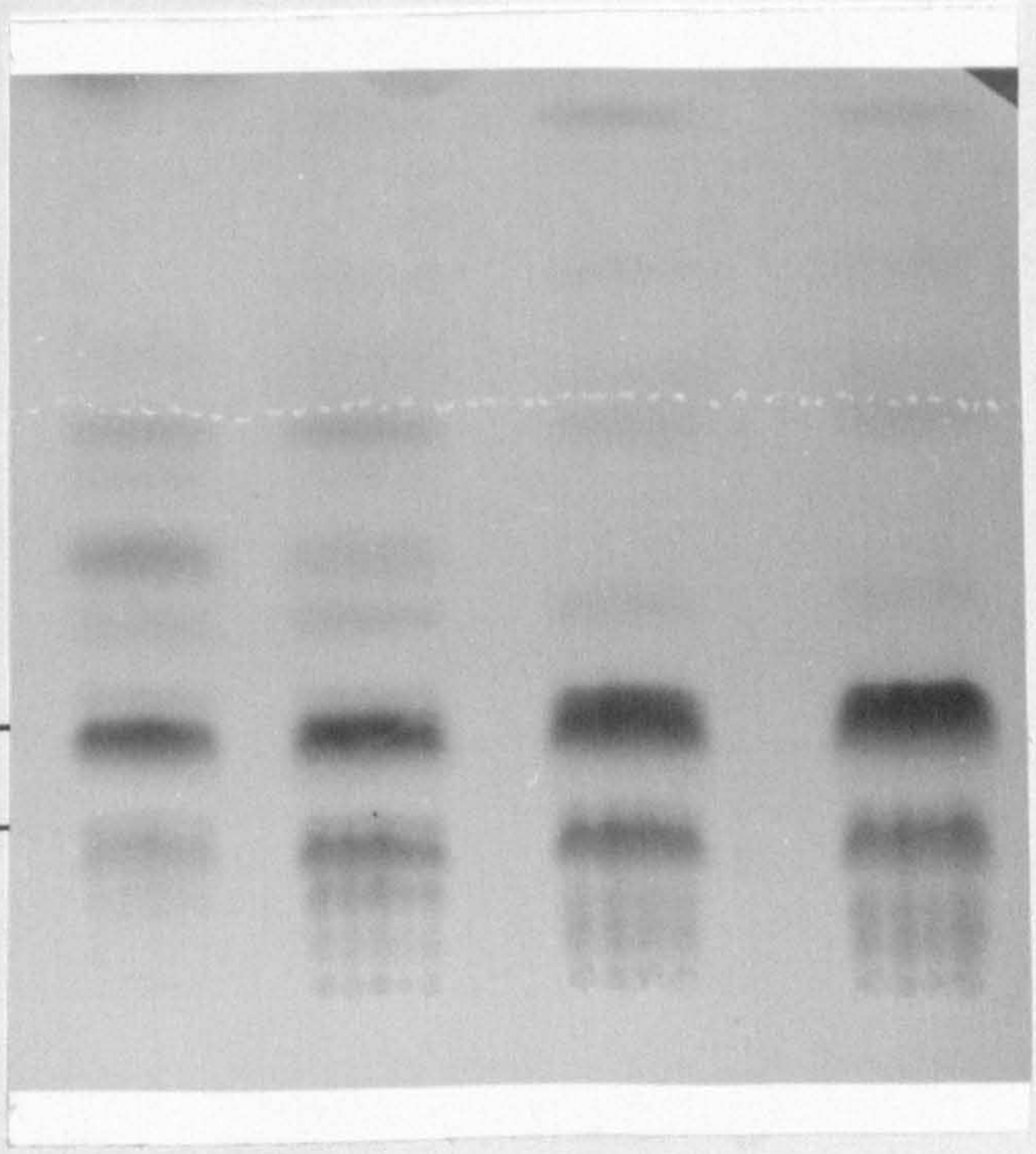
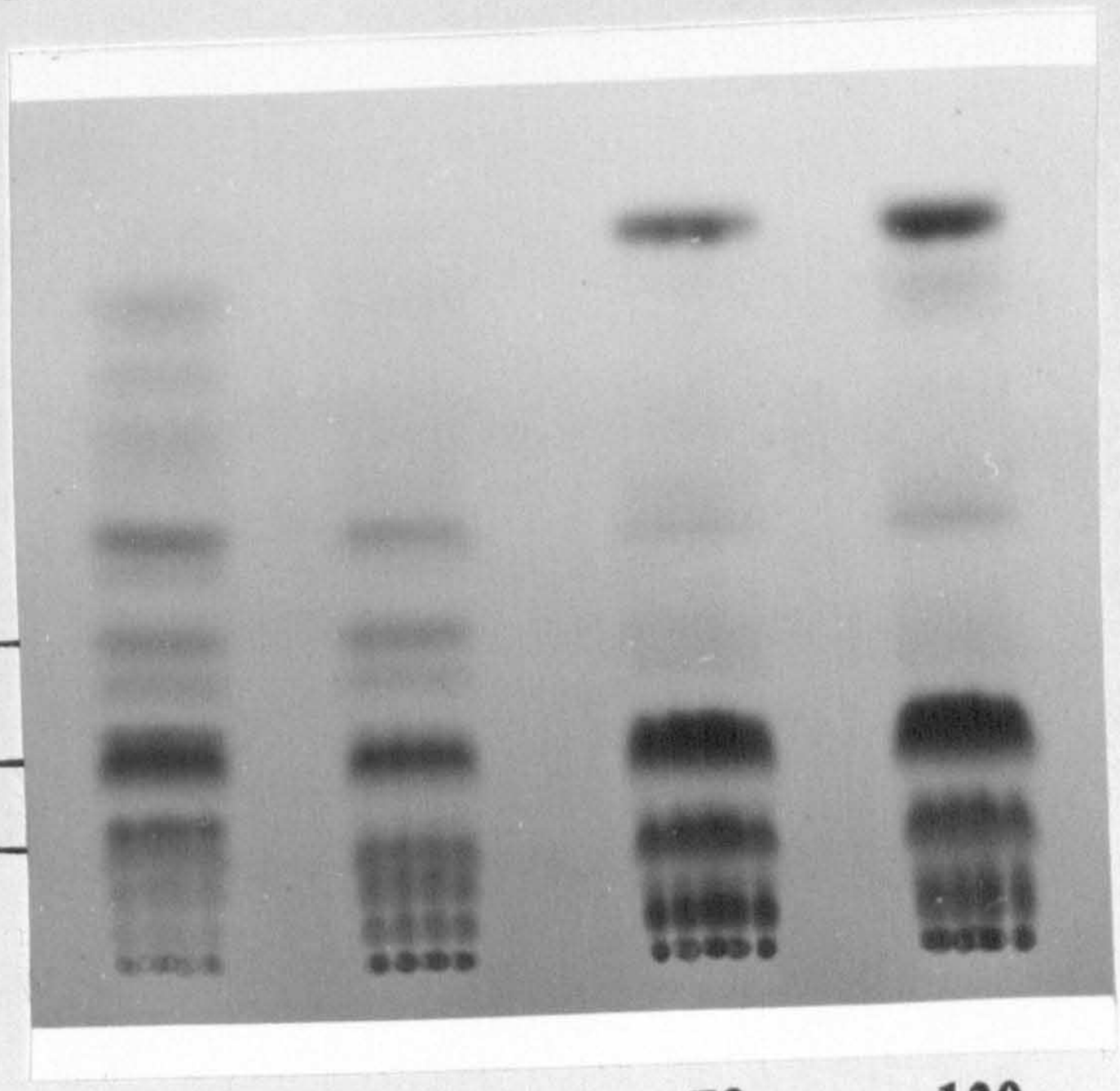


Fig.103 continued:

Series 1: Flask 3.



Series 1: t = 5hrs.

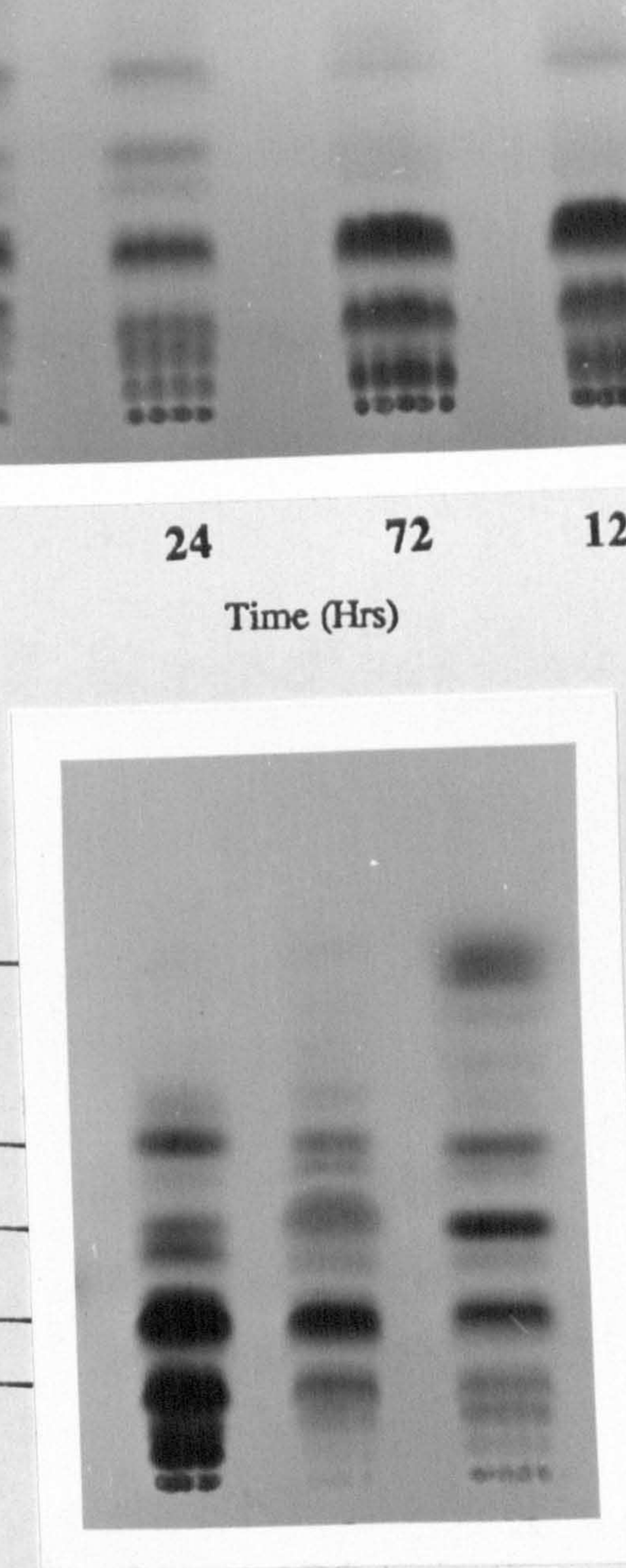
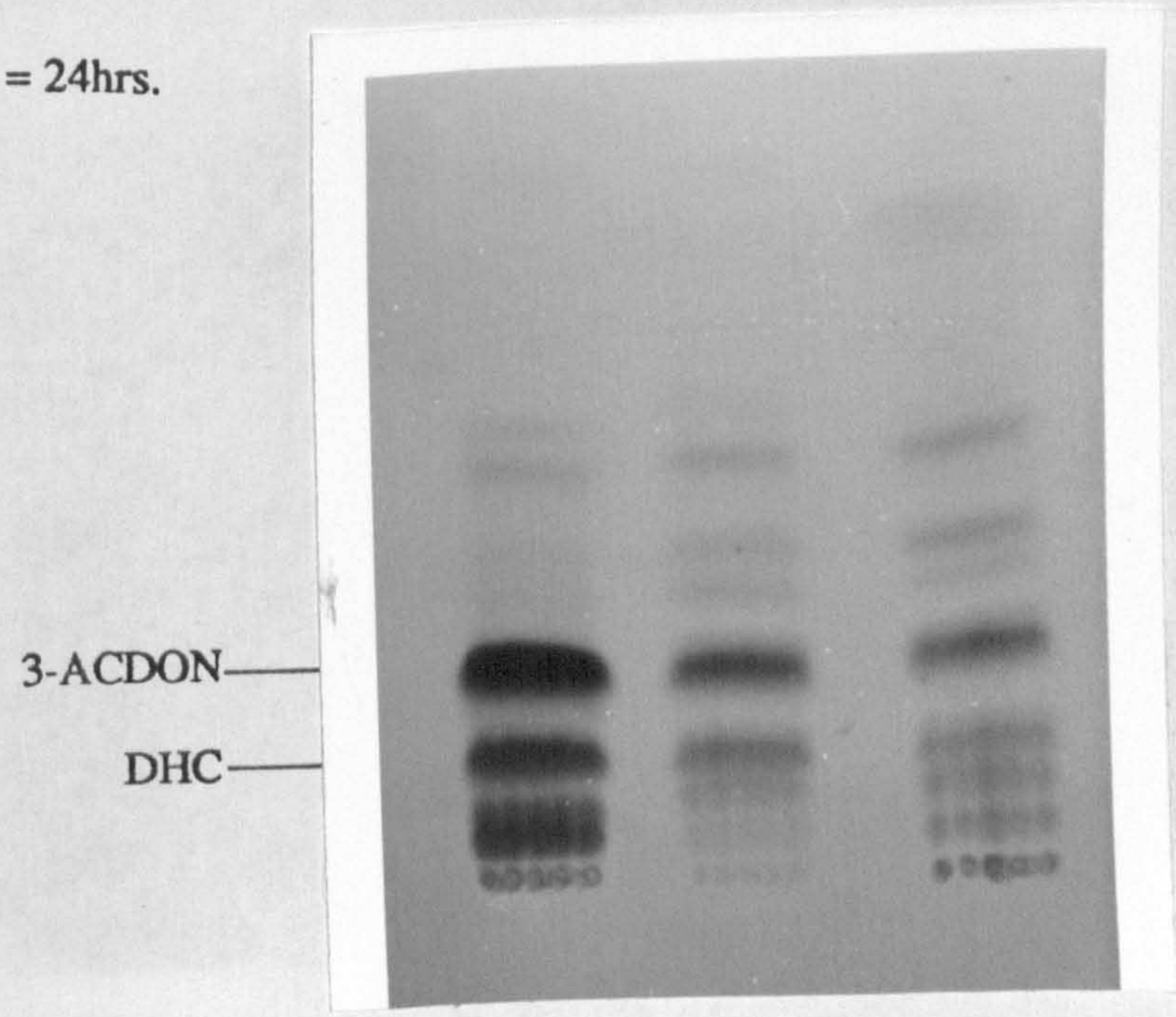


Fig.103 continued:

Series 1: t = 24hrs.



Series 1: t = 72hrs.

