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**ISOLATION AND CHARACTERIZATION OF SECONDARY
METABOLITES FROM FUSARIUM SPOROTRICHIOIDES DAOM
165006**

David A. Fielder, B.Sc.

A thesis submitted to the Faculty of Graduate Studies in partial
fulfillment of the requirements for the degree of

Master of Science

Department of Chemistry
Carleton University
November 1988

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The undersigned hereby recommend to the Faculty of Graduate Studies the acceptance of this thesis submitted by David A. Fielder, B.Sc. entitled:

Isolation and characterization of secondary metabolites from *Fusarium sporotrichioides* DAOM 165006

in partial fulfillment of the requirements for the degree of Master of Science.

Thesis Supervisor

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ABSTRACT

A crude fungal extract of Fusarium sporotrichioides DAOM 165006, grown in MOSS medium was found to produce two major trichothecenes; T-2 toxin (850 mg/L) and neosolaniol (400 mg/L), as well as many other minor metabolites. These included a number of novel hydroxy and acetoxy 11-epi-apotrichothec-9-enes. The 3-hydroxy epimers of 3,13-dihydroxy-11-epi-apotrichothec-9-ene have been previously detected in F. culmorum, F. crookwellense and F. roseum albeit in different ratios.

The 11-epi-apotrichothecenes and the trichothecenes are both derived from mevalonate. Trichodiene is thought to be a biosynthetic precursor of both classes of compounds. However, the trichothecenes possess a cis fused A/B ring system as opposed to the trans fused system of the 11-epi-apotrichothecenes which suggests two different oxidation and cyclization mechanisms of trichodiene must occur to account for their formation.

The isolation of a number of novel analogues of 11-epi-apotrichothecene suggest that they are a separate class of compounds involving this common biosynthetic intermediate, trichodiene.

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For my Parents,

who taught me the value of an education

ISOLATION AND CHARACTERIZATION OF SECONDARY
METABOLITES FROM FUSARIUM SPOROTRICHIOIDES DAOM

165006

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I INTRODUCTION

Mycotoxins are naturally occurring secondary metabolites produced by various genera of fungi. Since biblical times, fungi have been known to be responsible for the contamination of a wide range of agricultural crops while they are growing in the field and after they have been harvested. The contamination of feed and cereal grains by mycotoxins produced by the genus Fusarium destined for livestock and/or human consumption have caused many outbreaks of intoxication and death throughout the world. Toxigenic mold contamination is not only limited to cereal grains. Many other types of foods such as edible nuts, vegetables, dried fruits and milk products are known to be susceptible to infestation by strains of Aspergillus. It has only been in the last two decades, however, that mycotoxins have been recognized as a potential medical and economic threat [1]. As a result, much research principally in the analysis and chemistry of mycotoxins has been dedicated to the study of these naturally occurring toxins.

I-1 History

The term mycotoxicosis is defined as ' a poisoning of the host which follow the entry into the body of toxic substances of fungal origin ' [1,5]. The earliest known mycotoxicoses were associated with the genus Claviceps and are thought to be responsible for many outbreaks of illnesses over several centuries [2]. Man has been poisoned from consuming bread made from rye flour contaminated with sclerotia of Claviceps purpurea, while animals have been poisoned from feeding on grasses infected with the ergot fungus.

Since the turn of the century, fungal contamination of plants and food stuffs has resulted in various mycotoxicoses, with many clinical cases having been documented. One well known outbreak occurred between 1942 and 1947 when up to

60% of the population of Orenburg near Siberia was fatally affected after consuming overwintered cereal grains. The characteristic symptoms of this unknown ailment called 'Alimentary Toxic Aleukia' (ATA) were fever, necrotic angina, leukopenia, haemorrhaging, depression of bone marrow and death [3]. Although the ailment was first thought to be of bacterial origin, analysis of grain samples showed contamination by a number of fungal species. Toxicological studies later indicated that a number of metabolites including T-2 toxin isolated from these fungal species, could induce the clinical symptoms of ATA in experimental animals [3,4]. Other outbreaks of mycotoxicoses occurred in Europe during the 1930's and 40's, when livestock suffered from severe intoxication associated with moldy fodder and the 1950's when horses in the northern Japanese province of Hokkaido showed signs of severe mycotoxicoses leading to death. Further examples of mycotoxicoses are listed in TABLE 1 [1,4,5].

Year	Toxicosis	Country	Species	Food	Mycotoxin
1711	Ergotism	var.	<u>Cliceps purpurea</u>	rye barley	Ergot alkaloids
1890	Taumeldetreide	USSR	<u>Fusarium roseum</u> <u>Gibberella subinetti</u> <u>Cladosporium herbarum</u>	millet barley	trichothecenes
1942- 1947	Alimentary Toxic Aleukia (ATA)	USSR	<u>Fusarium sporotrichioides</u>	wheat barley millet	T-2 toxin
1952	Hyperkeratosis	USA	<u>Aspergillus clavatus</u>	millet	Patulin
1960	Turkey X	UK, USA	<u>Aspergillus flavus</u>	peanut meal	aflatoxin
1960- 1975	Bean Hull poisoning	Japan	<u>Fusarium solani</u>	bean hulls	T-2 toxin neosolaniol
1971	Moldy corn toxicosis	USA	<u>Fusarium tricinum</u>	corn	T-2 toxin

TABLE 1: Reported cases of Mycotoxicoses

I-2 CLASSES OF COMPOUNDS

The mycotoxins are a diverse group of compounds as shown in FIGURE 1. These secondary metabolites exhibit a wide range of biological properties. The aflatoxins produced by Aspergillus flavus and A. parasiticus were first discovered in the early 1960's [5]. It was the acute toxicity exhibited by this class of compounds which intensified the research on fungal toxins. Aflatoxin B, (1), the most commonly isolated compound isolated of its class, is the most potent hepatocarcinogen of natural origin known for experimental animals [4,6].

Ochratoxin A (2) belongs to another group of structurally related mycotoxins produced by other species of Aspergillus, and is known to be carcinogenic and teratogenic to mice and rats [6].

Patulin (3) is produced by species of Aspergillus and Penicillium, and is known to inhibit DNA, RNA and protein synthesis [2].

Zearalenone (4) is produced by various species of Fusarium, and causes estrogenic effects when livestock especially swine consume moldy corn [2,4].

Aflatoxin B₁ (1)

Ochratoxin A (2)

Patulin (3)

Zearalenone (4)

FIGURE 1: Examples of mycotoxins

II TRICOTHECENES

The trichothecenes mycotoxins make up the largest class of naturally occurring sesquiterpenes. They are produced by various genera of fungi imperfecti such as Trichothecium, Trichoderma, Fusarium, Myrothecium, Cephalosporium, Stachybotrys, Verticimonosporium and Cylindocarpon. Trichothecenes exhibit a wide range of biological properties, and are known to be the cause of moldy corn toxicosis of cattle and poultry and stachybotrytoxicosis [4].

The basic trichothecene structure consists of a tetracyclic skeleton (FIGURE 2). The numbering system of the ring skeleton begins at the oxygen of the tetrahydropyran ring and proceeds in a clockwise direction with the three methyl groups numbered 14, 15 and 16.

Characteristic of the trichothecenes is the double bond at the C-9, C-10 position and the epoxide at the C-12, C-13 position and consequently they are known as 12,13-epoxytrichothec-9-enes. Oxygenation can occur at positions C3, C4, C7, C8 and C15 resulting in a wide range of naturally occurring compounds.

FIGURE 2: The basic trichothecene skeleton

The trichothecenes have been classified by Tamm according to their various substitution at these positions (FIGURE 3) [4]. Group A contains a ketone functionality at C-8 and alcohols or simple esters at the other substituted positions. Group B trichothecenes possess free alcohols or simple esters at all substituted positions. Group C trichothecenes are characterized by the absence of any functional group at C-8, while Group D, which consists of only one compound, croticin, contains an epoxy group at C-7, C-8. Group E are the macrocyclic trichothecenes which contain a macrocyclic chain of di- or triesters linked at C-4 and C-15.

The first trichothecene to be isolated from Trichothecium roseum was trichothecin (6) by Freeman and Morrison in 1948 [7]. Based on traditional chemical methods of structure determination, including oxidation, hydrolysis and acid/base reactions, structure (5) was proposed. However, the structure was incorrect since a reactive oxygen was misinterpreted as being part of an oxirane ring instead of an epoxide. It was not until 1965 that the structure was correctly determined by analogy to the structure of trichodermol, when Godtfredsen et al. determined the structure of its p-bromobenzoate by X-ray crystallography [8,9].

To date, over 80 trichothecenes varying in both the degree of oxygenation in the molecule and the amount of esterification have been isolated and characterized.

(5)

(6)

GROUP A

GROUP B

GROUP C

GROUP D

GROUP E

FIGURE 3: Structural types of trichothecenes

III TOXICITY

Mycotoxins in general exhibit a wide range of biological properties including acute and chronic toxicity, carcinogenicity, cytotoxicity, immunosuppressive activity and phytotoxicity [3,6,21].

The literature reveals a plethora of trichothecene induced toxicoses. T-2 toxin is one of the most toxic trichothecenes that contaminate feed grains, such as corn and wheat. The acute symptoms for trichothecene poisoning are characterized by skin irritation, food refusal, vomiting, diarrhea, haemorrhage, neural disturbance, abortion and death [21].

Table 2 summarizes the 50% lethal doses (LD_{50}) of trichothecenes to a variety of experimental animals [3]. Verrucarin A and roridin A, two macrocyclic trichothecenes, show the highest acute toxicity, while trichothecin and trichodermol have an acute toxicity about 100 times less than that of other trichothecenes.

Type	Trichothecenes	Mouse				Newborn		Rat				Guinea pig			
		iv	ip	sc	po	sc	iv	ip	sc	po	ip	sc	po		
A	T-2 toxin		5.2		10.5	0.15				5.2			3.06		
	HT-2 toxin		9.2												
	Diacetoxyscirpenol	12	23.0			0.17	1.3	0.75		7.3					
	Neosolaniol		14.5												
	Monoacetoxyscirpenol								0.725						
	Triacetoxyscirpenol														
	Scirpenol							0.81							
	Trichodermol			500-1000											
	Trichodermin			> 500	> 1000										
B	Nivalenol		4.1						0.9						
	Fusarenon-X	3.4	3.4	4.2	4.5	0.2			0.5	4.4	0.5	0.1			
	Diacetylneovalenol		9.6												
	Deoxynivalenol		70.0		46.0										
	3-Acetyldeoxynivalenol		49.0		34.0										
	Trichothecin	< 100	< 250						< 250						
C	Crotocin	< 810	< 500	> 1000					< 100						
D	Roridin A	1.0	0.5												
	Verrucarin A	1.5	0.5					0.87							
	Verrucarin B	7.0													
	Verrucarin J		0.5												

		Rabbit		Cat	Dog		Swine	Duckling	Chick		Trout		
		iv	sc		iv	sc			iv	sc		1-day-old	7-day-old
A	T-2 toxin			0.5				1.21		1.75	4.97	4.0	6.1
	HT-2 toxin								6.25	7.22			
	Diacetoxyscirpenol	1.0			1.1		0.37			3.82	5.0		
	Neosolaniol									24.87			
	8-Acetylneosalaniol									3.22			
	3-Hydroxy HT-2 toxin								8.5				
	15-Acetyl T-2 toxin								10				
	T-2 tetraol								10				
	Fusarenon-X			< 5.0				2.0		33.79			
	Deoxynivalenol					> 0.1		27.0					
D	3-Acetyldeoxynivalenol					> 1.0		37.0					
	Verrucarin A	0.44											

TABLE 2: LD_{50} values (mg/kg) of trichothecenes

[taken from reference 3].

IV FUSARIUM SPOROTRICHIOIDES

In the past, there has been a lot of confusion with regard to the taxonomic classification of Fusarium species. As a result, there are a number of taxonomic systems presently in use throughout the world, with no one internationally recognized system currently being employed [22,23].

It has been found that in several older taxonomic systems, a number of strains had been misidentified or incorrectly used in the literature, while different isolates had been combined under the same name [23].

43 strains of F. sporotrichioides have been identified under the section Sporotrichiella, according to the system devised by Marasas [23]. The most toxic of the trichothecene metabolites, are produced by the species of the Sporotrichiella section, namely; F. sporotrichioides, F. sporotrichioides var. tricinctum and F. poae [4,22,23].

F. sporotrichioides is a soil microorganism occurring on a variety of plants, mainly cereal grains and grasses. Unlike other species of Fusarium, which proliferate in warm climates, this species thrives in cool temperate regions of the world such as Canada, the northern USA, Japan, northern Europe and the USSR. Infestation of grain by the organism usually occurs when it is left in the fields in autumn under wet conditions or overwintered under snow [23,24].

The quantity and type of trichothecenes and other secondary metabolites seem to vary between strains of F. sporotrichioides. However in many strains that have been widely studied, T-2 toxin is the most abundant and the most toxic of the trichothecenes produced [21]. Crude extracts containing T-2 toxin isolated from Fusarium cultures produce a wide range of symptoms associated with trichothecene poisoning in experimental animals [6,21]. As a powerful dermal toxin T-2 also causes localized irritation, inflammation and histopathological lesions [21].

Prior to 1980, only T-2 toxin and diacetoxyscirpenol (DAS) had been reported to have occurred naturally in Canada, but the Fusarium species that produced these toxins was not identified [2]. Recently, evidence suggested that F. sporotrichioides was the potential source although whether this was the sole species responsible is still unknown.

Since the concentration of toxic trichothecenes produced by F. sporotrichioides is fairly high, studies in a number of laboratories are being carried out to isolate and characterize the other secondary metabolites produced. The trichothecene-related compounds isolated thus far from F. sporotrichioides, numbering close to 40 can be divided into three predominant groups:

A: Trichothecenes

Most isolates of F. sporotrichioides produce various derivatives of T-2 toxin and neosolaniol which account for >90% of the toxins produced [23]. In general, this species is known to produce 3,4-oxygenated trichothecenes unlike species of F. graminearum and F. culmorum which mainly produce 3-oxygenated trichothecenes in liquid culture [24]. One exception is the isolation of nivalenol which is 3,4-oxygenated trichothecene [39].

B: Modified trichothecenes

Two modified trichothecenes; sambucinol and sambucol, which were first isolated from F. sambucinum, have also been detected in fungal extracts of F. sporotrichioides and F. culmorum [17,24]. Recent oxygenated analogues of the latter compound have been isolated from F. sporotrichioides MC 72083 [25]. The toxicity of these compounds is far less than that of T-2 toxin [18,24].

C: Trichothecene precursors

Several compounds thought to be biosynthetic precursors of the trichothecenes have been isolated from crude extracts of F. sporotrichioides. One of these compounds, trichodiol was first isolated from Trichothecium roseum [1,3]. Both trichodiol and its 3-OH derivative, trichotriol have been recently isolated from F. sporotrichioides MC 72083 [26,27]. The latter compound has been observed to cyclize in methylene chloride in the presence of traces of water to produce 3-OH trichothecene [18]. These three compounds have similar embrotoxicity to that of T-2 toxin ($LD_{50} = 55\text{ng/egg}$) [18].

V GLOBAL CONCERN

Mycotoxin contamination of food stuffs and feed grains is now a global problem producing economic losses for developed countries and health problems in developing countries that rely heavily on agriculture for their own food supply and/or for export trade. In 1985, the UN Food and Agriculture Organization estimated that some 25% of the world's food crops were ruined by mycotoxins [2,5]. Furthermore, it was also estimated that between 10 and 50 percent of the grain crops in Africa and the Far East were contaminated annually. It has also been estimated that during 1973-74, more than one million dollars was lost in the Canadian hog industry as a result of zearalenone contamination of feed grain [2,4].

VI MYCOTOXINS IN CANADA

During the period 1970-78, various samples of feed grains had been analyzed for mycotoxins [2,28]. In the autumn of 1973, domestic ducks, geese, horse and swine showed signs of T-2 toxicosis in the Peace River District of northern British Columbia and Alberta [2,4]. In 1975, there was another outbreak of mycotoxicosis in swine in the same region. Again, the symptoms were the same and consistent with T-2 toxicosis. During the same period (1970-78) in Ontario, contamination of corn by a number of mycotoxins was reported [29,30]. The most commonly detected mycotoxin was zearalenone [5].

In 1980, Fusarium mold was observed on the Ontario white winter wheat and the Quebec spring wheat crop. Analysis of random samplings indicated that the trichothecene DON (4-deoxynivalenol, vomitoxin) was present in average concentrations ranging from 0.01 -4.3 ug/g [2,4]. Levels as high as 8.5 ug/g were reported in some samples. These values were fairly significant since DON has been identified as the toxic agent responsible for feed refusal in swine at levels of 0.7-1.0 ug/g [2].

Soon after the detection of DON in the winter and spring wheat crops of 1980, it was realized that it was difficult to ascertain the nature and extent of mycotoxin contamination. There were two reasons for this. Firstly, there was a lack of quick analytical methods for detection and characterization of various toxins and secondly, there was a lack of pure standards [2].

