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ISOLATION AND IDENTIFICATION OF MINOR METABOLITES FROM
FUSARIUM ROSEUM (ATCC 28114).

by

Rose-Marie A. Meier, B.Sc.
(Carleton University)

A thesis submitted to the Faculty of Graduate
Studies and Research in partial fulfillment
of the requirements for the degree of
Master of Science.

Department of Chemistry
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June, 1985

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The undersigned recommend to the Faculty of Graduate Studies
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ABSTRACT

Minor metabolites produced by Fusarium roseum (ATCC 28114) in liquid culture were isolated and purified from the crude fungal extract. Through the use of ^1H and ^{13}C NMR, these compounds were assigned the following structures; 3α -acetoxy- 7α ,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one[•], 3α ,15-diacetoxy- 7α -hydroxy-12,13-epoxytrichothec-9-en-8-one[•], 3α -acetoxy-15-hydroxy-12,13-epoxytrichothec-9-en-8-one[★], 3α -acetoxy-12,13-epoxytrichothec-9-ene[★], 3α -acetoxy-7-hydroxy-12,13-epoxytrichothec-9-ene[★], 3α -acetoxy-8-hydroxy-12,13-epoxytrichothec-9-ene[★], 3α ,15-diacetoxy-12,13-epoxytrichothec-9-ene[★], 3α -acetoxy-15-hydroxy-12,13-epoxytrichothec-9-ene[★], 3α ,15-diacetoxy-7,8-dihydroxy-12,13-epoxytrichothec-9-ene[★], 3α ,15-diacetoxy-7-hydroxy-12,13-epoxytrichothec-9-ene[★], 3α ,15-diacetoxy-8-hydroxy-12,13-epoxytrichothec-9-ene[★], sambucoin[○], "apotrichothecene"[★], culmorin[★], and culmorone[★]. Considering the variety of compounds isolated, some biosynthetic routes to the trichothecenes have been proposed.

Legend: ★unaware of its previous isolation

★detected previously in extracts of F. culmorum

•detected previously in extracts of F. roseum

○detected previously in extracts of F. sambucinum

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NOMENCLATURE USED IN THIS THESIS

Common names for all compounds and systematic names for trichothecenes based on the trichothecene skeleton (3)

Deoxynivalenol (= DON, Vomitoxin) = 3 α ,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one (39)

3-Acetyldeoxynivalenol (= 3-AcDON) = 3 α -acetoxy-7 α ,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one (40)

3,15-Diacetyldeoxynivalenol (= 3,15-diAcDON) = 3 α ,15-diacetoxy-7 α -hydroxy-12,13-epoxytrichothec-9-en-8-one (41)

3-Acetyl-7-deoxydeoxynivalenol (= 3-Ac-7-deoxyDON) = 3 α -acetoxy-15-hydroxy-12,13-epoxytrichothec-9-en-8-one (42)

Isotrichodermin = 3 α -acetoxy-12,13-epoxytrichothec-9-ene (43)

7-Hydroxyisotrichodermin = 3 α -acetoxy-7-hydroxy-12,13-epoxytrichothec-9-ene (45)

8-Hydroxyisotrichodermin = 3 α -acetoxy-8-hydroxy-12,13-epoxytrichothec-9-ene (46)

Calonectrin = 3 α ,15-diacetoxy-12,13-epoxytrichothec-9-ene (47)

15-Deacetylcalonectrin = 3 α -acetoxy-15-hydroxy-12,13-epoxytrichothec-9-ene (49)

3,15-Diacetyl-7,8-dihydroxydeoxynivalenol (= DDD = 7,8-Dihydroxycalonectrin) = 3 α ,15-diacetoxy-7,8-dihydroxy-12,13-epoxytrichothec-9-ene (50)

7-Hydroxycalonectrin = 3 α ,15-diacetoxy-7-hydroxy-12,13-epoxytrichothec-9-ene (51)

8-Hydroxycalonectrin= 3 α ,15-diacetoxy-8-hydroxy-12,13-epoxy-
trichothec-9-ene (52)

Sambucoid (53)

"Apotrichothecene" (54)

Culmorin (55)

Culmorone (59)

INTRODUCTION

1-1 Mycotoxins

The term mycotoxin is derived from the Greek word "mykes" meaning fungus and the Latin word "toxicum" meaning poison (1). This term is used to describe naturally occurring toxic secondary metabolites, produced by fungi, which have been shown to represent a significant health hazard to humans as well as animals through ingestion of contaminated foodstuffs (2-9).

Mycotoxicosis, or mycotoxin induced diseases resulting from their ingestion, have plagued mankind since ancient times, the earliest recorded reference being in the Book of Amos Chapter 4, verse 9:

"I smote you with blight and mildew" (10)
(ca. 760 B.C. (1))

It is believed that many documented illnesses and epidemics, initially attributed to other causes, may actually have been due to mycotoxicosis (11). The Soviet

Union has been affected by cases of mycotoxicosis, for example during 1944, just over 10% of the population of Orenburg, near Siberia, was stricken with alimentary toxic aleukia (ATA) which is characterized by symptoms such as fever; a hemorrhagic rash; bleeding from the nose; throat and gums; necrotic angina; extreme leukopenia; agranulocytosis; sepsis and exhaustion of the bone marrow, sometimes causing death (12). This was thought to result from the ingestion of grain which had become mouldy due to overwintering in the fields (6, 7).

Another classic case of mycotoxicosis occurred in late 1960 when 100,000 young turkeys in the United Kingdom suddenly died from an unidentified disease termed "Turkey X Disease" (13). It was later established that this was a result of feed contaminated with the mould Aspergillus flavus, a common and widely distributed food spoilage fungus. When grown on suitable substrates, it produced a number of highly toxic metabolites known as the aflatoxins (9, 14, 15). These compounds have since been shown to be highly carcinogenic (8, 16), with Aflatoxin-B₁ (1) being recognized as one of the most active hepatocarcinogens yet discovered (8, 17, 18).

1

In addition to the contamination of animal feed with aflatoxins, they are also present in grains and nuts (19) preserved through reduction of their water content (20).

Table 1 presents a summarized list of documented cases involving mycotoxicosis as presented by L. Fishbein (9).

★
TABLE 1 Summarized List of Studies of Mycotoxins

Disease and date of outbreak	Affected organism	Pathogenic organism	Mycotoxin	Location
Ergotism, 1762, 1966	Humans, cattle, sheep	<i>Claviceps purpurea</i>	Ergot alkaloid	Europe
Alimentary toxic aleukia, 1913, 1942	Humans, cattle, horse, pig, dog	<i>Fusarium sporotrichoides</i>	Sporofusarin	Soviet Union
Fusariotoxiosis, 1928	Swine, horse, humans, duckling	<i>Fusarium nivale</i>	Nivalenol, fusarenon-X	Japan
Yellowed rice toxiosis, 1940	Chicken	<i>Fusarium surin</i> <i>Penicillium islandicum</i>	Diacetoxyscorpennol Luteoskyrin, cyclochlorotine	United States, Europe Japan
Turkey-X, 1960	Turkey, trout	<i>Aspergillus flavus</i>	Aflatoxins	Britain, United States
Hepatitis X, 1950	Dog	<i>Aspergillus nidulans</i>	Sterigmatocystin	South Africa
Hyperkeratosis, 1953, 1961	Calf	<i>Aspergillus clavatus</i>	Patulin	United States
Hepatitis X, 1952	Dog, swine (Wheat, Katsubushi)	<i>Penicillium urticae</i> , etc. <i>Penicillium rubrum</i> <i>Aspergillus ochraceus</i>	Penicillic acid Rubratoxin B Ochratoxin A	United States United States Japan, South Africa

★ Taken from p.179 of reference 9.

Mold contamination of food and feeds is a constantly recurring phenomenon and for years, this was regarded as harmless. It was not until the early sixties that an increased awareness prevailed concerning the toxic effects that fungal secondary metabolites were capable of inducing, even though fungi had long been recognized as active producers of antibiotic materials such as penicillin and streptomycin. As mentioned by Scott (21), it seemed somewhat peculiar that prior to this time, little or no thought had been given to the possibility that fungal metabolites could be toxic to higher organisms including man.

Fungi are known to produce different classes of mycotoxins (9, 22), including the rubratoxins (22), ochratoxins (22), furanocoumarins (22) and aflatoxins. Even though these different classes are grouped into the category of mycotoxins, they bear little or no physical resemblance to each other. One class, not previously mentioned, is the trichothecenes which are gaining increased significance due to their effect on global agriculture as well as their possible use as chemical warfare agents *. The trichothecenes exhibit a wide range of biological activities depending upon the functional groups present on this unique tricyclic skeleton.

*
Note:

The analysis of leaves, water, cereal grains, soil and yellow powder as well as blood, urine, and body tissues from chemical warfare victims from regions of Southeast Asia (Laos and Kampuchea; July 1981-March 1982), gave a positive result for the presence of the trichothecene mycotoxins, in levels which seemed improbable to have occurred naturally, raising suspicions as to their use as chemical warfare agents (23-28).

1-2 TRICHOTHECENES

1-2-a Introduction

The trichothecenes encompass a broad group of naturally occurring sesquiterpenoids produced by such fungal species as Fusarium, Trichoderma, Trichothecium, Myrothecium, Stachybotrys, Acremonium (Cephalosporium), Cylindrocarpon, and possibly Dendrochium (7, 29, 30). Each of these species produces a number of toxins with the fungi being found throughout the world in plants and agricultural products. The trichothecenes are generally colourless, crystalline, optically active compounds which are stable in the solid state. Their physical constants and spectral data as well as those for other mycotoxins have been collated by Cole and Cox (31).

The name trichothecane was proposed in 1967 by Godtfredsen et al. (32) for a group of naturally occurring sesquiterpenoids produced by various species of Fungi Imperfecti. (The imperfect state is synonymous with the anamorph (asexual) state (7)). Their tricyclic skeleton was found to be composed of a cyclohexane, cyclopentane and a

six-membered oxirane ring in addition to four methyl groups
(2).

2

The majority of these mycotoxins contain an epoxide bridging the C-12,13 positions plus an olefinic double bond linking the C-9,10 positions. Hence the name trichothecene, or more specifically, 12,13-epoxytrichothec-9-ene (3) (2). The numbering system for the trichothecene ring is shown in (3). The possibility for different substituents at the C-3,4,15,7 and 8 positions, i.e. R_1 - R_6 , results in a substantial number of potential trichothecenes, approximately 75 of which have been isolated to date, particularly during the last few years, due to increased scientific awareness of their potential as well as through the availability of improved separation and analytical techniques.

3

1-2-b Categorization

It is possible to categorize the trichothecenes into four groups according to their structural features, as first proposed by Ueno in 1977 (33). Hydroxy and acyloxy substituted trichothecenes were assigned to class A, the 8-keto derivatives to class B, 4,15-macrocyclic derivatives to class C and 7,8-epoxy derivatives to class D. Since then, Bamburg (34) has added an additional group known as class E, to represent the 4,15-macrocyclic trichothecodiene, verrucarin K. (Figure 1).

Appendix 1 (p. 192) presents a list of the trichothecenes discovered to date along with their presently accepted structures.

FIGURE 1: The Trichothecene Classes

class A

(hydroxy and acyloxy derivatives)

class B

(8-keto derivatives)

class C

(macrocyclic derivatives)

class D

(7,8-epoxy derivatives)

class E

(trichothecodiene derivatives)

1-2-c Historical Background (2).

In 1946, Brian and McGowan (35) isolated the first crystalline and seemingly homogeneous metabolite of the trichothecene type from a culture of Metarrhizium glutinosum S. Pope (a Myrothecium sp.). They named it Glutinosin and it was found to have a high fungistatic activity displaying powerful dermatitic properties, with a molecular formula represented by $C_{48}H_{60}O_{16}$. Glutinosin was later shown (36) to be a mixture of verrucarins A (4) and verrucarins B (5) in the ratio of approximately 4:1.

It was a result of the testing of numerous culture filtrates of Fungi Imperfecti which led to the observation that antifungal activity was also present in strains of Fusarium, Trichoderma, Gliocladium, Trichothecium, and Myrothecium, among others (2). As a result of efforts to characterize the active principles responsible for the antifungal activity, trichothecin, the first trichothecene to be obtained in a pure form, was isolated from a strain of Trichothecium roseum Link (syn. Cephalothecium roseum Corda) by Freeman and Morrison in 1948 (37, 38). The molecular formula was determined to be $C_{19}H_{24}O_5$. When subjected to alkaline hydrolysis, it gave isocrotonic acid and a neutral keto alcohol, $C_{15}H_{20}O_4$, which was named trichothecolone. The structures proposed for trichothecin (6) and trichothecolone (7) are shown here:

old structures

It should be noted that the 4-membered oxide ring had not been previously encountered in natural products prior to its proposal here (40).

Evidence supporting structures (6) and (7) was obtained through a variety of chemical reactions (39, 41) including oxidation, hydrolysis and treatments with acid and base. For example, the treatment of trichothecolone (7) with HCl led to the isolation of a chlorohydrin ($C_{15}H_{21}O_4Cl$) (8) while treatment of (7) with dilute H_2SO_4 led to the isolation of a glycol ($C_{15}H_{22}O_5$) (9).

7 (old structure)

x = HCL 8 ; R = Cl

x = 0.5 N H_2SO_4 9 R = OH

In the decade following the discovery of trichothecin (37), no new trichothecenes were reported until the year 1959 when in the period up to 1965, a further 13 compounds were isolated (2) as a result of increased investigations into the biologically active substances produced by these various species of fungi due to screening for cytostatic and antifungal activity.

In 1961, Brian et al. (42) reported on a new trichothecene which was highly toxic to plants as well as animals. They identified it as diacetoxyscirpenol, the diacetate of the alcohol scirpentriol, $C_{15}H_{22}O_5$. Scirpentriol itself was found to be less phytotoxic than diacetoxyscirpenol. No structure was proposed.

In 1962, Tamm and Gutzwiller published studies on verrucarín A (43) and proposed a partial structure. It had been recently isolated as one of a group of antibiotics with high cytostatic activity (44). Base catalyzed hydrolysis of verrucarín A gave a sesquiterpene alcohol, verrucarol, $C_{15}H_{22}O_4$ (10). From its U.V., I.R., and NMR data (2, 43-45) it was concluded that it possessed: 1) a stable ether linkage possibly in a cyclic structure, 2) a trisubstituted olefinic double bond carrying a methyl group

since upon reduction of the double bond, the tertiary methyl group was transformed into a secondary methyl group, 3) two hydroxyl groups which could be readily acetylated, and 4) an epoxy group.

Catalytic hydrogenation of the double bond to yield the dihydro derivative dihydroverrucarol, $C_{15}H_{24}O_4$, followed by $LiAlH_4$ reduction, resulted in the formation of a triol $C_{15}H_{26}O_4$. This new product contained the secondary and tertiary methyl groups of the starting material as well as a new tertiary methyl and hydroxyl group. These results were interpreted as indicating the presence of a terminal epoxy group in verrucarol (43, 45). Treatment of verrucarol (10) with concentrated HCl gave a chlorohydrin whereas treatment of verrucarol (10) with dilute H_2SO_4 led to the formation of a tetraol. These four hydroxyl groups were found to be stable to sodium periodate as well as lead tetraacetate leading them to believe that a 1,3-diol group originating from an oxetane ring was present instead of a 1,2-diol as would be expected from an oxiran ring. Therefore, in 1963, Gutzwiller and Tamm proposed the structure, as shown, for verrucarol (10) (46).

10 (old structure)

1-2-d Revision of the Trichothecene Skeleton to the Presently Accepted Structure

The turning point in the history of trichothecene development came during the years 1964-1965 when the correct ring structure for the trichothecenes was established as a result of the work by Danish scientists, Godfredsen and Vangedal. They were investigating the chemical nature of a new fungal metabolite, trichodermin, $C_{17}H_{24}O_4$ (45, 47), which had been isolated from a strain of Trichoderma viride. Based on the previous skeleton for the trichothecenes, one would have expected the structure of trichodermin (11) to be as represented below:

11 Expected "old" structure for trichodermin

After subjecting trichodermin to a variety of chemical reactions (45) such as alkaline hydrolysis to yield trichodermol, $C_{15}H_{22}O_3$, the NMR data, in each case, indicated an AB system composed of 2 doublets centered at 2.79ppm and 3.09ppm. The magnitude of the coupling constant ($J = 4\text{Hz}$) was believed to favor the presence of an epoxide rather than the methylene group in an oxetane ring (45).

If structure (11) was valid for trichodermin, the results of certain reactions of trichodermin and its derivatives were difficult to interpret. To ascertain the correct structure of trichodermin, a single crystal X-ray analysis of the trichodermol p-bromobenzoate ($C_{22}H_{25}O_4Br$) was conducted (48), with the results unambiguously indicating the presence of an epoxide ring as well as the relative configuration for the molecule. Structures (12) and (13) represent the currently accepted structures (40, 47), for trichodermin and trichodermol respectively.

12 R = Ac

13 R = H

Gutzwiller *et al.* (49) were able to interrelate verrucarol (14) with trichodermin (12) and trichothecolone (15) by converting all three to a common product, 4-O-mesyl trichothecolone (16) thus proving that verrucarol (14) had the same ring system as trichodermin (12) and trichothecolone (15) as illustrated in Figure 2. This in turn led to a revision of the other structures of the trichothecenes including diacetoxyscirpenol (50) and trichothecin (48).