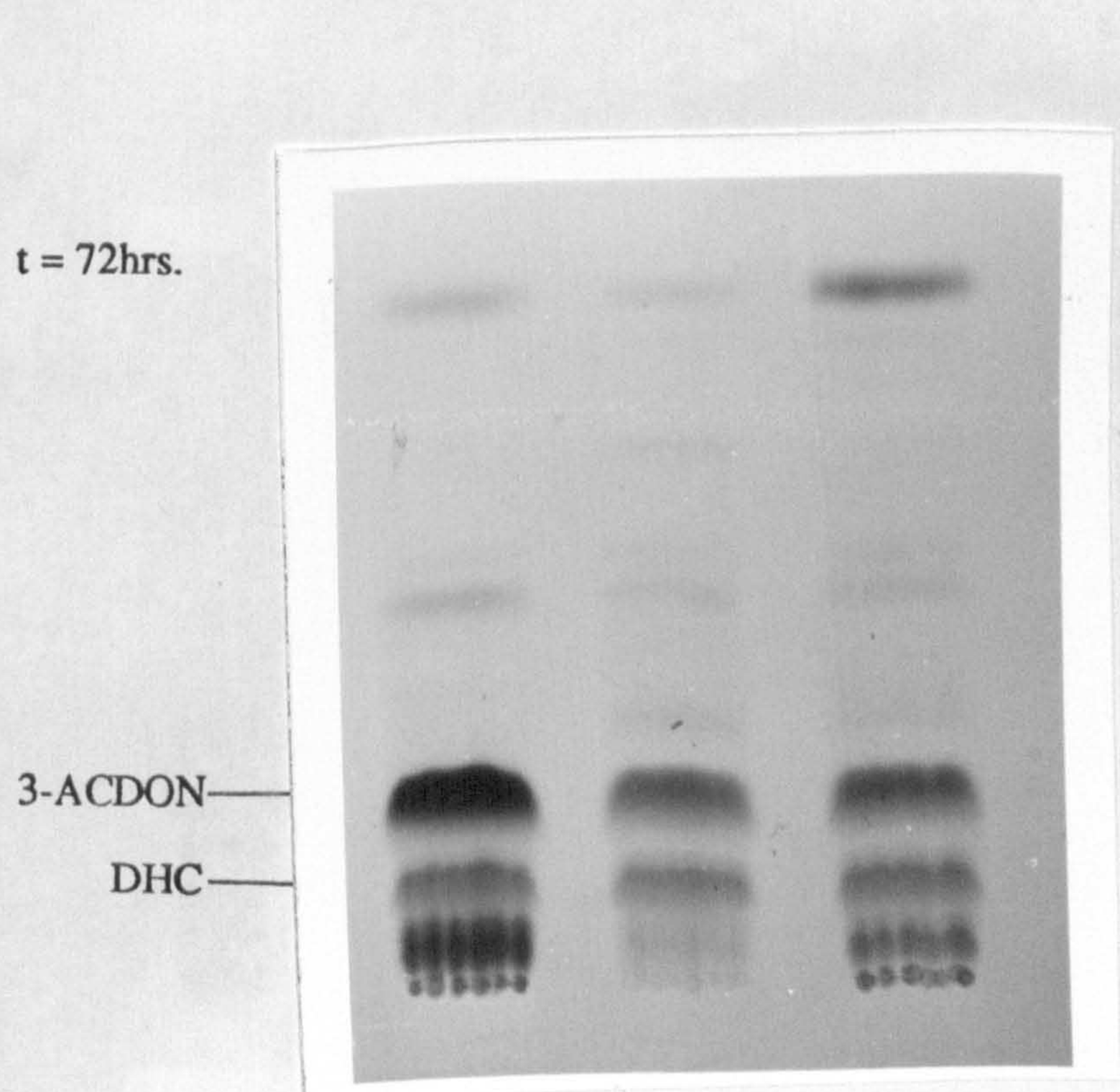
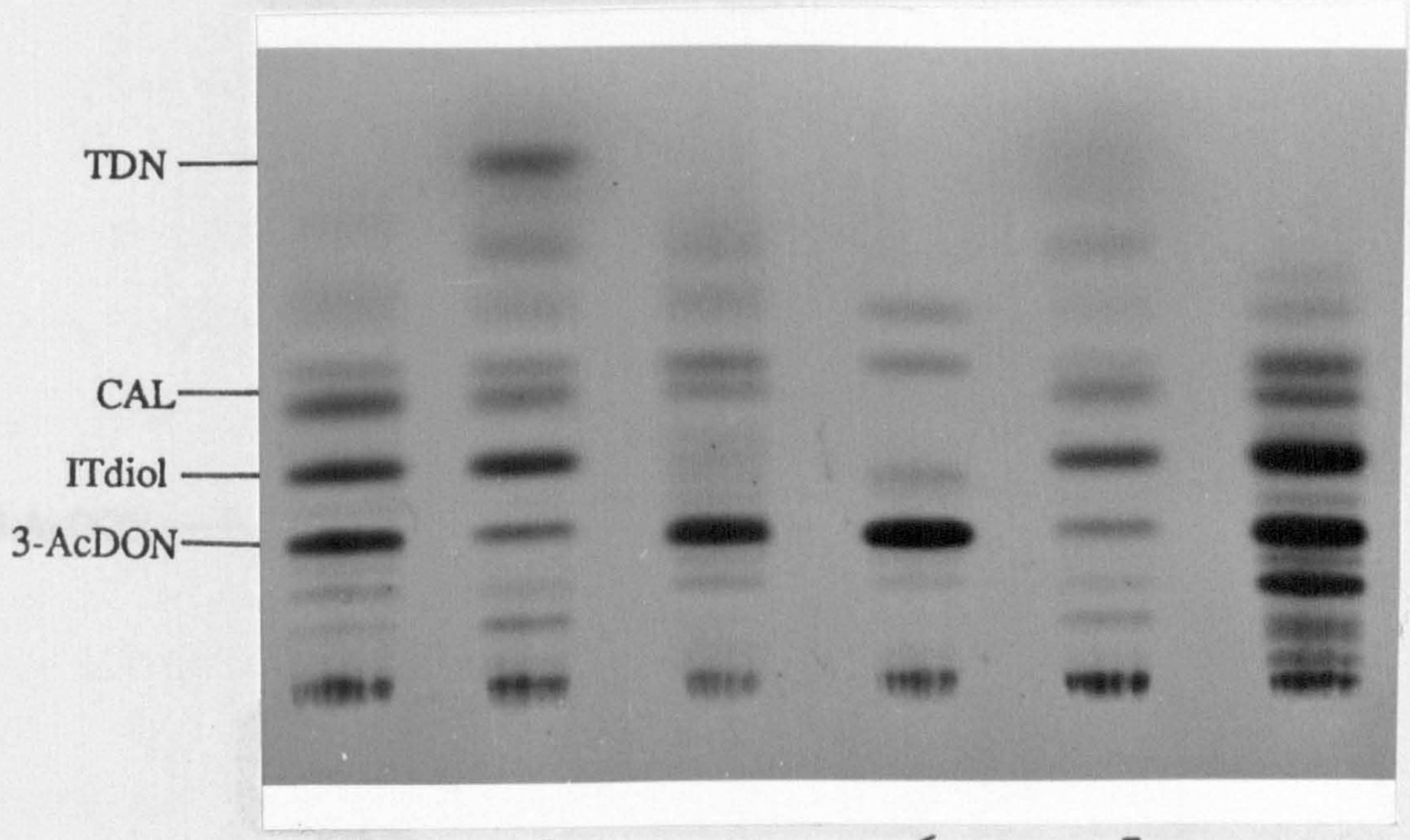


Fig.103 continued:

Series 2: t = 5 hours.



t = 24 hours.

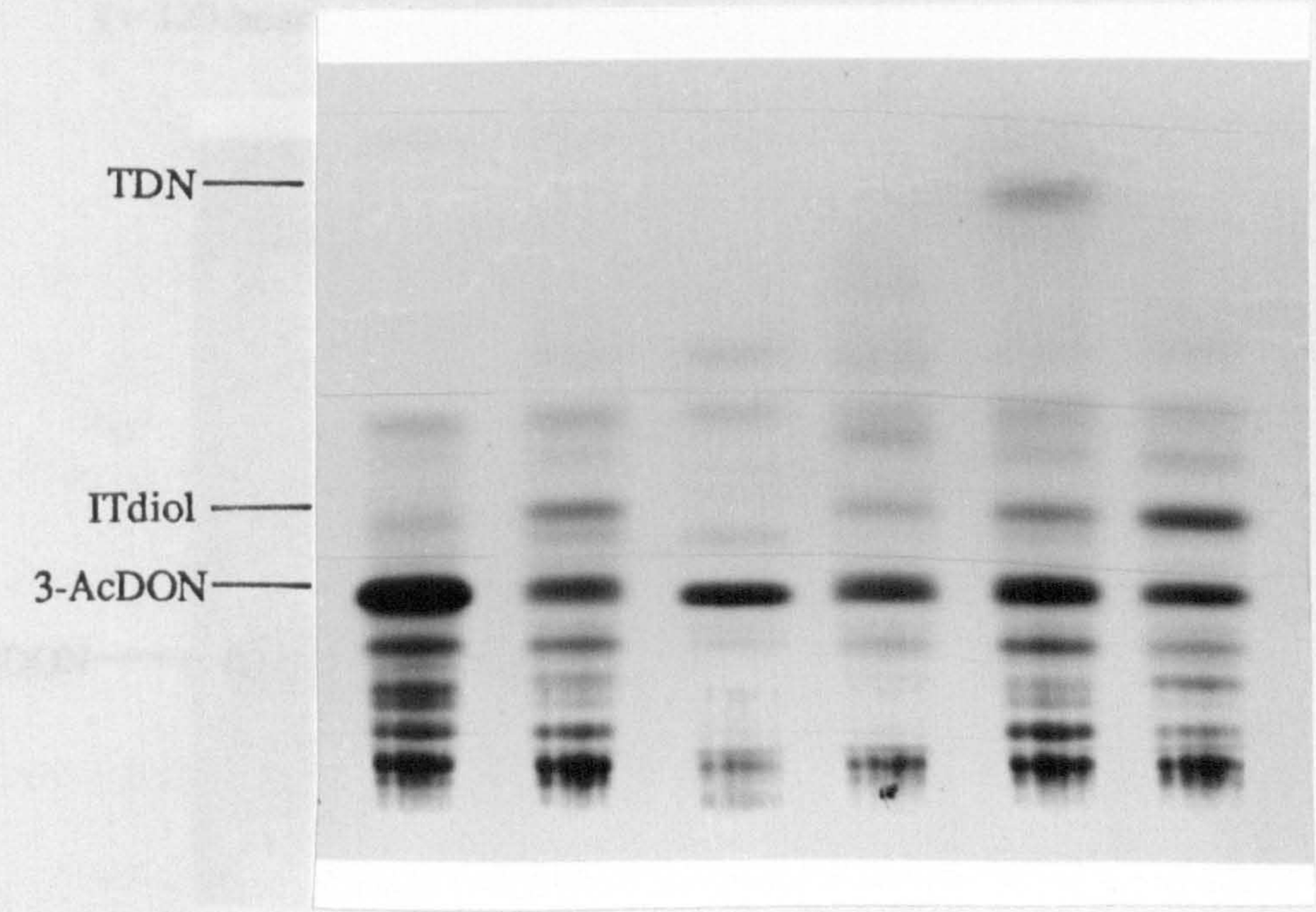
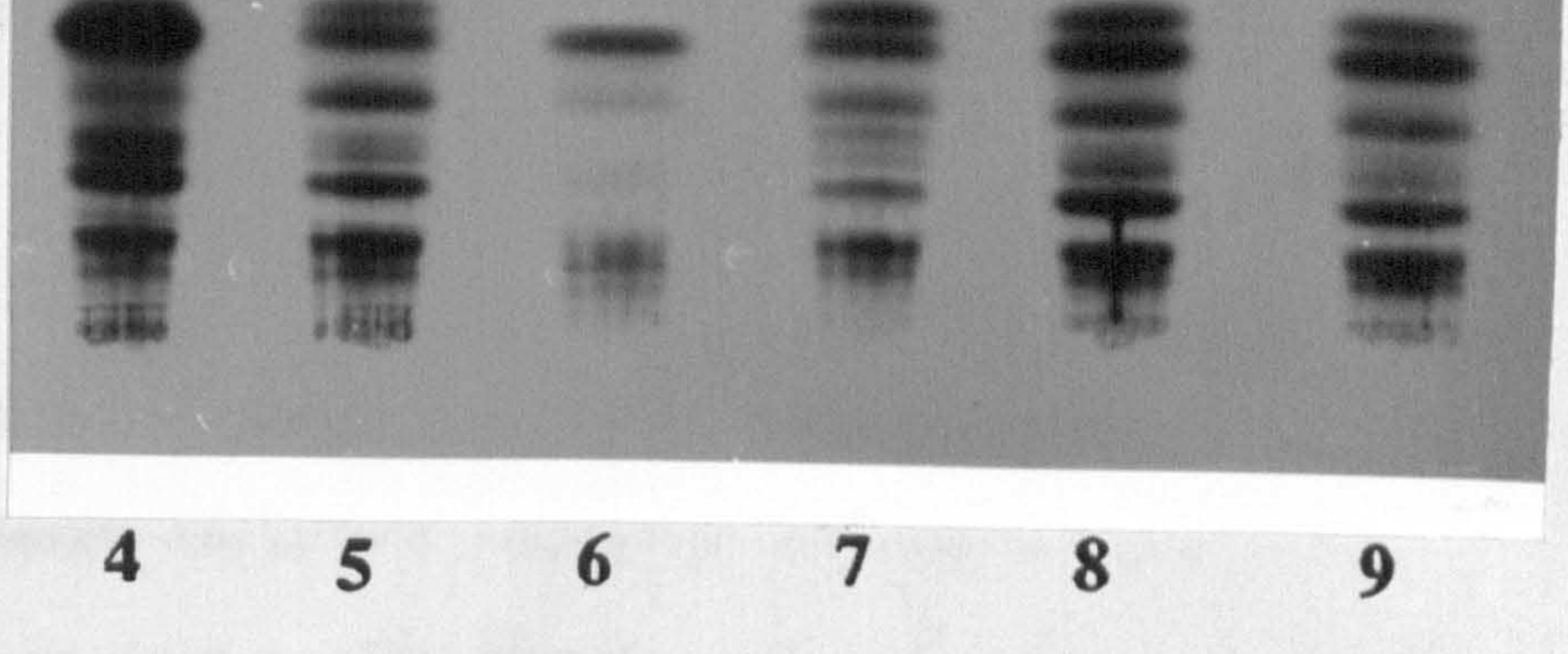
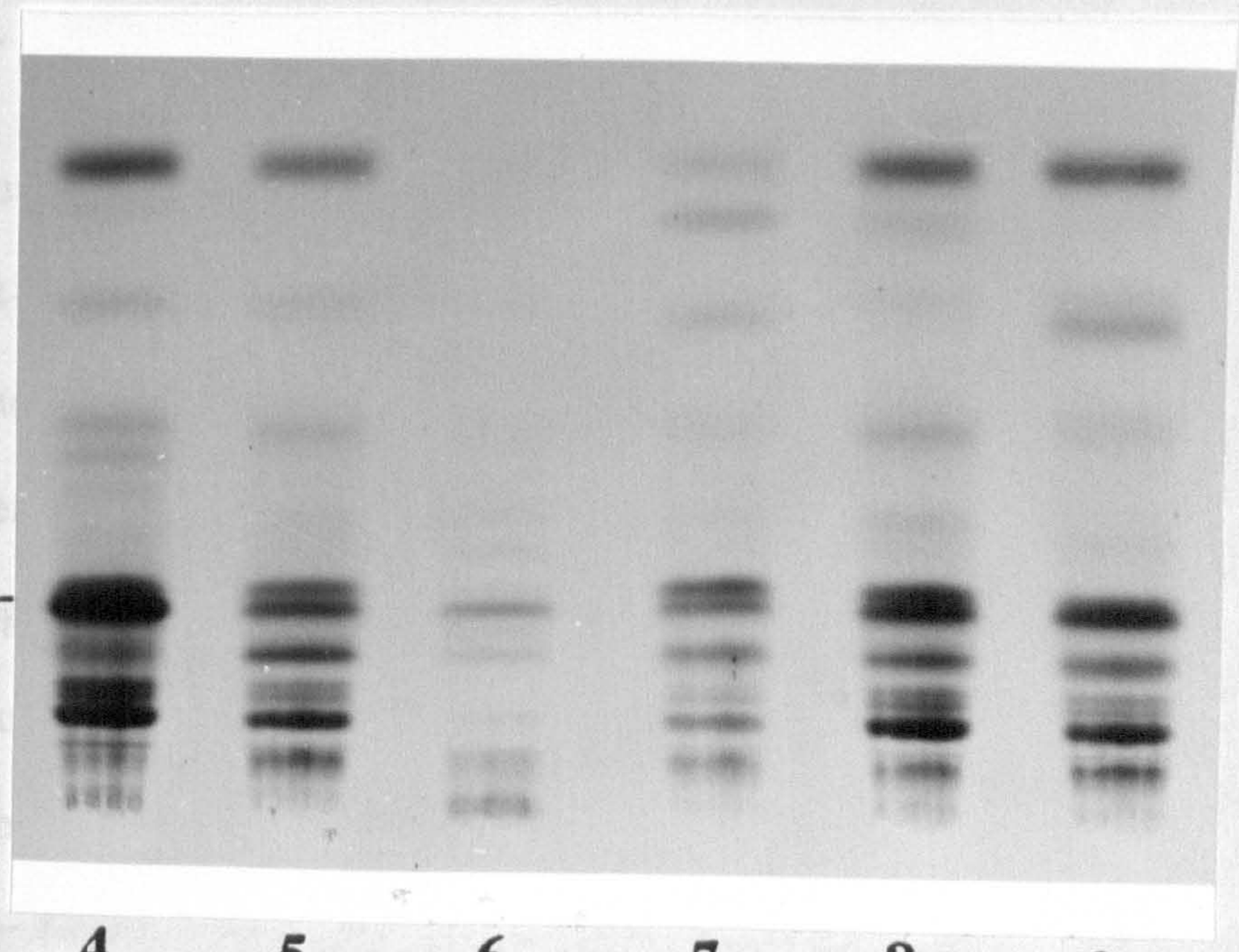