Feeding studies of crude fungal extracts from F. graminearum to swine in the early 1980's indicated that DON was not wholly responsible for the toxicity observed in these extracts [31]. Methods were then developed for the large scale production of this trichothecene to conduct further toxicological and feeding studies. As a result, a number of other trichothecenes and secondary metabolites have been isolated from the crude fungal extract and characterized in minor amounts.

Initial tolerance levels of DON in food set in Canada by the Health Protection Branch (HPB) was 3.0 ug/kg b.w and 1.5 ug/kg b.w for adults and infants respectively for a daily intake [2]. Final products derived from uncleaned Ontario soft white winter wheat and destined for human consumption was originally set at 0.3 mg/kg in 1982. At that time, very little toxicological data was available for DON. Based on revised data and estimated losses due to processing, the limit has been increased to 2 mg/kg [2].

VII CHEMISTRY OF TRICHOTHECENES

Most trichothecenes are colourless crystalline compounds which are optically active. They are fairly stable and can be stored in the laboratory for long periods of time without deterioration. Substituents at C-3, C-4 and C-7 have a specific stereochemistry, those at C-3 and C-7 have an α orientation, while at C-4, they have a β orientation. In general, substitution at C-8 has an α configuration although two compounds have recently been isolated having a β configuration [34,39].

VIII DETECTION OF TRICHOTHECENES

A crude fungal extract contains a variety of organic compounds including lipids, pigments, sugars, and the trichothecenes which are present in fairly low concentrations (ppb-ppm).

Generally speaking, Group C trichothecenes are soluble in aprotic solvents such as CHCl_3 , EtOAc , Et_2O and CH_2Cl_2 , while Group A and B trichothecenes, which bear many polar substituents, are mainly soluble in more polar solvents. Both groups tend to be insoluble in non-polar solvents and consequently an initial

extraction with hexane can be used to remove most fats and lipids from the crude extract [3,5].

VIII-1 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) was a widely used technique for the initial screening of trichothecenes. Since they lack a chromophore, a wide range of methods have been developed to chemically form either coloured or fluorescent derivatives after the TLC plate has been sprayed with various reagents [1,3,5,10]. The sensitivity of detection of these chromogenic reagents is between 0.02-0.50 ug/spot (Table 3) [11].

Procedure	Trichothecene	Limit of detection (ug per spot)	Colour
p-Anisaldehyde (MeOH, AcOH, H ₂ SO ₄ soln)	Deoxynivalenol	0.05	Yellow
	Diacetoxyscirpenol	0.10	Purple
	T-2 toxin	0.10	Brown
	HT-2 toxin	0.20	Brown
20% H ₂ SO ₄ soln	Deoxynivalenol	0.05	Yellow
	Diacetoxyscirpenol	0.20	Purple
	T-2 toxin	0.20	Grey
	HT-2 toxin	0.50	Grey
10% AlCl ₃	Deoxynivalenol	0.10	Blue
	Nivalenol	0.10	(fluor)
	Fusarenon-X	0.10	
4-(p-Nitrobenzyl) pyridine	All trichothecenes	0.02-0.2	Blue spots
Nicotinamide/2- acetylpyridine	All trichothecenes	0.02-0.5	Light blue (fluor)

TABLE 3: Chromogenic TLC reagents for the detection of trichothecenes
[taken from reference 11]

VIII-2 Gas Chromatography (GC)

In the past, gas chromatography (GC) analysis using a flame ionization detector (FID) was routinely used for the analysis of samples containing trichothecenes. GC had inherent advantages in sensitivity in comparison to TLC where resolution may not always have been satisfactory. Most trichothecenes are sufficiently volatile to be analyzed by GC directly or in preference they may be easily derivatized to volatile derivatives (TMS ethers, HFB esters, etc.) [1,11]. Most current GC routine analytical procedures utilizes an electron capture detector (ECD) which is more sensitive than an FID. Limits of detection have been reported to be as low as 10ppb [5].

GC coupled to a mass spectrometer (GC-MS) is now the preferred method for the screening of trichothecenes. The mass spectrum obtained is a clear identity to the compound. The sample can be quantitated and the compounds characterized by their fragmentation pattern by comparison to a library of standards.

The mass spectrum in the electron ionization (EI) mode may or may not reveal the molecular ion (M^+). However, this can usually be determined by operating the MS system in a chemical ionization (CI) mode, which will produce an $M+1$ molecular ion. CI is a softer ionization method which uses a gas such as CH_4 or NH_3 .

The use of mass spectral data for the structural elucidation of trichothecenes has some limitations since the fragmentation patterns are often complex and a characteristic pattern is not always observed [3,10]. In general for trichothecenes, the fragmentation patterns in the EI spectra show losses of acyl groups, water and methyl groups. A number of interpretations of fragmentation patterns have been documented [3,12].

IX SEPARATION METHODS

Chromatographic methods such as column and thin layer chromatography have long been used for the isolation and purification of trichothecenes as well as other natural products. A number of procedures have been developed that give reliable and reproducible results for quantitative analysis [1,10]. High performance liquid chromatography (HPLC) is becoming the standard procedure for final purification of trichothecenes mainly because it is more precise and sensitive than TLC and the methodology is reproducible. Separation of trichothecenes by HPLC can be accomplished using a variety of analytical and semi-preparative columns [1]. By using standards, the concentrations of known trichothecenes may be accurately determined.

X STRUCTURAL DETERMINATION

X-1 ^1H NMR

NMR plays an increasingly important role in structural determination of trichothecenes, complementing the classical methods. Since the advent of FT NMR in the early 1970's, the spectral analysis of trichothecenes can often be obtained on milligram amounts. The availability in the last few years of increasingly higher field instruments (up to 600 MHz) and the advent of two dimensional techniques and more sophisticated software permits for very specific structural information to be obtained from a variety of 2D NMR experiments, such as $^1\text{H}/^1\text{H}$ homonuclear (COSY) and $^1\text{H}/^{13}\text{C}$ heteronuclear correlation spectra (HETCOR).

There are a number of characteristic features in the ^1H NMR spectra of trichothecenes which aid in the structural elucidation of new trichothecenes and

trichothecene-like compounds. The range of chemical shifts for all the protons in the trichothecene molecule based on approximately 30 trichothecenes is shown in FIGURE 4 [13]. The 16-methyl group being attached to an olefinic carbon appears as a slightly broadened singlet (1.65-1.89 ppm) downfield from the other methyl signals. It shows allylic coupling to H-10 and long range coupling to H-8 β , as observed in the $^1\text{H}/^1\text{H}$ correlation spectrum (COSY). The C-14 and C-15 methyl groups are resolved in the ^1H spectrum and are easily differentiated from each other by long range coupling observed in the COSY spectrum. The 15-methyl group is usually found in the range 0.74-1.0 ppm upfield of the 14-methyl signal (0.8 ppm). The 15-methyl group shows long range coupling to H-7 β which can be seen on a COSY spectrum. When the 15-methyl group is oxidized to a hydroxymethylene group, the methylene protons exhibit a characteristic AB pattern between 3.5-4.0 ppm ($J_{\text{AB}} = 12$ Hz). The signal is shifted (ca. 0.5 ppm) downfield upon acetylation. The 13-methylene protons also exhibit a characteristic AB pattern between 2.70-3.40 ppm ($J_{\text{AB}} = 3.5$ -4.5 Hz) which is characteristic of the epoxide moiety. A multiplet at 5.4-5.8 ppm is attributed to the olefinic proton at H-10 which shows coupling to H-11 ($J_{10,11} = 5.2$ Hz) and to H-16 ($J_{10,16} = 1.5$ Hz). H-11 appears as a multiplet in the chemical shift range 3.9-5.0 ppm, depending on the substitution at C-8. H-2 appears as a doublet between 3.6-3.9 ppm and shows coupling to H-3 β . When the hydroxyl group at C-3 is acetylated, the H-2 resonance appears slightly downfield at 3.72-3.75 ppm [13].

respectively [12,14].

A ^1H decoupled ^{13}C spectrum yields information regarding only the ^{13}C chemical shifts. However, when the use of other NMR techniques such as the DEPT (Distortionless Enhancement by Polarization Transfer) experiment, allow the multiplicity of the carbon (i.e. CH_3 , CH_2 , CH and quaternary groups) to be determined [15]. Depending on the pulse angles used, the intensities of the various groups can either be zero, positively or negatively maximized. In normal DEPT spectrum, CH_3 and CH resonances are observed positive and CH_2 resonances are observed negative, while quaternary carbons are absent altogether. However, a DEPT experiment need not always be performed. Normally resonances with weak intensities can usually be assigned to quaternary carbons since they lack an $^1\text{H}/^{13}\text{C}$ nOe [15].

FIGURE 5: ^{13}C NMR chemical shift assignments for trichothecenes [12,14,31].

X-3 Relative Stereochemistry

Once a tentative structure has been assigned based on the MS, ^1H and ^{13}C NMR spectra, the stereochemistry of the substitution group at the asymmetric centres must be determined. This can be difficult especially if there are a number of similar functional groups present. Analysis of the COSY spectrum can provide some information. This two dimensional NMR experiment shows through bond coupling between various protons either geminal, vicinal or long range. However, with complex spectra especially in the aliphatic region, where methylene protons exhibit an AB pattern and are often coupled to neighbouring protons, other methods are required to sort out the complex coupling patterns. One of these methods is a $^1\text{H}/^{13}\text{C}$ heteronuclear correlation spectrum (HETCOR) which correlates a carbon with its proton (s). This may provide more structural information whereupon further analysis of the COSY may then be possible. Bond angles may be estimated from molecular models, and using the Karplus theory, approximate coupling constants can be calculated which can be compared to those obtained from the spectra. The magnitude of the coupling constant between two adjacent CH bonds ($\text{H}-\text{C}-\text{C}-\text{H}$) is dependent on the dihedral angle α between the two bonds [19].

X-4 Absolute Stereochemistry

The first three dimensional structure of a trichothecene determined by X-ray crystallography was that of the p-bromobenzoate of trichodermol [8,9]. A number of macrocyclic trichothecenes have since been analyzed by X-ray diffraction [4]. Even though the absolute stereochemistry has been assumed to be the same for all newly isolated trichothecene-type compounds, the X-ray analyses reported to date for these compounds in fact provides only the relative stereochemistry

[17,18].

Standard crystallographic studies of a single crystal will yield the molecular formula and the three dimensional structure from several measured intensities. However, in the case of an optically active compound which crystallizes in a lattice devoid of mirror symmetry, there is an ambiguity with regards to which of two possible enantiomorphs actually represent the 3D structure that has a particular chiral property (eg. optical rotation) [20]. It is possible to determine the absolute stereochemistry by X-ray crystallography based on a concept known as anomalous dispersion, or anomalous scattering.

By comparing the intensities of pairs of Friedel reflections, it is possible to determine which enantiomer exists. When no anomalous dispersion is observed, the intensities are identical, but when an anomalous dispersion is observed, the intensities are different. In general, an anomalous dispersion will be larger for atoms with an atomic mass (Z) near to that of the element producing the X-ray incident radiation. Oxygen having a Z value closer to copper than carbon, will show a larger anomalous dispersion and therefore in the case of trichothecenes, it is possible to determine the absolute configuration using Cu $K\alpha$ radiation. However, it is not possible to determine the absolute configuration from a source such as Mo $K\alpha$, where the intensities diffracted from a light atom compound (containing only carbon, oxygen and hydrogen) such as a trichothecene are almost negligible [19].

XI: SCOPE OF THIS RESEARCH WORK

In 1977, a number of race horses died in Saskatchewan from eating feed contaminated with F. sporotrichioides DAOM 165006 [11]. The ability of this species to produce such large quantities of T-2 toxin and, in general, more toxic trichothecenes than those produced by F. graminearum or F. roseum has lead to similar studies of this isolate as relatively little was known about other secondary metabolites produced by F. sporotrichioides. At the present time, acceptable limits need to be established for feed grains contaminated by F. sporotrichioides.

It is the aim of this research work to isolate and characterize these unknown compounds especially the ones present in minor amounts.

XII DISCUSSION

XII-1: Introduction

The study of minor secondary metabolites from F. sporotrichioides required the optimization of fermentation conditions for the large-scale production of T-2 toxin. The Canadian isolate (DAOM 165006) was obtained from feed in Saskatchewan which induced severe mycotoxicoses in horses. A large-scale fermentation (250L) in liquid culture (FIGURE 6) was carried out at the Atlantic Research Laboratories of NRC in Halifax, Nova Scotia, using parameters developed by CBRI

After the fermentation was complete, the aqueous broth was adjusted to pH 8.3 before being poured through a filter bed containing Celite and Hyflo. Extraction of the aqueous medium with CH_2Cl_2 removed the secondary metabolites and, after evaporation of the organic solvent, about 120g of crude fungal extract was obtained. The extract which contained solid material was taken up in CH_2Cl_2 and filtered, leaving behind a white precipitate (ca. 4g). Initial toxicity tests revealed it to be very toxic in nature. The sample was set aside pending further analysis.

Half of the crude fungal extract (50g) was chromatographed on silica gel. In order to determine the optimum conditions for separation of the crude extract, 3 runs (labelled A,B,C) were carried out and monitored by GC/ITD (ion trap detector). Due to the lack of sensitivity and resolution of TLC, this method of analysis was not found to be useful since, in each sample, many unknown compounds were present had similar R_f values.

Some 150 fractions were collected and analyzed by GC/ITD and then combined to give 24 fractions. The weights of these samples ranged from 1mg to 3500mg.

FIGURE 6:

FERMENTATION PROCEDURE OF
F. SPOROTRICHIOIDES DAOM 165006

The procedure for the workup of these samples is outlined below (FIGURE 7).

FIGURE 7: Separation of the crude fungal extract

Fractions with weights >500mg were chromatographed on Silica gel columns. Fractions between 100-500mg and containing only a small number of compounds were subjected to Vacuum Liquid Chromatography (VLC).

VLC is a rarely used chromatographic method of separation, and although its origins are not certain, it is thought to have been developed because of the impatience of Australian chemists with classical column chromatography [32,33].

This method of separation proved to be fast and effective as an intermediate cleanup procedure and was preferred over column chromatography in many instances. A sintered glass filter is packed under vacuum with TLC grade Silica gel (usually 100:1 adsorbent: sample). The filter is mounted on an adaptor connected to

a separatory funnel. The sample is applied to the silica gel and an aliquot of solvent (1/2 column volume was found to be optimum) is added to the top of the column. Vacuum (water aspirator) is then applied and the eluant is collected in the separatory funnel. The vacuum remains applied until the silica is nearly dry at which time the vacuum is disconnected and a sample flask is filled with the aliquot of eluant. The procedure is then repeated. Solvent systems were determined by TLC with an optimum $R_f=0.3$ being achieved. It was determined that 50% of the polarity of the TLC solvent system gave ideal separation for VLC.

For fractions which were <100mg, semi-preparative HPLC was used using a Nitrile column, and a UV detector.

A Reconstructed Ion Chromatograph (RIC) trace of the underivatized crude fungal extract from F. sporotrichioides DAOM 165006 is shown in FIGURE 8. The major compound detected was T-2 toxin (160mg/L) followed by neosolaniol (85 mg/L) and sambucinol (24 mg/L). A number of other metabolites were also detected including; 3,13-dihydroxy-11-epi-apotrichothec-9-ene, diacetoxyscirpenol (DAS) and analogues of neosolaniol. Peaks labelled SP denote unknown compounds detected and/or isolated.

The concentrations of the major metabolites (T-2, neosolaniol and sambucinol) are not representative of the optimal growth conditions for the production of T-2 toxin. Typical yields from this isolate of T-2 and neosolaniol are 850 mg/L and 400 mg/L, respectively [24]. The reason for the five fold decrease in concentration was due to a computer failure controlling this fermentation run, which shut down the fermentor after 72 hours. Maximum yields of T-2 toxin however, have been found to occur at 96-120 hours, after which time T-2 rapidly degrades, mainly to HT-2 [24].

FUSARIUM SPOROTRICHIOIDES (DAOM 165006)

RECONSTRUCTED ION CHROMATOGRAM (RIC)
OF THE CRUDE FUNGAL EXTRACT

XII-2A: STRUCTURE CHARACTERIZATION OF T-2 (7)

The major trichothecene metabolite isolated from the crude fungal extract of *F. sporotrichioides* DAOM 165006 was T-2 toxin (7). In several fractions, pure T-2 toxin crystallized out of solution. From 118g of crude extract, some 45g of T-2 toxin was isolated.

(7)

T-2 toxin serves as a good model for a complete NMR analysis from which the structure of other unknown trichothecenes from this isolate may be determined. FIGURE 9 shows a 250 MHz ^1H NMR spectrum of T-2 toxin.

The three resonances between 5 and 6 ppm are in the chemical shift range for protons attached to carbons bearing an oxygen functionality. The furthest downfield resonance (H-10) appears as a broad doublet showing allylic coupling to H-11 ($J_{10,11} = 5.9$ Hz) characteristic of most trichothecenes. The H-8 β proton also appears as a broad doublet ($J_{8,7\alpha} = 5.7$ Hz) with fine splitting due to coupling to H-7 α .

In T-2 and most naturally occurring trichothecenes, oxygenation at H-8 is in the α configuration. However, the first example of an 8 β derivative was recently isolated from *F. sporotrichioides* MC-72083 [35].