The years 1967 through 1969 saw the characterization of 8 new trichothecenes (2). Since then, many more have been isolated as shown in Appendix 1.

FIGURE 2: The Interrelation of Trichodermin (12) with Verrucarol (14) and Trichothecolone (15)

1-2-e Ring System Rearrangements (2,45)

It was the result of the ring system rearrangements, which appear to be characteristic for the trichothecenes, that greatly complicated the early stages of the structural elucidation problem. In light of the revised trichothecene skeleton, the reaction mechanisms can now be postulated as follows:

i) Rearrangement with mild acid.

(14) verrucarol

"apotrichothecene" ring system

ii) Rearrangement with mild alkali

1-3 Biosynthesis: (51)

Fishman et al. (1959) (52) and Jones and Lowe (1960) (53) were the first to study the biogenesis of the trichothecenes, specifically trichothecin (6), as a result of the intense structural work conducted by Freeman et al. (39, 34). In 1960, they stated that trichothecolone (7), the C₁₅ moiety of trichothecin (6), could not be derived by the classical head-to-tail linking of three isoprene units but rather, utilized the biochemical "isoprene unit" mevalonic acid as a major source of its carbon skeleton (53).

"old structures"

Through the use of [2-¹⁴C] mevalonate, they observed the incorporation of three of these molecules into the trichothecene nucleus of trichothecolone (7). As a result of extensive degradations, the positions of the labelled

carbon atoms were located at C-4, C-10 and C-14. The occurrence of a double 1,2-methyl group migration was revealed as having occurred during the biosynthetic formation of the skeleton. It was concluded that the farnesyl pyrophosphate (17) precursor of the trichothecenes (7), would be folded in the manner illustrated in Figure 3 (old structure), prior to cyclization.

Figure 3: Folding Pattern of the Farnesyl Pyrophosphate (17) Precursor of the Trichothecenes (old structures)

Folding Prior to Cyclization

(17) Folded Farnesyl pyrophosphate

Cyclization

Oxidation Steps

(7) Trichothecolone (old structure)

This work by Jones and Lowe (53) was carried out prior to knowledge of the correct structure for the trichothecene skeleton. In 1965, Godtfredsen and Vangedal (40) reinterpreted the results indicating that the radioactivity from [2- ^{14}C] mevalonate was distributed between C-4, C-8 and C-14 of trichothecolone (15). This was verified later by Hanson *et al.* (55), without the need for extensive chemical degradations, using [2- ^{13}C] mevalonate. After its incorporation into trichothecolone (15) by Trichothecium roseum, enrichment of the ^{13}C -NMR spectrum was seen only for the carbons at positions 4, 8 and 14. This led the authors to suggest that the farnesyl pyrophosphate precursor of the trichothecenes (17) is folded as shown in Figure 4 prior to cyclization.

When doubly labelled mevalonates of known $^3\text{H}:^{14}\text{C}$ ratios are used in feeding experiments, it becomes possible to determine the number and location of the mevalonoid hydrogen atoms that are incorporated and obtain information concerning the cyclization of the farnesyl pyrophosphate by determining the $^3\text{H}:^{14}\text{C}$ ratio of the metabolites and their degradation products. If specifically labelled geranyl and farnesyl pyrophosphates are employed, one can also determine

FIGURE 4: Folding Pattern of the Farnesyl Pyrophosphate (17)
Precursor of the Trichothecenes (presently accepted structure)

(17) Farnesyl pyrophosphate from $[2-^{13}\text{C}]$ -Mevalonate

Folding Prior to Cyclization

(17) Farnesyl Pyrophosphate

Cyclization

Oxidation Steps

15 (presently accepted structure)

which 5-carbon unit of farnesyl pyrophosphate a particular hydrogen originates from, thus disclosing any unsuspected hydride shifts. This was the case for Achilladelis et al. (56) who obtained information on the folding and cyclization of the farnesyl chain through the use of $[1-^3\text{H}_2, 2-^{14}\text{C}]$ farnesyl pyrophosphate (18) and $[2-^3\text{H}, 2-^{14}\text{C}]$ farnesyl pyrophosphate (19) as precursors of trichothecin (22) in Trichothecium roseum. The elimination of one tritium label was observed when $[1-^3\text{H}_2, 2-^{14}\text{C}]$ farnesyl pyrophosphate (18) was incorporated but for $[2-^3\text{H}, 2-^{14}\text{C}]$ farnesyl pyrophosphate (19), no tritium was lost. This result provided further support for the 6,7-trans-farnesyl pyrophosphate skeleton (20) acting as an intermediate as opposed to the 6,7-cis-farnesyl pyrophosphate skeleton (21).

By using $[2-^3\text{H}, 2-^{14}\text{C}]$ geranyl pyrophosphate (23) as the starting material, the resulting farnesyl pyrophosphate precursor to the trichothecenes would be labelled at the 6 position (24). It was observed that this tritium label at C-6 was retained during the biosynthesis and that it migrated to the 2-position of the trichothecene skeleton (25). (Figure 5).

FIGURE 5: Biosynthesis of the Trichothecene Skeleton (25) from [$2\text{-}^3\text{H}$, $2\text{-}^{14}\text{C}$] Geranyl Pyrophosphate (23)

As a result of these observations, the possibility of a bisabolene intermediate (26) was excluded, i.e., the tritium label at position C-6 of farnesyl pyrophosphate (24) would be lost. (Figure 6).

Figure 6:

The precise stereochemistry which the farnesyl pyrophosphate isomer must undergo prior to cyclization is still a controversial topic (57) with the problem concerning the isomerization of a trans-2,3 double bond (27) to a cis-2,3 double bond (28) so as to avoid the formation of trans cyclohexene (29).

Two theories are presently utilized to explain the isomerization of the trans-trans farnesyl pyrophosphate (27). The first one proposes that this trans-cis isomerization occurs via the intermediate, nerolidyl pyrophosphate (30).

30

The second theory suggests that the isomerization comes as a result of various redox schemes which share a common characteristic requiring removal of one of the C-1 hydrogen atoms. Evans et al. (58-60) presented results strongly favoring this theory through labelling studies conducted using a cell free system of Trichothecium roseum incubated with $[1,5,9\text{-}^3\text{H}_6, 4,8,12\text{-}^{14}\text{C}_3]\text{-trans,trans-farnesyl pyrophosphate}$ with a $^3\text{H}/^{14}\text{C}$ atom ratio of 6:3. Trichodiene was isolated with a $^3\text{H}/^{14}\text{C}$ atom ratio of 5.2:3. A variety of problems concerning the results by Evans have been discussed in the paper by Cane et al. (57).

In order to dispell any further confusion Cane reexamined the cell-free biosynthesis of trichodiene (57). A cell-free extract of Trichothecium roseum was incubated with $[1-^3\text{H}_2, 12,13-^{14}\text{C}_2]$ -trans,trans-farnesyl pyrophosphate (31) which had a $^3\text{H}/^{14}\text{C}$ ratio of 2:2. They obtained a crystalline derivative of the trans-diol derived from the endocyclic double bond of trichodiene (32) containing a $^3\text{H}/^{14}\text{C}$ atom ratio of 1.8:2 demonstrating that practically no loss of hydrogen from the C-1 position of the precursor had occurred. The position of the 2 tritium atoms was clearly established through degradations of the labeled trichodiene (32). This result therefore favors the involvement of the tertiary allylic isomer, nerolidyl pyrophosphate (30) for the isomerization of the trans, trans-farnesyl pyrophosphate (31) to the cis, trans-farnesyl pyrophosphate prior to cyclization. Thus, the biosynthesis of trichodiene (32) can be viewed as shown in Figure 7 (57).

FIGURE 7: Biosynthesis of Trichodiene (32) via Nerolidyl Pyrophosphate

a: Rotation about the 2,3 single bond

Recently the biosynthesis of mycotoxins produced by Fusarium graminearum has been investigated at the Chemistry and Biology Research Institute (CBRI) of Agriculture Canada. The incorporation pattern of $[1-^{13}\text{C}]$ and $[2-^{13}\text{C}]$ acetate precursors (33) and (34) respectively, was studied (61) for deoxynivalenol (3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one), also known as vomitoxin or DON (39), as well as other trichothecenes, using ^{13}C NMR spectroscopy. The results indicated that 3 mevalonate units (35) combine to form the trichothecene skeleton (38). This pattern of incorporation was seen to be consistent with the proposed biosynthetic pathway based on the formation of trichodiene (37) from farnesyl pyrophosphate (36) (51), in which 2 methyl group shifts, a 1,2 and a 1,5 are postulated to have occurred. The two methyl group shifts give rise to adjacent acetate labels at C-6 and C-15 ($[2-^{13}\text{C}]$ acetate) and C-5 and C-12 ($[1-^{13}\text{C}]$ acetate), (Figure 8) which was confirmed by the observed $^{13}\text{C}/^{13}\text{C}$ couplings.

FIGURE 8: Proposed Biosynthetic Pathway for DON (39) Showing Incorporation of [$^{13}\text{C} - 1$] acetate★ and [$^{13}\text{C} - 2$] acetate •

1-4 Toxicity (7)

The toxicity of many trichothecenes, in various species of domestic as well as laboratory animals, has been the focus of a substantial amount of research in the last few years as a result of increased awareness of their presence as natural contaminants in grains and processed foods (7, 34, 62, 63).

The LD₅₀s of several trichothecenes are listed in Table 3. The most toxic members were found to be T-2, 4-acetyl~~n~~ivalenol (fusarenon-x), nivalenol and diacetoxyscirpenol (DAS). Of those compounds tested, DON was found to be one of the least toxic.

The toxins were found to produce damage in a variety of organs and tissues with Table 4 listing some of the specific symptoms which were observed for DAS as well as T-2 toxin. The symptoms, as a result of exposure via oral, parenteral and cutaneous administration included, gastric and intestinal lesions; hematopoietic and immunosuppressive effects; central nervous system toxicity resulting in anorexia, lassitude and nausea; suppression of reproductive organ functions; and acute vascular effects leading to

TABLE 3
LD₅₀S OF SEVERAL TRICHOHECENE MYCOTOXINS IN
VARIOUS ANIMALS.*

Trichothecene	Animal	Route of Administration	LD ₅₀ mg/kg
T-2 Toxin	Chicken	Oral	4.0
	Chicken	Oral	3.6
	Rat		
	(Holtzman)	Oral	4.0
	Rat	Oral	5.2
	Trout		
	fingerlings	Oral	6.1
	Swine	Intravenous	1.21
	Guinea pig	Oral	3.1
	Mouse (ddYS strain)	Intraperitoneal	5.2
	Mouse	Oral	10.5
DAS	Chicken	Oral	5.0
	Swine	Intravenous	0.276
	Mouse	Intraperitoneal	23.0
	Mouse	Intravenous	12.0
	Rat	Intravenous	1.3
	Rat	Intraperitoneal	0.75
	Rat	Oral	7.3
	Rabbit	Intravenous	1.0
Nivalenol	Mouse (DDD or DDN strains)	Oral	4.1
4-Acetylnivalenol	Mouse (DDS strain)	Intraperitoneal	3.3
	Mouse	Subcutaneous	4.0
	Mouse (DDD strain)	Intravenous	3.4
	Mouse (DDD strain)	Per os	4.5
	Mouse (DDD strain)	Subcutaneous	4.0-4.5
	Rat (Wistar stock)	Oral	4.0
	Rat	Not reported	4.4
Solanolol	Mouse (ddYS)	Intraperitoneal	4.5
DON	Chick	Oral	4.0
Neosolanolol	Mouse (ddYS)	Intraperitoneal	4.5
HT-2 toxin	Mouse	Intraperitoneal	3.0
Diacetylnivalenol	Mouse	Intraperitoneal	3.5

* Taken from p. 101, reference 7.

TABLE 4
TOXIC EFFECTS OF TRICOTHECENES IN SEVERAL ANIMALS AT
DIFFERENT DOSE LEVELS.*

Trichothecene - T-2 toxin

Animal	Toxin Intake ^a	Effect
Cattle	0.16 mg/kg ^b	Enteritis; abomasal ulcers
	0.64 mg/kg	Death (calf); bloody feces, enteritis; abomasal and rumenal ulcers; coagulopathy
	0.6 mg/kg	Suppressed lymphoblastogenesis
Swine	12 ppm	Reduced pig and litter size
	8 ppm	No effects detected
Chickens		
Broiler	4 ppm	Oral necrosis
Broiler	4 ppm	Neural effects; seizures, loss of righting reflex
Broiler	4 ppm	Hepatic hematoma
Broiler	8 ppm	Coagulopathy
Broiler	4 ppm	Reduced gain
Broiler	1.5-3 mg/kg	Decreased rate of gain
Broiler	2.5 mg/kg	Thymic and bursal necrosis
Broiler	3 mg/kg	Malformed feathers
Laying hen	20 ppm	Decreased egg production and shell quality
Cats	0.6-1.0 mg/kg	Hematopoietic depression, hemorrhage, gastroenteritis
Trout	6.1 mg/kg	LD ₅₀ (fingerlings)
	8.0 mg/kg	Sloughing of intestinal mucosa
Rats	3.8-4.0 mg/kg	LD ₅₀ (21-day female)

Trichothecene - DAS

Animal	Toxin Intake ^a	Effect
Swine	0.38 ppm	Hemorrhagic enteritis
Chickens		
Broiler	2.5-3.5 mg/kg	Decreased rate of gain
Broiler	2.5-3.5 mg/kg	Decreased hematocrit
Broiler	2.5 mg/kg	Thymic and bursal necrosis
Rats	7.3 mg/kg ^c	LD ₅₀

* Toxin intake in mg/kg/day or feed content in ppm (1/2 g taken ad libitum).

^b Daily dose.

^c Single dose.

Taken from p. 100, reference.

hypotension and shock. In his latest review, Bamburg presents a detailed description of the symptoms associated with exposure to the trichothecene mycotoxins (34).

The National Research Council of Canada is presently formulating a bioassay method for mycotoxins using liquid yeast protoplast cultures. These cultures, containing a cell density of 1×10^6 cells/mL, are dosed with mycotoxins, at different concentrations, and the yeast survival rate is studied by determining the cell density. Tests indicate that DON exhibits a toxic as well as mitotic effect while T-2 toxin is approximately 10 fold more toxic than DON.

1-5. Mode of Action

Extensive studies (7, 33, 34, 64-66) have shown trichothecenes to be potent inhibitors of protein as well as DNA synthesis in eucaryotic cells. It is believed however that this effect on DNA synthesis is probably a result of the inhibition of protein synthesis (67).

Although many questions remain to be answered, the mechanism by which protein synthesis is inhibited by the

trichothecenes is relatively well understood (7, 33, 66, 90). Research has indicated that the trichothecene mycotoxin binds to the cellular ribosome which in turn causes an inactivation of the ribosomal cycle. As a result of the particular trichothecene which is bound, the cessation of protein synthesis may occur at the initiation, elongation or termination stage. This was first demonstrated by McLaughlin and coworkers (7, 66). For eucaryotic cells, protein synthesis has been shown to take place on polysomes, i.e., an mRNA molecule is simultaneously translated by several ribosomes into several polypeptide chains. The whole unit is called the polyribosome or polysome (Figure 9).

Figure 9: Model of Protein Synthesis and the Ribosome Cycle in Eukaryotic Cells.

I=initiation E=elongation T=termination

Numbers 1, 2, and 3 indicate the regions of action

The common requirement for the completion of each stage, i.e., initiation, elongation and termination, was found to be peptidyl transferase. Briefly, protein synthesis takes place in the following manner; the ribosomal subunits (60s and 40s) from the subunit pool join the mRNA at region 1 (Figure 9), in a complex process, during the initiation stage (I). Amino acids are then added one at a time, during the elongation stage (E), to the growing polypeptide chain to complete the protein (region 2) which when finished, will measure between 100 and 200 amino acids in length. Once the termination codon on the mRNA has been reached (termination stage T), elongation of the polypeptide chain ceases and the polypeptide chain is released from the ribosome and the ribosome is then released from the mRNA (region 3).

The trichothecenes can be divided into 3 groups (34) (Table 5) which appear to be strongly related to the size of the substrate. For example the larger trichothecenes, such as the macrocyclic ones, inhibit protein synthesis at the initiation stage whereas the smallest trichothecenes inhibit synthesis at the termination stage.

The binding site for peptidyl transferase as well as the trichothecene trichodermin was found to be on the 60s ribosome (65). This suggests that the toxic effects associated with the trichothecenes are a result of some interaction and perhaps inhibition of peptidyl transferase acting as the key step. Studies have suggested that the various trichothecenes may compete for the same binding site on the ribosome (19). To try and account for the diverse effects on protein synthesis by the trichothecenes, McLaughlin et al. (66, 68) proposed that a structure activity relationship exists. The following structural correlations have been made (34); trichothecenes bearing a relatively small substituent at the C-4 position, such as trichodermol and some of its derivatives, are inhibitors of termination. Instead, if an ester group is present at the C-4 position, the compounds become elongation inhibitors. Some terminators of elongation have no acyl group at the 4 position, but have other substituents present. If an ester group is present at C-15, and either C-3 or C-4 alcohol groups are acetylated, these compounds are initiation inhibitors (34). (Table 5).