Fig.103 continued:

Series 2: t = 72 hours.



t = 120 hours.



MATERIALS AND METHODS.

Instrumentation.

Nuclear magnetic resonance spectroscopy.

^{1}H NMR spectra were recorded at either 250MHz on a Bruker WM 250 spectrometer or at 400MHz on a Bruker AM 400 spectrometer. ^{13}C NMR spectra were recorded at 100MHz also on the Bruker AM 400 machine. Tetramethylsilane was used as an internal standard and all samples were dissolved in $CDCl_3$.

Mass spectrometry.

EI and FAB mass spectra were recorded on an MS 902 spectrometer with direct probe insertion. Ionisation was at 70eV and the source temperature was 200°C.

CI mass spectra were recorded on a VG 7070 EQ mass spectrometer with direct probe insertion. Ionisation was with CH_4 at 100eV.

Gas chromatography.

GC was carried out using a Perkin Elmer Sigma 2 gas chromatograph fitted with a non-polar J & W DB-5 (SE-52, CP-SIL 8 equivalent) capillary column (30m x 0.25 mm) and a flame ionisation detector. The carrier gas was helium (2ml/min). Typically the oven temperature was maintained at 100° for 1 min after injection, then temperature programmed to 270° at 10°/min.

Trimethylsilylation of alcohols was achieved with a standard mixture of derivatising agents (bis(trimethylsilyl)trifluoroacetamide (BSTFA) or BSTFA: chlorotrimethylsilane: 1-(trimethylsilyl)imidazole in a ratio of 3:2:3).

Gas chromatography-mass spectrometry.

GC-MS was carried out on a Carlo-Erba 4160 gas chromatograph fitted with a capillary column and linked to a VG 7070 EQ mass spectrometer. The column used was a J & W DB-5 non-polar capillary column (30m x 0.25mm). Typically the oven temperature was maintained at 100° for 1 min after injection, and then temperature programmed to 270° at 10°/min. Electron ionisation at 70eV was used in conjunction with positive ion detection.

GC-MS were recorded by Mr I Parker at the MAFF Food Research laboratory, Norwich.

Column chromatography.

Various sizes of glass column were used, all of which were fitted with Quickfit tops and teflon taps. The columns were packed under gravity with a slurry of the packing material (Merck silica gel 60 (70-230 mesh)) in the required eluant. Elution was completed under gravity, and fractions collected manually.

Thin layer chromatography.

TLC was the technique used for routine analyses. Commercially prepared aluminium backed plates (Merck silica gel 60 F_{254}; 200mm x 200mm x 0.2mm) were cut to the required size, and aliquots of solutions of the compounds to be analysed were applied using glass capillaries. Chromatography was completed vertically at ambient temperature. Separation of samples could be improved by twice running the TLC to a distance of 1.5cm above the baseline prior to allowing a complete development of the plate. Visualisation was achieved by use of

chromogenic spray reagents (see Table 5).

Preparative TLC was carried out in the same manner with visualisation being achieved by cutting off the edges of the plates and spraying these with 20% H_2SO_4 (aq). Bands were collected by scraping off the absorbent phase and eluting with a suitable organic solvent such as EtOAc or ether. Alternatively, for separating sample loads greater than 6mg, glass backed plates were used (200mm x 200mm x 0.5mm), with visualisation being achieved by covering most of the plate with a sheet of glass, spraying the edges of the plates as above and heating the sprayed portions with a hair dryer.

Centrifugal thin layer chromatography.

CTLC was carried out using a Harrison Research Chromatotron model 7924T chromatograph (TC Research, Norwich). Chromatography was completed under a nitrogen atmosphere with initial purging at 1000ml/min and subsequently at 15ml/min.

Three thicknesses of adsorbent layer (Merck silica gel PF with $CaSO_4$) were used (see Table 6), and solvent flow rate was altered according to plate thickness: 1mm plate, 2-4ml/min; 2mm plate, 6-8ml/min; 4mm plate, 8-10ml/min.

Plates were prepared by applying the adsorbent to the glass rotors as a slurry made by mixing silica and water cooled to 0-5°C according to the ratios below.

Plate size	1mm	2mm	4mm
Flask size	250ml	500ml	1000ml
Adsorbent	45g	65g	115g
Water	90ml	130ml	200ml

The slurry was immediately applied to the centre of a clean, dry, slowly rotating (1/2-1 revolutions/sec) glass rotor which was then repeatedly dropped from a height of 1cm onto a felt cloth in order to release any air bubbles from the slurry. After this, the rotor was covered and the silica allowed to set for 1hr. The rotor was further air dried whilst spinning (ca. 30rpm) and then oven dried (70°C) overnight. After allowing the rotor to cool the silica was cut to shape with the tools supplied.

Samples dissolved in the minimum volume of solvent were applied to the rotor via the wick and then eluted under gravity with the introduction of solvent by the same route. Fractions were collected manually, the size of the fractions being determined by the thickness of the layer-typically 7-10ml for a 4mm plate, 2-5ml for a 2mm plate and 1-2ml for a 1mm plate.

Evaporation of solvents.

Large volumes of organic solvents were removed under reduced pressure by use of a Buchi Rotovapor at 30-40°C. Smaller volumes (up to 5ml) were removed under a gentle flow of nitrogen.

Melting points.

Melting points were determined by use of a Buchi melting point apparatus.

Autoradiography.

Autoradiograms were obtained by placing TLC plates in contact with Hyperfilm-βmax (Amersham) for exposure times ranging from 1-25 days. Films were developed by immersion first in developer (Kodak D-19) for 5 mins, then in a water bath for 1 min, and finally in X-ray fixer (Kodak FX-40) for 5 mins. After

rinsing under running water for 10 mins the developed films were air dried at 35°.

Scintillation counting.

Scintillation counting was performed using a RackBeta 'spectral' 1219 scintillation counter (LKB scintillation products). The samples to be analysed (either aliquots of solutions or a spot scraped off a TLC plate) were dissolved or suspended in 10ml of liquid scintillant (Optiphase 'safe' (LKB Scintillation products)) and counted for a period of 10 mins. Efficiencies were determined via external standardisation.

Sterilisation.

Sterilisations were carried out by autoclaving in a Denley Cabbum Steriliser at 121°C for 20 mins.

Maintainance of fungi.

Fungi were grown on slopes of the appropriate agar (below) at 26°C for 5-7 days before being stored at 4°C in the dark. All slopes were subcultured bimonthly.

Fungus	Agar*
Fusarium culmorum CMI 14764	Czapek-Dox
Trichothecium roseum CMI 50661	Beer wort

* Composition and preparation as described in Appendix 1.

Production of trichothecenes from *Fusarium culmorum.*

Culture of fungus.

Czapek Dox (CD) agar plates were inoculated with mycelia/agar portions (approx. 5mm cubes) cut from mature CD slope cultures (see above), and incubated at 27°C in the dark. Mature growths were cut from excess peripheral agar and old inoculum source, and liquidised for 1 min in sterile distilled water (15ml per plate). This homogenate was used to inoculate Erlenmeyer flasks containing 20% volume of CD production medium (Appendix 2), using 15ml of homogenate per 200ml of medium. Cultures were incubated in the dark at 27°C on an orbital shaker (250rpm) for 7 days. The cultures were then filtered at the pump (Whatman No.4 paper), the filtrate saturated with NaCl and then extracted with EtOAc (3x400ml). The combined extracts were dried over anhydrous $MgSO_4$ and evaporated to an oil under reduced pressure.

Purification.

The crude toxin extract (6.5g) from an 8.0L culture was fractionated by CTLC (4mm plate) eluting with ether-acetone, 9:1, and collecting 10ml fractions (These fractions obtained from the first chromatographic separation are denoted with *, subsequent fractionation of pooled fractions follows). Fractions were combined as appropriate after analysis by TLC (ether-acetone, 9:1).

Fractions 5-9* contained two fast eluting trichothecenes which were purified by further CTLC (2mm plate) eluting with hexane-EtOAc, 8:2, and collecting 2ml fractions. This yielded isotrichodermin (ITD) (4mg in F.35-38) and calonectrin (CAL) (16mg in F.45-49) as viscous colourless oils which were identified by ^1H

NMR (Table 18) and EI-MS.

ITD m/z (rel.int): 292 ($[M]^+$, 30%), 277 (35), 249 (13), 232 (9), 202 (26), 187 (15), 173 (25), 159 (21), 145 (29), 134 (21), 124 (100), 107 (92), 93 (93).

CAL m/z (rel.int): 307 ($[M-Ac]^+$, 2%), 290 (32), 262 (22), 236 (18), 218 (30), 175 (12), 153 (23), 137 (30), 121 (31), 110 (100), 107 (56), 91 (51).

Fractions 10-14* contained mainly one, more polar toxin which was further purified by CTLC (4mm plate) eluting with ether-acetone, 9:1, and collecting 7ml fractions. Recrystallisation of pooled fractions 14-19 from ether yielded 3-acetyldeoxynivalenol (3-AcDON) (470mg) as white crystals. Identification was confirmed by ^{1}H NMR (Table 18) and EI-MS analysis.

3-AcDON m/z (rel. int.): 338 ($[M]^+$, 2%), 290 (14), 248 (7), 223 (17), 203 (15), 181 (19), 163 (36), 135 (33), 107 (38), 98 (60), 43 (100).

Fractions 15-22* contained the most polar trichothecene detected, which was then purified by CTLC (4mm plate) eluting with ether-acetone, 9:1, and collecting 10ml fractions. Recrystallisation of fractions 23-30 from ether yielded dihydroxycalonectrin (DHC) (160mg) as white, cubic crystals. ^{1}H NMR (Table 18) and EI-MS were used to identify the product.

DHC m/z (rel.int.): 382 ($[M]^+$, 0.1%), 322 (1), 304 (3), 291 (2), 262 (3), 223 (4), 189 (4), 173 (4), 159 0, 151 (4), 137 (8), 121 (13), 109 (13), 100 (19), 82 (46), 43 (100).

Production of trichodiene from *F. culmorum.*

F. culmorum cultures (20x400ml; 2L flasks) were incubated (250rpm) for 2 days at 27°C in the dark, and then each flask treated with 1ml of a xanthotoxin solution (8.7mg/ml in DMSO) to give a concentration of 0.1mM. After a further 6 days incubation, the cultures were filtered at the pump (Whatman No.4 paper) and the filtrate saturated with NaCl and then extracted with EtOAc (3x400ml). The combined extracts were dried ($MgSO_4$) and evaporated to dryness under reduced pressure to yield an orange oil (5.5g). TLC analysis (hexane) indicated the presence of TDN (Rf≈0.6) which gave a brown colour with the H_2SO_4 spray, turning a characteristic purple colour on exposure to air over about 30 mins at room temp. The extract was dissolved in a minimum of EtOAc (approx. 3ml) and loaded onto a silica gel column (150mm x 65mm diameter) packed in hexane. The column was eluted first with hexane (400ml) and then with EtOAc (600ml) collecting fractions of approx. 100ml. Fractions 1-4 were pooled and evaporated under reduced pressure to yield trichodiene (TDN) as a colourless, low viscosity oil (2.16g). It was identified by ^{1}H NMR (Table 10), ^{13}C NMR (Table 11) and EI-MS analysis.

TDN m/z (rel.int.): 204 ($[M]^{+}$,5%), 189 (3), 122 (3), 121 (8), 119 (4), 110 (18), 109 (100), 108 (83), 107 (18), 96 (29), 95 (21), 93 (22), 91 (11).

Production of [^{14}C]TDN.

a) Experiment to assess the relative merits of multiple batch addition of the [2-^{14}C]acetate precursor as compared to addition in a single batch.