The H-4 α resonance at 5.28 ppm shows coupling to H-3 β and to H-2 α . Substitution at C-4 occurs characteristically in the β configuration in most trichothecenes. In general, acetylation of the 4-OH results in a chemical shift range for H-4 of 5.0 to 5.4 ppm (FIGURE 4, pg 18). The H-11 resonance appears as a broad doublet at 4.32 ppm. In all trichothecenes, H-11 is in the α configuration producing a cis A/B ring junction. The methylene protons of H-15 exhibit an AB pattern, showing long range coupling to H-7 β . H-16 appears characteristically as a broad singlet.

The AB system of H-7 is found in the aliphatic region between 1.65-2.0 ppm. In most cases, one half of the AB system (H-7 α) is clearly defined, from which the geminal coupling can be determined. However, the H-7 β proton always couples to H-15 and H-8 β and usually the coupling constants are unresolved. H-2 appears as a singlet at 3.68 ppm and in the chemical shift range 3.6-3.9 ppm for trichothecenes in general.

The chemical shifts of the protons of the isovaleryl group were assigned from a $^1\text{H}/^{13}\text{C}$ heteronuclear correlation spectrum (HETCOR).

The ^{13}C NMR spectrum of T-2 toxin shows twenty-seven (27) resonances. These were assigned in part by ^{13}C labelled studies, and by a number of NMR experiments. Three resonances between 170-180 ppm are assigned to the two acetoxy groups at C-4 and C-15, while the third resonance is attributed to the carbonyl of the isovaleryl group at C-8. The two resonances at 123.8 and 136.3 ppm are assigned to the C-10 and C-9 olefinic carbons respectively, and have a chemical shift characteristic of all trichothecenes. The five resonances between 60-90 ppm are indicative of carbon atoms bearing oxygen functionalities. The rest of the resonances found in the aliphatic region have been assigned by NMR experiments such as DEPT and HETCOR. The ^1H and ^{13}C NMR chemical shifts for T-2 are shown in TABLES 4 (pg 18) and 5 (pg 19) respectively.

TABLE 4: 250 MHz ¹H NMR chemical shift assignments for T-2 analogues

Proton	T-2 (7)	3'-OH-T-2 (8)	4'-deoxy-T-2 (9)
2	3.68	3.67	3.43
3	4.14	4.14	4.34
4	5.28	5.27	2.05-2.25
7	1.89,2.31	1.90,2.40	1.96-2.30
8	5.26	5.26	5.70
10	5.78	5.79	5.73
11	4.32	4.35	4.24
13	2.78,3.04	2.78,3.04	2.81,3.02
14	0.79	0.78	0.74
15	4.03,4.27	4.04,4.26	3.91,4.15
16	1.73	1.73	1.69
2'	2.12	2.42	2.21
3'	2.00	----	2.05-2.30
4'	0.95	1.27	0.91
5'	0.94	1.27	0.89
CH ₃ Ac (15)	2.01	2.01	2.01
CH ₃ Ac (4)	2.12	2.12	----

TABLE 5: 62.8 MHz ^{13}C NMR Chemical shift assignments for T-2 analogues

Carbon	T-2 (7)	3'-OH-T-2 (8)	4-deoxy-T-2 (9)
2	78.7	78.7	79.6
3	78.2	78.3	68.9
4	84.2	84.5	42.0
5	48.3	48.4	45.5
6	42.9	42.9	41.7
7	27.7	27.7	27.1
8	68.0	68.5	68.5
9	136.3	135.8	136.0
10	123.8	124.3	124.3
11	67.3	67.2	67.6
12	64.2	64.2	65.3
13	47.0	47.1	46.4
14	6.6	6.7	12.0
15	64.5	64.6	64.6
16	20.1	20.1	20.2
1'	172.7	172.8	172.8
2'	43.4	46.6	43.4
3'	25.6	69.1	25.6
4'	21.9	28.9	22.3
5'	22.7	29.4	22.3
C=O(Ac)	20.8	20.8/20.9	20.8
CH ₃ (Ac)	170.3/172.5	170.3/172.6	170.3

XII-2B: STRUCTURE DETERMINATION OF 3'-HYDROXY T-2 (8)

A molecular ion m/z 482 and a similar EI fragmentation pattern to that of T-2 (MW 466) indicated a T-2 analogue with possibly an additional hydroxyl group had been isolated. The ^1H NMR spectrum indicated the chemical shifts of the protons in the A and C rings to be virtually identical to those of T-2. The major structural difference was determined to be in the isovaleryl moiety where the chemical shift of the methylene protons of H-2' were found downfield at 2.42 ppm in comparison to T-2 (2.12 ppm). This suggested that the C-3' was the site of oxygenation. Both methyl groups of the isovaleryl group had also been shifted downfield by 0.3 ppm, which confirmed C-3' as the site of the alcohol moiety. The ^{13}C chemical shift of C-3' had shifted downfield from 25.6 ppm for T-2 to 69.1 ppm, indicative of a carbon bearing an oxygen atom (TABLE 5, pg. 32).

All NMR and mass spectral data are in agreement with the structural data of 3'-hydroxy T-2 (8) previously obtained from this isolate and from *F. sporotrichioides* P-11 and DACM 165006 [26,24]. The ^1H and ^{13}C NMR chemical shifts and coupling constants are shown in TABLE 4 and 5 respectively.

(8)

XII-2C: STRUCTURE DETERMINATION OF 4-DEACETOXY-T-2 (9)

GC analysis of Fraction 21 (1381 mg) revealed many compounds. Cleanup of this fraction involved the use of various types of chromatography including vacuum liquid chromatography and HPLC. One of the compounds isolated which was an unknown and was obtained in sufficient quantity for NMR analysis.

The CI mass spectrum confirmed the molecular ion of m/z 324 from the EI mass spectrum. The EI fragmentation pattern showed some similarity to T-2 (molecular formula $C_{24}H_{34}O_9$), suggesting the molecular formula to be $C_{22}H_{32}O_8$.

The 1H NMR of this compound was almost identical to that of T-2, with all chemical shifts of the trichothecene skeleton differing by ca. 0.5 Hz (FIGURE 10). Most resonances in the ^{13}C NMR spectrum were assigned by comparing chemical shifts of T-2 and its analogues. The resonance at ca. 84-85 ppm, which is usually attributed to an acetoxy substituent at C-4 in T-2 was absent and a resonance at 42.0 ppm was observed. This chemical shift is indicative of a methylene group with a neighbouring carbon atom bearing an oxygen functionality. For both carbons, C-3 and C-4, chemical shifts were observed upfield of those of T-2, the most significant difference being the C-3 chemical shift at 68.9 ppm. The C-14 methyl group was observed at 12.0 ppm, a downfield shift from 6.5 ppm observed in T-2 analogues.

Based on the GC/MS and NMR data, the proposed structure of this compound is 4-deacetoxy-T-2 (9). Further evidence supporting this assigned structure is the comparison of NMR data to sporotrichiol (4-deoxy,15-deacetyl-T-2), recently isolated from *F. sporotrichioides* MC72083 [34]. The chemical shifts assignments for C-3, C-4, C-6 and C-14 are in agreement with those found for the proposed structure.

4-DEACETOXY T-2 TOXIN

(9)

**XII-2D: STRUCTURE DETERMINATION OF
4,15-DIACETYL-7-DEOXYNIVALENOL (10)**

4,15-diacetyl-7-deoxynivalenol (10) was found in Fraction 14, which contained mostly neosolaniol and a number of unknowns.

The EI mass spectrum revealed a weak molecular ion M^+ 380, which suggested the molecular formula $C_{19}H_{24}O_6$. Loss of two fragments of mass unit 60 each suggested a diacetate compound.

The 1H NMR spectrum showed a number of diagnostic resonances which suggested that the compound was a 8-keto trichothecene. One of the resonances attributed to H-7 at 2.85 ppm exhibited an AB pattern showing long-range coupling to the 15-methyl group in the COSY spectrum. The coupling pattern is usually more complex if an H-8 α is present. The absence of a CH_3 singlet at ca. 1.0 ppm and the appearance of an AB pattern centered at 4.14 ppm was indicative of a acetoxy moiety at C-15. This left one acetoxy and one hydroxyl group to be accounted for.

In comparison to other substituted 8-keto trichothecenes, the doublet at 5.06 ppm was attributed to H-4. Its downfield chemical shift suggested an acetate substituent was present at C-4. This left C-3 as the only other possible position for substitution. H-3 was observed as a broad multiplet at 4.21 ppm. Upon acetylation, the resonance was observed to move downfield by 0.5 ppm, thus confirming the position of substitution. The rest of the protons were assigned in comparison to other 8-keto trichothecenes.

The ^{13}C NMR spectrum verified that an 8-keto trichothecene had been isolated. The resonance at 198 ppm, indicative of a carbonyl carbon was assigned to C-8. Six resonances in the region 60- 90 ppm were assigned to oxygen-bearing carbons, again in comparison to compiled ^{13}C NMR data, as well as the rest of the resonances in the aliphatic region of the spectrum [1,3,14].

Based on MS, ^1H and ^{13}C NMR data, the proposed structure of this compound is 4,15-diacetyl-7-deoxynivalenol (**10**). This compound has also recently been isolated from *F. crookwellense* [36]

(10)

XII-2E: ISOLATION OF THE TWO 3-OH EPIMERS OF 3,13-DIHYDROXY-11-EPI-APOTRICHOTHEC-9-ENE (11,12)

3,13-dihydroxy-11-epi-apotrichothec-9-ene¹ was first isolated from F. graminearum in 1985 and was the second modified trichothecene after sambucoidin with the H-11 proton in the β - configuration, producing the trans A/B ring junction [35]. Analysis of the ¹H NMR spectrum indicated more than one compound was present, even though only one peak was visible by capillary GC. By changing from a non-polar DB-5 column to a more polar DX-4 column, the sample was resolved into two isomers in a 3:1 ratio. From X-ray diffraction data, these two compounds (11,12) were found to be epimeric at C-3 [35].

Both of these epimers were detected in the crude fungal extract of F. sporotrichioides in a 3:1 ratio (α : β), but have also been detected in other ratios in F. crookwellense and F. culmorum [35,28,37].

A method was found to resolve these epimers by HPLC using a Nitrile column. Using a 6% isopropanol/hexane solvent system, there was a 10 minute difference in elution time between the α and the β epimers (6.5 and 16.5 mins. respectively).

1: 'Apotrichothecene' was the name given to the rearranged products produced by acid hydrolysis of a number of trichothecenes [14]. A structurally similar compound to the 'apotrichothecene' structure, but having a different configuration at C-11 was first isolated from Fusarium roseum, hence the name 11-epi-apotrichothecene [35].

(11 R₁ = H, R₂ = OH)

(12 R₁ = OH, R₂ = H)

STRUCTURAL DETERMINATION OF THE 3-OH EPIMERS OF 3,13-DIHYDROXY-11-EPI-APOTRICHOTHEC-9-ENE (11,12)

The mass spectra of both epimers have virtually the same EI fragmentation pattern. They differ only in the intensity of the major ions, m/z 91, 124, 224, 237 and 250 (M^+) [38], confirming they are epimers.

The most striking feature of the two ^1H NMR spectra of the 3-hydroxy epimers is that although the two compounds differ only at the C-3 asymmetric centre, subtle conformational differences of each molecule have quite an effect on the chemical shifts (FIGURE 11, TABLE 7).

As in the trichothecenes, a resonance at 5.5 ppm is attributed to the olefinic proton H-10, but unlike the trichothecenes appears as a broad singlet. H-10 shows long range coupling to H-11, H-16 and H-8 β in the COSY spectrum. Unlike the characteristic $J(10,11)$ coupling constant observed for the trichothecenes (approx. 5.3 Hz), the coupling constant for the epimers is on the order of 2.3 Hz.

3 β ,13-DIHYDROXY-11-EPI-APOTRICHOTHEC-9-ENE PPM FROM (CH₃)₄ Si

The configuration of H-11 was determined by a number of nOe experiments. Irradiation of the 14-methyl group shows a strong nOe enhancement to H-11, H-13 and a small nOe to H-4 β in nOe difference spectra of both two epimers, thus confirming the β -configuration of H-11. The chemical shifts of H-13 protons differ for each epimer by 0.5 ppm possibly due to the 3 α -OH epimer hydrogen bonding to the hydroxyl group at C13. The chemical shift of the C-3 methine proton of the 3 β -OH epimer is observed further downfield at 4.51 ppm than that of the 3 α -OH epimer at 4.28 ppm. Consequently, the methylene protons of H-2 and H-4 of each epimer show slight differences in chemical shifts.

Irradiation of the H-15 methyl group reveals small positive nOe's to H-4 α , H-2 α and H-3 α in the 3 β -OH epimer only. The configuration of the hydroxyl group of the β - epimer was confirmed by irradiating H-3 α and observing a positive nOe to H-15. The chemical shifts of 14 and 15 methyl groups of the two epimers are also considerably different (TABLE 6, pg. 50). In both molecules, H-15 shows long range coupling to H-7 β , which is observed in the COSY spectrum.

The ^{13}C NMR spectra of the two epimers also show subtle differences in chemical shifts notably for C3, C12, C15 and C16 (TABLE 7, pg 51).

The absolute stereochemistry of the 3 α -OH epimer (11) was determined by X-Ray diffraction analysis. By comparing the experimental with the theoretical structure factor difference values the absolute configuration was determined [20].

XII-2F STRUCTURE DETERMINATION OF 13-HYDROXY, 3 α ,11-EPOXY- APOTRICHOTHEC-9-ENE (13)

Compound (13) was first detected in the crude extracts of *F. graminearum* but wasn't isolated in sufficient quantity for a full structural determination. Compound (11) (M^+ 252), also isolated from this strain, was known to degrade when left in the presence of ethyl acetate at room temperature, producing compound (13) as one of the major components.

Compound (13) was also present in trace amounts in the crude extract of *F. sporotrichioides* DAOM 165006 [24]. Since ethyl acetate was not used in the workup procedure at any stage of the isolation, it is possible that compound (13) is in fact a naturally occurring compound and not an artifact. It was also not observed in degradation experiments involving CHCl_3 or CH_2Cl_2 . After a number of chromatographic techniques were applied to Fraction 16, compound (13) was isolated and recrystallized from acetone/hexane to yield colourless plate-like crystals (mp. 90-92 °C). The EI spectrum revealed a molecular ion of m/z 250 which was confirmed by CI high resolution mass spectroscopy. The knowledge that 3 α ,13-dihydroxy-11-epi-apotrichothec-9-ene (11) rearranged rapidly ($t_{1/2}$ = 9 days) to compound (13) with a loss of 2 mass units suggested that the '11-epi-apotrichothecene' skeleton was still intact. The lack of an AB resonance at ca. 3.0 ppm in the ^1H NMR spectrum clearly indicated that the compound was not an epoxytrichothecene (FIGURE 12). A singlet at 4.51 ppm, previously attributed to H-11 in 3 α ,13-dihydroxy-11-epi-apotrichothec-9-ene (11), was also missing. The lack of a ketone peak in the IR suggested a possible ether bond had been formed

Upon acetylation, a mono derivative was obtained, resulting in a downfield shift of the H-13 AB system to 4.23 ppm. The doublet of doublets at 4.51 ppm attributed to H-3 had not changed in chemical shift in comparison to (11). The chemical shifts of the methyl groups had altered slightly but none were oxidized and

[illegible]

were in the expected chemical shift ranges.

Most of the resonances in the ^{13}C NMR spectrum could be assigned by comparison to the ^{13}C NMR spectrum of (11) (TABLE 7, pg. 51). The lack of a resonance for the C-11 carbon bearing a hydrogen atom at 81.4 ppm in (11) and a new resonance at 105.5 ppm suggested a ketal moiety at C-11 similar to the chemical shift observed for C-11 in sambucinol (17) (108.3 ppm).

Based on the MS, ^1H and ^{13}C NMR spectral data, the proposed structure for this compound is (13). The absolute stereochemistry of 13-hydroxy,3 α ,11-epoxy-apotrichothec-9-ene (13) was confirmed by X-Ray diffraction analysis (see Appendix 5).

(13)

**XII-2G STRUCTURE DETERMINATION OF 8-HYDROXY,2,13- DIACETOXY-11-
EPI-APOTRICHOTHEC-9-ENE (14)**

Fraction 14 yielded one major compound and a number of minor ones. The CI mass spectrum of the major compound revealed a molecular ion m/z 352, indicating a molecular formula $C_{19}H_{28}O_6$. Loss of 2 fragments of mass 60 in the EI spectrum was indicative of the molecule having two acetate groups. A single resonance at 2.01 ppm in the 1H NMR spectrum integrating for 6 protons confirmed the presence of these acetoxymethyl functional groups.

The 1H NMR spectrum of compound (14) showed some similarities to that of the 3-OH epimers of compound (11,12) (FIGURE 13). The broad resonance at 5.68 ppm was attributed to H-10 which shows long-range coupling to H-16 (1.1Hz). Long range coupling to H-16 and also to the resonance at 4.05 ppm (assigned to H-11) were observed in the $^1H/^1H$ homonuclear correlation spectrum (COSY). The AB pattern centered at 4.09 ppm ($J_{AB} = 11.8$ Hz) was attributed to the H-13 methylene protons with an acetoxymethyl moiety attached to the C-13 carbon. Hydroxyl groups at C-13 normally lead to an AB pattern upfield at about 3.5 ppm. The acetate group at C-13 was confirmed when acetylation of compound (14) yielded no downfield shift.