Table 5. Effects of trichothecenes on protein synthesis in eukaryotes *

Termination inhibitors

	R ₁	R ₂	Class
Trichoderma	OH	-	T
Trichodermone	-	-	T

Elongation inhibitors

	R ₁	R ₂	R ₃	Class
Trichodermin	H	H	-	E
Verrucarol	H	H	H	E
15-Desacetyl-calonectrin	Ac	H	H	E
3,15-Didesacetyl-calonectrin	Ac	H	OH	E
Trichothecin	-	-	-	-
(8-Keto)	H	Disacetyl	H	E
Trichothecolone	-	-	-	-
(8-Keto)	H	OH	H	E
Crotocin	-	-	-	-
(7,8-epoxy)	H	Disacetyl	H	E
Crotocin	-	-	-	-
(7,8-epoxy)	H	OH	H	E
Scirpene	OH	H	OH	E-I ₁
15-Acetoxy-scirpendiol	OH	OH	Ac	E-I ₂
3-Desacetyl-calonectrin	OH	H	Ac	E-I ₂

Initiation inhibitors

	R ₁	R ₂	R ₃	R ₄	R ₅	Class
Verrucarol	-	-	-	-	-	-
A, B, C	H	MAC ^a	H	H	-	I ₁
Foridin A	H	MAC ^a	-	-	-	I ₁
T-2 Toxin	OH	Ac	Ac	H	Disoval	I ₁
HT-2 Toxin	OH	OH	Ac	H	Disoval	I ₁
Neosolaniol	OH	Ac	Ac	H	OH	-
Diacetoxy-scirpenol	OH	Ac	Ac	H	H	I ₂ -E
Calonectrin	OAc	H	Ac	H	H	I ₂ -E
Nivalenol	-	-	-	-	-	-
(8-Keto)	OH	OH	OH	OH	O	I ₂
Fusar-enon-X	-	-	-	-	-	-
(8-Keto)	OH	Ac	OH	OH	O	I ₂ -E

^aMAC=macrocyclic ring connecting R₁ and R₂.

* Taken from p. 74 of reference 34

1-6 Trichothecene Producing Fungi (7)

Trichothecenes can be produced by species of perfect as well as imperfect fungi. (The perfect state is synonymous with the teleomorph (sexual) state. The imperfect state is synonymous with the anamorph (asexual) state, (7)). Those trichothecenes lacking a macrocyclic ring, produced by species of imperfect fungi, are members of the family Monoliaceae which includes the genera Fusarium, Acremonium, Trichoderma and Trichothecium.

The taxonomy of the Fusarium species is complex. Even when the species are grown from monospore cultures, they exhibit extreme variability in their morphological characteristics. This has led to the development of numerous taxonomic systems of fusaria in the literature of which Toussoun and Nelson give a short review (69).

Nelson et al. have recently published a manual for the identification of Fusarium species (70). Of the 30 species of Fusarium listed in this manual, 17 have been reported to produce toxins. These include F. Merismoides (=F. episphaeria, in part), F. decemcellulare (=F. rigidiusculum), F. nivale, F. poae (=F. tricinctum), F.

sporotrichioides (=F. tricinatum), F. chlamydosporum (= F. tricinatum), F. avenaceum (=F. roseum), F. semitectum (=F. roseum), F. equiseti (=F. roseum), F. scirpi (=F. roseum), F. acuminatum (=F. roseum), F. heterosporum (=F. roseum), F. culmorum (=F. roseum), F. graminearum (=F. roseum), F. lateritium, F. oxysporum, and F. solani. The species names in parentheses are those from the Snyder and Hansen system (69).

1-7 Present Situation (7, 34, 71)

The global situation of trichothecene mycotoxin production in animal feeds as well as foodstuffs destined for human consumption is reviewed in the text by Ueno (71).

Researchers have noted a correlation between major outbreaks of animal toxicosis and cold, wet harvest seasons since the high moisture content of the grains cannot decrease sufficiently to prevent mould growth and as a result, mycotoxin formation occurs. The presence of trichothecenes in corn which had been associated with an outbreak of toxicosis, was first quantitated by Hsu et al. (72). T-2 toxin was identified at levels of 2 ppm. In Canada, T-2 toxin has been identified in barley while

The most frequent types of mycotoxins occurring in Canada appear to be produced primarily by the Fusarium species, i.e. zearalenone (40) (77) or F-2 toxin. These are attributed mainly to Fusarium roseum and Fusarium graminearum. The prime mycotoxicosis associated with the ingestion of zearalenone contaminated corn appears to be the estrogenic syndrome in hogs.

Table 6. Natural occurrence of trichothecenes by location *

Country	Trichothecene	Amount (µg/g)	Substrate
Austria	Deoxynivalenol	1.3-7.9	Corn
Brazil	Verrucarins/roridins	Unknown	Soil ^b
Canada	T-2 toxin	25	Barley
	Deoxynivalenol	7.9	Corn
	Deoxynivalenol	8.5	Wheat
France	T-2 toxin	0.02	Corn
	Nivalenol	4.28	Corn
	Deoxynivalenol	0.6	Corn
Germany	Diacetoxyscirpenol	31.5	Corn
Hungary	Diacetoxyscirpenol	1.5	Corn
India	Diacetoxyscirpenol	14	Corn
	T-2 toxin	4	Corn
	T-1 toxin	Unknown	Sorghum
	Trichothecin	62	Anise
	Trichothecolone	19	Anise
	4-acetyltrichothecolone	14	Anise
	4-cinnamoyltrichothecolone	35	Anise
Japan	Nivalenol	7.3	Barley
	Nivalenol	0.1-22.9	Barley
	Deoxynivalenol	3	Barley
South Africa	Deoxynivalenol	0.25-4	Corn
USA	T-2 toxin	2	Corn
	T-2 toxin	0.076	Mixed feed
	Diacetoxyscirpenol	0.8	Mixed feed
	T-2 toxin	1	-
	Diacetoxyscirpenol	10-50	-
	Deoxynivalenol	10-50	-
	Deoxynivalenol	8	Corn
	Deoxynivalenol	40	Corn
	Deoxynivalenol	0.5-10	Corn
	Deoxynivalenol	37	Corn
	Deoxynivalenol	0.1-1.8	Corn
	Diacetoxyscirpenol	0.38-0.5	Mixed feed
Cambodia	Deoxynivalenol	7.4	Corn

* Taken from p. 61 of reference 34

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TABLE 7

AVERAGE LEVELS OF DON IN WHEAT

REGION	DON (ppm) (averages)			
	CROP YEAR			
	1979	1980	1981	1982
Ontario Soft Winter Wheat	0.06	0.42	0.22	0.74
Quebec Hard Spring Wheat		0.93	3.03	0.33
Western Hard Wheat (Alberta, Saskatchewan, Manitoba)	0.03	0.02	0.03	<0.03

For Ontario soft winter wheat, the levels of DON (39) reached a peak for the 1982 crop year when the accepted temporary upper limit was set at 0.3 ppm for primary products (biscuit flour and bran) used in non-staple foods.

Plate 1: Field corn inoculated with *Tr. aurea* (p. 100-101)

The most frequent types of mycotoxins occurring in Canada appear to be produced primarily by the Fusarium species, i.e. zearalenone (40) (77) or F-2 toxin. These are attributed mainly to Fusarium roseum and Fusarium graminearum. The prime mycotoxicosis associated with the ingestion of zearalenone contaminated corn appears to be the estrogenic syndrome in hogs.

Another important aspect associated with mycotoxin contamination of food, for hogs and cattle, is feed refusal, vomiting, loss of weight and internal hemorrhaging. These symptoms are believed to be linked to the trichothecene, DON (39).

In response to the presence of these mycotoxins in Canadian grains, work by researchers at Agriculture Canada was initially directed towards the study of the trichothecene mycotoxins, DON (39), 15-AcDON and Zearalenone (40), in field corn inoculated with Fusarium graminearum, during a growing season (78). Results indicated that the toxins were found throughout the plant but mainly in the infected part (Plate 1). Studies concerning the production of DON (39) and related compounds in liquid culture, by Fusarium graminearum, were initiated (79) in response to the requirement of large amounts which could be used in animal toxicological studies.

In order to develop methods of controlling mycotoxin formation in the field, it was deemed necessary to obtain an understanding of potential steps for inhibition. In response, a biosynthetic study was initiated using labelled

Plate 1: Field Corn inoculated with *Peronospora* sp. in the field

precursors to the trichothecenes, in conjunction with ^{13}C NMR. As a prerequisite to this study, ^1H and ^{13}C spectral assignments of unlabelled DON as well as other related metabolites were verified (30, 81). This required the isolation and purification of these secondary metabolites using a large scale fermentation of Fusarium graminearum roseum. Once the individual ^1H and ^{13}C data for each compound was obtained, these values could be used to identify the components present in the crude extracts from labelling studies. In addition to gaining biosynthetic information, studies could be conducted using a variety of growing conditions to investigate how the relative amounts as well as the overall concentration of these secondary metabolites are altered.

Once substantial amounts of these various secondary metabolites have been accumulated, a variety of toxicological studies may be conducted, with the possibility that the toxic effects noted in animals may not be solely due to DON (39) but rather may be a result of some other secondary metabolite or combination thereof, i.e. synergistic effect. For example, naturally contaminated corn, with levels of DON (39) at 1-2 ppm, has been shown to be highly toxic to animals maintained on this diet.

However, animals fed "clean" feed corn spiked with comparable levels of DON (39), do not exhibit the same toxic effects suggesting that unknown toxins present in the naturally contaminated corn may be responsible.

In Japan, F. graminearum has been shown to simultaneously produce DON (39) and nivalenol (3,4-oxygenated) with the toxicity of nivalenol being at least 10 times more toxic than DON (39). The presence of nivalenol in Canadian grains has not been thoroughly investigated partly because the analytical procedure, used to extract these toxins from grain products, does not successfully analyze for nivalenol. As a result, the toxicity of minor metabolites assumes greater importance.

2-DISCUSSION

In order to produce sufficient amounts of DON (39) to be used in further toxicological studies as well as to characterize some of the many minor secondary metabolites produced in hopes of obtaining additional toxicological as well as biosynthetic information, a large scale culture of Fusarium roseum (ATCC 28114) (82) was undertaken at the Atlantic Regional Laboratory of the National Research Council in Halifax, Nova Scotia. A schematic illustration of how this large scale preparation of DON (39) was accomplished (83) is presented in figure 10. The fermentation was carried out at 28°C for eight days at the end of which the pH was raised to 9.3 and the aqueous broth was extracted with dichloromethane. The aqueous layer which contained cell walls as well as certain metabolites from the culture medium was discarded. The resulting residue was partitioned between petroleum ether and methanol. The petroleum ether layer, containing the lipids, was discarded and the methanol layer was purified, after evaporating to dryness, through the use of a Florisil column in order that the phospholipids as well as pigments could be separated from the trichothecenes. The column was eluted first with dichloromethane resulting in the isolation of fraction A.

FIGURE 10: LARGE SCALE PREPARATION OF DEOXYNIVALENOL

Elution of the column with 0.5% methanol in dichloromethane and 5% methanol in dichloromethane resulted in the isolation of fractions B and C respectively. The major product in fraction A, 3-AcDON (40) was crystallized on addition of diethyl ether and recrystallized from ~~95%~~ ethanol. Hydrolysis of the needle-like crystals resulted in the isolation of the required DON(39). The remaining oil from fraction A, after removal of 3-AcDON (40), was a potential source of a multitude of minor products (Figure 12: GC trace), a number of which were isolated using the two methods presented in figures 11a and 11b.

2-1 ISOLATION OF MINOR METABOLITES BY METHODS 1 AND 2

The initial separation of various components in fraction A was accomplished using a silica gel column. The type of silica gel used was varied in an endeavour to improve the separation. For method 1, silica gel with a particle size of 63-200 μm (70-230 mesh) was used versus 25-40 μm for method 2. A solvent system based on varying concentrations of methanol in dichloromethane was chosen for method 1. This resulted in the isolation of only 4 major fractions, each of which contained a substantial number of

other products. As a consequence, an alternative solvent system of varying concentrations of ethyl acetate in hexane, was chosen for method 2, resulting in the isolation of some 50 major fractions, some of which were relatively pure. In order for the various components of these individual fractions to be identified, it was necessary to carry out further purifications. Due to the small amount of material available (2.2 mg - 497 mg, depending upon the fraction), column chromatography as well as preparative tlc plates seemed inappropriate at the time. The apparatus chosen, known by the trade name "Chromatotron", used a circular spinning tlc plate (720 rpm) precoated to a uniform thickness with silica gel. The solvated sample was injected onto the center of the rinsed plate and migrated as a circular band, towards the perimeter of the plate, due to centrifugal forces, where sample fractions were collected.

For method 1, a substantial amount of overlap between the compounds in the four major fractions was observed. The initial purification of fractions A-2 and A-3 was attempted due to the relatively large amount of material available in comparison to fractions A-1 and A-4. The first Chromatotron run usually resulted in the isolation of

several subfractions which could then be individually rechromatographed followed by recrystallization. Fraction A-2 was initially purified using the Chromatotron and a solvent system of 30% ethyl acetate in hexane which led to the isolation of six major fractions. The further purification of three of these fractions by chromatography resulted in the isolation of isotrichodermin (43), 3-AcDON (40), 3-Ac-7-deoxyDON (42) and 8-hydroxycalonectrin (52) which had R_f values of 0.74, 0.46, 0.43 and 0.37 respectively (75% ethyl acetate in hexane).

The initial purification of fraction A-3 using the Chromatotron with a solvent system of 50% ethyl acetate in hexane, resulted in the isolation of five major fractions of which two were further purified by chromatography. This led to the isolation of culmorin (55) and 3,15-diAc-7,8-dihydroxyDON or DDD (50) with R_f values of 0.38 and 0.33 respectively (75% ethyl acetate in hexane).

For method 2, purifications were generally attempted on samples which appeared crystalline and relatively pure. In two cases the only purification required was a recrystallization from ethyl acetate and hexane; i.e.,

FIGURE 11a: ISOLATION OF MINOR METABOLITES OF *F. ROSEUM*

(METHOD 1)

FIGURE 11b: ISOLATION OF MINOR METABOLITES OF F. ROSEUM

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sambucosin (53) and "apotrichothecene" (54). The other samples however required an initial purification using the Chromatotron. For example, the initial purification of sample number 13 was attempted using ethyl acetate and hexane as the eluting solvent. A seemingly pure oil (one spot by tlc, 75% ethyl acetate in hexane) was shown to be composed of two components by GC/MS. Further attempts at purifying the material were improved using a solvent system of 5% methanol in chloroform. The R_f values for the two components then showed a difference of 0.23 allowing for a successful separation. The identity of these two compounds was subsequently determined to be culmorone (59) and calonectrin (47) with R_f values of 0.67 and 0.69 (75% ethyl acetate in hexane) or 0.63 and 0.86 (5% methanol in chloroform) respectively.

The apparent success in the separation noted here suggested a change in the Chromatotron solvent system from ethyl acetate and hexane to methanol and chloroform. The majority of the remaining samples were purified using this new solvent system.

Two silica gel plates of the purified samples were run, one in 5% methanol in chloroform and the other in 75% ethyl acetate in hexane (Plates no. 2 and 3). The

PLATE 2.

Solvent System: 5%MeOH/ CHCl_3

D
PLATE 3.

Solvent System: 75% EtOAc/Hexane

TABLE 9

Summary of R_f Values for the Minor Metabolites Isolated from
F. roseum.

Column Number	Compound	Plate # 2	Plate # 3
		5% MeOH/CHCl ₃	75% EtOAc/Hexane
1	Culmorin (<u>55</u>)	0.39	0.38
2	Culmorone (<u>59</u>)	0.63	0.67
3	Unidentified material	0.34	0.33
4	DON (<u>39</u>)	0.26	0.19
5	3-AcDON (<u>40</u>)	0.61	0.46
6	3,15-diAcDON' (<u>41</u>)	0.84	0.63
7	3-Ac-7-deoxyDON (<u>42</u>)	0.62	0.43
8	DDD (<u>50</u>)	0.58	0.33
9	Isotrichodermin (<u>43</u>)	0.86	0.74
10	7-Hydroxyiso- trichodermin (<u>45</u>)	0.65	0.55
11	8-Hydroxyiso- trichodermin (<u>46</u>)	0.64	0.44
12	15-Deacetyl- calonectrin (<u>49</u>)	0.69	0.57
13	Calonectrin (<u>47</u>)	0.86	0.69
14	7-Hydroxy- Calonectrin (<u>51</u>)	0.75	0.50
15	8-Hydroxy- Calonectrin (<u>52</u>)	0.65	0.37
16	Sambucoin (<u>53</u>)	0.64	0.71

calculated R_f values are given in Table 9. With regards to these 2 plates, 75% ethyl acetate in hexane appeared to display a generally better separatory ability for some of the compounds as compared to 5% methanol in chloroform.

From the tlc plate number 2, it seems that an initial purification using methanol in chloroform would easily remove culmorin (55), which appears to constitute the major component of the fungal extract, the unidentified material in column 3 of the tlc plate, and DON (39). This would then facilitate the further purification of the mixture.

2-2 Capillary Gas Chromatography

As a further means of identifying the individual compounds isolated, their retention times on capillary GC were determined (Figure 13), enabling a positive identification of some of the many peaks in the capillary GC spectrum of the original oil, fraction A, (Figure 12).

FIGURE 13

Capillary G.C. Trace of the Isolated Minor Metabolites
from
Fraction A (*F. roseum*)

2-3-4-DEOXYNIVALENOL (DON, VOMITOXIN) (39)

SPECTRAL ASSIGNMENT

Through the combination of peak intensity data and $^1\text{H}/^1\text{H}$ coupling constants, the ^1H NMR spectra of the pure trichothecenes, being essentially first order, can be assigned with relative ease.