Two day old *F. culmorum* cultures (4x50ml; 250ml flasks) were exposed to 0.1mM xanthotoxin as above. Two flasks received 125µl (925KBq) portions of an aqueous solution of sodium [2-^{14}C]acetate (1.25ml; 9.25MBq) at the same time as the xanthotoxin addition. Aliquots (100µl; 185KBq) of a 250µl portion of the same sodium [2-^{14}C]acetate solution diluted to 1000µl with sterile distilled water, were added to the two remaining flasks at times 0, 24, 48, 72, and 120 hours after addition of the xanthotoxin. After a total incubation time of 172 hours each 50ml culture was harvested separately by filtration (Whatman No.4 paper) under reduced pressure, followed by extraction of the filtrate using EtOAc (3x50ml). The extracts were dried ($MgSO_4$), filtered, and evaporated to dryness under reduced pressure. Each extract was purified by column chromatography (125mm x 30mm diameter) eluting with hexane and collecting 10ml fractions. Fractions containing [^{14}C]TDN were pooled (typically F.7-12) and the solvent removed under reduced pressure. The yield of [^{14}C]TDN produced was recorded, and specific activities were measured using liquid scintillation counting procedures (Table 8).

b) Large scale production [^{14}C]TDN.

A culture of *F. culmorum* (200ml) was exposed to 0.1mM xanthotoxin 2 days after subculture, as above. Portions (1ml) of an aqueous solution of sodium [2-^{14}C]acetate (37MBq; 1.9GBqmM^{-1}; 5ml) were added at times 0, 8, 31, 77 and 100hr after addition of the xanthotoxin, and the culture was then worked up after a

further 44hrs incubation. Filtration (Whatman No.4 paper) was followed by extraction with EtOAC (3x70ml). The combined organic extracts were dried ($MgSO_4$) then evaporated to dryness under reduced pressure. The resultant oil was applied to a silica gel column (900mm x 65mm diameter), and elution with hexane yielded [^{14}C]TDN (164mg) in the first 200ml.

Identification and purity of the product was checked using GC and TLC analysis, and the specific activity determined using liquid scintillation counting (Table 9).

Production of 12,13-epoxytrichothec-9-ene and isotrichodermin from xanthotoxin-inhibited cultures of *F. culmorum.*

F. culmorum cultures (15x260ml, 1L flasks) were incubated (250rpm) at 27°C in the dark for 2 days, and then each flask was treated with 1ml of a xanthotoxin solution (39.3mg/ml in DMSO) to give an inhibitor concentration of 0.7mM. After a further 6 days incubation the cultures were worked up as above, yielding the crude toxin extract (3g). This was fractionated by CTLC (4mm plate) eluting with hexane (100ml) then hexane-EtOAC mixtures (85:15, 100ml; 66:34, 100ml) and collecting 25ml fractions*. These were analysed by TLC (hexane-EtOAc, 1:1), and combined as below.

Fractions 1-4* gave TDN (600mg).

Fractions 6-10* were combined and further purified by CTLC (1mm plate) eluting with hexane-EtOAc, 8:2, and collecting 1.5ml fractions. 12,13-Epoxytrichothecene (EPT) (4mg) was obtained from fractions 28-31.

Fractions 11-13* were combined and further purified by CTLC (1mm plate) eluting with toluene-EtOAc, 3:1, and collecting 1.5ml fractions. Fractions 21-26 were combined and further purification by CTLC (1mm plate; hexane-EtOAc, 1:1; 1ml fractions) afforded isotrichodermin (ITD) (mg) in fractions 24-28.

^{1}H NMR (Table 18) and EI-MS analysis confirmed the identity of the products.

EPT 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).

<u>Semi-synthesis of EPT from ITD.</u>

Isotrichodermol (5).

A solution of ITD (30mg) in MeOH (1.7ml) was treated with NH_4OH (2M; 1.2ml), stirring at room temp. for 24h. The reaction was terminated by carefully adjusting the pH to 7.0 with M HCl, and the MeOH removed under reduced pressure. The aqueous residue was extracted with CH_2Cl_2 (3x10ml), the organic layers combined, dried over $MgSO_4$, and evaporated to a colourless oil (23mg).

Purification by CTLC (1mm plate) eluting with hexane-EtOAC, 2:3, and collecting 1ml fractions gave isotrichodermol (9mg, 35%) in fractions 20-25.

^{1}H NMR ($CDCl_3$): δ5.50 (1H, *br dd*, J=5.4, 1.4Hz, H-10), δ4.45 (1H, *ddd*, J=9.0, 4.6, 4.6Hz, H-3), δ4.10 (1H, *d*, J=5.3Hz, H-11), δ3.51 (1H, *d*, J=4.6Hz, H-2), δ3.08 (1H, *d*, J=4.0Hz, H-13), δ2.85 (1H, *d*, J=4.0Hz, H-13), δ2.2-1.8 (6H, *m*, H-4,7,8), δ1.73 (3H, *br s*, H-16), δ0.82 and 0.75 (each 3H, *s*, H-15 and H-14).

EI-MS m/z (rel.int.): 250 ($[M]^+$, 10%), 235 (16), 232 (2), 220 (7), 189 (7), 171 (7), 149 (10), 133 (19), 124 (62), 108 (100), 93 (88).

12,13-Epoxytrichothecene-3-phenylthionocarbonate (69).

This esterification of isotrichodermol is extremely sensitive to both oxygen and moisture, the presence of either of which causes failure of the reaction. All equipment to be used was therefore baked dry at 140° for 24hrs, assembled whilst still hot, and allowed to cool whilst flushing through with N_2. A positive pressure of N_2 was maintained, and reagents were added to the reaction vessel by injection through rubber septa.

To a solution of isotrichodermol (9mg) in dry CH_2Cl_2 (1ml) containing 4-dimethylaminopyridine (4.7mg) and pyridine (18µl) was added phenylchlorothionocarbonate (25µl). The reaction was stirred at room temperature under a N_2 atmosphere for 20hrs, during which time the colour changed from orange to green. After addition of CH_2Cl_2 (40 ml), the solution was washed first with M HCl (2x25ml) and then with sat. $NaCl_{(aq)}$ (30ml). The organic layer was then dried over $MgSO_4$ and evaporated under reduced pressure to yield a green oil (70mg). This was purified by CTLC (1mm plate) eluting with petrol (40-60°)-EtOAc, 85:15, and collecting 1ml fractions. Fractions 29-33 were combined to give EPT-3-phenylthionocarbonate (6mg, 48%) as a pale yellow oil. The identity of the product was confirmed by ^1H NMR analysis.

^1H NMR ($CDCl_3$): δ7.46-7.13 (5H, *m*, Ph), δ5.66 (1H, *ddd*, J=9.2, 6.1, 4.7Hz, H-3), δ5.50 (1H, *br d*, J=5.4Hz, H-10), δ4.01 (1H, *d*, J=5.4Hz, H-11), δ3.97 (1H, *d*, J=4.7Hz, H-2), δ3.13 (1H, *d*, J=3.9Hz, H-13), δ2.90 (1H, *d*, J=3.9Hz, H-13),

δ2.4-1.4 (6H, *m*, H-4,7,8), δ1.73 (3H, *br s*, H-16), δ0.84 and 0.80 (each 3H, *s*, H-14 and H-15).

12,13-Epoxytrichothecene (3).

Again care was taken to ensure that the reaction was performed under strictly anhydrous conditions, and in an inert atmosphere.

To a solution of EPT-3-phenylthionocarbonate (6mg) in dry benzene (0.5ml) containing 2,2'-azo-bis(2-methylpropionitrile) (AIBN; 1mg) was added tri-n-butyltin hydride (6.5µl). The reaction was stirred under a N_2 atmosphere at 80° for 75 mins, cooled, then evaporated under reduced pressure to give an oil (14mg). This was chromatographed by CTLC (1mm plate) eluting with hexane-acetone, 2:1, and collecting 1ml fractions. Fractions 25-30 were combined and further purified by preparative TLC (1 plate; 20cm x 20cm x 0.2mm) in hexane-EtOAc,1:1, to yield EPT (1.2mg, 50%) as a colourless oil spectrally identical to the natural material (^{1}H NMR and MS).

<u>Semi-synthesis of TDN derivatives.</u>

9β,10β-EpoxyTDN.

To a solution of TDN (306mg) in dry CH_2Cl_2 (100ml) containing $Na_2HPO_4.2H_20$ (250mg) was added, with stirring, *m*-chloroperoxybenzoic acid [50-60%] (300mg) in four aliquots. The reaction was stirred at room temperature and progress monitored by TLC (hexane-EtOAc, 8:2). After 10 mins all the starting material had reacted, and the reaction was stopped by addition of H_2O (100ml), and then extracted with ether (3x70ml). The organic extracts were combined and then

washed successively with saturated solutions of $NaHCO_3$ (50ml) and NaCl (50ml), dried over $MgSO_4$, and evaporated to an oil under reduced pressure.

This was chromatographed by CTLC (2mm plate) in hexane-EtOAc, 1:1, collecting 2ml fractions. Fractions 21-26 were combined to give 9β,10β-epoxyTDN (225mg, 68%) as a viscous colourless oil. On TLC analysis (hexane-EtOAc, 8:2) this compound (Rf=0.57) gave a brown colour with the H_2SO_4 spray, turning to a purple colour on exposure to air over about 30 mins at room temperature.

It was characterised by ^1H NMR (Table 10), ^{13}C NMR (Table 11) and EI-MS.

9β,10β-EpoxyTDN m/z (rel.int.): 220 ($[M]^+$, 1%), 205 (1), 202 (1), 125 (36), 107 (46), 96 (100), 95 (81), 67 (15).

9β,10α-DihydroxyTDN.

To a solution of 9β,10β-epoxyTDN (60mg) in THF-H_2O, 1:1 (6ml) at 0°, was added dropwise 6% $HClO_4$ (aq) (0.9ml). The reaction was stirred at 0° for 18hrs, and then the pH adjusted to approx. 9 by addition of solid $NaHCO_3$. After treatment with a similar quantity of NaCl(s), water (10ml) was added and the mixture extracted with ether (2x20ml). The combined ethereal extracts were washed with sat. NaCl (aq) (30ml), dried over $MgSO_4$ then evaporated to an oil under reduced pressure.

Purification by CTLC (1mm plate) eluting with hexane-EtOAc, 1:2, collecting 1ml fractions, yielded 9β,10α-dihydroxyTDN (33mg, 51%) as colourless crystals (mpt=109°) on evaporation of fractions 16-21. The product was identified using ^1H NMR (Table 10), ^{13}C NMR (Table 11) and EI-MS analysis.

9β,10α-DihydroxyTDN m/z (rel.int.): 238 ([M]$^+$, 1%), 220 (1), 205 (1), 141 (6), 125 (70), 108 (33), 96 (100), 95 (32), 81 (85).

9β,10β;12,13-DiepoxyTDN.

A solution of TDN (153mg) in dry CH_2Cl_2 (50ml) containing $Na_2HPO_4.2H_2O$ (250mg) was treated with *m*-chloroperoxybenzoic acid [50-60%] (500mg), and the reaction stirred at room temp. TLC analysis (hexane-EtOAc, 8:2) indicated that the 9β,10β-epoxyTDN initially formed had all reacted after 6hrs, and the reaction was stopped by addition of H_2O (100ml), then extracted with ether (3x70ml). The organic extracts were combined then washed successively with saturated solutions of $NaHCO_3$ (50ml) and NaCl (50ml), dried over $MgSO_4$ and then evaporated under reduced pressure to give a colourless oil.

Purification by CTLC (1mm plate) eluting with hexane-EtOAc, 8:2, and collecting 1.5ml fractions, yielded 9β,10β;12,13-diepoxyTDN (94mg, 54%) in fractions 40-47 as a viscous colourless oil. It was identified by ^{1}H NMR (Table 10), ^{13}C NMR (Table 11), and EI-MS analysis.

9β,10β;12,13-DiepoxyTDN m/z (rel.int.): 236 ([M]$^+$, 1%), 208 (15), 205 (6), 193 (7), 161 (24), 131 (10), 125 (19), 124 (20), 107 (100), 99 (19), 93 (31), 81 (40).

<u>Attempted synthesis of 12,13-epoxyTDN.</u>

9,10-DibromoTDN.

To a solution of TDN (204mg) in CH_2Cl_2 (20ml) containing pyridine (39.5mg) at 0°C, was added dropwise over 10 mins a solution of bromine (158mg) in CH_2Cl_2 (20ml) at 0°C. After a further 3 mins stirring the reaction had decolourised, and

was then washed with sodium metabisulphite (aq) (20ml) followed by 5% HCl (aq) (20ml) and water (20ml). After drying over $MgSO_4$, the organic layer was evaporated to an oil under reduced pressure. This was chromatographed by CTLC (1mm plate) in hexane, collecting 1ml fractions. 9,10-DibromoTDN (184mg, 51%) was obtained from fractions 13-25 as a viscous colourless oil. On TLC analysis (hexane) it reacted with the PNBP/TEPA reagent giving an intense purple coloured spot (Rf=0.45).

It was characterised by ^1H NMR (Table 10), ^{13}C NMR (Table 11) and FAB-MS.

9,10-DibromoTDN m/z (rel.int.): 365,363,361 ($[MH]^-$, 20,35,17%), 283 (71), 281 (56), 269 (66), 267 (52), 203 (100), 201 (99), 189 (100), 175 (100), 147 (99), 145 (100), 143 (96), 121 (100), 110 (100), 108 (100), 95 (100).

9,10-Dibromo-12,13-epoxyTDN.

A solution of 9,10-dibromoTDN (184mg) in dry CH_2Cl_2 (20ml) containing $Na_2HPO_4.2H_20$ (66mg) was treated with *m*-chloroperoxybenzoic acid [50-60%] (143mg), and the reaction stirred at room temp. TLC analysis (hexane-EtOAc, 8:2) indicated that all the starting material had reacted after 3.5hrs, and the reaction was stopped by addition of H_2O (50ml), and extracted with ether (3x70ml). The combined organic extracts were washed successively with saturated solutions of $NaHCO_3$ (30ml) and NaCl (30ml), then dried over $MgSO_4$ and evaporated to an oil under reduced pressure.

This was purified by CTLC (1mm plate) eluting with hexane-EtOAc, 8:2, collecting 1ml fractions. Fractions 21-33 on evaporation gave

9,10-dibromo-12,13-epoxyTDN (118mg, 62%) as white crystals mpt=74-76°C.

The product was characterised by ^{1}H NMR (Table 10), ^{13}C NMR (Table 11) anf FAB-MS.

9,10-Dibromo-12,13-epoxyTDN m/z (rel.int.): 381,379,377 ($[MH]^-$, 13,20,11%), 301 (55), 299 (73), 281 (48), 269 (33), 267 (31), 219 (93), 201 (100), 189 (98), 187 (50), 154 (78), 133 (99), 111 (98).

Attempted debromination of 9,10-dibromo-12,13-epoxyTDN.

A solution of 9,10-dibromo-12,13-epoxyTDN (20mg) in dry acetone (305ml) was treated with an excess of anhydrous NaI (60mg), and the reaction stirred at 38°C. TLC (hexane-EtOAc, 8:2) and GC analysis indicated that after 3.5hrs numerous products had been formed, none of which predominated.

Debromination of 9,10-dibromoTDN.