The resonances of the three methyl groups could be easily distinguished similar to those of the trichothecenes. The 16-methyl resonance appeared as a broad singlet at 1.77 ppm showing long range coupling to H-8 β and H-10, which were observed in the COSY spectrum. The 14 and 15-methyl groups were easily distinguished since the 15-methyl resonance was further downfield from 14-methyl at 1.06 ppm, and showed long range coupling to H-7 β .

Since the location of one acetoxymethyl group was shown at C-13, this meant that one hydroxyl and one acetoxymethyl moiety still had to be accounted for. Their location as well those of the other protons, were partially resolved upon further interpretation of

8-HYDROXY,2,13-DIACETOXY,11-EPI-APOTRICHOTHEC-9-ENE

the COSY spectrum and the $^1\text{H}/^{13}\text{C}$ heteronuclear correlation spectrum (HETCOR). The broad resonance at 4.08 ppm overlapping with H-11 (4.05 ppm) was indicative of a methine proton on a carbon bearing an oxygen atom. This proton coupled to one half of an AB system attributed to H-7 α . Thus the resonance at 4.08 ppm was attributed to H-8. Upon acetylation of compound (14), a broad doublet was observed downfield 1.2 ppm verifying that an hydroxyl group was originally present at the C-8 position. Coupling of this proton with H-7 β ($J = 7.5$ Hz) indicates the 8-OH has an axial (α) configuration, the J value found was similar to $J_{7\beta,8\alpha}$ in the trichothecenes. If substitution had occurred at H-8 β , then the $J_{8\alpha,7\beta}$ would be of the order of 1 Hz. The relative stereochemistry was also verified by nOe experiments. When the olefinic proton at H-10 was irradiated, an enhancement of the H-8 β was observed.

To date, three compounds have been isolated bearing a C-8 β substituent, namely 8 β -hydroxytrichothecene, 8 β -hydroxysambucoidin and 8 β -OH-calonecristin. The first two compounds were recently isolated from *F. sporotrichioides* MC 72083 [34], while the last compound was isolated from *F. culmorum* [39].

A resonance at 5.07 ppm appeared as a triplet. This resonance is indicative of a carbon bearing an oxygen functionality. The carbon in fact must bear an acetoxy group since one of these groups still was unaccounted for. The only possible location for this group was either the C-2 or C-4 positions. C-3 was eliminated since H-3 would have to show coupling to two AB systems at C-2 and C-4, as in (11). Instead, this resonance showed coupling to the protons of only one AB system in the COSY spectrum.

In an effort to determine the location and relative stereochemistry of the acetoxy moiety, a number of nOe difference experiments were performed (FIGURE 14). Upon irradiating the C-14 methyl group, a large increase in intensity was observed for H-11 confirming the trans A/B ring junction similar to that of the 3,13-dihydroxy-11-*epi*-apotrichothec-9-enes (11,12). An enhancement was observed to both methylene protons of C-13 as well as two smaller enhancements for the resonances at 1.24 and 1.95 ppm which were attributed to H-4 β and H-7 β . These were resolved

FIGURE 14: NOE DIFFERENCE SPECTRUM OF 8-HYDROXY-2,13-DIACETOXY-11-EPI-APOTRICONOTHEC-ENE (14)

into a doublet of triplets and a quartet, respectively. The irradiation of the C-15 methyl group revealed a positive enhancement of H-10 and H-7 α . A positive enhancement was observed at 2.17 ppm revealing a pentet assigned to H-4 α . A resonance at 1.85 ppm revealing a doublet of quartets was assigned to H-3 α . Upon irradiation of the triplet at 5.10 ppm a small enhancement was observed to one half of the C-13 AB system revealing that the acetoxy moiety must be on the α -face (pseudo-axial) of the molecule. A positive enhancement was also observed to both protons of an AB system at 2.07 and 1.85 ppm respectively, confirming the location of the acetoxy group.

TABLE 6:

250 MHz ^1H NMR chemical shift assignments of 3α and 3β hydroxy epimers of 3,13-dihydroxy-11-epi-apotrichothec-9-ene (11,12) and 13-hydroxy- 3α ,11-epoxy-apotrichothec-9-ene (13).

POSITION	(11)	(12)	(13)
2α	1.74	2.23	2.13
2β	2.60	2.10	1.44
3	4.28	4.51	4.51
4α	2.13	2.45	2.46
4β	1.67	1.38	1.16
7α	1.37	1.36	1.34
7β	1.54	1.57	1.86
8	1.93 2.03	1.99m	1.80 2.03
10	5.52	5.50	5.35
11	4.15	4.11	-----
13	3.18 3.73	3.56 3.78	3.66 3.79
14	0.92	1.07	1.04
15	0.99	0.52	0.90
16	1.63	1.63	1.67

TABLE 7:

**62.8 MHz ^{13}C NMR chemical shift assignments of $3\alpha\text{-OH}$,
 $3\beta\text{-OH}$,13-dihydroxy-11-*epi*-apotriconthec-9-ene (11,12)
and 13-OH, 3α ,11-epoxy-apotriconthec-9-ene (13).**

POSITION	(11)	(12)	(13)
C-2 ^a	43.5	44.4	42.2
C-3	72.8	73.4	76.3
C-4 ^a	44.5	45.7	42.7
C-5	52.6	54.9	50.0
C-6	44.7	45.1	47.1
C-7	29.6	29.1	28.3 ^b
C-8	27.7	27.4	28.2 ^b
C-9	135.5	135.2	141.7
C-10	121.4	121.1	119.5
C-11	81.4	81.1	105.5
C-12	92.6	95.1	88.7
C-13	63.4	65.0	65.3
C-14	19.4	20.0	14.7
C-15	17.8	16.2	13.9
C-16	22.6	22.5	22.9

^{a,b}: assignments may be reversed

From the results of the nOe difference experiments, it could be concluded that the acetoxy group was at the C-2 α position. The GC/MS, ^1H and ^{13}C NMR data for compound (14) are consistent with the structure 8 α -hydroxy,2 α ,13-diacetoxy-11-epi-apotrichothec-9-ene (14).

(14)

XII-2H: STRUCTURAL DETERMINATION OF 2-ACETOXY,8,13-DIHYDROXY-11-EPI-APOTRICHOTHEC-9-ENE (15)

Fraction 16 containing 6 compounds. One of the unknown compounds was isolated by HPLC.

A molecular ion of m/z 310 revealed by CI/MS, which was confirmed by high resolution mass spectroscopy giving a molecular formula $\text{C}_{17}\text{H}_{26}\text{O}_6$ (310.178 found, 310.178 calc.). The fragmentation pattern in the EI mass spectrum showed many similarities to that of compound (13), primarily in having the same major ions. The IR spectrum showed a broad alcohol peak (3200-3450 cm^{-1}) and a carbonyl peak at 1732 cm^{-1} . Upon acetylation, a mass increase of 84 was observed in the MS, indicating the presence of two alcohol groups in the original compound.

The ^1H NMR spectrum of the acetylation product of compound (15) was identical to the that of the acetylation product (16) of (14). Similar to compound (14), the resonance at 4.09 ppm attributed to H-11 was partially obscured by a broad multiplet. Upon acetylation, a broad singlet was revealed for H-11, while the multiplet shifted downfield 0.5 ppm and appeared as a broad doublet. The doublet, integrating for one proton, was coupled to one of the protons of the H-7 AB system in the COSY spectrum, and showed long range coupling to H-16. The chemical shifts for H-7 β and H-8 could not be resolved because they overlapped with H-3, H-16 and the acetate resonances. The AB pattern centred at 3.5 ppm was attributed to H-13. Upon acetylation, the resonance shifted downfield to 4.0 ppm, revealing a free hydroxyl group at C-13.

The ^{13}C chemical shifts were assigned by comparison to compound (14) and the acetylated product of compound (14) (TABLE 9, pg. 57). The C-13 resonance was observed at 62.8 ppm, a upfield shift of 2 ppm from the C-13 resonance of compound (14) (64.4 ppm). A β -effect was also observed at C-12, with a downfield shift of 3 ppm to 95.0 ppm.

Compound (15) was isolated in trace amounts prior to the isolation of compound (14). The extensive NMR analysis of compound (14) enabled the structure of compound (15) to be assigned.

(15)

XII-21: STRUCTURE DETERMINATION OF 13-HYDROXY,2,15-DIACETOXY-11-EPI-APOTRICHOTHEC-9-ENE (17)

Fraction 20 (3564mg) consisted predominantly of T-2 toxin but also contained a few unknown compounds. The CI mass spectrum of one of these compounds (17) confirmed the molecular ion of M^+ 352 from the EI mass spectrum, suggesting a molecular formula $C_{18}H_{26}O_3$.

The absence of an AB system around 3.0 ppm suggests this compound was not a trichothecene. Further inspection of the 1H NMR spectrum suggested that an 11-epi-apotrichothecene structure for the compound had been isolated, but with a different oxygenation pattern than that of the previously isolated compounds.

A triplet at 5.18 ppm is indicative of an acetate at the C-2 or C-4 position, however in comparison to the chemical shift of H-2 in compound (14), it would tend to suggest that substitution is at the C-2 position. Substitution at C-3 would produce a multiplet showing coupling to H-2 and H-4 methylene protons. The absence of the H-15 resonance at 1.0 ppm and the appearance of 2 AB systems between 3-4 ppm indicates C-15 has an oxygen functionality. Since both H-13 and H-15 exhibit AB patterns, the ^{13}C NMR spectrum was used to determine which had the primary alcohol and which had the acetate group since the 1H spectrum couldn't reveal the position of substitution.

Making use of the α and β -effects of carbon chemical shifts when alcohols are acetylated, suggested the C-15 position was acetylated by. The C-12 chemical shift for compound (11), compound (14) and sambucinol (18) lie in the range 94-95 ppm. Upon acetylation of the C13-OH, an upfield shift of the C-12 chemical shift (β -effect) is observed in all compounds (92-93 ppm), while a downfield shift (α -effect) is observed for the C-13 hydroxyl carbon.

Two ^{13}C resonances of 62.4 ppm and 65.5 ppm were assigned to the C-13 and C-15 carbons respectively. The chemical shift of C-6 (β carbon to C-15) and C-12 (β carbon to C-13) are 45.4 ppm and 95.0 ppm respectively. Comparison of the

chemical shifts of C-6 and C-12 of compound (11), compound (14) and sambucinol (18) (TABLEs 9 and 13), suggests that C-15 is the probable position of the acetate.

The rest of the ^1H and ^{13}C NMR chemical shifts were assigned by comparison to other 11-epi-apotrichothecenes, although a number of resonances in the ^1H NMR spectrum couldn't be resolved due to the complexity of the aliphatic region.

The GC/MS, ^1H and ^{13}C NMR data for compound (17) are consistent with the structure 13-hydroxy,2 α ,15-diacetoxy-11-epi-apotrichothec-9-ene.

(17)

TABLE 8:

250 MHz ¹H NMR chemical shift assignments for 8-hydroxy, 2,13-diacetoxy-11-epi-apotrichothec-9-ene (14), 2-acetyl,8,13-dihydroxy-11-epi-apotrichothec-9-ene (15), 2,8,13-triacetoxy-11-epi-apotrichothec-9-ene (16) and 13-hydroxy,2,15-diacetoxy-11-epi-apotrichothec-9-ene (17).

Proton	(14)	(15)	(16)	(17)
H-2	5.07	5.16	5.09	5.18
H-3 α	1.85	1.95	1.96m	1.95-2.20
H-3 β	2.07	2.05	2.14m	" "
H-4 α	2.17	2.24	2.10	1.50-2.00
H-4 β	1.24	1.22	1.22	" "
H-7 α	1.63	1.63	1.60	1.17-2.20
H-7 β	1.94	1.91	2.10	" "
H-8 β	4.08	4.10	5.21	1.80-2.20
H-10	5.68	5.66	5.82	5.53
H-11	4.05	4.09	4.10	4.29
H-13	3.82,4.39	3.31,3.76	3.98,4.28	3.48,3.79
H-14	0.97	0.94	0.98	0.99
H-15	1.06	1.08	1.03	3.97,4.15
H-16	1.77	1.77	1.67	1.63
Ac	2.06	2.05	2.04/2.07	2.05/2.06

TABLE 9:

62.8 MHz ^{13}C chemical shift assignments for 8-hydroxy, 2,13-diacetoxy-11-epi-apotrichothec-9-ene (14), 2-acetyl,8,13-dihydroxy-11-epi-apotrichothec-9-ene (15), 2,8,13-triacetoxy-11-epi-apotrichothec-9-ene (16) and 13-hydroxy,2,15-diacetoxy-11-epi-apotrichothec-9-ene (17).

Carbon	(14)	(15)	(16)	(17)
C-2	72.1	75.7	75.7	76.0
C-3	31.7	31.8	31.7	29.3
C-4	32.6	32.6	32.6	32.1
C-5	55.3	55.7	55.7	58.4
C-6	43.5	43.9	43.8	45.4
C-7	38.5	38.5	36.1	28.1
C-8	68.8	68.8	70.1	27.8
C-9	136.2	136.1	133.3	135.3
C-10	123.1	122.7	125.5	120.8
C-11	82.4	82.3	81.9	82.6
C-12	95.0	92.4	92.7	95.0
C-13	62.8	64.4	64.3	62.4
C-14	19.4	19.9	19.7	15.0
C-15	16.7	16.5	15.9	65.5
C-16	19.0	19.5	19.2	22.3
Ac:	170.4/ 21.0/21.1	171.1/ 21.0	171.1/ 21.0/21.1	170.9/ 20.9/21.0

XII-2J: STRUCTURE IDENTIFICATION OF SAMBUCINOL (18)

Sambucinol (**18**), a modified trichothecene, was first isolated from *F. sambucinum* in 1984 [17]. It has been found in extracts of all *Fusaria* species studied so far, and was a major metabolite of *F. culmorum* [39]. The molecule does not have an epoxide at the C-12, C-13 position (characteristic of the trichothecenes) but possesses a ketal moiety at the C-11 position. This was the first of the trichothecene-related compounds to have substitution at the C-3 carbon in the β -configuration.

GC/MS analysis of Fractions 23-24 and comparison to a library of standards indicated that sambucinol had been isolated. The melting point of this sample (234-235°C) was in agreement with that of another sample previously isolated in our laboratory, while Tamm had previously reported a melting point of 227°C [17]. A COSY NMR experiment elaborated upon a number of originally assigned chemical shifts and coupling constants [39].

The ^1H NMR spectrum of (**18**) shows the three methyl groups in the aliphatic region. The singlet at 3.90 ppm is attributed to H-2. No coupling is observed to H-3, which appears as a doublet of doublets at 4.22 ppm, coupling to H-4 only. An AB pattern is not observed for the methylene protons of H-13, but appears upon acetylation.

Most resonances in the ^{13}C NMR spectrum were assigned by comparison to the trichothecenes. A clue which helped determine the structure of (**18**) was the resonance at 108.3 ppm which suggested a ketal functionality at C-11. All MS and NMR data are in agreement with previously published data by Tamm [17] and Greenhalgh [39].

(18)

XII-2K: STRUCTURE DETERMINATION OF 3,13-DIACETYLSAMBUCINOL (19)

After an extensive cleanup by column and vacuum liquid chromatography, an unknown was isolated from Fraction 14. The molecular formula $C_{19}H_{26}O_6$ was deduced from the high resolution mass spectrum (calc.350.187, found 350.198). The lack of an AB system at ca. 3.0 ppm and the similarity in other diagnostic regions of the 1H NMR spectra immediately suggested the compound was a modified trichothecene (FIGURE 15).

The olefinic proton of H-10 at 5.45 ppm was observed as a broad singlet showing long range coupling to H-16 and H-8 α in the COSY spectrum. Two acetate resonances were present at 2.01 and 2.09 ppm respectively. The sharp AB system centered at 4.43 ppm was attributed to C-13. The downfield shift from ca. 3.5 ppm for an alcohol moiety at C-13 was indicative of this position being acetylated. The doublet of doublets at 5.01 ppm is in the chemical shift range of a carbon bearing an oxygen. In comparison to sambucinol, the downfield chemical shift of C-3 is indicative of acetylation.

3,13-DIACETYL-SAMBUCINOL

Ac Ac

From the molecular formula, two more oxygen atoms had to be accounted for. A downfield singlet at 3.92 ppm suggested another oxygen functionality. No coupling to H-3 was observed, although long range coupling to H-4 ($J = 0.7$ Hz) was observed in the COSY spectrum. This resonance was attributed to H-2, which was in good agreement with sambucinol (18) (3.90 ppm). The chemical shifts of the three methyl groups C-14, C-15 and C-16 were similar to those of the trichothecenes, with H-16 appearing as a broad singlet showing long range coupling to both H-10 and H-8.