In order to spectroscopically identify the metabolites isolated from the Fusarium roseum culture, a comparison was made between their ^1H and ^{13}C NMR spectra and that for DON (39). Resonances in the NMR spectra will be identified by referring to the standard numbering system of the trichothecenes, e.g. H-13⁴ refers to the methylene hydrogens of the epoxide moiety.

^1H NMR ASSIGNMENT OF DON (39)

A 250 MHz ^1H NMR spectrum of DON (39) is shown in Figure 14, p. 73. The fact that no resonances overlap,

greatly simplified the calculation of the $^1\text{H}/^1\text{H}$ coupling constants and ultimately the structural assignment.

For example:

i) a resonance characteristic of all trichothecenes is represented by the methylene protons of the epoxide moiety (H-13) which appear as an AB quartet centered at 3.11 ppm ($J = 4$ Hz) in a region free from other resonances.

ii) the proton at position 10, i.e., H-10, (6.61 ppm) appears as a doublet of quartets, due to coupling with the H-11 proton at 4.80 ppm ($J = 5.9$ Hz) combined with long range coupling to the H-16 methyl protons at 1.86 ppm. This long range coupling of the methyl protons of H-16 results in a methyl resonance characterized by very fine splitting and reduced intensity in comparison with the H-14 methyl group (1.13 ppm) allowing one to easily distinguish between the two respective methyl groups.

iii) the H-3 proton appears as a multiplet at 4.53 ppm, representing an ABXY system, as a result of coupling with the H-2 methine proton ($J = 4.5$ Hz; 3.62 ppm) and coupling with the H-4 methylene protons (2.07 ppm and 2.21 ppm). The H-4 methylene protons appear as an octet, ($J = 10.7, 4.5, 14.8$ Hz) characteristic of an ABX system.

iv) the H-15 methylene protons appear as an AB quartet at 3.73 and 3.89 ppm respectively ($J_{AB} = 11.7$ Hz), due to non-equivalence, as a result of restricted rotation.

v) the 7-OH proton of DON (39) appears to be less labile than the other two OH protons (3-OH and 15-OH) perhaps as a result of hydrogen bonding with the carbonyl oxygen at position 8 (83). Fine coupling ($J = 2.1$ Hz) is observed between the methine proton of H-7 (4.83 ppm) and the 7-OH proton (3.82 ppm) in $CDCl_3$ but not in CD_3OD where the doublet at 4.83 ppm collapses to a singlet and the doublet at 3.82 ppm disappears, due to proton exchange, allowing for an unambiguous assignment of these peaks. The 1H assignments for DON (39) are seen in Table 10, p. 84.

^{13}C NMR ASSIGNMENT OF DON (39)

The assignment of the peaks in the ^{13}C NMR spectrum of DON (39) (Figure 15, p. 74) was accomplished through the use of spin-echo as well as $^1H/^{13}C$ 2D correlation spectra.

The spin-echo method was used to determine the multiplicities for the various chemical shifts observed in the ^{13}C NMR spectrum. From this it was concluded that the

fifteen resonances present were composed of two methyl, three methylene, five methine and five quaternary carbons. The exact assignment of the individual resonances was accomplished using the results from $^1\text{H}/^{13}\text{C}$ 2D correlation spectra. For example, the ^1H methyl resonances occurring at 1.1 ppm (position 14) and 1.9 ppm (position 16) were assigned through correlation to the methyl resonances in the ^{13}C NMR at 13.9 and 14.9 ppm respectively. This same technique was utilized in assigning the resonances of C-7 and C-11 which are adjacent in both ^{13}C and ^1H spectra. A correlation was made between the known ^1H assignments for H-7 (4.83 ppm) and H-11 (4.80 ppm) and the ^{13}C resonances at 70.2 and 74.4 ppm, respectively. It was also possible to distinguish the quaternary positions, C-5 and C-6 through long range coupling which gave rise to weak crosspeaks between the C-5 chemical shift (46.0 ppm) and the H-4 protons at 2.1 ppm and between the C-6 chemical shift (52.1 ppm) and the H-7 or H-11 protons. The ^{13}C assignments for DON (39) (80) are seen in Table 11, p. 85.

FIGURE 14: DEOXYNIVALENOL
(250 MHz ^1H)

ppm FROM TMS

FIGURE 15: (62.8 MHz ^{13}C NMR Spectrum)

DEOXYNIVALENOL

2-4 IDENTIFICATION OF THE ISOLATED MYCOTOXINS

The spectroscopic analysis of the compounds isolated is discussed in terms of structurally related compounds as opposed to their order of isolation, allowing for a clearer comparison to be drawn.

2-4-1. 3-KETO DERIVATIVES

¹H assignments Table 10, p. 84.

¹³C assignments Table 11, p. 85.

3-ACETYLDEOXYNIVALENOL (3-AcDON) (40)

SPECTROSCOPIC ANALYSIS

From high resolution mass spectroscopy, the molecular ion was observed at 338.124 with the best fitting empirical formula being $C_{17}H_{22}O_7$. Compared to DON (39), the molecular formula for this compound is $C_{20}H_{26}O_7$, or 42 mass units larger suggesting the presence of an acetoxy group.

The λ_{max} for this U.V. active sample occurs at 219 nm, characteristic of an α, β -unsaturated carbonyl group.

The ^1H NMR spectrum of this sample shows resonances typical of an H-13 epoxide, H-14 methyl, and H-16 allylic methyl moieties of a trichothecene ring system similar to DON (39). Trichothecenes containing an epoxy moiety, show a characteristic AB system with a J_{AB} of 4 Hz whose chemical shift and separation of the AB doublets (Δ) are sensitive to the nature of the other substituents of the molecule, particularly those at positions 3, 4, 7, and 8 (80). For this sample, the two doublets are centered at 3.08 ppm and 3.14 ppm ($\Delta = 0.06$ ppm) compared to a value of 3.07 ppm and 3.15 ppm ($\Delta = 0.08$ ppm) for DON (39). The characteristic H-3 multiplet is shifted downfield compared to DON (39) (4.53 ppm (DON) to 5.17 ppm). This is also the case for the H-2 doublet and the H-4 multiplet (3.62 ppm (DON) to 3.87 ppm, 2.07 ppm and 2.21 ppm (DON) to 2.15 ppm and 2.35 ppm, respectively). The other resonances remain basically the same except for H-10 and H-11 both of which experience slight upfield shifts (6.61 ppm (DON) to 6.56 ppm, and 4.80 ppm (DON) to 4.66 ppm, respectively). This information, coupled with the fact that a single resonance representing a three proton singlet is observed at 2.11 ppm in the ^1H NMR but absent in the ^1H NMR of DON (39), suggests an acetoxy moiety. The existence of such a moiety is further supported by a band in the carbonyl region of the IR at 1745 cm^{-1} .

The presence of an α, β -unsaturated carbonyl, as suggested by the IR (1685 cm^{-1}), combined with the fact that little or no change is apparent for the resonances representing H-7, H-15 and H-16 in the ^1H NMR, strongly implies little or no change to the A-ring of DON (39).

Even though the resonances representing H-4 are shifted slightly downfield, in comparison to DON (39), the ABX pattern remains the same eliminating the possibility of an acetoxy substituent there. Thus, these observations would suggest a structure in which the 3-OH position of DON (39) has been acetylated. The stereochemistry of the acetoxy group at the 3 position, appears to be identical to that for the hydroxy group at the 3 position in DON (39) since, the $J_{2,3}$ value remains the same.

In comparing the ^{13}C NMR spectra for this compound and that for DON (39), seventeen carbon atoms are observed in comparison to fifteen in DON (39). These two additional carbons are represented by a methyl group at 20.9 ppm and a carbonyl at 170.3 ppm, confirming the presence of an acetoxy moiety. The majority of the resonances observed however, have identical chemical shifts to DON (39) differing by less than 1 ppm, with the exception of three resonances located

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at 40.5 ppm (methylene), 71.3 ppm (methine) and 79.1 ppm (methine). (For DON, C-2 = 80.6 ppm, C-3 = 68.6 ppm and C-4 = 43.0 ppm). The largest downfield shift would be expected for the carbon to which the acetoxy group is directly attached, i.e., C-3. Smaller shifts would be expected for C-2 and C-4. Subsequently, the resonance at 71.3 ppm is assigned to C-3 while 79.1 and 40.5 ppm are assigned to C-2 and C-4, respectively.

Therefore, the spectroscopic data displayed by this compound would suggest a structure represented by 3-acetyldeoxynivalenol (40).

3,15-DIACETYLDEOXYNIVALENOL (3,15-diAcDON) (41).SPECTROSCOPIC ANALYSIS

From high resolution mass spectroscopy, the molecular ion was observed at 380.148 with the best fitting molecular formula being $C_{19}H_{24}O_8$.

This U.V. active sample displayed a λ_{max} at 220 nm suggesting an α, β -unsaturated carbonyl group. The possibility of a trichothecene ring system for this sample was confirmed by the presence of such typical resonances in the 1H NMR as the H-13 epoxide, the H-14 methyl and the H-16 allylic methyl moieties of DON (39). Using this observation, a comparison between the molecular formulas of DON and this compound indicated an increase of $C_4H_4O_2$ or 84 mass units.

Two new resonances, in the 1H NMR, both representing three proton singlets occurring at 1.88 and 2.13 ppm suggest the presence of two acetoxy moieties. This is further verified by a broad band in the IR at 1745 cm^{-1} , as well as the four additional resonances in the ^{13}C NMR representing two methyl groups at 20.9 and 20.6 ppm and two

carbonyl groups at 170.2 and 170.1 ppm, respectively. The characteristic H-3 multiplet, H-2, doublet and H-4 multiplet are shifted downfield relative to DON (39) by 0.69, 0.29, and 0.07 ppm, respectively. However, both H-10 and H-11 exhibit slight upfield shifts of 0.04 and 0.10 ppm, respectively, as was the case for 3-AcDON (40), suggesting that one of the acetoxy groups is present at the 3 position of the trichothecene skeleton. This is confirmed by the positions, in the ^{13}C NMR, of C-2, C-3 and C-4 which exhibit resonances nearly identical with those of 3-AcDON, (Table 11, p. 85).

The functionality of the carbon atoms in trichothecenes can be determined from the ^{13}C NMR spectrum using the correlations established by Cole and Cox (1983, 84). Thus, the C-9 and C-10 chemical shifts of 135.6 and 138.4 ppm, respectively, are consistent with C-8 being a carbonyl group which is supported by the band at 1685 cm^{-1} in the IR, characteristic of an α, β -unsaturated carbonyl group.

The chemical shift of H-7 and its 7-OH resonance in the ^1H NMR differs from DON (39) and 3-AcDON (40) by less than 0.6 ppm indicating a similar functionality at this

position (Table 10, p. 84). However, in the ^1H NMR, the H-15 quartet has been shifted downfield relative to DON (39) (3.73' and 3.89 ppm (DON) to 4.22 and 4.28 ppm, respectively). The ^{13}C NMR also indicates a slight downfield shift of 0.7 ppm for C-15 which is apparent for 3-AcDON (40) as well as 15-AcDON, compared to DON (39) (80). Slight upfield shifts in the ^{13}C NMR for C-6 and C-11 by 0.6 and 1.0 ppm respectively, provides further evidence to support the suggestion of an acetoxy group at the 15 position.

The ^1H NMR data for this compound is similar to that reported by Yoshizawa et al (1978, 85) for 3,15-diacetyldeoxynivalenol (41).

3-ACETYL-7-DEOXYDEOXYNIVALENOL (3-AC-7-deoxyDON) (42)

SPECTROSCOPIC ANALYSIS

A molecular ion of 322 is displayed by this UV active sample ($\lambda_{\text{max}} = 219 \text{ nm}$) with the best fitting molecular formula ($\text{C}_{17}\text{H}_{22}\text{O}_6$) differing from that of DON (39) by C_2H_2 or 26 mass units and 3-AcDON (40) by one oxygen or 16 mass units.

The presence of the trichothecene ring system containing one acetoxy group at the 3 position is again confirmed by the characteristic peaks in the ^{13}C as well as ^1H NMR as compared to DON (39), 3-AcDON (40) and 3,15-diAcDON (41), (Tables 10 and 11, p. 84 and p. 85, respectively).

From the ^{13}C NMR spectrum, the chemical shifts of C-9 and C-10 of 138.3 and 137.7 ppm, respectively, are consistent with C-8 being a carbonyl group (84) as verified by the IR (1680 cm^{-1}). The H-15 resonance appears as a broad singlet as a result of either accidental equivalence or rotational averaging. In this case, it is shifted upfield relative to DON (39) as well as to 3-AcDON (40) by 0.17 and 0.14 ppm respectively, indicating a possible structural change at the 7 position. This is confirmed by the fact that H-7 and its 7-OH are no longer present at their characteristic positions of 2.35 and 4.78 ppm (^1H of 3Ac-DON (40)) but rather, H-7 which now integrates for two protons, has been shifted upfield to 2.42 and 2.86 ppm and exhibits a strong AB character with a coupling constant of 16.8 Hz. The 7-position of the trichothecene skeleton is now represented by a methylene group as indicated by the C-7 position in the ^{13}C NMR which is upfield relative to DON

(39). (70.2 ppm methine (DON) to 38.3 ppm methylene). This shift is also apparent for C-6 (52.1 ppm (DON) to 47.5 ppm).

Bennett et al (86) isolated the corresponding deacyl analogue from corn inoculated with Fusarium cultures (NRRL 6206 and NRRL 6207) (87). The ^1H NMR spectrum of this latter compound reported H-7 as a doublet (2.87 ppm, $J = 15.6$ Hz).

With an empirical formula of $\text{C}_{17}\text{H}_{22}\text{O}_6$, the observed spectral data indicate the structure to be 3-acetyl-7-deoxydeoxynivalenol (3-Ac-7-deoxyDON) (42).

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TABLE 10

250-MHz ^1H NMR Chemical Shift Assignments and Coupling Constants for the 8-Keto-Epoxytrichothecenes.

Trichothecene Proton Position	DOM (32)	3-AcDOM (40)	3,15-diAcDOM (41)	3-Ac-7-deoxyDOM (42)
2	3.62 ($J_{2,3} = 4.5$)	3.87 ($J_{2,3} = 4.5$)	3.91 ($J_{2,3} = 4.5$)	3.81 ($J_{2,3} = 4.6$)
3	4.53 ($J_{3,4} = 10.7, 4.5$; $J_{3,2} = 4.5$)	5.17 ($J_{3,4} = 11.2, 4.5$; $J_{3,2} = 4.5$)	5.22 ($J_{3,4} = 10.9, 4.6$; $J_{3,2} = 4.6$)	5.18 ($J_{3,4} = 10.2, 4.4$; $J_{3,2} = 4.6$)
4	2.07 ($J_{4,3} = 10.7, 4.5$) 2.21 ($J_{AB} = 14.8$)	2.15 ($J_{4,3} = 11.2$) 2.35 ($J_{AB} = 15.1$; $J_{4,3} = 4.5$)	2.14-2.28 ($J_{4,3} = 4.7, 11.0$)	2.17 ($J_{4,3} = 11.2$) 2.40 ($J_{AB} = 14.7$; $J_{4,3} = 4.1$)
7	4.83 ($J_{7,OH} = 2.0$) 3.82 (OH)	4.78 ($J_{7,OH} = 2.0$) 3.81 (OH)	4.81 ($J_{7,OH} = 2.0$) 3.76 (OH) ($J_{7,OH} = 2.0$)	2.42 ($J_{AB} = 16.8$) 2.86
8				
10	6.61 ($J_{10,11} = 5.9$; $J_{10,16} = 1.5$)	6.56 ($J_{10,11} = 5.9$; $J_{10,16} = 1.6$)	6.57 ($J_{10,11} = 5.8$; $J_{10,16} = 1.6$)	6.55 ($J_{10,11} = 5.7$; $J_{10,16} = 1.4$)
11	4.80 ($J_{11,10} = 5.9$)	4.66 ($J_{11,10} = 5.8$)	4.70 ($J_{11,10} = 5.8$)	4.52 ($J_{11,10} = 5.7$)
13	3.07 ($J_{AB} = 4.3$) 3.15	3.08 ($J_{AB} = 4.2$) 3.14	3.10 ($J_{AB} = 4.3$) 3.15	2.86 ($J_{AB} = 3.9$) 3.09
14	1.13 (s)	1.11 (s)	1.09 (s)	0.83 (s)
15	3.73 ($J_{AB} = 11.7$) 3.89	3.73 ($J_{AB} = 11.7$) 3.83	4.22 ($J_{AB} = 12.0$) 4.28	3.64 (s)
16	1.86 ($J_{16,10} = 1.5$)	1.85 ($J_{16,10} = 1.1$) 2.11 (s)	1.89 ($J_{16,10} = 0.8$) 1.88 (s), 2.13 (s)	1.80 ($J_{16,10} = 1.4$) 2.12 (s)
CH ₃ (Ac)				

TABLE 11

62.8 MHz ^{13}C NMR Chemical Shift Assignments for the
8-Keto-Epoxytrichothecenes

Carbon Number	DON (<u>39</u>)	3-AcDON (<u>40</u>)	3,15-diAcDON (<u>41</u>)	3-Ac-7-deoxyDON (<u>42</u>)
2	80.6	79.1	78.9	78.1
3	68.6	71.3	71.1	71.2
4	43.0	40.5	40.4	38.5
5	46.0	45.9	45.8	45.0
6	52.1	52.0	51.5	47.5
7	70.2	70.2	70.1	38.3
8	202.3	199.8	199.2	198.7
9	135.7	135.8	135.6	138.3
10	138.5	138.4	138.4	137.7
11	74.4	74.5	73.4	68.2
12	65.7	65.1	64.9	65.3
13	47.2	47.5	47.4	48.3
14	13.9	14.1	13.6	11.2
15	61.4	62.2	62.1	64.1
16	14.9	15.2	15.3	15.3
C = O (Ac)		170.3	170.2, 170.0	170.5
CH ₃ (Ac)		20.9	20.9, 20.6	20.9

Spectra recorded in CDCl_3 , δ in ppm relative to chloroform.