A solution of 9,10-dibromoTDN (3mg) in dry acetone (0.7ml) was treated with an excess of anhydrous NaI (6mg) and the reaction stirred at 38°C. TLC (hexane) and GC analysis indicated that after 4hrs all the starting material had reacted yielding TDN as the major product (95% of products running on GC, RT=5.7 mins).

<u>9β-Hydroxytricho-10,12-diene.</u>

9β-Hydroxy,10α-phenylselenoTDN.

A solution of diphenyldiselenide (211mg) in dry ethanol (10ml) was reacted, under a nitrogen atmosphere, with 5.3ml of a solution prepared by dissolving $NaBH_4$ (100mg) in dry ethanol (10.6ml). An additional 5 drops of the $NaBH_4$ solution

were required to decolourise the yellow reaction mixture, and then a solution of 9β,10β-epoxyTDN (198mg) in dry ethanol (10ml) was added and the reaction heated at 65°C for 18hrs. After cooling, the reaction was treated with 10% aqueous HCl (36ml) causing the formation of a white precipitate. The suspension was neutralised using saturated $NaHCO_3$ (aq) and then extracted with ether (3x30ml). The combined organic extracts were washed successively with saturated solutions of $NaHCO_3$ (30ml) and NaCl (30ml), dried over $MgSO_4$ then evaporated under reduced pressure to a yellow oil. This was chromatographed using CTLC (2mm plate) in hexane-ether, 2:1, collecting 1ml fractions immediately after the elution of a yellow coloured band running near the solvent front.

On evaporation fractions 8-16 gave 9β-hydroxy,10α-phenylselenoTDN (213mg, 63%) as a viscous colourless oil which crystalised on standing. Mpt=52-54°C. The product was identified by ^1H NMR analysis (Table 10).

A solution of 9β-hydroxy,10α-phenylselenoTDN (100mg) in THF (7ml) at 0°C was treated with a solution of $NaIO_4$ (169mg) in 70% aqueous methanol (5ml). The reaction was stirred at 0° for 1.5hrs then refluxed (60°) for a further 2.5hrs. After cooling, the mixture was treated with saturated NaCl (30ml) and extracted with ether (3x40ml). The combined organic extracts were washed with sat. NaCl (50ml), dried over $MgSO_4$ and evaporated to an oil under reduced pressure. Purification by CTLC (1mm plate) in hexane-ether, 1:3, collecting 1ml fractions yielded 9β-hydroxytricho-10,12-diene (27mg, 48%) as white waxy crystals, mpt=33-35°C.

The product was characterised by ^1H NMR (Table 10), ^{13}C NMR (Table 11), $^1H/^{13}C$ COSY (Fig.43) and EI-MS.

9β-Hydroxytricho-10,12-diene m/z (rel.int.): 202 ([M-H_2O]$^+$, 7%), 187 (5), 125 (28), 107 (100), 96 (42), 95 (44), 91 (29), 81 (37).

Production of 9α-hydroxy- and 9-methoxy- tricho-10,12-diene from 9β-hydroxy,10α-phenylselenoTDN.

A solution of 9β-hydroxy,10α-phenylselenoTDN (100mg) in THF (5.4ml) at 0°C was treated with a solution of $NaIO_4$ (183mg) in 70% aqueous methanol (6ml). The reaction was stirred at 0° for 1.5hrs then heated in a sealed Reactivial at 75° for a further 2.5hrs, and then worked-up as above. TLC analysis (hexane-ether, 1:3) indicated the formation of a major product (A: Rf≈0.6) and two minor, more polar products (B: Rf≈0.45; C: Rf≈0.35). These were purified by CTLC (1mm plate) eluting with hexane-ether, 1:3, and collecting 0.5ml fractions. Appropriate fractions were combined as indicated by TLC analysis (hexane-ether, 1:3) giving A (15mg, 24%) in F. 17-20, B (5.5mg, 9%) in F. 29-32 and C (3mg, 5%) in F. 34-36.

A, B and C were identified as 9-methoxytricho-10,12-diene, 9β-hydroxytricho-10,12-diene and 9α-hydroxytricho-10,12-diene, repectively, by ^{1}H NMR (Table 10) and EI-MS analysis. ^{1}H NMR analysis also demonstrated that A was a mixture of 9β- and 9α-methoxytricho-10,12-diene in a ratio of 85:15.

9-Methoxytricho-10,12-diene (A), m/z (rel.int.): 219 ([M-CH_3]$^+$, 1%), 202 (12), 187 (5), 156 (5), 139 (52), 108 (40), 107 (100), 96 (38), 95 (51).

9α-Hydroxytricho-10,12-diene (C), m/z (rel.int.): 202 ([M-H_2O]$^+$, 17%), 125 (72), 107 (100), 96 (39), 95 (59), 91 (33), 81 (55).

Production of 9β- and 9α-methoxytricho-10,12-dienes from 9β-hydroxytricho-10,12-diene.

A solution of 9β-hydroxytricho-10,12-diene (2mg) in a 1:1 mixture of THF-70% aqueous methanol (2ml) was adjusted to approximately pH 4 by addition of 1N HCl (15µl). The reaction was heated in a sealed Reactivial at 75° for 1.5hrs. After cooling, TLC analysis (hexane-ether, 1:3) indicated that about 50% of the starting material had reacted producing 9-methoxytricho-10,12-diene. Further TLC analysis (hexane-ether, 85:15) indicated that this was a mixture of the 9β- and 9α-methoxy isomers in a ratio of approximately 4:1.

Production of 9α-hydroxytricho-10,12-diene from 9β-hydroxytricho-10,12-diene.

A solution of 9β-hydroxytricho-10,12-diene (3.1mg) in a 1:1 mixture of THF-H_2O (4ml) was adjusted to approximately pH 4 by addition of 1N HCl (20µl) and the reaction heated in a sealed vessel at 75°. TLC analysis (hexane-ether, 1:3) indicated that after 1.5hrs 9α-hydroxytricho-10,12-diene was present, with the ratio of β:α being approximately 3:1. The reaction was monitored periodically for up to 18hrs, but no observable change in this ratio was detected.

Large scale synthesis of 9α-hydroxytricho-10,12-diene.

A solution of 9β-hydroxy,10α-phenylselenoTDN (120mg) in THF (10ml) at 0° was treated with a solution of $NaIO_4$ (202mg) in H_2O (4ml). The reaction was stirred at 0° for 1.5hrs then heated in a sealed vessel at 75° for a further 3hrs. Work-up and purification as for 9β-hydroxytricho-10,12-diene yielded 9α-hydroxytricho-10,12-diene (8.0mg, 11%) in fractions 18-22 as white crystals,

mpt=87-89°C. This was characterised by ^{1}NMR (Table 10), ^{13}C NMR (Table 11) and EI-MS.

9β-Hydroxytricho-10,12-diene (44.5mg, 63%) was also isolated from the reaction in fractions 10-16.

<u>Screening TDN derivatives for activity as inhibitors of post-TDN trichothecene biosynthesis in *F. culmorum*.</u>

CD production medium cultures (30ml; 42hrs old) were filtered, and the mycelia washed with distilled water (x3) then resuspended in water (6ml) in a 25ml flask. The flasks received the appropriate TDN derivative (in 100µl acetone) and were then incubated at 27°,250rpm in the dark. After 30 mins, [^{14}C]TDN was added (in 50µl acetone) and incubation continued for a further 5.5hrs.

Series		TDN derivative		[^{14}C]TDN	
		Name	Quantity	mg	MBqmM^{-1}
A	1	---	---	0.5	7.69
	2	9β,10β-EpoxyTDN	5mg	0.5	7.69
	3	9β,10β;12,13-DiepoxyTDN	5mg	0.5	7.69
B	4	---	---	0.5	3.92
	5	9,10-DibromoTDN	5mg	0.5	3.92
	6	9,10-Dibromo-12,13-epoxyTDN	5mg	0.5	3.92
C	7	---	---	0.25	3.92
	8	9β,10α-DihydroxyTDN	2.5mg	0.25	3.92
	9	9β-Hydroxytricho-10,12-diene	2.5mg	0.25	3.92

Experiments in which derivatives were present (ie. 2, 3, 5, 6, 8 and 9) were

simultaneously repeated using mycelia which had been boiled for 10 mins then cooled.

The cultures were harvested by filtration (Whatman No.4 paper) under reduced pressure, followed by extraction of the filtrate with EtOAc (15ml). The organic extracts were dried ($MgSO_4$), filtered and then evaporated to dryness under reduced pressure. Extracts were redissolved in EtOAc (50µl) and aliquots (10µl) analysed by TLC (hexane-EtOAc, 1:1) and autoradiography (series A: 24hr exposure; series B: 48hr exposure; series C: 96hr exposure).

Autoradiograms: Series A, Fig.48; series B, Fig.104; series C, Fig.105.

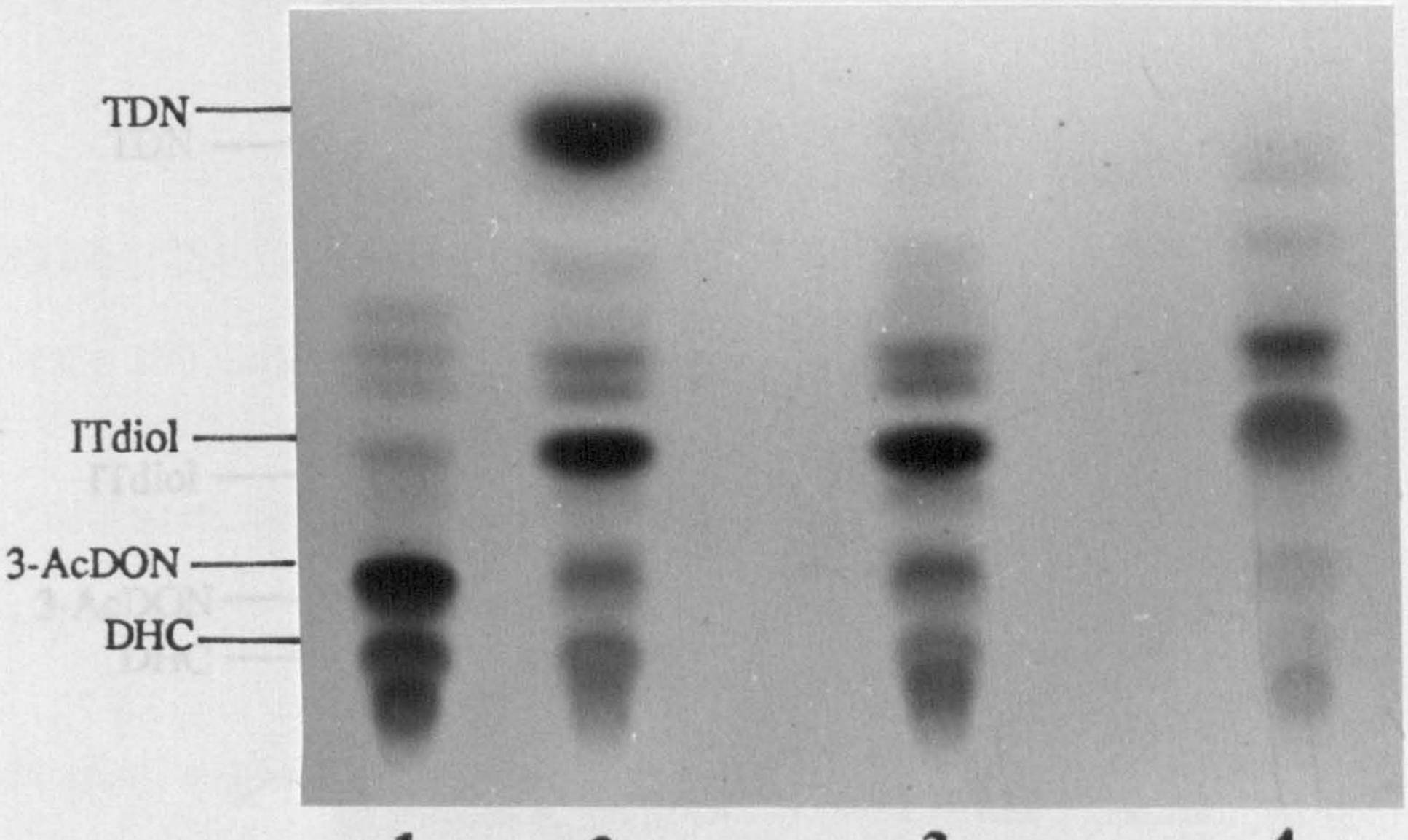
Production of isotrichodiol.

CD production medium cultures (3 x 200ml) of *Fusarium culmorum* (42hrs old) were filtered under reduced pressure (Whatman No.4 paper), and the mycelia washed with distilled water (3x50ml) then resuspended in distilled water (160ml) in a 1L flask. TDN (156mg) in acetone (0.6ml) was added and the culture was incubated at 27°C, 250rpm in the dark for 30 mins. A further portion of TDN (44mg in 0.2ml acetone) was added and the incubation continued for 5.5hrs. The mycelia was filtered and washed with EtOAc (2x20ml), and the combined filtrate and washings extracted with EtOAc (3x50ml). The combined organic extracts were dried over $MgSO_4$, and evaporated under reduced pressure to an oil (120mg). This was fractionated by CTLC (1mm plate) eluting with hexane-EtOAc, 1:1, and collecting 1ml fractions. Isotrichodiol (ITdiol) (11.8mg) was obtained as white crystals mpt=138-140°C from fractions 33-36 by recrystallisation from EtOAc.

On TLC (hexane-EtOAc, 1:1) it appeared as a light brown spot (Rf=0.35) with the H_2SO_4 spray, and reacted with the PNBP/TEPA reagent producing a bright sky

Fig.104: Autoradiograms showing the effect of a range of TDN derivatives on the incorporation of [^{14}C]TDN into trichothecene toxins in *Fusarium culmorum*.