The H-15 resonance was assumed to be upfield of H-14, although no long range coupling was observed between H-15 and H-7_a. The COSY spectrum revealed two AB systems, for the H-7 and H-8 methylene protons. The individual chemical shifts couldn't be resolved due to the complexity of the coupling to H-16 and H-8 α . Both H-7 α and H-8 protons also overlap. The coupling between H-7 α and H-8 were measured at the resolved H-7 α resonance. The third AB system was attributed to H-4 and showed coupling to the resonance at 5.01 ppm.

The resonances at 146 and 115 ppm in the ^{13}C NMR spectrum were similar those of C-9 and C-10 of sambucinol (145.8 and 116.1 ppm respectively). In comparison to the trichothecenes, these chemical shifts are reversed due to the ketal at the C-11 position. The C-12 quaternary carbon is found downfield of the C-2 methine carbon at 95 ppm. All other resonances were assigned in comparison to sambucinol (TABLE 11, pg. 64).

All NMR and MS data were in agreement with the spectral data of the synthetically produced sambucinol diacetate (19). Similarly to the trichothecenes, one would expect various acetylated derivatives of modified trichothecenes, however GC analysis of the crude fungal extract failed to detect the mono-acetylated derivative of sambucinol.

(19)

TABLE 10:

250 MHz ¹H NMR chemical shift assignments for sambucinol (18), 3,13-diacetylsambucinol (19) and 3,13-diacetyl-isosambucinol (20)

Proton	(18)	(19)	(20)
H-2	3.90	3.92	3.86
H-3	4.22	5.01	4.98
H-4 α	1.48	1.50	1.47
H-4 β	2.57	2.62	2.70
H-7	1.34, 1.93	1.40-2.08	1.63-2.32
H-8	1.80, 2.02	1.80-2.10	5.27
H-10	5.42	5.45	2.11-2.42
H-13	4.03, 4.10	4.34, 4.51	4.40, 4.45
H-14	1.04	1.00	1.04
H-15	0.81	0.85	0.88
H-16	1.73	1.73	1.65
Ac	-----	2.01/2.09	2.01/2.09

TABLE 11:

62.8 MHz ^{13}C NMR chemical shift assignments for sambucinol (18), 3,13-diacetylsambucinol (19) and 3,13-diacetyl-isosambucinol (20)

Carbon	(18)	(19)	(20)
2	88.2	84.2	82.3
3	72.5	74.6	73.8
4	45.1	42.3	42.6
5	51.0	50.7	50.2
6	47.7	47.7	48.6
7	29.7	30.3	34.7
8	28.9	29.0	119.5
9	146.0	145.8	129.2
10	116.3	116.1	32.5
11	108.1	108.3	110.0
12	93.5	92.2	93.7
13	59.4	60.0	60.1
14	16.2	16.3	17.6
15	14.9	14.8	16.2
16	22.6	22.8	22.3
Ac	---	20.9/21.7	20.7/20.9
C=O	---	170.1/170.8	170.2/170.8

XIII BIOSYNTHETIC IMPLICATIONS

XIII-1 BIOGENESIS OF 11-EPI-APOTRICHOTHECENE

Apotrichothecene was the name given to the structure (21); formed by acid rearrangement of acylated verrucarol [14]. This was one of the first reactions performed on a trichothecene. In 1985, a compound was isolated from *F. roseum*, which appeared to be the 3,13-dihydroxy derivative of apotrichothecene. The relative stereochemistry was thought to be the same as the trichothecenes (ie. with a *cis* A/B ring junction) [35].

(21)

(11)

However, this assignment was proved incorrect, and latter revised to be (11), by nOe experiments [37]. A strong nOe enhancement was observed for H-11 when the H-14 methyl group was irradiated, showing H-11 to have a β -configuration. This was confirmed by X-ray diffraction analysis. In light of these results, a more appropriate name would seem to be 3 α ,13-dihydroxy-11-epi-apotrichothec-9-ene.

11-epi-apotrichothecene has been shown to be derived via the mevalonate pathway with the use of ^{13}C -acetate, as are the trichothecenes [38]. However, since the trichothecenes and apotrichothecene have a different relative stereochemistry at the C-11 asymmetric centre, it would appear that two different cyclization mechanisms must occur as shown in FIGURE 16. It is known that 11-epi-apotrichothecene has the same biosynthetic precursor as the trichothecenes, which is thought to be trichodiene (22). Oxygenation of trichodiene to trichodiol (23) followed by cyclization would afford trichothecene (24) with the *cis* fused A/B ring junction. Trichotriol, a hydroxylated analogue of (23) at C-3 and thought to be a biosynthetic intermediate, was recently isolated from *F. sporotrichioides* MC 72083. It readily cyclized in an NMR tube to form 3 α -hydroxytrichothecene thereby suggesting it may also be a precursor to the trichothecenes [25].

The biosynthesis of 11-epi-apotrichothecene (25) would involve the formation of a different intermediate, possibly (26). Cyclization involving the opening of the epoxide at C-12 by 11-OH would produce (25) with the *trans* fused A/B ring junction. Opening of the epoxide at C-13 would lead to the formation of sambucoid (28) via the intermediate (27).

In general, the 11-epi-apotrichothecenes have a tendency to be less stable in solution in comparison to the trichothecenes. They are more susceptible to oxidation and rearrangement under mildly acidic conditions. 3-hydroxy epimers of 11-epi-apotrichothecene (11,12) both rearrange, albeit at different rates to produce a number of compounds. The other analogues of 11-epi-apotrichothecene degrade in

ethyl acetate, CH_2Cl_2 and CHCl_3 , and can even degrade in cold storage. Analogues with free hydroxyl groups tend to be more susceptible to degradation, acetylation of these compounds makes them more stable. They are known to be cytotoxic but the toxicity appears to be much less than that of the trichothecenes [39].

3 α ,13-dihydroxy-11-epi-apotrichothecene has been reported to be a end metabolite [37]. However the isolation of several oxidative products of the 3-OH epimers of 11-epi-apotrichothecene [38], and the isolation of a number of novel analogues of 11, epi-apotrichothec-9-ene tends to suggest that the apotrichothecenes are a parallel class of naturally occurring metabolites. Oxidation occurs at C-2, C-3, C-8 and C-15 of the 11-epi-apotrichothecenes isolated to date, similar to the trichothecenes (FIGURE 17). *F. sporotrichioides* DAOM 165006 produces 3,4 oxygenated trichothecenes in liquid culture [24]. Although no C-4 oxygenated 11-epi-apotrichothecenes have yet been isolated, it is quite possible that C-4 and other oxygenated analogues may be produced by this strain.

FIGURE 17: Sites of oxidation for 11-epi-apotrichothec-9-enes

XIII-2 BIOGENESIS OF SAMBUCINOL (18)

In 1984, sambucinol (18) and sambucoin (28) were first isolated from *F. sambucinum* by Tamm [17]. Both compounds are considered modified trichothecenes. Sambucoin (26) was the first compound to exhibit the *trans* A/B ring junction. Tamm proposed two possible biosynthetic pathways for the formation of sambucinol, both starting with trichodiene (22) (FIGURE 18, pg. 70).

Pathway A involves the oxidation of trichodiene (22) to trichodiol (23). Pyran formation forms the 12,13-epoxytrichothecene (24), also a known naturally occurring secondary metabolite. At this point, oxidation at the C-11 must occur followed by ketalization, opening the epoxide. Finally, oxidation at C-3 occurs to form sambucinol (18).

Pathway B doesn't involve the formation of a trichothecene, but involves 2 allylic hydroxylations (at C-2 and C-11) followed by epoxidation formation to yield the first intermediate (29). Oxidation of the allylic alcohol to the α,β -unsaturated ketone followed by epoxide opening gives the second key intermediate (30). Finally, ketalization and hydroxylation at C-3 produces sambucinol (18).

However, recent biosynthetic studies involving ^{13}C enriched acetate disproves pathway A since the production of sambucinol was observed to level off after 72 hrs. while the production of 3-Ac-DON increased [40].

The isolation of 3,13-dihydroxy-11-*epi*-apotrichothecene also possessing the *trans* A/B ring junction has led to a number of hypotheses regarding mechanisms of cyclization of trichodiene, as previously discussed [39]. The observation that compound (11) can cyclize to form 13-hydroxy-3 α ,11-epoxy-*epi*-apotrichothecene (14) suggests that possibly a 2 α -OH derivative of 11-*epi*-apotrichothecene could be the precursor of sambucinol (18) as shown in FIGURE 18.

FIGURE 18: PROPOSED BIOGENESIS OF SAMBUCINOL (18)
taken from reference 17.

XIII-3 CHEMISTRY OF SAMBUCINOL (18)

In the effort to understand the chemistry of sambucinol, a number of experiments were performed to determine the chemical reactivity of the ketal moiety. The retrosynthesis could yield a precursor having oxygenation at the C-2 position similar to the proposed 2 α -OH apotriconothecene derivative.

Since ketals are susceptible to mild acid hydrolysis, a number of mineral and organic acids were reacted with sambucinol. However, the ketal moiety was found to be very stable and sambucinol failed to react. The use of a Lewis acid, BF₃·Et₂O was attempted to open the ketal followed by trapping of the carbocation with acetic anhydride. Unfortunately, HPLC analysis of the reaction mixture indicated at least 12 compounds were formed, mainly di- and triacetates. In an effort to isolate a crystalline compound, sambucinol diacetate was prepared prior to the reaction involving the Lewis acid.

Acetic anhydride was replaced by acetic acid and GC analysis of the product mixture indicated the presence of a 45:55 mixture of isomers, one of which being sambucinol diacetate. The ¹H NMR spectrum of the new isomer indicated it was almost structurally identical to the starting material.

XIII-4 STRUCTURE DETERMINATION OF 3,13-DIACETOXY-ISOSAMBUCINOL (20)

Most ¹H NMR resonances were assigned by comparison to those of 3,13-diacetylsambucinol (**19**) (TABLE 10). The broad multiplet at 5.27 ppm was upfield of that of the broad singlet at 5.45 ppm observed for H-10 in (**19**). This resonance was attributed to the olefinic proton at H-10, since long-range coupling to H-16 was still evident in the COSY spectrum. A poorly resolved multiplet between 2.11- 2.45 ppm was assigned to H-8.

The ^{13}C spectrum had been altered considerably. Most significant was the upfield shift of C-9 from 145.8 ppm in sambucinol diacetate to 129.2 ppm. The resonance attributed to C-10 had shifted downfield to 119.5 ppm, indicating a change in the A ring. The only other difference in the spectrum was the resonances of C-5 and C-6 which differed by 1-3 ppm.

In an effort to determine the structure of this compound, a number of experiments involving deuterated acetic acid and acetic anhydride were carried out to determine the location of the acetate groups, since it was surmised that the ketal moiety may have been opened, followed by an acetate migration or a loss and then addition of acetate. In the first experiment, the di-deuteroacetate of sambucinol was prepared and then subjected to the same reaction conditions. The only noticeable difference in the ^1H spectrum was a collapsed AB system assigned to C-13. The mass spectrum revealed the loss of one d_3 -acetate, however, a number of fragmentation ions as high as M^+6 were observed.

Based on the NMR data, the structure of this unknown compound was thought to be **(31)**. One possible mechanism for the reaction that took place was opening of the C-11,C-2 ether bond after attack by the Lewis acid. Once the carbocation is formed, attack by the 13-OH would lead to the formation of compound **(31)**, while attack of the 2-OAc would form the starting material. This mechanism seemed to support the experimental results of two isomers being present in nearly equal amounts, and was further substantiated when compound **(31)** was subjected to the initial conditions ($\text{AcOH}/\text{BF}_3\cdot\text{Et}_2\text{O}$) and a 50:50 ratio **(31:19)** was observed by GC.

Unfortunately, X-ray analysis failed to support the structure of **(31)**, and indicated **(20)** had formed by a Lewis acid catalyzed isomerization. Reinterpretation of the NMR spectra revealed the chemical shifts of H-8 and H-10 were incorrectly assigned due to poor resolution of H-10 in the aliphatic region.

(31)

(20)

An AB system in the aliphatic region had initially been attributed to H-8, this resonance showed long-range coupling to H-16. The lack of $J(8,7)$ coupling because of a complex aliphatic region led to H-8 being initially assigned incorrectly. In fact, re-interpretation of the ^1H spectrum of the di-deuterated product showed a resolved AB system (1.66, 2.30 $J_{AB} = 13.3$ Hz), reassigned to H-10.

Deuteration of (31) could be explained with the acid-catalyzed reaction. Once the carbocation had formed, migration of the double bond would result in the incorporation of deuterium at positions C-8, C-10 and C-16, accounting for the

observed fragmentation pattern in the EI spectrum.

Had a COSY experiment been performed on this compound, long-range coupling would have been seen to H-16 and H-10. The C-8 and C-10 resonances reversed are in agreement, for each type of carbon present. The large change in chemical shift in C-8 and C-9 can only be attributed to a conformational change in the molecule.

XIV FURTHER WORK

Most compounds that were present in trace amounts (1-20mg) have been isolated from F. sporotrichioides DAOM 165006. However, there are many compounds yet to be characterized. A number of other compounds have been isolated during the writing of this thesis, but at the present time no new structures have been proposed.

Further isolation of larger quantities of new compounds isolated are needed for biological studies, although the unstable nature of the 11-epi-apotrichothecenes isolated thusfar will make this task difficult. Although ca. 50g of crude extract is still available, fermentation conditions did not produce optimum yields of the major metabolites and consequently there are very low concentrations of the minor metabolites.

The isolation of new analogues of the 11-epi-apotrichothec-9-ene class of compounds may lead to the discovery of possible biosynthetic intermediates.

XV EXPERIMENTAL

I Physical Analyses

UV and IR

All ultraviolet spectra were obtained on a Varian DMS 200 UV-visible spectrophotometer. Infrared spectra were obtained on a Bomem Mitehelson 100 FT-IR. Samples were applied as a film on a NaCl plate.

GC/MS

Mass spectra of the samples were obtained on a Finnigan GC/MS system, model 4500, operating in the electron impact (EI) mode, with a DS400 data system. The underivatized crude fungal extract and chromatographed samples were separated on a DB-5 fused silica capillary column (20m x 0.32mm i.d.), 0.25um film. All samples were injected on column. The GC was temperature programmed from 140 °C to 260 °C at 15 °C/min. with helium as the carrier gas at 10 psi. Routine analysis was done by using a Varian Vista 5500 coupled with a Finnigan ion trap (GC/ITD), operating with a split injector (10:1). The GC was temperature programmed from 120-280 °C at 20 °C/min. holding for 12 min. at the end of each run. GC/MS data was acquired by an IBM PC using an Ion Trap Detection System (ITDS) software program.

NMR

¹H and ¹³C NMR spectra were run on a Bruker WM 250 and AM-500 NMR spectrometer. The ¹H spectra were acquired with 16K data points, a 2200 Hz spectral window, 60° pulses, and 5-6 second repetition time. Chemical shifts were referenced to deuteriochloroform at 7.24 and 77.0 ppm, respectively, for ¹H and ¹³C and reported (ppm) relative to tetramethylsilane (Me₄Si). ¹³C chemical shift assignments were made by using the DEPT pulse sequence to determine the number of attached protons corresponding to each resonance. Confirmation of ¹H chemical shifts assignments

was made by $^1\text{H}/^1\text{H}$ homonuclear correlation spectra (COSY). The (90-t,-45 FID) pulse sequence (COSY-45) was accumulated by using 512 x 1K FID's of 16 scans each. Sine bell windows (shifted $\pi/4$ in F_1) were used for a final resolution of 1.4 Hz/point. Protons attached to carbons were made by using $^1\text{H}/^{13}\text{C}$ heteronuclear correlation spectra (HETCOR).

X-Ray Crystallography

A single crystal was analyzed using an automated Picker 4-circle diffractometer. Accurate cell parameters and intensity data were obtained at room temperature using graphite-monochromatized $\text{Cu K}\alpha$ radiation. The structures were solved by direct methods using MULTAN [38]. The absolute configuration determination was carried out by measuring the intensities of selected pairs of Friedel reflections and their symmetry-related reflections, which showed the highest difference between calculated (+) and (-) structure factors.

HPLC

Preparative HPLC was conducted on a Varian Vista 5500 series chromatograph using a linear gradient solvent system, using a UV 200 detector operating at 205nm. The column used was a 5 μm CN column (0.3 x 25cm) CSC-S (Serial No. 058816) with a flow rate of 4ml/min. Silica gel for vacuum liquid chromatography was Merck TLC grade silica gel 60 (No. 7731). For preparative column separations, Merck Lichroprep Si 60 silica gel (cat. 9390, 25-40 μm) was used. The silica gel was activated by heating it in a muffle furnace (500°C/24hrs), prior to use.

All solvents used for isolation and purification were HPLC grade glass distilled purchased from Caledon or Anachemia.

II Fermentation

Conditions were optimized for the production of T-2 toxin by F. sporotrichioides DAOM 165006 with a New Brunswick Scientific Magnaferm fermentor equipped with oxygen and pH controls and a CO₂ analyzer (ADC), interfaced with a Hewlett-Packard data acquisition system.

30 flasks (500ml) of medium were prepared and inoculated with 2ml of spore suspension, for 3 days at 25 °C on a rotary shaker at 250 rpm.