2-4-II/III HYDROXY AND ACYLOXY DERIVATIVES

Isotrichodermin Derivatives

^1H assignments, Table 12, p. 93.

^{13}C assignments, Table 13, p. 94.

ISOTRICHODERMIN (43). SPECTROSCOPIC ANALYSIS

The empirical formula for this sample was determined from high resolution mass spectroscopy to be $\text{C}_{17}\text{H}_{24}\text{O}_4$, with a molecular weight of 292, indicative of a compound with few additional substituents.

An epoxytrichothecene ring system with one acetoxy group is confirmed by the characteristic peaks in the ^1H NMR spectrum. From the relative positions of H-3 and C-3 (Tables 12 and 13, p. 93 and p. 94, respectively), it is concluded that the acetoxy substituent is again present at the 3 position.

Four methyl resonances are observed in the ^1H NMR, and confirmed by their ^{13}C resonances, two of which can be assigned to the 14 and 16 positions. Through a process of

elimination, the third resonance is assigned to an acetoxy substituent and the fourth to position 15 since the characteristic AB doublets of H-15 (methylene moiety), normally found between 3.8 and 4.2 ppm, as well as the ^{13}C resonance at approximately 63 ppm, depending upon the substituent, are no longer present.

The C-9 and C-10 chemical shifts of 139.5 and 119.4 ppm, respectively, are consistent with a methylene group at position C-8 (84). The resonances typical of H-7 and its 7-OH at 4.8 and 3.8 ppm, respectively (^1H NMR), as well as C-7 at 70.2 ppm (^{13}C NMR) are again absent as was the case for 3-Ac-7-deoxyDON (42). However, a complex of multiplets in the ^1H NMR (0.9-2.1 ppm), integrating for six protons, can be assigned to H-7, H-8 and H-4.

The ^1H and ^{13}C NMR as well as MS data are compatible with the structure 3-acetoxy-12,13-epoxy-trichothec-9-ene (43) also known as isotrichodermin:

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The isomer of (43) was isolated in 1978 by T. Riison *et al.* (88) and is known as trichodermin (44) in which the acetate group is located at the 4 position rather than the 3 as observed here. A comparison of the ^{13}C resonances between these two compounds indicates differences of greater than 1 ppm are apparent for C-3, C-4, C-5, and C-14. The changes are most dramatic for C-3 and C-4 where a reversal of the two resonances appears to have occurred.

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7-HYDROXYISOTRICHODERMIN (45). SPECTROSCOPIC ANALYSIS

An empirical formula of $\text{C}_{17}\text{H}_{24}\text{O}_5$ was calculated for this sample using high resolution mass spectroscopy. In comparison to isotrichodermin (43), an increase of 0₁ or sixteen mass units is observed, equivalent to an oxygenation.

An acetoxy substituent at the 3 position of the epoxytrichothecene ring system is again apparent from the ^1H

and ^{13}C NMR spectra (Tables 12 and 13, p. 93 and 94). In addition to this acetoxy methyl, three additional methyl resonances, verified using ^{13}C multiplicity sorting, are visible in the ^1H NMR. Three of the four exhibit resonances similar (<0.02 ppm) to those observed for isotrichodermin (43), i.e. the H-14 methyl, the H-16 allylic methyl, and the acetoxy methyl. This fourth resonance can be assigned to the H-15 methyl since the AB quartet characteristic of a substituted H-15 and its associated ^{13}C resonance, are no longer visible at that position. This H-15 methyl (^1H NMR) is shifted downfield relative to isotrichodermin (43) indicating a spatially close electron withdrawing substituent, i.e. OH group, present at either the 7 or the 8 position.

In the ^{13}C NMR, resonances for C-9 and C-10 at 138.0 and 119.6 ppm, respectively, indicate a methylene group at the 8 position through spectral correlations established by Cole and Cox (84). The hydroxy group is therefore assigned to position 7 with the H-7 resonance at 4.46 ppm and the C-7 resonance at 74.7 ppm. Using a $2\text{D}^1\text{H}/^1\text{H}$ correlation NMR spectrum (p.232), these assignments are further verified. For example, the octet representing the AB system of 8 is assigned to the resonances at 1.9 and 2.19 ppm as opposed to

H-4, which, from this correlation spectrum, is assigned to the doublet at 2.06 ppm. Coupling is observed between the peaks at 1.9 and 2.19 ppm (H-8, AB system) and 4.46 ppm (H-7) as well as the peak at 5.40 ppm (H-10). If the OH had been assigned to position 8, the resonance at 4.46 ppm would show coupling with H-10 which is not the case here.

Carbon-6, C-7, and C-8 were assigned in the ^{13}C NMR to resonances at 46.2, 74.7, and 41.4 ppm, respectively, since the largest change in chemical shift, compared to isotrichodermin (43), would be expected for the carbon to which the hydroxy group is directly attached, i.e. C-7, which displays a shift of 50.3 ppm downfield. This data is therefore consistent with the structure represented by 7-hydroxyisotrichodermin (45).

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8-HYDROXYISOTRICHODERMIN (46). SPECTROSCOPIC ANALYSIS

From high resolution mass spectroscopy, an empirical formula of $C_{17}H_{24}O_5$, differing by only one oxygen or 16 mass units from isotrichodermin (43) was calculated for this compound.

This epoxytrichothecene again displays the resonances characteristic for an acetoxy substituent at the 3 position, (Tables 12 and 13, p. 93 and p. 94, respectively). As for 7-hydroxyisotrichodermin (45) and isotrichodermin (43) four methyl groups are apparent, by its 1H and ^{13}C NMR spectra. Two display chemical shifts in the 1H NMR spectrum slightly downfield, from isotrichodermin (45), i.e. H-15: 0.74 ppm (Isotrichodermin) to 0.98 ppm and H-16: 1.71 ppm (Isotrichodermin) to 1.86 ppm. Comparing this compound ($C_{17}H_{24}O_5$) to 7-hydroxyisotrichodermin (45) ($C_{17}H_{24}O_5$), H-15 as well as H-14 are shifted upfield by 0.06 ppm and 0.05 ppm respectively, while H-16 is shifted somewhat downfield by 0.16 ppm.

Using the literature correlations (84), the ^{13}C NMR resonances for C-9 and C-10 at 138.8 and 122.1 ppm respectively, indicate a hydroxyl group at the 8 position of

this structure, which would account for the additional oxygen calculated from the high resolution mass spectrum. H-8 appears in the ^1H NMR as a broad triplet occurring at 4.11 ppm. The AB part of this ABX system, i.e. H-7, is assigned to resonances at 1.69 and 2.21 ppm while the AB group at H-4 is assigned to the four peaks occurring between 2.07 and 2.11 ppm (^1H NMR) as a result of extrapolating the data from the $^1\text{H}/^1\text{H}$ 2D NMR of 7-hydroxy-isotrichodermin (45).

These results (^1H NMR, ^{13}C NMR and MS) are therefore consistent with the structure represented by 8-hydroxy-isotrichodermin (46).

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TABLE 12

250-MHz ^1H NMR Chemical Shift Assignments and Coupling
Constants for the Isotrichodermin Derivatives.

Trichothene Proton Position	Isotrichodermin (42)	7-Hydroxyisotrichodermin (43)	8-Hydroxyisotrichodermin (46)
2	3.73 ($J_{2,3} = 4.6$)	3.76 ($J_{2,3} = 4.5$)	3.72 ($J_{2,3} = 4.6$)
3	5.15 ($J_{3,4} = 10.0$; $J_{3,2} = 4.6$)	5.16 ($J_{3,4} = 9.2$; 6.7; $J_{3,2} = 4.5$)	5.16 ($J_{3,4} = 9.8$; 5.5; $J_{3,2} = 4.5$)
4	1.0-2.0 (m)	2.06 ($J = 2.7$)	2.07-2.11 ($J_{4,3} = 9.6$; 5.8)
7	1.0-2.0 (m)	4.46 ($J_{7,8} = 10.1$; $J_{7,\text{OH}} = 6.0$ $J_{7,8} = 6.3$)	1.69 ($J_{AB} = 14.3$; $J = 1.4$)
8	1.0-2.0 (m)	1.09 (OH) ($J_{\text{OH},7} = 6.3$) 1.9 (unresolved)	2.21 ($J_{7,8} = 6.0$) 4.11 ($J_{8,7} = 5.4$; t)
10	5.44 ($J_{10,11} = 5.5$; $J_{10,16} = 1.5$)	2.19 ($J_{8,7} = 5.9$; $J_{AB} = 17.2$) 5.40 ($J_{10,11} = 5.5$; $J_{10,16} = 1.2$)	5.59 ($J_{10,11} = 5.9$)
11	3.96 ($J_{11,10} = 5.5$)	4.10 ($J_{11,10} = 5.5$)	4.04 ($J_{11,10} = 5.7$)
13	2.84 ($J_{AB} = 4.0$)	3.08 ($J_{AB} = 4.4$)	2.87 ($J_{AB} = 4.0$)
14	3.07	3.10	3.07
15	0.80 (s)	0.82 (s)	0.77 (s)
16	0.74 (s)	1.04 (s)	0.98 (s)
	1.71	1.70 (br.s.)	1.86 (br.s.)
CH_3 (ac)	2.11 (s)	2.10 (s)	2.12 (s)

TABLE 13

62.8 MHz ^{13}C NMR Chemical Shift Assignments for
Isotrichodermin Derivatives

Carbon Number	Isotrichodermin (<u>43</u>)	7-Hydroxy- Isotrichodermin (<u>45</u>)	8-Hydroxy- Isotrichodermin (<u>46</u>)
2	78.2	79.0	78.1
3	71.7	71.4	71.5
4	38.6	39.5	38.1
5	45.3	44.6	45.4
6	40.2	46.2	39.6
7	24.4	74.7	33.6
8	28.2	41.4	68.0
9	139.5	138.0	138.8
10	119.4	119.6	122.1
11	71.5	69.0	71.3
12	65.2	64.9	65.2
13	48.4	47.7	48.5
14	11.0	10.2	11.0
15	15.9	15.0	19.0
16	23.1	22.4	20.4
C=O (Ac)	170.6	170.6	170.6
CH ₃ (Ac)	20.9	20.9	20.9

Spectra recorded in CDCl_3 , δ in ppm relative to chloroform.

2-4-III CALONECTRIN DERIVATIVES

^1H assignments, Table 14, p. 113.

^{13}C assignments, Table 15, p. 114.

CALONECTRIN (47). SPECTROSCOPIC ANALYSIS

A molecular ion of $351 (\text{M} + \text{H})^+$ was identified by Fast Atom Bombardment (FAB) mass spectroscopy. High resolution mass spectroscopy however, led to an empirical formula of $^{13}\text{C}_{17}\text{H}_{22}\text{O}_4$, indicating a difference of 60 mass units between the two techniques. Since two acetoxy groups are present in this molecule, as indicated by ^1H and ^{13}C NMR resonances, the loss of acetic acid would account for this difference, resulting in a corrected empirical formula of $\text{C}_{19}\text{H}_{26}\text{O}_6$. Through the use of the link scan technique, the ion at 290 was shown to be the daughter ion from the molecular ion, H^+ , at 350.

The presence of four methyl groups in this epoxy-trichothecene is confirmed by ^1H and ^{13}C resonances of which two represent the acetoxy methyls, one of which is located at the 3 position (by comparing this compound to 3-AcDON (40) the carbon resonance at 20.8 ppm can be assigned to the

$\text{CH}_3(\text{Ac})$ at the 3 position). The remaining two resonances are associated with the methyl groups at positions 14 and 16. The H-16 methyl displays its characteristic reduced intensity due to long range coupling to H-10.

The basic epoxytrichothecene skeleton (48) can be represented by the formula $\text{C}_{15}\text{H}_{22}\text{O}_2$.

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The addition of two acetoxy groups would increase this formula by $\text{C}_4\text{H}_4\text{O}_2$ to the value of the empirical formula, $\text{C}_{19}\text{H}_{26}\text{O}_6$, indicating that no other additional substituents can be present.

The occurrence of an acetoxy group at C-8 can be eliminated since the C-10 resonance at 119.0 ppm is indicative of a methylene substituent at that position (84). This acetoxy moiety must therefore occur at the 15 rather than the 7 position since the latter would result in a compound containing five methyl groups as opposed to the

observed value of four. The AB system representing H-15 (^1H NMR) occurs at 3.82 and 4.06 ppm respectively, while its ^{13}C resonance occurs at 63.7 ppm being very similar to that of 3,15-diAcDON (41) (62.1 ppm). However, in the case of 3,15-diAcDON (41), the ^{13}C resonance is shifted somewhat upfield possibly due to the γ substituted OH (7 position).

The ^1H resonances for the methylene groups at positions 4, 7 and 8, occur as an unresolved multiplet between 1.78 and 2.14 ppm. One group of four resonances, however, can be resolved. These occur between 2.14 and 2.19 ppm with associated coupling constants of 5.3 and 10.0 Hz, respectively, which appear to represent the coupling constants between H-3 and H-4. There is however a change in the pattern produced which could have resulted from a change in the stereochemistry of the acetoxy group at position 3. However, the coupling constant for H-2, i.e. $J_{2,3}$, remains unchanged in comparison to the compounds previously examined. The pattern of H-4 therefore, appears to be specific to this group of compounds.

The fact that the ^1H and ^{13}C resonances are comparable to those observed in the literature (89,55,84), confirms the

assignment of the resonances. However, the melting point (m.p.) as well as the optical rotation ($[\alpha]_D$) for this compound differ from the literature values, which can be attributed to the presence of minor impurities, i.e. for this compound, a m.p. of 75.5-78.5 °C and an $[\alpha]_D$ of +7.97° (c 0.121, CHCl₃) was obtained, versus a m.p. of 83.0-85.0°C and an $[\alpha]_D$ of +14.6° (c 1.0, CHCl₃) from the literature (89).

Therefore, the data presented here are comparable to that displayed by the compound calonectrin (47) (89).

15-DEACTYLCALONECTRIN (49). SPECTROSCOPIC ANALYSIS

From high resolution mass spectroscopy, a molecular ion of 309.167 (M)⁺ was observed with the molecular formula being calculated to be C₁₇H₂₄O₅.

This compound displays resonances typical of the many characteristic epoxytrichothecene substituents already observed in the previous compounds, i.e. a 3-acetoxy group a 9,10 double bond and two methyl groups located at the 14 and 16 positions, (Tables 14 and 15, p. 113 and p. 114, respectively). The addition of an acetoxy moiety to the basic epoxytrichothecene skeleton, results in a molecular formula of $C_{17}H_{24}O_4$, i.e., one less oxygen than the calculated empirical formula, $C_{17}H_{24}O_5$. This suggests an hydroxy substituent at either the 7,8 or 15 positions but not the 4 position due to the presence of the typical ^{13}C resonances for C-2, C-3, and C-4, (Table 15, p. 114).

The C-10 resonance (119.5 ppm) suggests a methylene substituent at the 8 position (84), eliminating the possibility of the substitution by an hydroxyl group. Since the compound has only two methyl groups, in addition to the acetoxy methyl, it suggests an hydroxyl substituent at the 15 position.

The H-15 AB system consists of two doublets centered at 3.47 and 3.66 ppm, respectively, each doublet being split by coupling with the 15-OH proton. In comparison with calonectrin (47), the position of H-15 in the 1H NMR is

shifted upfield, as expected, for this hydroxy substituted methylene group. However, the difference is not as apparent in the ^{13}C spectrum (C-15 of calonectrin (47) occurs at 63.7 ppm while C-15 for this sample occurs at 62.8 ppm), perhaps as a result of the more freely rotating acetoxy group in calonectrin (47), i.e. the hydroxyl group in this molecule appears to be hydrogen bonded since coupling is observed between the methylene group at C-15 and the 15-OH proton as a result of the proton of the hydroxyl group being unable to exchange freely with its environment.

The three methylene groups representing H-4, H-7 and H-8 appear in the ^1H NMR as an unresolved multiplet between 1.73 and 2.30 ppm. One group of four peaks occurring between 2.17 and 2.23 ppm can be resolved with associated coupling constants of 5.3 and 10.1 Hz, indicating in all probability that these resonances represent H-4 which is coupling with H-3. As in calonectrin (47), this compound displays an atypical pattern for H-4, apparently characteristic to this class of trichothecenes.

Comparing the ^{13}C spectra of this compound, and isotrichodermin (43), the most prominent difference is the absence of a methyl resonance and the appearance of a

methylene resonance. Comparing the actual values, C-15 shifts from 15.9 ppm (isotrichodermin (43)) to 62.8 ppm, C-6 is also shifted downfield by 4.0 ppm for this sample, but C-7 as well as C-11 experience upfield shifts of 3.6 and 3.2 ppm respectively. Compared to calonectrin (47), one observes the absence of the C-15 resonance (15.9 ppm) in the ^{13}C NMR and the appearance of a methylene resonance at 62.8 ppm representing the hydroxyl substituted methylene group at C-15.