Series B:

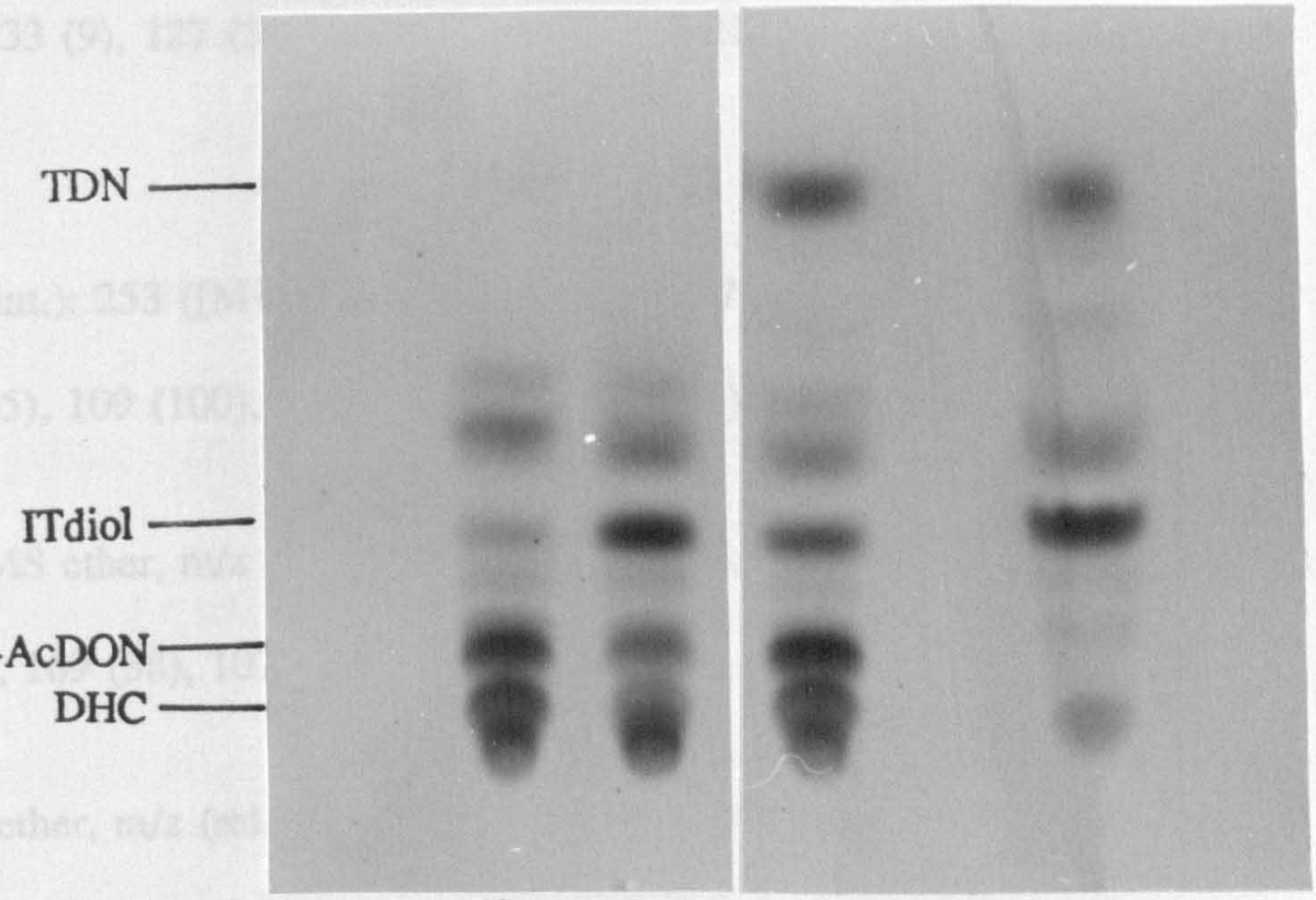


Lane 1. Fed [^{14}C]TDN (0.25mg)
Lane 2. Fed [^{14}C]TDN (0.25mg) + unlabelled TDN (2.5mg)
Lane 3. Fed [^{14}C]TDN (0.25mg) + 9β-hydroxytricho-10,12-diene (2.5mg)
Lane 4. Fed [^{14}C]TDN (0.25mg) + 9β,10α-dihydroxyTDN (2.5mg)

6hr incubation TLC Hexane-EtOAc, 1:1

Fig.105: Autoradiograms showing the effect of a range of TDN derivatives on the incorporation of [^{14}C]TDN into trichothecene toxins in *Fusarium culmorum*.

Series C:



Lane 1. Fed [^{14}C]TDN (0.5mg)
Lane 2. Fed [^{14}C]TDN (0.5mg) + unlabelled TDN (5mg)
Lane 3. Fed [^{14}C]TDN (0.5mg) + 9,10-dibromoTDN (5mg)
Lane 4. Fed [^{14}C]TDN (0.5mg) + 9,10-dibromo-12,13-epoxyTDN (5mg)

6hr incubation TLC Hexane-EtOAc, 1:1

blue colour.

ITdiol was fully characterised by ^{1}H and ^{13}C NMR (Table 16), $^{1}H/^{1}H$ COSY 45 (Fig.53A), $^{1}H/^{1}H$ COSY 90 (Fig.53B), $^{1}H/^{13}C$ COSY (Fig.56), DNOES (Fig.54), EI-MS, CI-MS, and by GC-MS of the products after trimethlysilylation.

EI-MS m/z (rel.int.): 252 ($[M]^{+}$, 4%), 237 (11), 234 (5), 224 (50), 219 (10), 177 (10), 151 (10), 133 (9), 127 (3), 125 (13), 124 (42), 121 (28), 107 (43), 93 (47), 84 (100).

CI-MS m/z (rel.int.): 253 ($[M+1]^{+}$, 10%), 235 (23), 217 (37), 199 (30), 137 (18), 127 (25), 125 (35), 109 (100), 97 (58), 93 (43), 81 (73).

EI-MS mono-TMS ether, m/z (rel.int.): 324 ($[M]^{+}$, 1%), 309 (2), 306 (0.7), 296 (1), 219 (4), 211 (6), 169 (38), 107 (96), 84 (32), 78 (100).

EI-MS di-TMS ether, m/z (rel.int.): 381 ($[M-CH_3]^{+}$, 0.3%), 339 (0.9), 313 (0.3), 306 (1), 291 (1), 169 (20), 156 (100), 73 (89).

Transformation of [^{14}C]TDN to trichothecenes.

Fusarium culmorum cultures (9 x 200ml in 1L flasks; 42hrs old) were filtered under reduced pressure, the mycelia washed thoroughly with distilled water (3x150ml) and divided into 3 equal portions. Three 1L flasks containing 160ml distilled H_2O received one portion, and to each of these cultures was added low activity [^{14}C]TDN (156mg) in acetone (0.6ml). After 30 mins incubation at 27°, 250rpm a further portion of the [^{14}C]TDN (44mg in 0.2ml acetone) was added to each, and incubation was continued for a further 5.5hrs. The mycelia was filtered ane washed with EtOAc (3x60ml), and the combined filtrate and washings were

extracted with EtOAc (4x100ml). The combined extracts were dried ($MgSO_4$), filtered and evaporated to a yellow oil (390mg). This was chromatographed by CTLC (2mm plate) eluting first with hexane-EtOAc, 8:2 (60ml), and then with hexane-EtOAc, 1:1, collecting 2ml fractions. Fractions containing pure compounds were combined (as below) as indicated by TLC analysis (hexane-EtOAc, 1:1). All compounds were identified by EI-MS and ^1H NMR analysis (Table 18).

Fractions 18-22 yielded recovered TDN (233mg; Rf=0.72).

Fractions 34-41 yielded EPT (1.5mg; Rf=0.55) as a viscous colourless oil.

Fractions 47-51 yielded ITD (1.0mg; Rf=0.49).

Fractions 55-59 yielded CAL (2.5mg; Rf=0.45) as a viscous colourless oil.

Fractions 74-80 yielded ITdiol (23.0mg; Rf=0.35) as white crystals.

Fractions 87-91 yielded 15-deacetylcalonectrin (15-deacetylCAL) (3.5mg; Rf=0.28) as a viscous colourless oil.

15-DeacetylCAL EI-MS m/z (rel.int.): 308 ($[M]^+$, 1%), 290 (2), 278 (3), 265 (3), 217 (11), 199 (8), 159 (10), 140 (26), 123 (17), 109 (32), 93 (25), 43 (100).

Fractions 97-100 yielded 7α-hydroxycalonectrin (7-hydroxyCAL) (4.0mg; Rf=0.22) as a viscous colourless oil. This compound gave a characteristic pink colour with the 20% H_2SO_4 spray.

7-HydroxyCAL EI-MS m/z (rel.int.): 348 ($[M-H_2O]^+$, 1%), 306 (9), 288 (13), 215 (10), 187 (13), 137 (23), 121 (40), 107 (56), 95 (50), 43 (100).

Fractions 102-END contained two toxins which were further purified by CTLC (1mm plate) eluting with ether-acetone, 9:1, collecting 1ml fractions. 3-AcDON (17.0mg; Rf=0.18) was obtained as white crystals from fractions 16-21, and DHC (21.0mg; Rf=0.11) as colourless crystals from fractions 26-30.

The Rf values listed are for TLC in hexane-EtOAc, 1:1. TLC-autoradiography showed that all the compounds were radiolabelled.

Production of [^{14}C]ITdiol.

F. culmorum cultures (4 x 200ml; 42hrs old) were filtered under reduced pressure and the mycelia (approx. 14g wet weight) washed with distilled H_2O (3x60ml) and resuspended in water (200ml). [^{14}C]TDN (280mg; 124KBqmM^{-1}) in acetone (0.6ml) was added, and the culture incubated at 27°, 250rpm for 6hrs. The mycelia was filtered, washed with EtOAc (3x30ml) and the combined filtrate and washings extracted with EtOAc (3x70ml). The combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure to an oil (154mg). This was chromatographed by CTLC (1mm plate) eluting first with hexane (20ml), and then with hexane-EtOAc, 8:2 (20ml), and hexane-EtOAc, 1:1 (60ml), collecting 1ml fractions. Fractions 82-84 were combined to yield [^{14}C]ITdiol (8.0mg; 83.7KBqmM^{-1}) as white crystals.

Biotransformations of [^{14}C]ITdiol.

a) Normal conditions.

A filtered and washed mycelial mat from 1 x 200ml *Fusarium culmorum* culture (42hrs old) was divided into four portions. Two 25ml flasks containing 7.5ml H_2O

each received one portion, and to these cultures were added [^{14}C]ITdiol (2.1mg; 0.67KBq) in acetone (0.1ml) or [^{14}C]TDN (2.1mg; 0.67KBq) in acetone (0.1ml). Mycelium in a third flask was boiled for 10 mins, cooled, and then [^{14}C]ITdiol as above was added. The three cultures were incubated at 28°, 250rpm for 6hrs, filtered under reduced pressure and the mycelia washed with EtOAc (15ml). The filtrate was extracted with the EtOAc washings, and the extracts dried over $MgSO_4$, filtered and evaporated to dryness under reduced pressure. The extracts were dissolved in EtOAc (50µl) and aliquots (10µl) analysed by TLC (hexane-EtOAc, 1:1, and ether-acetone, 9:1) and autoradiography (15 days exposure; Fig.59).

Large scale: A filtered and washed mycelial mat from 2 x 200ml *Fusarium culmorum* culture (42hrs old) was washed in H_2O (200ml), filtered and resuspended in distilled H_2O (250ml). [^{14}C]ITdiol (18mg; 52.9KBqmM^{-1}) in acetone (0.6ml) was added and the culture incubated at 28°, 250rpm for 7hrs, filtered and the mycelia washed with EtOAc (100ml). The combined filtrate and washings were extracted with EtOAc (4x100ml) and the extracts dried over $MgSO_4$, filtered, and evaporated to an oil (54mg) under reduced pressure. This was chromatographed by CTLC (1mm plate) eluting with ether-acetone, 94:6, collecting 1.5ml fractions. TLC analysis (ether-acetone, 9:1) indicated that fractions 42-49 contained mainly DHC. These were combined to give an oil (4.6mg) which was purified by preparative TLC (1 plate; 20cm x 20cm x 0.2mm) in hexane-EtOAc, 4:6, to yield white crystals (3.0mg). Further similar purification by preparative TLC in $CHCl_3$-MeOH, 96:4, yielded DHC (2.6mg; 31.3KBqmM^{-1}).

Fractions 27-37, containing mainly 3-AcDON and 7-hydroxyCAL, were combined (12mg) and chromatographed by preparative TLC (2 plates; 20cm x

20cm x 0.2mm) in $CHCl_3$-MeOH, 96:4. This yielded 3-AcDON (2.5mg) which was purified further by preparative TLC (1 plate) in hexane-EtOAc, 4:6, giving pure 3-AcDON (1.4mg; 41.6KBqmM^{-1}) as white crystals.

7-HydroxyCAL (0.9mg) was further purified by preparative TLC using the same system, yielding the pure compound (0.6mg; 19.4KBqmM^{-1}) as a viscous colourless oil.

The purity of the compounds was checked by TLC-autoradiography in 3 different solvent systems (hexane-EtOAc, 4:6; $CHCl_3$-MeOH, 96:4; ether-acetone, 94:6).

b) In the presence of xanthotoxin.

Aliquots (5ml) of a *F. culmorum* culture (30hrs old) were placed in four 25ml flasks. Three flasks were treated with xanthotoxin (in 0.1ml DMSO) to give concentrations of 0.1, 0.4 and 0.7mM respectively. The four flasks were incubated at 28°, 250rpm for 18hrs, after which time [^{14}C]ITdiol (0.5mg; 83.7KBqmM^{-1}) was added to each. The cultures were incubated for a further 6hrs, filtered under reduced pressure and the mycelia washed with EtOAc (15ml). The filtrate was extracted with the EtOAc washings, and the extracts dried over $MgSO_4$, filtered and evaporated to dryness under reduced pressure. The extracts were dissolved in EtOAc (20µl) and all analysed by TLC (hexane-EtOAc, 1:1) and autoradiography (15 days exposure; Fig.60).

c) In the presence of nitrogen.

Aliquots (5ml) of a *F. culmorum* culture (48hrs old) were placed in three 25ml flasks. Two flasks were purged with N_2 gas for 30 mins after which time they

received [^{14}C]ITdiol (0.5mg; 83.7KBqmM^{-1}) in acetone (50µl) and in one case ATP (16.7mg in 0.2ml H_2O) in addition. The third flask received the [^{14}C]ITdiol but was not purged with N_2. The cultures were incubated for 6hrs at 27°, 250rpm then filtered under reduced pressure and the mycelia washed with EtOAc (15ml). The filtrate was extracted with the EtOAc washings, and the extracts dried over $MgSO_4$, filtered and evaporated to dryness under reduced pressure. The extracts were dissolved in EtOAc (20µl) and all analysed by TLC (hexane-EtOAc, 1:1) and autoradiography (15 days exposure; Fig.60).

Biotransformations of 9β,10β-epoxyTDN and 9β,10β;12,13-diepoxyTDN.

a) A filtered and washed mycelial mat from 1 x 200ml CD production medium culture of *F. culmorum* (46hrs old) was divided into 16 equal portions. Two 25ml flasks containing 5ml H_2O each received one portion, and to these cultures were added [^{14}C]-9β,10β-epoxyTDN (0.25mg; 3.92MBqmM^{-1}) or [^{14}C]-9β,10β;12,13-diepoxyTDN (0.25mg; 3.92MBqmM^{-1}). Mycelia in two further flasks were boiled for 10 mins, cooled, and then ^{14}C-labelled samples as above were added. The cultures were incubated at 27°, 250rpm for 6hrs, filtered under reduced pressure and the filtrates extracted with EtOAc (15ml). The extracts were dried over $MgSO_4$, evaporated to dryness under reduced pressure and redissolved in EtOAc (50µl). Aliquots (10µl) were analysed by TLC (hexane-EtOAc, 1:1 and 8:2) and autoradiography (5 days exposure; Fig.67). Product formation was measured using scintillation counting procedures.

b) Large scale feeding of 9β,10β-epoxyTDN.