The ingredients for 25L of Moss media were dissolved in 2-3L of water and added to the 250L fermentor. Table sugar was substituted for sucrose. 125L of filtered H₂O was pumped into the fermentor followed by a 90 min. sterilization cycle. The inoculum was added. The fermentation conditions were as follows: temperature, 27°C; stirring rate, 285 rpm; air flow, 1.6 h min⁻¹; the initial pH was 5.36 after the addition of the inoculum and then was set to 4.0 and maintained by the addition of sterile 0.8 N NaOH. After 7 days, the culture filtrate was adjusted to pH 8.3. The aqueous medium was poured over a filter bed containing 300g of Celite and 3kg of Hyflo. After the mycelium was washed, the combined filtrates (140L) were treated with NaCl (10kg) and extracted with methylene chloride (3 x 30L). The combined extracts were evaporated in a cyclone evaporator (5 °C (100 torr)) to ca. 2.5L and the concentrate was taken to dryness in vacuo. One half of the crude fungal extract (50g) was chromatographed in three portions on TLC grade Silica gel.

Extraction and isolation procedure

The crude fungal extract (117.44g) was dissolved in CH₂Cl₂ (500ml) and the insoluble inorganic salts filtered off (3.78g). The crude extract was then subjected to silica gel chromatography. The extract-solvent mixture was concentrated and the dark red oil (112g) was taken up in CH₂Cl₂ (220ml) to yield a non-viscous solution (0.5g/ml). The oil was applied to a column (15cm x 1.5m) containing Lichroprep Si-60

Silica gel (1.5kg) with a flow rate of 33ml/min. (2L/min.). The crude extract were chromatographed in three batches which were labeled Runs A, B and C respectively. The following eluting solvents were used:

RUN A 20ml (10g)

CH₂Cl₂ (2L), 1.25% MeOH/CH₂Cl₂ (2L), 1.66% MeOH/CH₂Cl₂ (2L), 3.40% MeOH/CH₂Cl₂ (5L), 4.2% MeOH/CH₂Cl₂ (2L), 7.2% MeOH/CH₂Cl₂ (2L), 13.0% MeOH/CH₂Cl₂ (2L), 20.0% MeOH/CH₂Cl₂ (2L) and finally MeOH (2L). 51 fractions were collected, each of 500ml.

RUN B 40ml (20g)

CH₂Cl₂ (2L) followed by 1.5% MeOH/CH₂Cl₂ (2L), 2.0% MeOH/CH₂Cl₂ (2L), 3.0% MeOH/CH₂Cl₂ (2L), 4.0% MeOH/CH₂Cl₂ (2L), 4.5% MeOH/CH₂Cl₂ (4L), 8.0% MeOH/CH₂Cl₂ (4L), 10.0% MeOH/CH₂Cl₂ (2L), 12.0% MeOH/CH₂Cl₂ (2L), 15.0% MeOH/CH₂Cl₂ (2L), 20.0% MeOH/CH₂Cl₂ (2L), and finally MeOH (2L). 51 fractions were collected.

RUN C 40ml (20g)

same as RUN B except: 5.5% MeOH/CH₂Cl₂ followed by 7.0% MeOH/CH₂Cl₂. 50 fractions were collected.

All samples were analyzed by GC/IT and were combined to give 24 fractions labeled DF1- DF24. the weights and the description of each fraction are outlined on the following pages.

Sample	Fraction	Weight
DF0	0-12A	Discarded
DF1	13-14A	188mg
DF2	15-18A	1862mg
DF3	19-20A	890mg
DF4	21-22A	636mg
DF5	23-24A	650mg
DF6	25-28A	830mg
DF7	29-30A	189mg
DF8	31-36A	961mg
DF9	37-42A	596mg
DF10	42-51A	906mg
DF11	1-4B	554mg
DF12	5-7B	5217mg
DF13	8-11B	2718mg
DF14	12-16B	3285mg
DF15	17-18B	1693mg
DF16	19-40B	3346mg
DF17	41-51B	753mg
DF18	0-19C	Discarded
DF19	20-21C	171mg
DF20	22-24C	3564mg
DF21	25-27C	1381mg
DF22	34-35C	1334mg
DF23	36-37C	776mg
DF24	38-51C	3377mg

Sample DF1 fractions 13-14A gave a light orange oil (188mg) consisting predominantly of T-2, by GC/MS analysis.

Sample DF2 fractions 15-18 gave a light orange oil (1862mg) consisting of primarily T-2 with traces of three unknowns.

Sample DF3 fractions 19-20A contained crystals and an oil (890 mg) consisting primarily of neosolaniol with traces of Sporo 16, Sporo 18 and a number of other unknowns.

Sample DF4 fractions 21-22A contained crystals and an oil (636mg) comprising a mixture of sambucinol and neosolaniol.

Sample DF5 fractions 23-24A contained crystals and an oil (1650mg), some of which was sambucinol, which was recrystallized from acetone yielded 112mg.

Sample DF6 fractions 25-28A gave an orange oil (830mg) consisting predominantly of T-2 with HT-2 and neosolaniol present.

Sample DF7 fractions 29-30A gave a red oil (189mg) which was a mixture of Gram 1, Apo B, and a number of unknowns.

Sample DF8 fractions 31-36A gave a dark red oil (961mg) consisting of Apo isomer 1B, Apo A, Apo B and a number of unknowns.

Sample DF9 fractions 37-42A gave a dark red oil (596mg) consisting of sambucinol, neosolaniol, HT-2 and some unknowns.

Sample DF10 fractions 43-51A gave a black oil (906mg) consisting of Sporo 16, neosolaniol, sambucinol, T-2 and an unknown.

Sample DF11 fractions 1-4B contained crystals and an oil (554mg) containing T-2.

Sample DF12 fractions 5-7B contained crystals of T-2 (5217mg).

Sample DF13 fractions 8-11B gave an orange oil (2718mg) which contained DAS, NEO 1, NEO 2, T-2 and an unknown.

Sample DF14 fractions 12-16B contained crystals and an oil (3285mg) consisted of neosolaniol, Sporo 5, Sporo 6, Sporo 4 and a number of unknowns.

Sample DF15 fractions 17-18B contained crystals and an oil (1693mg) containing sambucinol, neosolaniol and HT-2.

Sample DF16 fractions 19-40B contained crystals of (of sambucinol (3346mg).

Sample DF17 fractions 41-51B gave a black oil (753mg) consisting of T-2 and an unknown.

Sample DF18 fractions 20-21C gave an orange oil (171mg) consisting of phalates and trace amounts of sambucol and a number of unknowns.

Sample DF19 fractions 22-24C contained crystals of T-2 (3564mg).

Sample DF20 fractions 25-27C gave an orange oil (1381mg) consisting of predominantly T-2 with trace amounts of NEO 1, NEO 2, DAS and an unknown present.

Sample DF21 fractions 28-33C 28-33C contained crystals and an oil (3567mg) containing neosolaniol as the major compound with sambucinol and SPORO6 present.

Sample DF22 fractions 34-35C contained crystals (1334mg) identified as sambucinol.

Sample DF23 fractions 36-37C gave a red oil (776mg) containing sambucinol, T-2 and a number of unknowns.

Sample DF24 fractions 38-50C gave a black oil (3377mg) containing T-2, phthalates and an unknown.

ISOLATION AND IDENTIFICATION OF T-2 TOXIN (7)

Fractions 22-24 (16.13g) yielded crystals upon evaporation of the solvent, which was recrystallized from acetone/hexane to afford colourless needle crystals.

mp: 148-149°C

$[\alpha]_D$: +15 °C (c = 2.58, 95% EtOH, 26 °C)

GC Rt: 15.06 min. (604 scans)

MS: $C_{24}H_{34}O_9$; m/z 57 (91, (intensity relative to base fragment)), 85 (68), 105 (54), 121 (base), 138 (35), 180 (56), 205 (20), 278 (31), 364 (15), 466 (M').

IR: (NaCl) cm^{-1} , 3441, 2960, 1732

UV: end absorption

1H NMR: δ 0.79 (3H, H-14), 0.95 (3H, H-4', $J_{4,3} = 7.0$ Hz), 0.94 (3H, H-5', $J_{5,4} = 7.0$ Hz), 1.73 (3H, H-16), 1.89, 2.31 (2H, H-7, $J_{AB} = 15.3$), 2.00 (2H, H-3'), 2.12 (2H, H-2'), 2.78, 3.04 (2H, H-13, $J_{AB} = 3.9$ Hz), 3.68 (1H, H-2, $J_{2,3} = 4.9$ Hz), 4.03, 4.27 (2H, H-15, $J_{AB} = 12.6$ Hz), 4.14 (1H, H-3, $J_{3,2} = 4.9$ Hz, $J_{3,4} = 2.9$ Hz), 4.32 (1H, H-11, $J_{11,10} = 5.9$ Hz), 5.28 (2H, H-4), 5.28 (H-8, $J_{8,7} = 5.7$ Hz), 5.78 (1H, H-10, $J_{10,11} = 5.9$ Hz), 2.01, 2.12 (CH₃-Ac).

^{13}C NMR: δ 6.6 (C-14), 22.7 (C-16), 21.9 (C-5'), 22.7 (C-4'), 25.6 (C-3'), 27.7 (C-7), 42.9 (C-6), 43.4 (C-2'), 47.0 (C-13), 48.3 (C-5), 64.2 (C-12), 64.5 (C-15), 67.3 (C-11), 68.0 (C-8), 78.2 (C-3), 78.7 (C-2), 34.2 (C-4), 123.8 (C-10), 136.3 (C-9), 172.7 (C-1'), 170.3, 172.5 (C=O (Ac))

2 of/de 2

MicroD

ISOLATION AND CHARACTERIZATION OF 3'OH T-2 (8)

Fraction 21 (3567mg), a dark orange oil was chromatographed on silica gel (50:1 adsorbant :sample) by vacuum liquid chromatography (VLC). Further cleanup of fraction 21-3 (29mg) by HPLC yielded an oil (10mg).

GC Rt: 11.43 min. (469 scans)

MS: $C_{24}H_{34}O_{10}$ m/z 59 (76), 83 (33), 95 (24), 105 (57), 121 (base), 138 (25), 180 (26), 382 (M^+).

1H NMR: δ 0.78 (3H, H-14), 1.27 (3H, H-4'), 1.27 (3H, H-5'), 1.73 (3H, H-16), 1.90, 2.40 ($J_{7,8} = 5.2$ Hz), 2.42 (2H, H-2'), 2.78, 3.04 (2H, H-13, $J_{AB} = 3.9$ Hz), 3.67 (1H, H-2, $J_{2,3} = 4.9$ Hz), 4.04, 4.26 (2H, H-13, $J_{AB} = 12.6$ Hz), 4.14 (1H, H-3, $J_{3,2} = 4.9$ Hz, $J_{3,4} = 2.9$ Hz), 4.35 (1H, H-11, $J_{11,10} = 5.9$ Hz), 5.26 (1H, H-8, $J_{8,7} = 5.3$ Hz), 5.27 (1H, H-4, $J_{4,3} = 2.9$ Hz), 5.79 (1H, H-10, $J_{10,11} = 5.9$ Hz), 2.02, 2.12 ($CH_3(Ac)$).

^{13}C NMR: δ 6.8 (C-14), 20.2 (C-16), 20.9 (C-4',5'), 27.7 (C-7), 36.3 (C-2'), 43.0 (C-6), 47.1 (C-13), 48.9 (C-5), 64.3 (C-12), 64.6 (C-15), 67.4 (C-11), 68.1 (C-8), 69.1 (C-3'), 78.4 (C-3), 78.8 (C-2), 84.6 (C-4), 123.8 (C-10), 136.4 (C-9), 173.4 (C-1'), 170.1, 172.6 (C=O (Ac)).

ISOLATION AND CHARACTERIZATION OF 4-DEOXY T-2 (9)

Fraction 20 (2564mg), an orange oil was chromatographed by VLC which removed the major component, T-2 toxin. Fraction 20-10 (35 mg) was purified by HPLC (6% isopropanol/hexane) to afford a colourless oil (yield 4 mg).

GC Rt: 12.01 min. (481 scans)

MS: $C_{22}H_{32}O_7$, CI m/z 43 (43), 199 (31), 247 (base), 307 (59), 409 (23), 482 (M^+).

1H NMR: δ 0.74 (3H, H-14), 0.89, 0.91 (6H, H-4', 5'), 1.69 (3H, H-16), 1.90-2.30 (2H, H-7 J unresolved), 2.05-2.25 (2H, H-4 J unresolved), 2.00-2.20 (2H, H-3', J unresolved), 2.21 (2H, H-2'), 2.81, 3.02 (2H, H-13, $J_{AB} = 3.9$ Hz), 3.43 (1H, H-2), 3.91, 4.15 (2H, H-15, $J_{AB} = 12.2$), 4.24 (1H, H-11 $J_{11,10} = 5.8$ Hz), 4.34 (1H, H-3, $J_{3,2} = 4.7$ Hz), 5.70 (1H, H-8, $J_{8,7} = 5.7$ Hz), 5.73 (1H, H-10, $J_{10,11} = 5.8$ Hz),

^{13}C NMR: δ 12.0 (C-14), 20.2 (C-16), 22.3 (C-4', 5'), 25.6 (C-3'), 27.1 (C-7), 41.7 (C-6), 42.0 (C-4), 43.4 (C-2'), 45.5 (C-5), 48.4 (C-13), 64.6 (C-15), 65.3 (C-12), 67.6 (C-11), 68.5 (C-8), 68.9 (C-3), 79.6 (C-2), 124.3 (C-10), 136.0 (C-9), 172.8 (C-1').

ISOLATION AND CHARACTERIZATION OF 4,15-DIACETYL-7-DEOXYNIVALENOL (10)

Fraction 14 (3.285g) was chromatographed on TLC grade silica gel (55:1; adsorbant :sample) in a glass column (7x 40cm). The solvent system used ranged from 2-10% MeOH/ $CHCl_3$. Fraction 14-2 (170mg) was analyzed by GC/IT and shown to contain two major unknowns. Both compounds were isolated by HPLC (6% isopropanol/hexane, CN column). One of these compounds was isolated as a clear colourless oil. Yield: 9mg.

GC Rt: 12.43 min. (509 scans)

$[\alpha]_D$: + 15.8° (chloroform, 24.6 °C)
 MS: $C_{19}H_{24}O_8$ m/z 380 (M⁺), 109 (base), 121 (97), 100 (64), 173 (52), 189 (45), 278 (21).
¹H NMR: δ 0.80 (3H, H-14), 1.82 (3H, H-16, $J_{16,10}$ = 1.5 Hz), 2.46, 2.85 (2H, H-7, J_{AB} = 15.9 Hz, $J_{7,15}$ = 1.6 Hz), 2.79, 3.07 (2H, H-13, J_{AB} = 3.9 Hz), 3.21 (1H, 3-OH, $J_{OH,3}$ = 2.6 Hz), 3.77 (1H, H-2, $J_{2,3}$ = 4.9 Hz), 4.10, 4.17 (2H, H-15, J_{AB} = 12.5 Hz), 4.21 (1H, H-3, $J_{3,2}$ = 4.9 Hz, $J_{3,4}$ = 2.9 Hz, $J_{3,OH}$ = 2.6 Hz), 4.51 (1H, H-11, $J_{11,10}$ = 5.9 Hz), 5.06 (1H, H-4, $J_{4,3}$ = 2.9 Hz), 6.59 (1H, H-10, $J_{10,11}$ = 5.9 Hz, $J_{10,16}$ = 1.5 Hz).
¹³C NMR: δ 6.1 (C-14), 15.3 (C-16), 38.2 (C-7), 46.7 (C-13), 47.5 (C-6), 48.6 (C-5), 64.4 (C-12), 64.5 (C-15), 68.4 (C-11), 78.5 (C-3), 79.0 (C-2), 83.8 (C-4), 136.9 (C-10), 138.9 (C-9), 196.7 (C-8), 20.5, 20.8 (Ac-CH₃), 170.2, 172.2 (C=O).

ISOLATION AND CHARACTERIZATION OF

3 α ,13-DIHYDROXY-11-EPI-APOTRICHOTHEC-9-ENE (11)

GC analysis of Fraction 16-538 (76mg) indicated at least six compounds were present. Purification by preparative HPLC (elution with 6% isopropanol/hexane) afforded three compounds. 11, (28mg) was recrystallized from isopropanol/hexane.

GC Rt: 20.1 min. (DB-4X column)
 m.p: 139-141°C
 $[\alpha]_D$: -33.2° (EtOH, 23°C)
 MS: $C_{15}H_{24}O_3$ (calc. m/z 252.173, found 252.173) 107(100), 91(70), 139(64), 81(62), 55(56), 77(50), 67(47), 252 (M⁺, 38), 97(34), 224(28).

124(27), 237(26), 119(22).