Therefore, the data observed here is in agreement with the structure reported by Gardner *et al.* (89) for 15-deacetylcalonectrin (49).

3,15-DIACETYL-7,8-DIHYDROXYDEOXYNIVALENOL (DDD OR
7,8-DIHYDROXYCALONECTRIN) (50). SPECTROSCOPIC ANALYSIS

From high resolution mass spectroscopy, a molecular ion of $382.179 (\text{M})^+$ was observed with the empirical formula being calculated to be $\text{C}_{19}\text{H}_{26}\text{O}_8$.

The ^1H NMR of this material shows resonances typical of an epoxytrichothecene. The presence of two acetoxy moieties is confirmed by the characteristic peaks in the ^1H and ^{13}C NMR (Tables 14 and 15, p. 113 and 114, respectively) as well as the two carbonyl bands in the IR at 1735 and 1715 cm^{-1} . One of the acetoxy groups is present at the 3 position of the trichothecene skeleton as confirmed by the positions of C-2, C-3 and C-4 in the ^{13}C NMR as well as the position of the H-3 multiplet in the ^1H NMR which is observed at a comparable location relative to 3-AcDON (40).

The ^{13}C spin echo NMR spectrum shows resonances for 19 carbon atoms; 4- CH_3 , 5-O-CH, 1-CH, 6-C- including 2 keto groups, and 3- CH_2 - according to multiplicity sorting.

The four methyl groups are represented by the two acetoxy methyls in addition to the two methyl groups present at C-16 and C-14, through comparison to assigned spectral data for other trichothecenes (80). This would suggest that position 15 has been substituted as verified by the presence of an AB system centered at 4.29 ppm in the ^1H NMR. Compared to DON (39), this AB quartet is shifted downfield by 0.48 ppm. However, for 3,15-diAcDON (41), only a slight downfield shift of 0.04 ppm is apparent.

Comparing this 3,15-diacetylated trichothecene skeleton ($C_{19}H_{26}O_6$), i.e., calonectrin (47) to the calculated empirical formula ($C_{15}H_{26}O_8$), indicates a difference of two oxygens or 32 mass units suggesting the presence of two additional hydroxyl substituents.

The upfield shifts of H-11 and H-10 resonances relative to those of DON (39) by 1.03 and 0.48 ppm, respectively, combined with the lack of UV absorption at 218 nm is consistent with the absence of a ketone group at the C-8 position. Using the ^{13}C correlations (84), the C-9 and C-10 chemical shifts of approximately 139.3 and 122.1 ppm, respectively, are consistent with C-8 containing a hydroxyl substituent. This would suggest that the second hydroxyl group is present at the 7 position since the other resonances common to the trichothecene skeleton including the ABX system of H-4 appear at their characteristic positions with comparable coupling constants, (Table 14, p. 113). The multiplets 4.50 and 3.99 ppm (1H NMR), part of an AB system ($J_{AB} = 5.8$ Hz), are assigned to H-7 and H-8, respectively. This assignment was confirmed by the observed coupling between H-8 and H-16 resonances in a long-range COSY spectrum ($^1H/^1H$ shift correlation). In CD_3OD , these multiplets collapsed into two doublets (3.84 and 4.34 ppm)

while the two doublets at 3.00 and 2.71 ppm disappeared. The latter two resonances are therefore due to the 7-OH and 8-OH protons, which exchange in CD_3OD but not CDCl_3 , as was the case for the 7-OH in DON (39). The H-15 AB system also appears as a singlet (4.30 ppm) in CD_3OD instead of an AB quartet.

An unambiguous assignment of the various resonances in the ^{13}C NMR was accomplished using a $^1\text{H}/^{13}\text{C}$ 2D correlation spectrum. The results obtained here correct some of the previous assignments reported by Greenhalgh *et al.* (81). For example, the C-16 methyl group, in close proximity with the two acetate methyl groups, can now be correctly assigned to the resonance at 20.3 ppm. This was also the case for the four methine resonances located at 69.7, 70.2, 70.4 and 71.0 ppm, now assigned correctly to C-7, C-11, C-8, and C-3 respectively.

Therefore, the MS, ^1H and ^{13}C NMR data, combined with the absence of UV absorption at 218 nm, are compatible with the structure 3,15-diacetyl-7,8-dihydroxydeoxynivalenol (DDD) also known as 7,8-dihydroxycalonectrin (50).

As a further confirmation of structure, the allylic hydroxyl group at position 8 was oxidized to an α,β -unsaturated carbonyl compound using MnO_2 . The resulting product displayed a ^1H NMR identical with that of 3,15-diAcDON (41).

The H-7' and H-8 protons display a coupling constant of 5.6 Hz whose magnitude suggests a trans stereochemistry between these two groups. Since coupling is apparent between H-8 and H-10 using long range COSY, the relative stereochemistry as shown for 50 is suggested.

In order to determine the absolute configuration of these compounds, a circular dichroism (CD) experiment was proposed since the 1,2 diol present in DDD (50) would lend itself to these studies if benzoylated (91). The benzoylation reaction was accomplished using freshly distilled benzoylchloride and pyridine which when refluxed with DDD (50) for a period of 48 hours, resulted in a brownish/black liquid. Following a rigorous purification sequence, a molecular ion (M^+) of 590 was observed in the mass spectrum indicating that the dibenzoylated material was present as substantiated by ^1H NMR. The ^1H spectrum for this relatively pure material shows resonances similar to

those observed in DDD (50) with the exception being that the two hydroxyl peaks at 3.00 and 2.76 ppm are absent and the quartet and triplet characteristic of H-7 and H-8 located at 4.50 and 3.99 ppm respectively, have collapsed to two doublets further downfield at 6.16 and 6.01 ppm. The CD spectrum of this sample remains to be completed.

7-HYDROXYCALONECTRIN (51). SPECTROSCOPIC ANALYSIS

A molecular ion of 366 (M^+) was the result of solid probe mass spectroscopy while high resolution mass spectroscopy led to an empirical formula of $C_{17}H_{22}O_5$. As observed for calonectrin (47), this difference of 60 mass units between the two techniques, is believed to represent the loss of acetic acid, which when accounted for, results in an increase of the empirical formula by $C_2H_4O_2$ units to $C_{19}H_{26}O_7$. From the link scan technique, the ion at 306 ($C_{17}H_{22}O_5$) was shown to be the daughter ion from the molecular ion, M^+ , at 366.

From typical 1H and ^{13}C NMR resonances, (Tables 14 and 15, p. 113 and p. 114, respectively) this epoxytrichothecene substituted with an acetoxy group at the 3 position, also contains an acetoxy substituent at the 15 position as verified by the AB pattern present at 4.05 and 4.31 ppm, in the 1H NMR spectrum, as compared to that of calonectrin (47), i.e. 3.82 and 4.06 ppm, respectively. In addition, a resonance in the ^{13}C NMR at 64.4 ppm, exhibits a similar chemical shift to the C-15 of calonectrin (47), i.e. 63.5 ppm.

Comparing the molecular formula of a diacetylated epoxytrichothecene, i.e., calonectrin (47) ($C_{19}H_{26}O_6$), to the molecular formula calculated here ($C_{19}H_{26}O_7$), indicates an increase of one oxygen or 16 mass units, for this sample, suggesting an hydroxyl substituent which according to previous examples, could be located at either the 4, 7 or 8 position. An hydroxy substituent at the 4 position appears unrealistic as a result of resonances in the ^{13}C NMR characteristic of C-4 being a methylene (39.6 ppm) and C-3 being substituted with an acetoxy group (71.1 ppm). Therefore, the hydroxyl substituent can be present at either the 7 or 8 position. Using the ^{13}C correlations (84), C-10 and C-11 display resonances of 139.4 and 119.0 ppm, respectively, indicating a methylene group at the 8 position. This would suggest that the hydroxyl group is present at the 7-position, as supported by a peak at 71.0 ppm (^{13}C NMR) as well as the downfield shifts of C-6 to 46.4 ppm from 43.0 in calonectrin (47) and C-8 to 41.7 ppm from 28.2 ppm in calonectrin (47). One would therefore expect to see the H-7 resonance around 4.5 ppm, as was the case for 7-hydroxyisotrichodermin (45) where H-7 appeared at 4.46 ppm. A multiplet is observed at 4.55 ppm.

Using the $^1\text{H}/^1\text{H}$ 2D correlation NMR obtained for 7-hydroxyisotrichodermin (45) to assign the resonances for H-4 and H-8 in this compound, one proton of H-8 is assigned to the quartet at 2.41 ppm but the second proton of H-8 is unresolved at approximately 2.1 ppm due to the number of overlapping resonances in this area. In addition, the characteristic ABX pattern of H-4 is difficult to discern however, two strong doublets displaying a coupling constant of approximately 9.8 Hz can be resolved.

Therefore, the ^1H and ^{13}C NMR spectra as well as the MS data, are compatible with the structure 7-hydroxycalonectrin (51).

8-HYDROXYCALONECTRIN (52). SPECTROSCOPIC ANALYSIS

A molecular ion of 366 (M^+) was the result of solid probe mass spectroscopy while high resolution mass spectroscopy led to an empirical formula of $C_{17}H_{22}O_5$.

This epoxytrichothecene, substituted at the 3 position with an acetoxy substituent, also contains an acetoxy group at the 15 position as suggested by the AB pattern centered at 4.14 ppm (1H NMR). This AB pattern however, has started to collapse as compared to calonectrin (47) and 7-hydroxycalonectrin (51) and as a result, the J_{AB} value could not be calculated.

Comparing the molecular formula of a diacetylated epoxytrichothecene, i.e., calonectrin (47) ($C_{19}H_{26}O_6$), to the molecular formula calculated here ($C_{19}H_{26}O_7$), indicates an increase of one oxygen or 16 mass units, for this sample, suggesting an hydroxyl substituent at either the 4, 7, or 8 positions. As in 7-hydroxycalonectrin (51), an hydroxyl group at the 4 position of this sample is not the case as a result of resonances in the ^{13}C NMR characteristic of C-4 being a methylene (39.1 ppm) and C-3 being substituted with an acetoxy group (71.1 ppm).

Using the ^{13}C correlations (84), C-9 and C-10 display resonances of 139.5 and 121.6 ppm, respectively, indicating that the C-8 position is substituted with an hydroxyl group and according to the empirical formula, C-7 must be a methylene group. This is further substantiated by the peak at 67.0 ppm (^{13}C NMR), representing C-8, as well as the downfield shifts of C-16 to 20.3 ppm from 23.2 ppm in calonectrin (47).

In the ^1H NMR, the resonances representing H-8 appear at approximately 4.14 ppm and are unresolved due to the overlapping resonances of H-11 and H-15. Compared to 7-hydroxycalonectrin (51) this compound exhibits an upfield shift for H-8 from 4.55 ppm (H-7 in 7-hydroxycalonectrin (51)) to 4.14 ppm which was also the case for 7-hydroxyisotrichodermin (45) and 8-hydroxyisotrichodermin (46), i.e. 4.46 ppm (H-7) to 4.11 ppm (H-8), respectively.

It became increasingly difficult to identify the resonances representing H-4 and H-7 in the ^1H NMR of this compound and as a result, they were assigned to the multiplets in the area between 1.90 and 2.30 ppm.

Therefore, the ^1H and ^{13}C NMR spectra as well as the MS data are compatible with the structure 8-hydroxycalonectrin (52).

TABLE 14
250-MHz ^1H NMR Chemical Shift Assignments and Coupling
Constants for the Calonectrin Derivatives.

Trichothene cane Proton Position	Calonectrin (47)	7-Hydroxycalonectrin (51)	8-Hydroxycalonectrin (52)	DDD (50)	15-Desacetylcalonectrin (49)
2	3.74 ($J_{2,3} = 4.6$)	3.78 ($J_{2,3} = 4.4$)	3.73 ($J_{2,3} = 4.6$)	3.77 ($J_{2,3} = 4.4$)	3.72 ($J_{2,3} = 4.7$)
3	5.15 ($J_{3,4} = 9.6$, 7.4; $J_{3,2} = 5.0$)	5.17 ($J_{3,4} = 10.0$, 8.0; $J_{3,2} = 4.5$)	5.15 ($J_{3,4} = 10.0$, 5.1, $J_{3,2} = 5.0$)	5.15 ($J_{3,4} = 11.1$, 4.5; $J_{3,2} = 4.4$)	5.13 ($J_{3,4} = 10.0$, 5.0, $J_{3,2} = 4.6$)
4	1.78-2.19 ($J_{4,3} = 10.0$, unresolved)	1.97-2.18 ($J_{4,3} = 9.8$; unresolved)	1.90-2.30 (unresolved)	2.13 ($J_{4,3} = 11.2$)	1.73-2.30 (unresolved)
7	1.78-2.19 (unresolved)	4.55 ($J_{7,8} = 11.2$; $J_{7,OH} = 5.5$) 1.14 (OH) (d)	1.90-2.30 (unresolved)	2.34 ($J_{AB} = 15.0$; $J_{4,3} = 4.6$) 4.50 ($J_{7,8} = 5.4$, $J_{7,OH} = 9.6$) 3.00 (OH) ($J_{7,OH} = 9.7$)	1.73-2.30 (unresolved)
8	1.78-2.19 (unresolved)	1.9-2.0 (unresolved) 2.41 ($J_{AB} = 17.6$; $J_{8,7} = 5.9$)	4.14 (unresolved)	3.99 ($J_{8,7} = 5.8$, $J_{8,OH} = 7.5$) 2.76 (OH) ($J_{8,OH} = 8.0$)	1.73-2.30 (unresolved)
10	5.45 ($J_{10,11} = 5.4$; $J_{10,16} = 1.3$)	5.38 ($J_{10,11} = 5.4$; $J_{10,16} = 1.3$)	5.58 ($J_{10,11} = 5.7$; $J_{10,16} = 1.1$)	5.58 ($J_{10,11} = 5.8$; $J_{10,16} = 0.9$)	5.46 ($J_{10,11} = 5.3$, $J_{10,16} = 1.5$)
11	4.00 ($J_{11,10} = 5.6$)	4.11 ($J_{11,10} = 5.4$)	4.14 (unresolved)	4.32 ($J_{11,10} = 5.8$)	3.96 ($J_{10,11} = 5.3$)
13	2.84 ($J_{AB} = 4.0$) 3.08	3.09 ($J_{AB} = 4.4$) 3.23	2.89 ($J_{AB} = 4.0$) 3.08	3.09 ($J_{AB} = 4.3$) 3.20	2.84 ($J_{AB} = 4.0$) 3.07
14	0.81 (s)	1.14 (s)	0.83 (s)	1.12 (s)	0.90 (s)
15	3.82 ($J_{AB} = 12.2$) 4.06	4.05 ($J_{AB} = 12.2$) 4.31 ($J_{15,16} = 1.1$)	4.14 (AB quartet)	4.14 ($J_{AB} = 12.3$) 4.44	3.47 ($J_{AB} = 11.6$, 3.66 $J_{15,OH} = 4.5$, $J = 3.2$)
16	1.70 (br.s.)	1.72 (br.s.)	1.85 ($J_{16,10} = 0.56$)	1.86 ($J_{16,10} = 1.0$)	1.71 ($J_{16,10} = 1.5$)
CH ₃ (Ac)	2.03 (s)	2.08 (s)	2.03 (s)	2.02 (s)	2.09 (s)
CH ₃ (Ac)	2.10 (s)	2.09 (s)	2.11 (s)	2.10 (s)	

TABLE 15

62.8-MHz ^{13}C Chemical Shift Assignments for
Calonectrin Derivatives

Carbon Number	15- Deacetyl- calonectrin (<u>49</u>)	7- Hydroxy- calonectrin (<u>51</u>)	8- Hydroxy- calonectrin (<u>52</u>)	Calonectrin (<u>47</u>)	DDD (<u>50</u>)
2	78.2	78.9	78.1	77.9	78.5
3	71.5	71.1	71.1	71.1	71.0
4	39.4	39.6	39.1	39.1	41.3
5	45.3	46.1	45.3	45.2	46.0
6	44.2	46.4	42.3	42.8	46.2
7	20.8	71.0	30.0	20.9	69.7
8	28.5	41.7	67.0	28.0	70.4
9	140.5	139.4	139.5	140.3	139.3
10	119.5	119.0	121.6	118.9	122.1
11	68.3	70.0	67.8	68.0	70.2
12	65.2	64.3	64.9	64.9	64.6
13	48.5	47.8	48.6	48.2	47.8
14	12.3	15.9	11.7	11.9	15.0
15	62.8	64.4	65.0	63.5	63.3
16	23.1	22.4	20.3	23.0	20.3
C=O (Ac)	170.6	170.0	170.5	170.4	170.4
CH ₃ (Ac)	20.8	21.0	20.9	20.4	20.9
C=O (Ac)		170.0	170.4	170.7	170.9
CH ₃ (Ac)		20.8	20.8	20.8	21.1

Spectra recorded in CDCl_3 , δ in ppm relative to chloroform.

IV SAMBUCOIN (53). SPECTROSCOPIC ANALYSIS

^1H assignments, Table 16, p. 121.

^{13}C assignments, Table 17, p. 122.

A molecular ion of 250 (M^+) was the result of solid probe mass spectroscopy while high resolution mass spectroscopy led to an empirical formula of $\text{C}_{15}\text{H}_{22}\text{O}_3$ with the number of double bond equivalents (DBE) being calculated to be five.