A filtered and washed mycelial mat from 300ml CD production medium cultures of

F. culmorum (42hrs old) was resuspended in H_2O (60ml) contained in a 250ml flask. 9β,10β-EpoxyTDN (40mg) in acetone (0.5ml) was added, and the culture incubated at 27°, 250rpm for 6hrs. The culture was then filtered under reduced pressure and the mycelia washed with EtOAc (30ml). The combined filtrate and washings were extracted with EtOAc (3x30ml) and the extract dried over $MgSO_4$ and evaporated to an oil under reduced pressure. This was chromatographed by CTLC (1mm plate) in hexane-EtOAc, 1:1, collecting 1ml fractions and washing the plate with EtOAc (70ml). Fractions 32-43 were combined and purified by preparative TLC (1 plate; 20cm x 20cm x 0.2mm) in hexane-EtOAc, 8:2, to yield 9β,10β;12,13-diepoxyTDN (2.0mg) spectrally (^{1}H NMR and EI-MS) identical to the synthetic standard.

The EtOAc wash was evaporated to an oil under reduced pressure, and rechromatographed by CTLC (1mm plate) in hexane-EtOAc, 1:4, collecting 1.5ml fractions. Fractions 47-50 were combined to yield 3α-hydroxy-9β,10β;12,13-diepoxyTDN (3.2mg) as a viscous colourless oil. It was identified using ^{1}H and ^{13}C NMR (Table 23), ^{1}H/^{1}H COSY (Fig.69) and EI-MS.

m/z (rel.int.): 252 ([M]$^+$, 1%), 221 (50, 203 (1), 161 (2), 149 (10), 125 (16), 124 (15), 117 (2), 107 (80), 97 (34), 81 (31), 43 (100).

c) Large scale feeding of 9β,10β;12,13-diepoxyTDN.

The experiment above was repeated feeding 9β,10β;12,13-diepoxyTDN (40mg). This yielded 3α-hydroxy-9β,10β;12,13-diepoxyTDN (8.0mg).

Acid catalysed rearrangements of ITdiol.

a) ITdiol (7.7mg) in acetone (0.5ml) was added to 200ml sterile YEPD-5G medium (Appendix 2) previously adjusted to pH 4.5 with dil.HCl, and incubated at 27°, 250rpm for 3 days. The products were extracted with EtOAc (3x70ml) and the combined extracts were dried over $MgSO_4$ and evaporated to an oil under reduced pressure.

TLC analysis (hexane-EtOAc, 1:1) indicated that all the starting material had been consumed, and that 3 compounds (2 more polar, 1 less polar) had been formed. Purification by preparative TLC (1 plate; 20cm x 20cm x0.2mm) in hexane-EtOAc, 1:1, yielded EPT (2.5mg, 35%) as a viscous colourless oil spectrally (^{1}H NMR and EI-MS) identical to the natural material.

b) ITdiol (2.0mg) in EtOAc (0.3ml) was added to 12ml of 100mM potassium phosphate buffer (Appendix 3) at pH 5.8, and incubated at 28°, 250rpm for 6 days. The products were extracted with EtOAc (20ml), and the extract dried over $MgSO_4$ and evaporated to an oil under reduced pressure. This was analysed by GC-MS of the products following trimethylsilylation.

EI-MS of di-TMS ether of 9α-trichotriol:

m/z (rel.int.): 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).

EI-MS of di-TMS ether of pre-sambucoin:

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), 147 (6), 124 (100), 93 (17), 73 (55).

c) ITdiol (13.2mg) in EtOAc 0.5ml) was added to 200ml potassium phosphate buffer at pH 5.9 and incubated at 28°, 250rpm for 6 days. The products were extracted with EtOAc (3x70ml), and the combined organic extracts dried over $MgSO_4$ and evaporated to an oil under reduced pressure. Preparative TLC (2 plates; 20cm x 20cm x 0.2mm) in hexane-EtOAc, 1:1, yielded pre-sambucoin (2.2mg, 17%) as a viscous colourless oil. EPT (2.2mg) was also isolated.

Pre-sambucoin was characterised by ^1H and ^{13}C NMR (Table 24), $^1H/^1H$ COSY (Figs.74 and 75) and EI-MS.

Pre-sambucoin m/z (rel.int.): 252 ($[M]^+$, 26%), 237 (34), 234 (11), 149 (12), 124 (100), 107 (55), 93 (67).

Chemical oxidation of pre-sambucoin.

A solution of trifluoroacetic anhydride (33µl) in dry CH_2Cl_2 (0.4ml) at -78° was treated by dropwise addition of a solution of DMSO (27µl) in dry CH_2Cl_2 (0.4ml) also at -78°. After stirring at this temp. for 10 mins, pre-sambucoin (3mg) in dry CH_2Cl_2 (0.2ml) was added and the reaction stirred for a further 30 mins. Triethylamine (30µl) was added and the reaction temperature allowed to slowly increase to -25° over 40 mins, and then increase more rapidly to room temp. The mixture was diluted with CH_2Cl_2 (15ml), washed with sat. NaCl (aq) (20ml), dried over $MgSO_4$, filtered and evaporated to an oil under reduced pressure.

TLC analysis (hexane-EtOAc, 1:1) indicated the formation of sambucoin, and this was purified by preparative TLC (1 plate; 20cm x 20cm x 0.2mm) in hexane-EtOAc, 1:1, to yield sambucoin (1.2mg, 40%) as a white solid.

Sambucoin ^1H NMR ($CDCl_3$): δ5.20 (1H, *br s*, H-10), 4.20 (1H, *d*, J=11.3Hz,

H-13), 3.96 (1H, *br s*, H-11), 3.45 (1H, *d*, J=11.3Hz, H-13), 2.58 (1H, *ddd*, J=19.9, 10, 8Hz, H-3), 2.28 (1H, *ddd*, J=19.9, 11.8, 2.9Hz, H-3), 2.01 (1H, *ddd*, J=13.5, 10, 3Hz, H-4), 1.97-1.90 (2H, *m*, H-7,8), 1.78 (1H, *ddd*, J=13.7, 11.8, 8Hz, H-4), 1.64 (3H, *br s*, H-16), 1.57 (1H, *br dd*, J=10.8, 6.6Hz, H-8), 1.46 (1H, *ddd*, J=13.4, 5.3, 2.3Hz, H-7), 1.15 (3H, *s*, H-14), 0.65 (3H, *s*, H-15).

Sambucoin EI-MS m/z (rel.int.): 250 ($[M]^+$, 40%), 235 (30), 180 (18), 125 (36), 124 (32), 108 (100), 93 (72), 84 (32).

Biotransformation of pre-sambucoin.

A filtered and washed mycelial mat from a 100ml CD production medium culture of *F. culmorum* (3 days old) was resuspended in sterile CD production medium (50ml). [^{14}C]-pre-sambucoin (1.0mg; 0.41MBqmM^{-1}) was added, and the culture incubated at 28°, 250rpm for 18hrs and then harvested by filtering under reduced pressure, and extracting the filtrate with EtOAc (2x50ml). The extract was dried over $MgSO_4$ and evaporated to an oil. This was spiked with unlabelled sambucoin (6.5mg) and then chromatographed by CTLC (1mm plate) in hexane-EtOAc, 1:1, collecting 1 ml fractions. Fractions 18-21 were combined to yield colourless crystals (6.9mg) which were further purified by preparative TLC (2 plates; 20cm x 20cm x 0.2mm) in ether-acetone, 9:1. Recrysallisation from EtOAc yielded sambucoin (3.2mg; 3.83KBqmM^{-1}).

Isolation of trichothecolone and 9β-trichodiol from *Trichothecium roseum* 50661.

Growth of fungus.

The surface of a mature beer wort agar (BWA) slope culture of *T. roseum* was flooded with sterile distilled H_2O (approx. 1-2ml), and the pink spores suspended in the H_2O by agitation with a sterile wire loop. Using the wire loop the spore suspension was transferred to the surfaces of BWA plates, and the plates incubated at 26° in the dark until mature (5-7 days). The plates were flooded with sterile distilled H_2O (2ml) and the spores dislodged by agitation. The spore suspension was used to inoculate sterile CD seed medium (Appendix 2), using the suspension prepared from 1 plate per 200ml of medium. The seed cultures were incubated at 27°, 250rpm for 2 days, and then aliquots (50ml) were used to inoculate 2L Erlenmeyer flasks containing sterile CD corn steep medium (400ml) (Appendix 2). The cultures were incubated for a further 21 days.

Work-up and purification.

A 6L culture was filtered under reduced pressure and the mycelia transferred to a metal container and frozen with liquid nitrogen. Boiling hot water was added and when the mycelia had thawed the slurry was extracted by stirring with EtOAc (400ml). The filtrate was saturated with NaCl and extracted with EtOAc (3x400ml). All the organic extracts were combined, dried over $MgSO_4$ and evaporated to an oil (2.5g) under reduced pressure. This was chromatographed by CTLC (4mm plate) in CH_2Cl_2-MeOH, 9:1, collecting 7ml fractions*. Fractions 9-16* were combined and rechromatographed by CTLC (2mm plate) in ether-acetone, 9:1, collecting 5ml fractions. Trichothecolone (150mg) was obtained as

white crystals mpt=180-182°C on evaporation of fractions 13-18 (lit. mpt=183-184°C[113]).

Trichothecolone ^{1}H NMR ($CDCl_3$): δ6.47 (1H, *dq*, J=5.8, 1.5Hz, H-10), 4.30 (1H, *dd*, J=7.5, 3.1Hz, H-4α), 3.92 (1H, *d*, J=5.3Hz, H-2β), 3.83 (1H, *d*, J=5.8Hz, H-11), 3.12 (1H, *d*, J=3.8Hz, H-13), 2.87 (1H, *d*, J=15.5Hz, H-7β), 2.83 (1H, *d*, J=3.8Hz, H-13), 2.68 (1H, *dd*, J=15.9, 7.5Hz, H-3α), 2.32 (1H, *dd*, J=15.5, 1.4Hz, H-7α), 2.01 (1H, *ddd*, J=15.9, 5.3, 3.1Hz, H-3β), 1.82 (3H, *br s*, H-16), 0.98 (3H, *d*, J=0.9Hz, H-15), 0.79 (3H, *s*, H-14).

Trichothecolone EI-MS m/z (rel.int.): 246 ($[M-H_2O]^+$, 4%), 233 (25), 221 (27), 203 (25), 187 (24), 175 (32), 161 (37), 147 (46), 133 (29), 122 (80), 107 (57), 95 (56), 79 (60), 41 (100).

Fractions 17-30° were combined and rechromatographed by CTLC (2mm plate) in $CHCl_3$-MeOH, 9:1, collecting 2ml fractions. Fractions 36-42 were further purified by CTLC (1mm plate) in ether-acetone, 9:1, collecting 1ml fractions, which yielded 9β-trichodiol (16.1mg) as colourless crystals from fractions 42-49. On TLC analysis ($CHCl_3$-MeOH, 9:1) 9β-trichodiol appeared as a brown spot (Rf=0.37) with the H_2SO_4 spray, and reacted with the PNBP/TEPA reagent producing a bright sky blue colour.

It was fully characterised by ^{1}H NMR (Table 25), ^{13}C NMR (Table 26), ^{1}H/^{1}H COSY (Fig.82), ^{1}H/^{13}C COSY (Fig.85), EI-MS and GC-MS of the di-TMS ether.

9β-Trichodiol EI-MS 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).

EI-MS di-TMS ether of 9β-trichodiol, m/z (rel.int.): 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).

Acid catalysed cyclisation of 9β-trichodiol.

9β-Trichodiol (3.5mg) in EtOAc (0.1ml) was added to 10ml of 100mM potassium phosphate buffer at pH 5.8 and incubated at 27°, 250rpm for 5 days. TLC analysis (hexane-EtOAc, 1:1) indicated the formation of a single, less polar product which was extracted with EtOAc (20ml). The extract was dried over $MgSO_4$, filtered and evaporated to an oil under reduced pressure. Preparative TLC (1 plate; 20cm x 20cm x 0.2mm) in hexane-EtOAc, 1:1, afforded EPT (2.1mg, 64%) as a viscous colourless oil which was spectrally (^{1}H NMR and EI-MS) identical to the natural material.

Isolation of 15-deacetylDHC, isotrichotriol, 8α-hydroxyITdiol and 9α- and 9β-trichodiol from *Fusarium culmorum* CMI 14764.

a) The crude toxin extract (1.2g) from a 1.6L culture of *F. culmorum* was chromatographed by CTLC (2mm plate) eluting first with hexane-EtOAc, 1:1 (30ml), and then with hexane-EtOAc-MeOH, 5:5:1 (120ml), collecting 1ml fractions*.

Fractions 78-85* were combined, and purification by preparative TLC (6 plates; 20cm x 20cm x 0.2mm) in $CHCl_3$-MeOH, 9:1, yielded 9β-trichotriol (11.3mg) as a viscous colourless oil. On TLC analysis ($CHCl_3$-MeOH, 9:1) it gave a faint yellow coloured spot (Rf=0.23) with cold H_2SO_4 spray which turned brown on heating to 140°, and reacted with the PNBP/TEPA reagent producing a bright sky blue colour. It was characterised by ^{1}H NMR (Table 25), ^{13}C NMR (Table 26), ^{1}H/^{1}H COSY

(Fig.88), $^{1}H/^{13}C$ COSY (Fig.89) and EI-MS analysis.

9β-Trichotriol 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).

Fractions 64-75* were combined and rechromatographed by CTLC (mm plate) eluting with $CHCl_3$-MeOH, 9:1, and collecting 1ml fractions. Fractions 37-42 were purified by preparative TLC (5 plates; 20cm x 20cm x 0.2mm) in $CHCl_3$-MeOH, 9:1, to yield 9β-trichotriol (7.4mg). Fractions 19-29 were purified by repeated preparative TLC (20 x 20 x 0.5mm plates) in $CHCl_3$-MeOH, 9:1, which yielded 15-deacetylDHC (24mg) as a viscous colourless oil. 15-DeacetylDHC was identified by ^{1}H NMR (Table 28) and EI-MS analysis.

15-DeacetylDHC m/z (rel.int.): 337 (9%), 319 (12), 304 (6), 289 (15), 221 (29), 134 (33), 124 (36), 120 (57), 108 (66), 99 (100), 94 (57), 81 (100).

Production of [^{14}C]-9β-trichotriol.

Portions (70µl) of a solution of sodium [2-^{4}C]acetate (9.25MBq; 1.98GBqmM^{-1}; 1.25ml) were added to 1L flasks containing *F. culmorum* cultures (4x200ml) 2, 3 and 4 days after subculture, so that each flask received a total of 1.57MBqmM^{-1}. The cultures were incubated for a total of 7 days and harvested as before by extraction of the filtrate with EtOAc (3x300ml). Work-up and purification as above yielded [^{14}C]-9β-trichotriol (7.0mg; 0.30MBqmM^{-1}) and [^{14}C]-15-deacetylDHC (6.5mg; 0.42MBqmM^{-1}).

b) Xanthotoxin inhibition.