UV: end absorption

IR: (cm⁻¹) 3426

¹H NMR: δ 0.92 (3H, CH₃-14), 0.99 (3H, CH₃-15), 1.54 (1H, H-7, $J_{7\alpha,8\alpha} = 2.7$ Hz), 1.37 (1H, H-7, $J_{AB} = 13.1$ Hz), 1.54 (1H, H-7, $J_{7\alpha,8\alpha} = 2.7$ Hz, $J_{7\alpha,8\beta} = 5.6$ Hz, $J_{7\beta,8\alpha} = 9.8$ Hz), 1.63 (3H, CH₃-16, $J_{16,10} = 1.2$ Hz, $J_{16,11} = 2.4$ Hz), 1.67 (1H, H-4 β , $J_{4\beta,2\beta} = 1.7$ Hz, $J_{4\beta,3} = 1.7$ Hz), 1.74 (1H, H-2 α , $J_{2\alpha,3\beta} = 2.2$ Hz, $J_{2\alpha,4} = 1.7$ Hz), 1.93, 2.03 (2H, H-8, $J_{8\alpha,7\alpha} = 2.8$ Hz), 2.13 (1H, H-4 α , $J_{AB} = 12.9$ Hz, $J_{4\alpha,3} = 10.1$ Hz), 2.60 (1H, H-2 β , $J_{AB} = 12.2$ Hz, $J_{2\beta,3} = 6.2$ Hz, $J_{2\beta,4\beta} = 1.7$ Hz), 3.18, 3.73 (2H, H-13, $J_{AB} = 11.4$ Hz), 4.15 (1H, H-11, $J_{11,10} = 2.5$ Hz, $J_{11,16} = 1.0$ Hz), 4.28 (1H, H-3, $J_{3,2\alpha} = 2.2$ Hz, $J_{3,2\beta} = 6.2$ Hz, $J_{3,4} = 1.3$ Hz, $J_{3,4\beta} = 10.2$ Hz), 5.52 (1H, H-10, $J_{10,11} = 2.5$ Hz, $J_{10,16} = 1.3$ Hz)/

¹³C NMR: δ 17.8 (C-15), 19.4 (C-14), 22.5 (C-16), 29.6 (C-7), 27.7 (C-8), 43.5 (C-2), 44.5 (C-4), 44.7 (C-6), 52.6 (C-5), 63.4 (C-13), 72.8 (C-3), 81.4 (C-11), 92.6 (C-12), 121.4 (C-10), 135.5 (C-9).

ISOLATION AND CHARACTERIZATION OF

3 β ,13-DIHYDROXY-11-EPI-APOTRICHOTHEC-9-ENE (12)

This compound was purified by HPLC (6% isopropanol/hexane) and isolated as an oil from fraction 16-538 (10mg).

GC Rt: 13.5 min. (DB-4X column)

- MS: $C_{15}H_{24}O_3$ (calc. 252.173, found 252.174) m/z 107(100), 124(66), 81(64), 91(64), 55(46), 67(40), 237(28), 139(26), 224(25), 252(M⁺, 16).
- UV: end absorption
- ¹H NMR: δ 0.52 (3H, CH₃-15), 1.07 (3H, CH₃-14), 1.36 (1H, H-7,m), 1.38 (1H, H-4 β , $J_{4\beta,3\alpha}$ = 5.8 Hz, $J_{4\beta,2\beta}$ = 1.3 Hz), 1.57 (1H, H-7, J_{AB} = 13.5 Hz), 1.63 (3H, CH₃-16, $J_{16,10}$ = 1.5 Hz), 2.10 (1H, H-2 β , J_{AB} = 14.9 Hz, $J_{2\beta,3\alpha}$ = 4.1 Hz), 2.23 (1H, H-2 α , $J_{2\alpha,3\alpha}$ = 6.3 Hz), 2.45 (1H, H-4 α , $J_{4\alpha,3\alpha}$ = 6.4 Hz, J_{AB} = 14.9 Hz), 3.56, 3.78 (2H, H-13, J_{AB} = 10.9 Hz), 4.11 (1H, H-11), 4.51 (1H, H-3, $J_{3,2\beta}$ = 6.3 Hz), 5.50 (1H, H-10, $J_{10,16}$ = 1.5 Hz).
- ¹³C NMR: δ 16.2 (C-15), 20.0 (C-14), 22.5 (C-16), 29.1 (C-7), 27.4 (C-8), 45.7 (C-2), 45.1 (C-6), 45.7 (C-4), 54.9 (C-5), 65.0 (C-13), 73.4 (C-3), 81.1 (C-11), 95.1 (C-12), 121.1 (C-10), 135.2 (C-9).

ISOLATION AND CHARACTERIZATION OF 13-HYDROXY,3 α ,11-EPOXY-APOTRICHOTHEC-9-ENE (13)

Fraction 16-1312 (190mg), a yellow oil contained a number of compounds. These compounds were purified by preparative HPLC (4% isopropanol/hexane) followed by rechromatography using the same solvent system. One major compound (16-13121, 16mg) was isolated and recrystallized from acetone/hexane.

- m.p: 90-92 °C
- GC Rt: 6.36 min. (264 scans)
- $[\alpha]_D$: -50.0° (EtOH, 25.5°C)
- MS: $C_{15}H_{22}O_3$ calc. m/z 250.159, found 250.157, 235(100), 161(75), 250(M⁺,42), 124(40), 82(40), 55(38), 109(26), 67(20), 95(20).
- IR: NaCl (cm⁻¹); 3200-3400

UV: end absorption

^1H NMR: δ 0.90 (3H, CH_3 -15, $J_{15,7\text{B}} = 0.8$ Hz), 1.04 (3H, CH_3 -14), 1.16 (1H, H-4B), 1.34 (1H, H-7, $J_{7,8} = 9.9$ Hz, $J_{7,8} = 9.9$ Hz, $J_{7,15} = 9.9$ Hz), 1.67 (3H, CH_3 -16, $J_{16,10} = 0.6$ Hz), 1.80, 2.03 (2H, H-8, $J_{AB} = 7.2$ Hz), 1.86 (1H, H-7, $J_{AB} = 12.9$ Hz, $J_{7\alpha,8} = 1.6$ Hz), 2.13 (1H, H-2 α , $J_{AB} = 11.5$ Hz, $J_{2\alpha,4\text{B}} = 2.0$ Hz), 2.46 (1H, H-4 α , $J_{4\alpha,2\alpha} = 2.00$ Hz, $J_{4\alpha,3\text{B}} = 3.4$ Hz, $J_{AB} = 13.5$ Hz), 3.66, 3.79 (2H, H-13, $J_{AB} = 12.1$ Hz), 4.51 (1H, H-3, $J_{3,2\alpha} = 1.8$ Hz, $J_{3,4\alpha} = 3.4$ Hz, $J_{3,4\text{B}} = 1.8$ Hz), 5.35 (1H, H-10, $J_{10,8} = 3.3$ Hz, $J_{10,16} = 1.6$ Hz).

^{13}C NMR: δ 13.9 (C-15), 14.7 (C-14), 22.9 (C-16), 28.2 (C-8), 28.3 (C-7), 42.2 (C-2), 42.7 (C-4), 47.1 (C-6), 50.0 (C-5), 65.3 (C-13), 76.3 (C-3), 88.7 (C-12), 105.5 (C-11), 119.5 (C-10), 141.7 (C-9).

ISOLATION AND CHARACTERIZATION OF 8-HYDROXY, 2,13-DIACETYL-11-EPI-APOTRICHOTHEC-9-ENE (14)

Fraction 14 (3285 mg) was chromatographed on silica gel by column chromatography followed by HPLC cleanup of fraction 14-2 (170mg). This afforded a clear colourless oil (66 mg).

GC Rt.: 9.09 min. (366 scans)

MS: $\text{C}_{19}\text{H}_{28}\text{O}_6$; EI 43 (base), 55 (48), 69 (27), 81 (25), 93 (52), 109 (49), 123 (51), 140 (48), 149 (28), 308 (27), 352 (M^+); CI 43 (65), 93 (76), 109 (32), 123 (80), 140 (25), 153 (70), 215 (83), 233 (80), 275 (28), 293 (62), 335 (base), 353 (59, $\text{M}+1$).

- ¹H NMR: δ 0.97 (3H, H-14, s), 1.06 (3H, H-15), 1.24 (1H, H-4 β , J_{AB} = 13.1 Hz, $J_{4\beta,3\beta}$ = 6.8 Hz), 1.63 (1H, H-7 α , J_{AB} = 14.2 Hz), 1.77 (3H, H-16 br. s), 1.85 (1H, H-3 α , J unresolved), 1.94 (H-7 β , $J_{7\beta,8\beta}$ = 7.5 Hz), 2.07 (1H, H-3 β , J unresolved), 2.17 (1H, H-4 α , $J_{4\alpha,3\beta}$ = 6.8 Hz), 3.82, 4.39 (2H, H-13, J_{AB} = 11.8 Hz), 4.05 (1H, H-11 br. s), 4.08 (1H, H-8 α , $J_{8\alpha,7\beta}$ = 7.5 Hz), 5.07 (1H, H-2, $J_{2\beta,3\alpha}$ = 12.1 Hz, $J_{2\beta,3\beta}$ = 6.3 Hz), 5.68 (1H, H-10, $J_{10,16}$ = 1.1 Hz).
- ¹³C NMR: δ 16.7 (C-15), 19.0 (C-16), 19.4 (C-14), 31.7 (C-3), 32.6 (C-4), 38.5 (C-7), 43.5 (C-6), 55.3 (C-5), 62.8 (C-13), 68.8 (C-8), 72.1 (C-2), 82.4 (C-11), 95.0 (C-12), 123.1 (C-10), 136.2 (C-9), 170.4 (C=O), 21.0, 21.1 (CH₃-Ac).

ISOLATION AND CHARACTERIZATION OF 2-ACETYL, 8,13-DIHYDROXY-11-EPI-APOTRICHOTHEC-9-ENE (15)

Fraction 16 (3346 mg) was chromatographed on silica gel by column chromatography to yield 8 sub-fractions. HPLC cleanup of fraction 16-4 (400mg) gave 16-42383 (11mg) as a pale yellow oil.

- GC Rt: 8.41 min. (528 scans)
- $[\alpha]_D$: + 43.2° (chloroform, 24.6 °C)
- MS: C₁₇H₂₆O₅; (310.178 calc. 310.178 found), m/z 43 (base), 55 (30), 81 (26), 95 (34), 97 (60), 109 (33), 123 (95), 140 (36), 149 (18), 266 (38), 310 (M⁺).
- IR: (cm⁻¹); 3509, 1740

¹H NMR: δ 0.94 (3H, H-14), 1.08 (3H, H-15, $J_{15,7} = 0.7$ Hz), 1.22 (1H, H-4 β , $J_{4\beta,3\beta} = 7.7$ Hz, $J_{4\beta,3\alpha} = 5.3$ Hz), 1.63 (1H, H-7 α , $J_{AB} = 14.1$ Hz), 1.77 (3H, H-16, $J_{16,10} = 1.6$ Hz), 1.91 (1H-H-7 β , J unresolved), 1.95 (1H, H-3 α , J unresolved), 2.05 (1H, H-3 β , J unresolved), 2.24 (1H, H-4 α , $J_{4\alpha,3\alpha} = 8.2$ Hz, $J_{AB} = 13.3$ Hz), 3.31, 3.76 (2H, H-13, $J_{AB} = 11.6$ Hz), 4.09 (1H, H-11, br. s), 4.10 (1H, H-8 β , J unresolved), 5.66 (1H, H-10, $J_{10,16} = 1.5$ Hz).

¹³C NMR: δ 16.5 (C-15), 19.5 (C-16), 19.9 (C-14), 31.8 (C-3), 32.6 (C-4), 38.5 (C-7), 43.9 (C-6), 55.7 (C-5), 64.4 (C-13), 68.8 (C-8), 75.7 (C-2), 82.3 (C-11), 92.4 (C-12), 122.7 (C-10), 136.1 (C-9), 171.1 (C=O), 21.0 (Ac-CH₃).

SYNTHESIS OF 2,8,13-TRIACETYL-11-EPI-APOTRICHOTHECENE (16)

Ac₂O (50ul) was added to a stirred solution of compound (14) (4 mg) dissolved in pyridine. After 12 hrs of stirring at room temperature, the solvents were removed under a stream of N₂, affording a clear, colourless oil (4 mg, quantitative).

MS: C₂₁H₃₀O, m/z 43 (base), 93 (38), 109 (29), 122 (48), 121 (47), 133 (24), 215 (19), 233 (24), 335 (48), 395 (M⁺).

IR: (cm⁻¹), 2950, 1741

¹H NMR: δ 1.03 (3H, H-15), 0.98 (3H, H-14), 1.22 (1H, H-4 β , $J_{AB} = 11.1$ Hz), 1.60 (1H, H-7 β , $J_{AB} = 14.8$ Hz), 1.67 (3H, H-16, $J_{16,8\beta} = 1.2$ Hz), 1.96 (1H, H-3 α , J unresolved), 2.10 (1H, H-7 β , J unresolved), 2.12 (1H, H-4 α , $J_{4\alpha,3\alpha} = 10.3$ Hz, $J_{4\alpha,3\beta} = 7.6$ Hz), 3.98, 4.28 (2H, H-13, $J_{AB} = 11.8$ Hz), 4.10 (1H, H-11, br. s), 5.09 (1H, H-2, $J_{2\beta,3\alpha} = 12.0$ Hz, $J_{2\beta,3\beta} = 6.1$ Hz).

Hz), 5.21 (1H, H-8B, $J_{8,7B} = 7.3$ Hz), 5.82 (1H, H-10, $J_{10,15} = 1.5$ Hz).

^{13}C NMR: δ 15.9 (C-15), 19.2 (C-16), 19.7 (C-14), 31.7 (C-3), 32.6 (C-4), 36.1 (C-7), 43.8 (C-6), 55.7 (C-5), 64.3 (C-13), 75.7 (C-2), 81.9 (C-11), 92.7 (C-12), 125.5 (C-10), 133.3 (C-9), 21.0, 21.1 ($\text{CH}_3\text{-Ac}$), 171.1 (C=O (Ac)).

ISOLATION AND CHARACTERIZATION OF 13-HYDROXY,2,15-DIACETOXY-11-EPI-APOTRICHOTHEC-9-ENE (17)

Fraction 20 (3564 mg) was chromatographed on silica gel by vacuum liquid chromatography (VLC), yielding 12 fractions. HPLC cleanup (5% isopropanol/hexane) of fraction 20-5 (112 mg) isolated 17 (3mg) as a pale oil.

GC Rt: 8.42 min. (348 scans)

MS: $\text{C}_{19}\text{H}_{26}\text{O}_6$; m/z 43 (base), 55 (15), 69 (11), 81 (14), 95 (15), 107 (27), 124 (44), 155 (9), 219 (21), 337 (12), 352 (M^+).

^1H NMR: δ 0.99 (3H, H-14), 1.17-2.20 (2H, H-7, J unresolved), 1.50-2.20 (2H, H-4 J unresolved), 1.95-2.20 (2H, H-3, J unresolved), 1.63 (3H, H-16), 3.48, 3.79 (2H, H-13, $J_{AB} = 12.2\text{Hz}$), 3.97, 4.15 (2H, H-15, $J_{AB} = 11.8\text{Hz}$), 4.29 (1H, H-11, J unresolved), 5.18 (1H, H-2 J unresolved), 5.53 (1H, H-10).

^{13}C NMR: δ 15.0 (C-14), 22.3 (C-16), 27.8 (C-8), 28.1 (C-7), 29.3 (C-3), 32.1 (C-4), 45.4 (C-6), 58.4 (C-5), 62.4 (C-13), 65.5 (C-15), 76.0 (C-2), 82.6 (C-11), 95.0 (C-12), 120.8 (C-10), 135.3 (C-9), 20.9/21.0 ($\text{CH}_3\text{-Ac}$), 170.9 (C=O).

ISOLATION AND CHARACTERIZATION OF SAMBUCINOL (18)

Fractions 23-24 (DF-5, 1650mg) yielded crystals upon evaporation of the solvent. Colourless needle crystals were obtained after recrystallization from acetone (112mg).

GC Rt: 7.18 min. (292 scans)

m.p.: 234-235 °C

$[\alpha]_D$: -37.4° (23 °C, pyridine)

MS: $C_{15}H_{22}O_4$ m/z 82 (78), 109 (29), 94 (24), 124 (base), 161 (22), 67 (21), 251 (20), 266 (22, M⁺).

¹H NMR: δ 0.81 (3H, H-15, $J_{1,5,7} = 0.5$ Hz), 1.04 (3H, H-14), 1.48 (1H, H-4 α , $J_{AB} = 15.1$ Hz, $J_{4\alpha,2} = 0.9$ Hz, $J_{4\alpha,3} = 4.3$ Hz), 1.73 (3H, H-16, br. s), 1.34, 1.93 (2H, H-7, $J_{AB} = 13.0$ Hz, $J_{7,8} = 5.7, 1.7$ Hz), 1.80, 2.02 (2H, H-8, J unresolved), 2.21 (1H, 13-OH, $J_{13,OH} = 6.9, 3.5$ Hz), 2.40 (1H, 3-OH, $J_{3,OH} = 6.5$ Hz), 2.57 (1H, H-4 β , $J_{AB} = 15.1$ Hz, $J_{4\beta,3} = 8.1$ Hz), 3.90 (1H, H-2, br. s), 4.03, 4.10, (2H, H-13, $J_{AB} = 11.2$ Hz, $J_{13,OH} = 6.9, 3.5$ Hz), 4.22 (1H, H-3, $J_{3,OH} = 6.5$ Hz, $J_{3,4} = 8.1, 4.3$ Hz), 5.52 (1H, H-10, m).