The first peculiarity of this compound observed in the ^1H NMR spectrum is the absence of an AB quartet centered at approximately 2.9 ppm representing the methylene group, H-13, of the epoxide, a substituent common to the trichothecenes so far examined. This is supported by the lack of the characteristic quaternary C-12 resonance at approximately 65.0 ppm as well as the C-13 methylene resonance at approximately 47.0 ppm.

Resonances for 15 carbon atoms are observed in the ^{13}C NMR spectrum with multiplicity sorting experiments. (DEPT) showing resonances for: 3 CH_3 , 4 CH_2 , 1 $\text{CH}_2\text{-O}$, 1 CH-O , 1 -CH , 1 C=O , and 4 $\text{-}\overset{\textstyle |}{\underset{\textstyle |}{\text{C}}}\text{-}$ carbons of which one is a $\text{-}\overset{\textstyle |}{\underset{\textstyle |}{\text{C}}}\text{-}$ and one is a $\text{-}\overset{\textstyle |}{\underset{\textstyle |}{\text{C}}}\text{-O}$, (Table 17, p. 122).

Two resonances in the ^1H NMR spectrum, at 5.18 and 3.94 ppm, respectively, appear to represent H-10 and H-11 of a trichothecene skeleton through comparison with other spectra. Since both resonances appear as broad singlets, it is impossible to calculate their respective coupling constants. However, through the use of a $^1\text{H}/^1\text{H}$ 2D correlation experiment, these two peaks are observed to couple with one another as well as with a methyl group at 1.64 ppm, displaying reduced intensity - a feature common to the methyl group at position 16.

Comparing the ^1H NMR spectrum of this compound with those previously studied, one observes a variety of differences such as: 1) the absence of any acetoxy groups as supported by a lack of methyl resonances in the region of 2.1 ppm, 2) the absence of the characteristic H-3 multiplet near 4.5 ppm as observed for DON (39), 3) the absence of the H-2 doublet at 3.7 ppm and 4) the absence of any multiplets between 4.5 and 4.0 ppm representing the hydroxylated 7 and/or 8 positions. This last observation would suggest that H-7 and H-8 are unsubstituted. However, the ^{13}C literature correlations (84), might suggest an hydroxy substituent at position 8 due to the resonances for C-9 and C-10, at 135.4 and 122.7 ppm, respectively.

However, in view of this "trichothecene-like" material, lacking an epoxy group, these correlations may no longer be valid.

Comparing the electron impact mass spectrum of this compound with those of the other trichothecenes isolated, the greatest similarity is seen with isotrichodermin (43) especially visible for the peaks at 93, 108 and 124 which are common to both spectra but differ slightly in terms of intensity. It is possible that the peak representing 124 may be due to the following fragmentation:

This would suggest that the trichothecene A ring, with an oxygen moiety at the 11 position and a methyl substituent at the 6 position, remains intact for this compound as shown below:

From the IR of this material, a band at 1735 cm^{-1} suggests a carbonyl group in a 5 membered ring.

In the ^1H NMR, an AB system is observed at 3.42 and 4.18 ppm with a coupling constant of 11.2 Hz suggesting 2 non-equivalent protons adjacent to an oxygen substituent.

From the $^1\text{H}/^1\text{H}$ 2D correlation NMR spectrum, (p.237) the presence of four AB systems is apparent; one at 2.26 and 2.56 ppm which couples to the system at 1.74 and 1.99 ppm and one at 1.43 ppm which couples to the system between 1.8 and 1.9 ppm. The second set, i.e., 1.43 ppm and 1.8-1.9 ppm, can be assigned to H-7 and H-8, respectively, due to the observed coupling between H-8 and H-16 and H-8 and H-10.

Spectroscopic data similar to that presented here were recently published in a paper by Mohr et al. (92) for which they identified this sample as sambucoin (53). In addition, an X-ray study presented in that paper revealed the stereochemistry of this compound with a major difference being the formation of a trans ring junction between rings A and B, as opposed to the usual cis ring junction normally observed for the trichothecenes.

53

Thus, the AB system observed at 3.42 and 4.18 ppm is assigned to H-13, while the AB system at 2.26 and 2.56 ppm is assigned to H-3 since it is shifted downfield slightly due to the adjacent carbonyl group. The AB system at 1.74 and 1.99 ppm, coupling to H-3, is thereby assigned to H-4.

Therefore, the ^{13}C , and ^1H NMR spectra as well as the MS data, are in agreement with the structure of sambucosin (53) presented by Mohr et al. (92).

TABLE 16

250 MHz ^1H NMR Chemical Shift Assignments and Coupling
Constants for Sambucosin (53)

Proton Position	Sambucosin (53)
3	2.26 ($J_{AB} = 19.9$; $J_{3,4} = 7.9, 10.0$) 2.56 ($J_{AB} = 19.95$; $J_{3,4} = 3.0, 11.8$)
4	1.74 ($J_{AB} = 13.8$; $J_{4,3} = 7.9, 11.7$) 1.99 ($J_{AB} = 13.6$; $J_{4,3} = 3.2, 10.2$)
7	1.43 ($J_{AB} = 13.3$; $J_{7,8} = 2.3, 5.4$)
8	1.8-1.9 (br. band)
10	5.18 (br.s.)
11	3.94 (br.s.)
13	3.42 ($J_{AB} = 11.2$) 4.18
14	0.63 (s)
15	1.13 (s)
16	1.64 (br.s.)

Spectra was recorded in CDCl_3 ,
0 in ppm relative to chloroform.

TABLE 17

62.8 MHz ^{13}C NMR Chemical Shift Assignments and Coupling
Constants for Sambucosin

Carbon Number	Sambucosin (<u>53</u>)
2	216.0
3	34.3
4	26.8
5	46.4
6	37.6
7	{ 27.52
8	27.47
9	135.4
10	122.7
11	75.6
12	74.6
13	66.4
14	14.1
15	15.8
16	22.4

Spectra was recorded in CDCl_3 , δ in ppm relative to chloroform.

2-4-V "Apotrichothecene" (54). Spectroscopic Analysis

^1H NMR assignments, Table 18, p. 127

^{13}C NMR assignments, Table 19, p. 128

From the fast atom bombardment mass spectrum, the molecular ion was observed at m/z 253 ($M + H$)⁺.

Of the 15 resonances present in the ^{13}C NMR spectrum, there are 3 CH_3 , 4 CH_2 , 1 $\text{CH}_2\text{-O}$, 2 CH-O , 2 -C- , 1 C-O , 1 $=\text{C}$ and 1 $=\text{CH}$.

3 The characteristic AB quartet centered at approximately 2.9 ppm, representing the methylene group of the epoxide is not present in the ^1H NMR spectrum of this material. However, other resonances common to the trichothecenes are visible, e.g., three (3H) singlets are present, one of which (1.64 ppm) displays reduced intensity due to fine coupling, a common feature of the H-16 methyl group. Two broad singlets displaying fine coupling at 5.52 and 4.15 ppm, respectively, integrating for one proton each, appear to represent the two protons at positions 10 and 11 of the trichothecene skeleton.

From the X-Ray data of this sample (an equivalent sample from Agriculture Canada was used), the structure was confirmed and called "apotrichothecene" (54) due to the similarity of its carbon skeleton and the apotrichothecenes obtained as rearrangement products upon treatment of trichothecenes with acid. (p. 20 of introduction). However, in light of the compound sambucinol (60) (p. 142), a more appropriate name appears to be seco-deoxy-sambucinol.

54

The assignment of H-2, H-4, H-7 and H-8 resonances are based on comparison to the other trichothecenes isolated. The ^1H NMR assignment of the H-2 proton at 2.60 ppm is based on the assumption that one of the protons of H-2 will be shifted further downfield since in addition to the adjacent 3-OH, there is a spatially close 13-OH. The multiplet at 4.29 ppm (^1H NMR) is assigned to H-3. An AB quartet (2H) is apparent at 3.17 and 3.71 ppm, respectively. One of the AB doublets is broadened while the other exhibits sharp peaks with fine coupling. This AB system was assigned to H-13.

The sample of "apotrichothecene" (54) isolated has been shown by capillary G.C. to be composed of 2 compounds in an 11:1 ratio. The sample isolated at Agriculture Canada contained approximately equal amounts of these same two isomers by comparison of their ^1H NMR spectra. The X-Ray study performed at NRC (Halifax, Nova Scotia), revealed that these 2 compounds were stereoisomers, involving the OH at the 3 position.

54a 54b

In order to elucidate the stereochemistry of the 2 isomers isolated in this work, the J values for the AB system of H-13, were examined. It showed a strong AB system for one isomer but in the other isomer, it had started to collapse. In an epoxy moiety, the two protons of the methylene group, held in a rigid orientation, being non-equivalent would result in a strong AB system. This could occur if the oxygen at position 13 could hydrogen bond to the oxygen at position 3, requiring both hydroxyl groups to be in the same orientation (54a).

54a

Therefore, the data is consistent with structure (54a) unfortunately, the sample was insufficient to confirm the assignments by $^1\text{H}/^1\text{H}$ 2D correlation NMR spectroscopy.

TABLE 18

250-MHz ^1H NMR Chemical Shift Assignments and Coupling Constants for "Apotrichothecene" (54).

Proton Position	δ (ppm)
2	1.50-1.57 (1H, unresolved multiplet) 2.60 (1H, $J = 1.6, 12.2$ Hz, dq)
3	4.29 (1H, unresolved multiplet)
4	1.60-1.78 (1H, $J = 2.0, 11.9, 18.8$ Hz, octet) 2.13 (1H, $J = 10.1, 13.0$ Hz, dd)
7	1.35 (1H, $J = 2.9, 5.9$ Hz, q)
8	1.98-2.03 (2H, $J = 2.1$, br. s.)
10	5.52 (1H, $J = 1.6, 3.2$ Hz, br. s.)
11	4.12 (1H, $J = 2.1$ Hz, br. s.)
13	3.17 (1H, $J = 10.7$ Hz, br. d.) 3.71 (1H, $J = 2.1, 11.4$ Hz, dd)
14	0.93 (3H, s)
15	1.00 (3H, s)
16	1.64 (3H, $J_{16,10} = 1.2$, t)

TABLE 19

62.8 MHz ^{13}C NMR Chemical Shift Assignments for
"Apotrichothecene" (54).

Carbon Number	δ (ppm)
2 (1)	43.6
3	72.8
4 (1)	44.6
5	44.8
6	52.6
7 (2)	29.4
8 (2)	27.8
9	135.4
10	121.3
11	81.4
12	92.6
13	63.5
14	17.9
15	19.5
16	22.6

Spectrum recorded in CDCl_3 ; δ in ppm from chloroform.

- (1) Assignments for carbons 2 and 4 may be reversed.
(2) Assignments for carbons 7 and 8 may be reversed.

TABLE 21

62.8-MHz ^{13}C NMR Chemical Shift Assignments for
Culmorin (55) and Culmorone (59).

Carbon Number	Culmorin (<u>55</u>)	Culmorone (<u>59</u>)
1	49.7	60.0
2	51.3 (1)	50.0 (1)
3	36.3 (2)	34.8 (2)
4	22.6	21.9
5	41.3 (2)	41.4 (2)
6	32.9	33.9
7	52.2	57.8
8	79.5	78.0
9	52.2 (1)	51.7 (1)
10	36.0	41.2
11	71.0	216.5
12	13.1	12.4
13	22.0	22.4
14, 15	28.8, 29.2	28.4, 29.6

Spectrum was recorded in CDCl_3 ; δ in ppm relative to
chloroform.

- (1) Assignments for carbons 2 and 9 may be reversed.
(2) Assignments for carbons 3 and 5 may be reversed.

Rather, resonances such as four quaternary methyl groups are observed, two of which exhibit reduced intensity as a result of geminal coupling as verified from the $^1\text{H}/^1\text{H}$ 2D NMR correlation spectrum (p.234). A six proton multiplet between 1.2 and 1.4 ppm is consistent with the presence of three methylene groups which are shown to couple only to each other from the $^1\text{H}/^1\text{H}$ 2D study. At 3.80 ppm, a doublet (1H), ascribed to a methine proton of a CH-OH group, is shown to couple with a doublet (1H) at 1.72 ppm ($J = 5.0$ Hz) while at 4.33 ppm, a sextet (1H), ascribed to a methine proton of a CH-OH group is shown to couple with a doublet (2H) at 1.63 ppm ($J = 6.8$ Hz) as well as a doublet (1H) at 1.88 ppm ($J = 4.5$ Hz). This data would suggest the following three partial structures, 56, 57, and 58, respectively.

565758

Not visible in the 250 MHz ^1H NMR spectrum but apparent in the $^1\text{H}/^1\text{H}$ 2D correlation spectrum is the coupling between one of the two protons associated with the

doublet at 1.63 ppm and the doublet (1H) at 3.80 ppm suggesting long range coupling through the quaternary carbon atom ("w" coupling).

The ^{13}C NMR spectrum of this material confirms the presence of four methyl group carbon atoms as first observed in the ^1H NMR spectrum. In addition, four methylene carbons, two methine carbons near 50 ppm, i.e., CH-C-O; two methine carbons at 71.0 ppm and 79.5 ppm, i.e., CH-O and three quaternary carbons can be identified.

Oxidation of this material using Collins reagent resulted in a product displaying a molecular ion of 236 (M) $^+$. No OH band was observed in the IR, however, a broad band in the carbonyl region at 1745 and 1755 cm^{-1} was observed which suggests the presence of two cyclopentanone units. The ^1H NMR spectrum of this material shows singlets (1H) at 2.69 ppm and 1.80 ppm as well as an AB quartet (2H) at 2.26 ppm and 1.19 ppm ($J = 18.7$ Hz).

The data presented here are in agreement with that presented by Barton and Werstiuk in 1968 (93) for the sesquiterpenoid, culmorin (55) first isolated in 1937 by Ashley et al. (94) from a strain of Fusarium culmorum.

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The problem in deducing the structure from the ^1H NMR spectrum is ambiguity resulting from the doublet (1H) at 1.88 ppm (H-1) only couples to the multiplet (1H) at 4.33 ppm (H-11) while the doublet (1H) at 1.72 ppm (H-7) only couples with the doublet (1H) at 3.80 ppm (H-6). From this, one would assume a quaternary carbon adjacent to both methine groups at 1.88 ppm and 1.72 ppm, respectively. However, Barton and Werstiuk (93) were able to show that the structure is such that the dihedral angle between H-1 and H-7 is about 90° , such that coupling is not observed between these two adjacent protons.

Apparent in the $^1\text{H}/^1\text{H}$ 2D correlation spectrum is coupling between H-10 and one methyl group as well as coupling between H-1 and one methyl group allowing for the unambiguous assignment of the two methyl groups to positions 12 and 13, respectively. However, the two methyl groups representing positions 14 and 15, could not be individually identified.

In order to correctly assign the ^{13}C NMR spectrum of culmorin, (55) a $^1\text{H}/^{13}\text{C}$ 2D correlation NMR was run. Based on the assumption that the ^1H NMR had been correctly assigned, the ^{13}C NMR could then be assigned as represented in Table 21, p. 141.

The ^1H , ^{13}C and MS data are therefore in agreement with the structure 55 for culmorin.

CULMORONE (59). SPECTROSCOPIC ANALYSIS:⁴

A molecular ion of 236 (M^+) was the result of solid probe mass spectroscopy while high resolution mass spectroscopy led to an empirical formula of $C_{15}H_{24}O_2$.

In the IR spectrum, an OH group is indicated in this compound by a band at 3420 cm^{-1} . Two small bands of approximately equal intensity at 1380 and 1370 cm^{-1} , represent the geminal dimethyls. In addition, there are two strong bands present in the carbonyl region, one at 1725 cm^{-1} and the other at 1745 cm^{-1} . The former however, is more intense and broader than the later, which is believed to represent an impurity since only one carbonyl resonance is apparent in the ^{13}C NMR spectrum.

From the empirical formula, four double bond equivalents can be calculated, one of which represents the carbonyl group. The resulting three suggest a tricyclic species as was the case for culmorin (55).

Compared to culmorin (55), the molecular weight for this species is only two mass units less and there appears

to be only one hydroxyl, in addition to a carbonyl group, suggesting that this compound may represent an oxidized product of culmorin (55), i.e., culmorone. The ^{13}C NMR spectrum is compatible with this idea with one less CH-O and one additional C=O resonance, compared to culmorin (55). There are however two possibilities for the structure, depending on which hydroxyl group (position 8 or 11) may have been oxidized i.e., (59a) or (59b).

The ^1H NMR spectrum of culmorone indicates the presence of four methyl groups, two of which display the characteristic reduced intensity of geminal dimethyls as seen in culmorin (55). A doublet (1H) ($J = 4.8$ Hz) is visible at 1.24 ppm (Table 20, p 140). A similar coupling constant is not apparent amongst the resolvable peaks in the 250 MHz ^1H NMR spectrum however, a $^1\text{H}/^1\text{H}$ 2D correlation NMR (p.235) indicates that there is strong coupling between this 1H doublet at 1.24 ppm and the broad unresolved singlet.

representing 1H at 4.03 ppm. An unresolved peak at 1.71 ppm couples only to the peak at 4.03 ppm and collapses in the presence of $\text{CH}_3\text{OH}-d_4$ suggesting the presence of an OH group with the following partial structure:

A multiplet (6H) between 1.38 ppm and 1.49 ppm suggests the presence of three adjacent methylene groups, as was the case for culmorin (55), which are shown to be flanked by two quaternary carbon atoms due to their lack of coupling with any other resonances in the $^1\text{H}/^1\text{H}$ 2D correlation NMR spectrum.