The extract from a culture of *F. culmorum* (8.0L) which had been exposed to a xanthotoxin concentration of 0.1mM (see above) was loaded onto a silica gel column (90mm x 65mm diameter) packed in hexane. The column was eluted first with hexane (400ml) and then with EtOAc (600ml), collecting 100ml fractions. Fractions 1-4 were combined to yield TDN (2.04g) and fractions 8-10 were combined to yield a yellow oil (413mg). This was rechromatographed by CTLC (2mm plate) eluting first with $CHCl_3$-MeOH, 95:5 (70ml) and then with $CHCl_3$-MeOH, 90:10 (80ml) and $CHCl_3$-MeOH, 80:20 (70ml), collecting 2ml fractions*.

Fractions 71-78* were combined and rechromatographed by CTLC (1mm plate) in ether-acetone, 8:2, collecting 1ml fractions. Fractions 25-29 were further purified by preparative TLC (2 plates; 20cm x 20cm x 0.2mm) first in ether-acetone, 9:1, and then in CH_2Cl_2-MeOH, 9:1, which yielded 8α-hydroxyITdiol (2.9mg) as a viscous colourless oil. Similarly fractions 36-43 were chromatographed by preparative TLC (2 plates; 20cm x 20cm x 0.2mm) in ether-acetone, 9:1, to yield isotrichotriol (4.6mg) as a white solid. On TLC analysis (CH_2Cl_2-MeOH, 9:1) both compounds reacted with the PNBP/TEPA reagent producing sky blue coloured spots (Rf isotrichotriol = 0.37; Rf 8α-hydroxyITdiol = 0.40). Identification was by ^1H NMR (Table 29) and EI-MS.

Isotrichotriol, 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).

8α-HydroxyITdiol, m/z (rel.int.): 268 ([M]$^+$, 1%), 250 (3), 224 (3), 149 (14), 140 (13), 123 (30), 110 (32), 100 (100), 95 (26).

Fractions 86-END* were combined and rechromatographed by CTLC (2mm plate) in ether-acetone, 9:1, collecting 2ml fractions. Further purification of fractions 48-70 by preparative TLC (1 plate; 20cm x 20cm x 0.2mm) in CH_2Cl_2-MeOH, 95:5, yielded 9α-trichotriol (1.3mg) as a viscous colourless oil. On TLC analysis (CH_2Cl_2-MeOH, 9:1) it reacted with PNBP/TEPA producing a sky blue coloured spot (Rf=0.25). Identification was by ^{1}H NMR (Table 25) and EI-MS analysis.

9α-Trichotriol 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).

Studies in the post-ITdiol biosynthesis of trichothecenes.

Attempted cyclisation of 9β-trichotriol using a cell-free extract prepared from *F. culmorum*.

A filtered and washed mycelial mat (12g wet weight) from 2 x 200ml cultures of *F. culmorum* was suspended in 100mM potassium phosphate buffer (pH 8.0; 80ml) containing 250mM sucrose. The mixture was cooled to 0°C and liquidised for 15 sec in a blender. The fragmented mycelia were then ruptured at a peak pressure of 10-20,000 psi in a microfluidiser (Microfluidic Corp. Model 110T, Newton) precooled with ice. The homogenate was centrifuged at 2,500 x g for 15 mins at 4°C and the supernatant used immediately.

One 25ml flask containing 4.5ml of 100mM potassium phosphate buffer at pH

7.15 received 0.75ml of the cell-free extract, and to this was added [^{14}C]-9β-trichotriol (0.2mg; 0.30MBqmM^{-1}) in acetone (40µl). Extract in a second flask was boiled for 10 mins, cooled, and then [^{14}C]-9β-trichotriol as above was added. The two cultures were incubated at 28°, 250rpm for 2hrs and then extracted with EtOAc (20ml). The extracts were dried over $MgSO_4$, filtered and evaporated to dryness under reduced pressure. All of the extracts were analysed by TLC (ether-acetone, 9:1) and autoradiography (7 days exposure; Fig.92). Product formation was measured using scintillation counting procedures.

<u>Feeding experiment to compare the biotransformation of [^{14}C]-9β-trichotriol to [^{14}C]ITdiol.</u>

A filtered and washed mycelial mat from 1 x 200ml culture of *F. culmorum* (4 days old) was divided into 12 equal portions (≈0.5g wet weight). Two 50ml flasks containing 10ml H_2O each received one portion, and to these cultures were added [^{14}C]ITdiol (0.5mg; 0.63MBqmM^{-1}) in acetone (50µl) or [^{14}C]-9β-trichotriol (0.5mg; 0.30MBqmM^{-1}) in acetone (50µl). The cultures were incubated at 28°, 250rpm for 7hrs, filtered (Whatman No.4 paper) under reduced pressure and the mycelia washed with EtOAc (30ml). The filtrates were extracted with the washings, and the extracts dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The extracts were dissolved in EtOAc (40µl for the [^{14}C]-9β-trichotriol extract; 50µl for [^{14}C]ITdiol) and aliquots (20µl for [^{14}C]-9β-trichotriol; 15µl for [^{14}C]ITdiol) were analysed by TLC (hexane-EtOAc, 1:1 and ether-acetone, 9:1) and autoradiography (14 days exposure; Fig.93). Product formation was measured using scintillation counting procedures.

Competitive feeding experiments using [^{14}C]ITdiol.

a) A filtered and washed mycelial mat from 1 x 200ml culture of *F. culmorum* (4 days old) was divided into 12 equal portions (≈0.5g wet weight). 50ml Flasks containing 10ml distilled H_2O each received one portion, and to these cultures was added the substrate being tested (0.5mg) in acetone (50µl), as listed below. The cultures were incubated at 28°, 250rpm and [^{14}C]ITdiol (0.5mg; 0.63MBqmM^{-1}) in acetone (50µl) was added to each after 15 mins and the incubation continued for a further 6.75hrs. The cultures were worked-up as for the previous experiment, and the extracts were dissolved in EtOAc (50µl) and aliquots (15µl) analysed by TLC (hexane-EtOAc, 1:1, and ether-acetone, 9:1) and autoradiography (14 days exposure; Fig.94).

	Substrate	Flask/lane No.
Series A:	None	2
	9β-Trichodiol	3
	9β-Trichotriol	4
Series B:	None	5
	Isotrichotriol	6

b) A filtered and washed mycelial mat from 1 x 200ml culture of *F. culmorum* (62hrs old) was divided into 32 equal portions (≈0.1g wet weight). 25ml flasks containing 5ml distilled H_2O each received one portion, and to these cultures was added the substrate being tested (0.6mg) in acetone (50µl), as listed below. The cultures were incubated at 28°, 250rpm and [^{14}C]ITdiol (0.1mg; 0.63MBqmM^{-1}) in acetone (50µl) was added to each after 15 mins and the incubation continued for a

further 5.75hrs. The cultures were worked-up as for the previous experiment, and the extracts were dissolved in EtOAc. All the extract was analysed by TLC (hexane-EtOAc, 1:1) and autoradiography (23 days exposure; Fig.95).

Substrate	Flask/lane No.
None	2
ITdiol	3
9β-Trichodiol	4
9β-Trichotriol	5
Isotrichotriol	6

<u>Further studies in the post-cyclisation biosynthesis of trichothecenes.</u>

a) Biotransformation of 15-deacetylDHC.

The filtered and washed mycelial mat from 1 x 200ml culture of *F. culmorum* (60hrs old) was divided into 6 equal portions (~0.7g wet weight). A 50ml flask containing 10ml distilled H_2O received one portion, and [^{14}C]-15-deacetylDHC (1.0mg; 0.42MBqmM^{-1}) was added. Mycelia in a second flask was boiled for 10 mins, cooled, and then [^{14}C]-15-deacetylDHC as above was added. The two cultures were incubated at 28°, 250rpm for 4hrs, filtered under reduced pressure and the filtrates extracted with EtOAc (30ml). The extracts were dried over $MgSO_4$, evaporated to dryness under reduced pressure and redissolved in EtOAc (40µl). Aliquots (20µl) were analysed by TLC (ether-acetone, 9:1, and hexane-EtOAc, 1:1) and autoradiography (6 days exposure; Fig.99). Product formation was measured by scintillation counting procedures.

b) Biotransformation of DHC.

The filtered and washed mycelial mat from 1 x 200ml culture of *F. culmorum* (60hrs old) was divided into 6 equal portions (≈0.7g wet weight). A 50ml flask containing 10ml distilled H_2O received one portion, and [^{14}C]DHC (1.0mg; 31.0KBqmM^{-1}) was added. Mycelia in a second flask was boiled for 10 mins, cooled, and then [^{14}C]DHC as above was added. The two cultures were incubated at 28°, 250rpm for 6hrs, filtered under reduced pressure and the filtrates extracted with EtOAc (30ml). The extracts were dried over $MgSO_4$, evaporated to dryness under reduced pressure and redissolved in EtOAc (20µl). All the extract was analysed by TLC (ether-acetone, 9:1) and autoradiography (23 days exposure; Fig.100). Product formation was measured using scintillation counting procedures.

<u>Inhibition of *Fusarium culmorum* cultures using semi-synthetic derivatives of TDN.</u>

Series 1: Cultures of *F. culmorum* (25ml) received the TDN derivatives listed below as a solution in acetone (0.4ml) 43hrs after subculture. Portions (25µl; 925KBq) of an aqueous solution of sodium [2-^{14}C]acetate (1.96GBqmM^{-1}) were added to each flask 30 mins later, and the incubation was continued at 28°, 250rpm for a further 5 days.

Flask	TDN derivative	Quantity	Concentration
1	None	-	-
2	9β,10α-DihydroxyTDN	12mg	2.01mM
3	9,10-Dibromo-12,13-epoxyTDN	24mg	2.52mM

At times 5, 24, 72 and 120hrs after addition of the derivative, 1ml samples were

taken and extracted with EtOAc (3x0.5ml). The combined organic extracts were dried over $MgSO_4$, evaporated to dryness under a flow of nitrogen and redissolved in EtOAc (40µl). Aliquots (10µl) were analysed by TLC (hexane-EtOAc, 1:1) and autoradiography (4 days exposure; Fig.103). Product formation was measured using scintillation counting procedures (Tables 33 and 34).

Series 2: Cultures of *F. culmorum* (25ml) received the TDN derivatives listed below as a solution in acetone (0.4ml) 43hrs after subculture. Portions (20µl; 740KBq) of an aqueous solution of sodium [2-^{14}C]acetate (37MBq; 1.57GBqmM^{-1}; 1.0ml) were added to each flask 30 mins later, and the incubation was continued at 28°, 250rpm for a further 5 days.

Flask	TDN derivative	Quantity	Concentration
4	None	-	-
5	TDN	10mg	1.96mM
6	9β,10β-EpoxyTDN	11mg	2.00mM
7	9β,10β;12,13-DiepoxyTDN	12mg	2.03mM
8	9,10-DibromoTDN	18mg	1.97mM
9	9β-Hydroxytricho-10,12-diene	11mg	2.00mM

At times 5, 24, 72 and 120hrs after addition of the derivative, 1ml samples were taken and extracted with EtOAc (3x0.5ml). The combined organic extracts were dried over $MgSO_4$, evaporated to dryness under a flow of nitrogen and redissolved in EtOAc (50µl). Aliquots (10µl) were analysed by TLC (hexane-EtOAc, 1:1) and autoradiography (7 days exposure; Fig.103). Product formation was measured using scintillation counting procedures (Tables 33 and 34).

Appendix 1.

Czapek-Dox Agar (Oxoid preprepared).

$NaNO_3$	2.00g
KCl	0.50g
Magnesium glycerophosphate	0.50g
$FeSO_4$	0.01g
K_2SO_4	0.35g
Sucrose	30.00g
Agar No.3	12.00g

Water was added to make 1L and the agar then sterilised at 121°C for 15 mins.

Beer wort agar (Oxoid preprepared).

Malt extract	15.00g
Peptone	0.78g
Maltose	12.75g
Dextrin	2.75g
Glycerol	2.35g
K_2HPO_4	1.00g
NH_4Cl	1.00g
Agar No.1	15.00g

Water was added to make 1L and the agar then sterilised at 121°C for 15 mins.

Appendix 2.

Czapek-Dox production medium.

$NH_4H_2PO_4$	1.0g
K_2HPO_4	3.0g
$MgSO_4.7H_2O$	0.2g
NaCl	5.0g
Sucrose	40.0g
Glycerol	10.0g
Water to 1 litre	

Sterilised at 121°C for 20 mins.

Czapek-Dox seed medium.

Malt extract	2.0g
Yeast extract	2.0g
Peptone	2.0g
KH_2PO_4	2.0g
$MgSO_4.7H_2O$	2.0g
$FeSO_4.7H_2O$	0.2g
NH_4Cl	3.0g
Glucose	20.0g
Water to 1 litre	

Sterilised at 121°C for 20 mins, with glucose kept in a separate solution.

Czapek-Dox corn-steep medium.

Ammonium tartrate	2.00g
$MgSO_4.7H_2O$	0.50g
K_2HPO_4	1.00g
KCl	0.50g
$FeSO_4.7H_2O$	0.01g
$ZnSO_4$	0.10g
Corn-steep liquor	10.00g
Glucose	50.00g
Water to 1 litre	

pH was adjusted to 5.5 with NaOH (1M). Sterilised at 121°C for 20 mins, with glucose kept in a separate solution.

YEPD-5G medium.

Yeast extract	1.0g
Peptone	1.0g
Glucose	50.0g
Water to 1 litre	

Sterilised at 121°C for 20 mins, with glucose kept in a separate solution.

Appendix 3.

100mM potassium phosphate buffer.

pH 9.6:	$K_2HPO_4.3H_2O$	22.82g
	Water to 1 litre	
pH 4.7:	KH_2PO_4	13.60g
	Water to 1 litre	

Solutions mixed to produce the desired pH.

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ERRATUM

Page 38. Lines 3 and 9: For '[1,2-^{13}C]acetate' read '[1,2-$^{13}C_2$]acetate'.

Line 15: For '[3,4-^{13}C]MVA' read '[3,4-$^{13}C_2$]MVA'.

Line 23: For '[4,6-^{13}C]MVA' read '[4,6-$^{13}C_2$]MVA'.

Page 39. Fig.21A: For '[1,2-^{13}C]acetate' read '[1,2-$^{13}C_2$]acetate'.

Fig.21B: For '[3,4-^{13}C]MVA' read '[3,4-$^{13}C_2$]MVA'.

Page 40. Fig.23: For '[4,6-^{13}C]MVA' read '[4,6-$^{13}C_2$]MVA'.

Page 45. Line 19: For '[3,4-^{13}C]MVA' read '[3,4-$^{13}C_2$]MVA'.

Page 85. Line 5: For 'at m/z 267 and 269' read 'at m/z 267, 269 and 271'.

Page 85. Line 24: For 'AB quartet' read 'AB system'.

Page 132. Line 10: For 'AB quartet' read 'AB system'.

Page 133. Line 11: For 'AB quartet' read 'AB system'.

Page 179. Line 24: For 'AB quartet' read 'AB system'.

Page 217. Line 10: For '7 days' read '7 hours'.

Page 258. Line 10: For '[MH]$^-$' read '[M-H]$^-$'.

Page 259. Line 4: For '[MH]$^-$' read '[M-H]$^-$'.

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