¹³C NMR: δ 14.9 (C-15), 16.2 (C-14), 22.6 (C-16), 28.9 (C-8), 29.7 (C-7), 45.1 (C-4), 47.7 (C-6), 51.0 (C-5), 59.4 (C-13), 72.5 (C-3), 88.2 (C-2), 93.5 (C-12), 108.1 (C-11), 116.3 (C-10), 146.0 (C-9).

ISOLATION OF 3,13-DIACETYLSAMBUCINOL (19)

Fraction 14 (3.285g) contained one major compound, neosolaniol and a number of other compounds. After extensive cleanup by column and VL

chromatography, one of the unknown compounds isolated, was purified by HPLC (6% isopropanol/hexane). Yield 3 mg.

mp: 104-105°C
 GC Rt: 5.38 min. (218 scans)
 $[\alpha]_D$: +12.3° (CHCl₃, 24.6 °C)
 MS: C₁₉H₂₆O₆ (350.187 calc. 350.198 found), m/z 43 (base), 95 (23), 109 (33), 122 (24), 133 (41), 159 (53), 171 (48), 189 (45), 215 (30), 275 (14), 335 (14), 350 (15 M⁺).
 IR: (CM⁻¹); 2950, 1741
 UV: end absorption
¹H NMR: δ 0.85 (3H, H-15), 1.00 (3H, H-14), 1.73 (3H, H-16 br. s), 1.50 (1H, H-4α, J_{4α,3} = 8.2 Hz, J_{AB} = 15.4 Hz), 1.63-2.32 (2H, H-7, J unresolved), 2.11-2.42 (2H, H-8 J unresolved), 3.92 (1H, H-2), 4.34, 4.51 (2H, H-13, J_{AB} = 12.6 Hz), 5.01 (1H, H-3α, J_{3α,4β} = 4.9 Hz, J_{3α,4α} = 8.2 Hz), 5.45 (1H, H-10 br. s), 2.01, 2.09 (CH₃-Ac).
¹³C NMR: δ 14.8 (C-15), 16.3 (C-14), 22.8 (C-16), 29.0 (C-8), 30.3 (C-7), 42.3 (C-4), 47.7 (C-6), 50.7 (C-5), 60.0 (C-13), 74.6 (C-3), 84.2 (C-2), 92.2 (C-12), 108.3 (C-11), 116.1 (C-10), 145.8 (C-9), 170.1, 170.8 (C=O), 20.9, 21.7 (Ac-CH₃).

PREPARATION OF 3,13-DIACETYLSAMBUCINOL (19)

Sambucinol (**18**) (32mg 0.12 mol) was dissolved in dry pyridine (1000uL). Acetic anhydride (250uL) was added to the stirred solution. After 18 hours, the reagents were removed by a stream of nitrogen, leaving a pale yellow oil. The oil was dissolved in an excess of MeOH and 5 uL of H₂O was added. On evaporation of

the solvents, plate-like crystals had formed (Yield: 43mg, quantitative).

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MS and NMR data: page 95

PREPARATION OF 3,13-DIACETYL-ISOSAMBUCINOL (20)

3,13-Dihydroxysambucinol (19) (42.6 mg, 0.116 mmol) was dissolved in acetic anhydride (250 μ L). To the stirred solution was added $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (25 μ L). After 24 hours, the pale yellow solution was quenched with H_2O (500 μ L) and extracted with ether (3 x 250 μ L). The ethereal phase was washed with NaHCO_3 (5%), dried over Na_2SO_4 and evaporated in vacuo, affording a pale red oil (Yield: crude 31.4 mg). The oil was chromatographed by HPLC (CN column, 2% isopropanol/hexane, flow rate; 4ml/min, UV; 205nm.) which separated two compounds in a 45:55 ratio. the first compound to elute was (19) (R_t = 10.5 min), which was recovered. Compound 20 had a retention time of 15.0 min.

m.p: 74-75°C

GC R_t : 7.40 min. (296 scans)

$[\alpha]_D$: +40.8° (pyridine, 23°C)

MS: $\text{C}_{19}\text{H}_{26}\text{O}_6$ (350.178 calc. 350.175 found), 43 (base), 122 (36), 133 (41), 146 (21), 159 (55), 171 (50), 189 (45), 350 (42, M^+).

IR: (CM $^{-1}$); 1738

UV: end absorption

^1H NMR: δ 0.88 (3H, H-14), 1.04 (3H, H-15), 1.47 (1H, H-4 α , J_{AB} = 15.8 Hz, $J_{4\alpha 3}$ = 8.2 Hz), 1.63- 2.32 (2H, H-7, J unresolved), 1.65 (3H, H-16), 2.11- 2.42 (2H, H-10, J unresolved), 2.70 (1H, H-4 β , $J_{4\beta 3}$ = 3.2 Hz),

3.86 (1H, H-2), 4.45, 4.40 (2H, H-13, $J_{AB} = 12.7$ Hz), 4.98 (1H, H-3 α , $J_{3\alpha 4} = 3.2$ Hz, $J_{3\alpha 4} = 8.2$ Hz), 5.27 (1H, H-8, br. s), 2.01, 2.09 (CH₃-Ac).

¹³C NMR: δ 16.2 (C-15), 17.6 (C-14), 22.3 (C-16), 32.5 (C-10), 34.7 (C-7), 42.6 (C-4), 48.9 (C-6), 50.2 (C-5), 60.1 (C-13), 73.8 (C-3), 82.8 (C-2), 93.7 (C-12), 110.0 (C-11), 119.5 (C-8), 129.2 (C-9), 170.2, 170.8 (C=O), 20.7, 20.9 (Ac-CH₃).

XVI: REFERENCES CITED

1. Tamm Ch. and Tori M. in **Mycotoxins-developments in Food Science-8**, edit. Betina V., Elsevier, Amsterdam, Chp. 8, pp. 131-183, (1984).
2. **Mycotoxins- A Canadian perspective**, ed. P.M. Scott; H.L. Trenholm and M.D. Sutton, NRCC Publications, Ottawa, (1985).
3. **Trichothecenes- developments in Food Science 4**, edit. Ueno Y., Elsevier, Amsterdam, (1983).
4. **Mycotoxins-in human and animal health**, (eds. Rodricks J.V, Hesselstine C.W., Mehlman M.A.), Pathotox Publishers Inc., Park Forest South, Illinois, pp. 187-295, (1977).
5. **Mycotoxins- formation, analysis and significance**, J.E. Smith and M.O. Moss, J. Wiley & Sons, Chichester, (1985).
6. Mirocha C.J., Sadanand P.V. and Christensen C.M. in: **Mycotoxic Fungi, Mycotoxins, Mycotoxicoses**, vol 1, (eds. Wyllie T.D. and Morehouse L.G.), Markel Dekker Inc., New York, Section 2.5, (1977).
7. G.G Freeman and R.I. Morrison, **Nature**, **162**, 30 (1948).
8. S. Abrahamsson and B. Nilsson, **Proc. Chem. Soc.**, **188**, (1964).
9. W. Godtfredsen and S. Vangedal, **Acta Chem. Scand.**, **19**, 1088, (1965).
10. Snyder P.A, **J. Food Protection**, **49**, pp 544-569, (1986).

11. J. Gilbert, The detection and analysis of *Fusarium* mycotoxins, in: Applied Mycology of *Fusarium*, ed. M.O. Moss and J. E. Smith, Cambridge University Press, Cambridge, pp. 175-193, (1984).
12. Advances in Chemistry Series, vol. 149, edit. Rodricks J.V., pp 1-144, American Chemical Society, (1976).
13. Savard M.E.; Blackwell B.A.; Greenhalgh R., Can. J. Chem., 65, pp 2254-2262, (1987).
14. Grove J.F. J. Chem. Soc. Per. Trans I, 647-654, (1986).
15. J.K.M. Sanders and B.K Hunter, Modern NMR Spectroscopy, Oxford University Press, Oxford, (1987).
16. D.L. Pavia, G.M. Lampman and G.S. Kriz, Introduction to Spectroscopy, Saunder College Publishing, Philadelphia, (1979).
17. Mohr P.; Tamm C.; Zurcher W., Helv. Chim. Acta, 67, pp. 406-412, (1984).
18. Corley D.G.; Rottinghaus G.E.; Tempesta M.S., J. Org. Chem., 52, pp. 4405-4408, (1987).
19. Jean-Pierre Charland, Personal communication, National Research Council (NRC), Ottawa.
20. Crystal Structure Analysis, 2nd edit., Glusker J.P. and Trueblood K.N., Oxford University Press, New York, (1985).

21. J.R. Bamburg and F.M. Strong, in: **Microbial toxins**, vol. 7 (eds. Ajl S.J., Ciegler A., Kadis S., Montie T.C., Weinbaum G.), Academic Press, New York, chp. 4-7, (1971).
22. Zoffe A.Z. **Fusarium species**, chp. 3, John Wiley & sons, New York, (1986).
23. W.F.O. Marasas, P.E. Nelson and T.A. Toussoun, **Toxigenic Fusarium species**, Pennsylvania State University Press, pp. 12-92, (1984).
24. Greenhalgh R.; Blackwell B.A.; Savard M.; Miller J.D; Taylor A., **J. Agric. Food Chem.**, 36, pp. 216-219, (1988).
25. Corley D.G.; Rottinghaus G.E.; Tempesta M.S., **J. Nat. Prod.**, 50, 897-902, (1987).
26. Visconti A.; Mirocha C.J.; Bottalico A.; Chelkowski J., **Mycotoxin Research**, 1, pp. 3-10, (1985).
27. S. Nozoe and Y. Machida, **Tetrahedron Lett.**, 2671-2674, (1970).
28. Greenhalgh R.; Blackwell B.A.; Pare J.R.; Miller J.D.; Levandier D.; Meier R-M.; Taylor A.; ApSimon J.W., **J. Proceeding, Sixth International Symposium on Mycotoxins and Phytotoxins**, Elsevier Press, Amsterdam, pp 137-152, (1986).
29. Greenhalgh, R.; Meier, R-M ; Blackwell, B.A.; Miller, J.D.; Taylor, A.; ApSimon, J.W., **J. Agric. Food Chem.** 34, 115-118, (1986).

30. P.M. Scott, P.Y. Lau and S.R. Kanhere, J. Assoc. Off. Anal. Chem., **64**, pp. 1364-1370, (1981).
31. H.L. Trenholm, R.M.G. Hamilton, D.W. Friend, B.K. Thompson and K.E. Hartin, J. Am. Vet. Med. Assoc., **185**, pp. 527-531, (1984).
32. J.C. Coll and B.F. Bowden, J. Nat. Prod., **49**, pp. 892-906, (1984).
33. S.W. Pelletier, H.P. Chokshi and H.K. Desai, J. Nat. Prod., **49**, pp. 934-936, (1986).
34. Corley D.G.; Rottinghaus G.E.; Tracy J.K.; Tempesta M.S., Tetrahedron Lett., **27**, pp 4133-4136, (1986).
35. R-M. Meier, MSc Thesis, Carleton University, (1985).
36. D.L. Lauren, A. Ashley, B.A. Blackwell, R. Greenhalgh, J.D. Miller and G.A. Neish, J. Agric. FoodChem., (1987), in press.
37. Zamir L.O.; Devor K.A.; Nadeau Y.; Sauriol F., J. Biol. Chem., **262**, pp. 15348-15353, (1987).
38. R. Greenhalgh et al., to be published.
39. Greenhalgh R.; Levandier D.; Adams W.; Miller J.D.; Blackwell B.A.; McAlees A.J.; Taylor A., J. Agric. Food Chem., **34**, pp. 98-102, (1986).
40. Miller J.D. and B.A. Blackwell, Can. J. Bot., **64**, 1-5, (1986).

APPENDIX 1**¹H NMR SPECTRA**

Chemical shifts are referenced to deuteriochloroform at 7.25 ppm and reported relative to tetramethylsilane (Me₄Si).

3'-HYDROXY T-2 TOXIN

[illegible]

4,15-DIACETYL-7-DEOXYNIVALENOL

3 β ,13-DIHYDROXY-11-EPI-APOTRICHOTHEC-9-ENE

13-HYDROXY-3 α ,11-EPOXY-APOTRICHOTHEC-9-ENE

2-ACETOXY,8,13-DIHYDROXY,11-EPI-APOTRICHOTHEC-9-ENE

13-HYDROXY-2,15-DIACETOXY,11-EPI-APOTRICHOTHEC-9-ENE

SAMBUCINOL

3,13-DIACETETLSAMBUCINOL

3,13-DIACETYL-SAMBUCINOL

3,13-DIACETYL-ISOSAMBUCINOL

APPENDIX 2

^{13}C NMR SPECTRA

Chemical shifts are referenced to deuteriochloroform at 77.0 ppm and reported relative to tetramethylsilane (Me_4Si).

T 2 TOXIN

3'-HYDROXY T-2 TOXIN

4-DEACETOXY T-2 TOXIN

4,15-DIACETYL-7-DEOXYNIVALENOL

3,13-DIHYDROXY-11-EPI-APOTRICHOTHEC-9-ENE

8-HYDROXY,2,13-DIACETOXY,11-EPI-APOTRICHOTHEC-9-ENE

2-ACETOXY,8,13-DIHYDROXY,11-EPI-APOTRICHOTHEC-9-ENE

2,8,13-TRIACETOXY,11-EPI-APOTRICHOTHEC-9-ENE

13-HYDROXY-2,15-DIACETOXY,11-EPI-APOTRICHOTHEC-9-ENE

SAMBUCINOL

3,13-DIACETYL-SAMBUCINOL

3,13-DIACETYL-ISOSAMBUCINOL

APPENDIX 3 **$^1\text{H}/^1\text{H}$ and $^1\text{H}/^{13}\text{C}$ SPECTRA**

8-HYDROXY,2,13-DIACETOXY,11-EPI-APOTRICHOTHEC-9-ENE

132

8-HYDROXY,2,13-DIACETOXY,11-EPI-APOTRICHOTHEC-9-ENE

134

APPENDIX 4**MASS SPECTRA**

3'-HYDROXY T-2 TOXIN **4-DEACETOXY T-2 TOXIN**

3 α ,13-DIHYDROXY-11-EPI-APOTRICHOTHEC-9-ENE **3 β ,13-DIHYDROXY-11-EPI-APOTRICHOTHEC-9-ENE**

13-HYDROXY,3 α ,11-EPOXY-11-EPI-APOTRICHOTHEC-9-ENE **13-HYDROXY,2,15-DIACETOXY,11-EPI-APOTRICHOTHEC-9-ENE**

2-ACETOXY,8,13-DIHYDROXY,11-EPI-APOTRICHOTHEC-9-ENE **8-HYDROXY,2,13-DIACETOXY,11-EPI-APOTRICHOTHEC-9-ENE**

2,8,13-TRIACETOXY,11-EPI-APOTRICHOTHEC-9-ENE **8-HYDROXY,2,13-DIACETOXY,11-EPI-APOTRICHOTHEC-9-ENE**

APPENDIX 5

ORTEP X-RAY PROJECTIONS

3*B*,13-DIHYDROXY-11-EPI-APOTRICHOTHEC-9-ENE (ORTEP 11)

(ORTEP 11)

13-HYDROXY-3 α ,11-EPOXY-APOTRICHOTHEC-9-ENE

APPENDIX 6**Compounds isolated from F. sporotrichioides DAOM 165006**

* : never previously isolated

** : previously detected but not characterized

T-2 TOXIN (7)

 $C_{24}H_{34}O_6$, MW 466

3'-HYDROXY T-2 TOXIN (8)

 $C_{24}H_{34}O_{10}$, MW 382

4-DEACETOXY T-2 TOXIN (9) *

 $C_{22}H_{32}O_7$, MW 384

4,15-DIACETYL-7-DEOXYNIVALENOL (10)

 $C_{19}H_{24}O_6$, MW 380 3 α ,13-DIHYDROXY-11-EPI-

APOTRICHOTHEC-9-ENE (11) **

 $C_{15}H_{24}O_3$, MW 252 3 β ,13-DIHYDROXY-11-EPI-

APOTRICHOTHEC-9-ENE (12) **

 $C_{15}H_{24}O_3$, MW 252

13-HYDROXY,3 α ,11-EPOXY-
APOTRICHOTHEC-9-ENE (13) **
 $C_{15}H_{22}O_3$ MW 250

13-HYDROXY-2,15-DIACETOXY-11-
EPI-APOTRICHOTHEC-9-ENE (17) *
 $C_{19}H_{22}O_4$ MW 266

8-HYDROXY,2,13-DIACETOXY-11-
EPI-APOTRICHOTHEC-9-ENE (14) *
 $C_{19}H_{26}O_6$ MW 352

2-ACETOXY,8,13-DIHYDROXY-11-
EPI-APOTRICHOTHEC-9-ENE *
 $C_{17}H_{26}O_6$ MW 310

SAMBUCINOL (18)
 $C_{15}H_{22}O_4$ MW 266

3,13-DIACETYL-SAMBUCINOL (20) *
 $C_{19}H_{26}O_6$ MW 352

END

18-02-90

FIN