An AB quartet (2H) at 1.82 ppm and 2.59 ppm, respectively, ($J = 18.5$ Hz) was also apparent in the oxidized version of culmorin, i.e., culmorin diketone (62), at 1.91 ppm and 2.26 ppm, respectively ($J = 18.7$ Hz), and had been assigned to H-10. Similarly, these resonances at 1.82 ppm and 2.59 ppm, respectively, are assigned to H-10. One proton of this AB system representing H-10 couples with one methyl group, presumably that at position 12, while the second proton displays long range coupling with the methine proton at 4.03 ppm.

In the $^1\text{H}/^1\text{H}$ 2D correlation NMR (p.235), a singlet (1H) at 2.33 ppm couples with three methyl groups, i.e., H-12 as well as H-14 and H-15 (reduced intensity). In addition, it also couples to the AB system (2H), assigned to H-10, (unequal intensity), as well as displaying weak coupling to the doublet (1H) at 1.24 ppm. These resonances exhibited in the ^1H NMR, suggest the structure culmorone yet some doubt remains as to the correct assignment of the carbonyl group to position 8 or 11 as indicated below:

59b

H-1	H-7	H-8	H-11
1.24 (d)	2.33 (s)	-	4.03 (br. s.)

59a

H-1	H-7	H-8	H-11
2.33 (s)	1.24 (d)	4.03 (br. s.)	-

It should be noted that the singlet (1H) at 2.33 ppm couples to both the α and β protons of H-10. In culmorone, if the C=O is at position 8 (59b) this singlet (1H) at 2.33 ppm would represent H-7, which could not couple to H-10 due

to the large spatial separation between the two protons, However, if culmorone had been oxidized at the 11 position (59a) the singlet (1H) at 2.33 ppm would represent H-1 which could couple to H-10, where the stronger coupling between H-1 and the β proton of H-10 (w coupling) is apparent by a stronger cross peak in the $^1\text{H}/^1\text{H}$ 2D correlation NMR. In addition, only one of the protons of H-10 (β at 1.82 ppm) couples with the 1H broad singlet at 4.03 ppm, assigned to H-8, due to long range "w" coupling, i.e., the α proton of H-10 at 2.59 ppm, does not couple to H-8.

To assign the ^{13}C NMR resonances, a $^1\text{H}/^{13}\text{C}$ 2D correlation NMR was carried out (p.236). The resonances are in agreement with the structure in which the C=O is located at the 11 position of culmorin (55).

In their extensive studies of culmorin (55), Barton and Werstiuk (93) prepared the two hydroxy-ketones, i.e., culmorone isomers 59a and 59b, by oxidizing culmorin with sufficient chromic acid to oxidize one hydroxyl group only. This resulted in a mixture which upon purification led to the isolation of two compounds, 59a and 59b, with melting

points of 120-121°C and 107-108°C, respectively. The sample isolated from *F. roseum* had a melting point of 116-119°C. In addition, some spectroscopic data was included by Barton and Werstiuk (93), more specifically; for the 8-keto derivative (59b), the H-11 resonance occurs at 4.55 ppm but for the 11-keto derivative (59a) the H-8 resonance occurs at 4.05 ppm. In this case, the natural product displays a resonance at 4.03 ppm suggesting the 11-keto derivative (59a) according to Barton's results.

Therefore, from the ^1H , ^{13}C and MS data, this product can be represented by the structure of the 11-keto derivative of culmorine (55), i.e., culmorone (59).

59

TABLE 20

250-MHz ^1H NMR Chemical Shift Assignments and Coupling Constants for Culmorin (55) and Culmorone (59).

Carbon Number	Culmorin (<u>55</u>)	Culmorone (<u>59</u>)
1	1.90 ($J_{1,11} = 4.5$)	2.33
3	1.2-1.4 (unresolved)	1.38-1.49 (unresolved)
4	1.2-1.4 (unresolved)	1.38-1.49 (unresolved)
5	1.2-1.4 (unresolved)	1.38-1.49 (unresolved)
7	1.72 ($J_{7,8} = 5.0$)	1.24 ($J_{7,8} = 4.8$)
8	3.80 ($J_{8,7} = 5.0$)	4.03 (br. s.) 1.71 (OH, br. s.)
10	1.63 ($J_{10,11} = 6.8$)	1.82 (β) ($J_{AB} = 18.5$; 1.49)
11	4.33 } ($J_{11,10} = 7.0$; $J_{11,1} = 4.5$)	2.59 (α)
12	0.85 (s)	1.01 (s)
13	0.79 (s)	0.88 (s)
14	0.89 (s)	0.95 (s)
15	0.98 (s)	0.98 (s)

Spectra were recorded in CDCl_3 ; δ in ppm from chloroform.

Resonances between position 14 and 15 may be reversed.

TABLE 21

62.8-MHz ^{13}C NMR Chemical Shift Assignments for
Culmorin (55) and Culmorone (59).

Carbon Number	Culmorin (<u>55</u>)	Culmorone (<u>59</u>)
1	49.7	60.0
2	51.3 (1)	50.0 (1)
3	36.3 (2)	34.8 (2)
4	22.6	21.9
5	41.3 (2)	41.4 (2)
6	32.9	33.9
7	52.2	57.8
8	79.5	78.0
9	52.2 (1)	51.7 (1)
10	36.0	41.2
11	71.0	216.5
12	13.1	12.4
13	22.0	22.4
14, 15	28.8, 29.2	28.4, 29.6

Spectrum was recorded in CDCl_3 ; δ in ppm relative to
chloroform.

- (1) Assignments for carbons 2 and 9 may be reversed.
(2) Assignments for carbons 3 and 5 may be reversed.

2-5 BIOSYNTHETIC IMPLICATIONS:

With the exception of culmorin (55) and culmorone (59), the sesquiterpenes isolated exhibit structural similarities which invite some unifying statements concerning their biosynthetic origin with respect to the Fusarium species.

Upon examination of the trichothecenes isolated from F. roseum and F. culmorum (101) cultures, four basic carbon skeletons are apparent. These include (i) isotrichodermin (43) which represents the simplest trichothecene isolated with an acetoxy group at the 3 position (ii) "apotrichothecene" (54) (iii) sambucoin (53) and (iv) sambucinol (60). The latter two compounds were first isolated by Mohr et al. (92) from cultures of F. sambucinum and, has since been detected in fermentations from F. sporotrichiodes (102). The fact that sambucoin (53) and sambucinol (60) were detected in species of F. Sambucinum implies a similarity in the biosynthetic pathways followed by these three species.

To suggest a biosynthetic pathway, we must assume that trichodiene (61), first isolated in 1972 by Machida and Nozoe (95), from strains of Trichothecium roseum, arising from farnesyl pyrophosphate via the bisabolyl cation (57) (62), also acts as the biosynthetic intermediate in the Fusarium species.

As suggested in Figure 16, slight modifications in the oxygenation steps as well as in the cyclization route followed from trichodiene (61), may lead to the 4 different carbon skeletons observed, i.e., 43, 54, 53 and 60.

For example, both "apotrichothecene" (54) and sambucinol (60), being structurally similar, appear to follow pathway C (Figure 16) in which "apotrichothecene" (54) may serve as a biogenetic precursor to the more highly

FIGURE 16: Proposed Route from Trichodiene (61) to the Four Basic Trichothecene Skeletons

oxygenated sambucinol (60). It is interesting to note that sambucinol (60) was not detected in the crude isolate, Fraction A, from F. roseum for the still culture conditions used.

Interestingly from X-Ray studies, the AB ring stereochemistry for the epoxytrichothecenes has been shown to be cis whereas for sambucoin (53), this stereochemistry was shown to be trans (92). It is quite possible that this trans stereochemistry may be the result of an oxygen substituent already present in a fixed orientation at the 11 position. Through the use of molecular models, the plausability of this cyclization process can be evaluated as follows:

Oxygenation Patterns

During our studies of the minor metabolites produced by F. roseum, all of the trichothecenes isolated contained an oxygen substituent at the 3 position, with the exception of sambucoin (53), and an epoxide group or evidence suggesting its prior existence, i.e., rearrangement product, at the 12 position. No examples of oxygenation at the 4 position were detected yet oxygenation at the 7,8 and 15 positions was frequently observed.

Recently, Ichinoe et al. (96) showed that Gibberella Zeae (F. graminearum) produced either nivalenol (3,4-oxygenated) or deoxynivalenol (3-oxygenated) metabolites. The fact that we isolated only 3-oxygenated and not 4-oxygenated trichothecenes, from F. roseum, suggests that the 3 and 4 positions of the trichothecene ring system are the primary sites for biological oxidation which may occur very early in the biosynthetic pathway, either just before or immediately after cyclization of trichodiene (61). This specificity may be the result of the presence of enzyme systems common to a particular species of fungi. Further oxidation of the trichothecene ring at positions 7,8 and 15

appears to be a more general reaction common to all fungal species, as is documented by Tamm and Breitenstein (51).

In terms of the 4 carbon skeletons presented in Figure 16, one could speculate that a bisepoxide of trichodiene (63) serves as their biogenetic precursor. It would then be possible for this epoxide, linking the 2,3 positions, to be opened with either hydroxide or hydride ion to yield the required intermediate.

63

Biosynthetic Scheme for 3-AcDON (40)

In terms of the epoxytrichothecenes isolated, they have in common an acetoxy group at the 3-position of the trichothecene ring. Since this is the only exocyclic oxygen substituent in isotrichodermin (43), it suggests that isotrichodermin (43) could be a biosynthetic precursor for

3-AcDON (40), with the other more highly oxygenated metabolites acting as intermediates. (Figure 17). It is possible that 3,15-diAcDON (41), isolated during these experiments, could be formed from DDD (50) as was demonstrated chemically using MnO_2 .

FIGURE 17: Biosynthetic Scheme for 3-AcDON (40) in *E. roseum*

CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

The extract from F. roseum led to the isolation and identification of 15 metabolites of which 9 are new compounds never before isolated from natural sources (Appendix 6 p.247). The structures of the trichothecenes isolated suggest a biosynthetic pathway based on ring A oxidation after cyclization.

Other ring structures (e.g. culmorin (55) and sambucoin (53) have been detected for the first time in this species as well an oxidized version of culmorin (58), i.e., culmorone (59), has been characterized.

A multitude of other metabolites from this extract remain to be characterized. For example, a compound present in significant quantities by G.C. has been purified but, the limited amount obtained has not allowed for its complete characterization.

The isolation of trichodiene (61) or of a more highly oxidized derivative would be significant in helping to confirm the biosynthetic route to the trichothecenes. With regards to the metabolites identified, "apotrichothecene"

(54) requires further study necessitating the isolation of additional material. In addition to confirming its structure, "apotrichothecene" (54) may be shown to represent a biosynthetic precursor of the trichothecene mycotoxin sambucinol (60).

3- EXPERIMENTAL

Abbreviations used in the experimental part of this thesis.

m = multiplet.

br. s. = broad singlet

dd = doublet of doublets

d = doublet

s = singlet

q = quartet

dt = doublet of triplets

Melting points (m.p.) were determined on a Buchi SMP-20 apparatus and are uncorrected.

The Infrared (IR) spectra were recorded on a Perkin-Elmer 683 Infrared Spectrophotometer.

Ultraviolet (UV) spectra were recorded on a DMS-90 UV-Visible spectrophotometer (Varian).

The optical rotation was recorded using sodium light at 589 nm with a Perkin-Elmer 141 Polarimeter.

Proton nuclear magnetic resonance (NMR) spectra were recorded at Agriculture Canada on a Bruker WM 250 NMR

spectrophotometer operating at 250 MHz. Carbon-13 nuclear magnetic resonance (NMR) spectra were recorded at Agriculture Canada on a Bruker WM 250 NMR spectrophotometer operating at 62.8 MHz. Chemical shifts are measured by reference to CDCl_3 at 77.0 ppm for ^{13}C and 7.24 ppm for ^1H and are reported relative to tetramethylsilane (TMS).

Mass spectra (MS) were determined at Agriculture Canada (a) by using a Finnigan MAT 312 mass spectrometer with the FAB probe, the samples were applied in glycerine, unless stated otherwise, and (b) by direct insertion into the source of a DuPont 21-110b instrument, precise mass measurements were obtained by peak matching with an ion in the spectrum of perfluorokerosene as a standard and c) by using gas-chromatography/mass spectrometry (GC/MS).

Thin layer chromatography (tlc) analysis was carried out on precoated 250 micron, multiple channel silica gel plates (Whatman). Detection was accomplished by viewing under a UV source or by heating the plate at 100-200 °C after spraying with a 1% solution of ceric ammonium nitrate in 50% aqueous sulphuric acid. For isolation purposes precoated 20 cm X 20 cm silica gel plates, GF-254, (Merck)

were used with a thickness of 0.25 mm (# 5760) for analytical and 2.0 mm (# 5717) for preparative purposes.

Medium pressure liquid chromatography (MPLC) separations were carried out on a home built "ApLab" system. Columns were supplied from Beckman and packed with LiChroprep TMSi 60-partical size 25-40 μ m (BDH Chemicals Canada Ltd.). The solvent was pumped through the columns using a FMI Lab Pump, Model RPSY (Fluid Metering Inc., New York, U.S.A.).

High pressure liquid chromatography (HPLC) separations were carried out on a Waters Associates Liquid Chromatograph linked to a variable wavelength UV detector (Spectroflow Monitor SF770 from Schoeffel Instrument Corp.) using a normal phase silica gel column (Waters).

Capillary gas chromatography (GC) was carried out using a Varian Vista 6000 Capillary G.C. The capillary column used was a Dufawax Series DX-2 Medium Polar; 75% dimethyl-polysiloxane and 25% polyethylene glycol.. Temperature range 50-250/270 °C, 15 meters in length $\times$ 0.32 mm, film thickness 0.25 μ m, supplied by J. and W. Scientific Inc. (Chromatographic Specialities Ltd.).

Temperature Program

Injector Program: 70-240 °C at 125 °C/min. Hold for 5 minutes.

Column Program: 70°C Hold 0.1 minutes.

70-200 °C at 15 °C/min.

200-240 °C at 5 °C/min. Hold 20 minutes.

Total time = 36.8 minutes.

Chromatographic separations were also carried out using a Chromatotron (Model number 7924, Harrison Research, Inc.). All solvents used were reagent grade and glass distilled before use.

As described previously by Greenhalgh et al. (83), Fusarium roseum (ATCC 28114) (82) was cultured, the cultures were harvested and the broth was extracted using dichloromethane. After evaporating this extract to dryness, the resulting concentrate was partitioned between methanol and petroleum ether. The methanol phase was concentrated to yield 8.5 g of an oil which when subjected to further purification (81), using a Florisil column (169 g) eluted with 750 mL portions of (A) methylene chloride, (B) 0.5% methanol in methylene chloride and (C) 5% methanol in methylene chloride, resulted in the isolation of three

fractions; fraction A afforded an oil (5.5 g) from which 3-AcDON (40) (2.9 g) was obtained, fractions B and C also afforded yellow oils weighing 1.24 and 1.89 g respectively (81).

These fractions were provided for use by the Atlantic Regional Laboratory, National Research Council, Halifax, Nova Scotia.

The purification of fraction A was carried out by two methods.

3-1 METHOD I

The oil (1.69 g), which remained in fraction A after the removal of 3-AcDON (40), was chromatographed on a silica gel column (20 g, Kieselgel 60, 63-200 μ or 70-230 mesh) and was eluted first with 10% toluene in methylene chloride (250 mL) and then with 0.5% methanol in methylene chloride (250 mL), 1.5% methanol in methylene chloride (250 mL), 3% methanol in methylene chloride (250 mL), and finally with 13% methanol in methylene chloride. Fractions (50 mL) ~~were collected and monitored by tlc;~~ fractions 6-10, 11-12, 13-17, and 18-20 were combined and the solvent was

removed under reduced pressure to give mixtures A1 (80 mg), A2 (740 mg), A3 (552 mg) and A4 (178 mg) respectively. Further purification of mixtures A2 and A3 was accomplished using a Chromatotron system. Silica gel plates (Kieselgel 60) of 2 mm thickness, unless specified otherwise, were used to effect this separation.

MIXTURE A-2

As a typical separation, a sample dissolved in ethyl acetate (520 mg of mixture A-2) was injected onto the Chromatotron and eluted with 30% ethyl acetate in hexane. The eluate was collected in 10 mL fractions and the progress was monitored by tlc (3:1 ethyl acetate in hexane). The appropriate fractions were combined (Fr. 2-13, 14-19, 20-26, 27-33, 34-43 and 44-60) and the solvent was removed under reduced pressure to yield 6 major fractions of varying purity.

1) ISOLATION OF ISOTRICHODERMIN (43)

Fraction 2-13 from the mixture A-2 was further purified by rechromatographing the material (205 mg) using the Chromatotron and a solvent system of 20% ethyl acetate

in hexane. After combining the appropriate fractions, 37 mg of an impure solid was obtained. This purification procedure was repeated twice more with a solvent system of 15 and 10% ethyl acetate in hexane, respectively. This final purification resulted in the isolation of a crystalline material which upon recrystallization from ethyl acetate and hexane yielded 3.4 mg of colourless crystals.

ISOTRICHODERMIN (43) SPECTROSCOPIC DATA

R_f = 0.74 (75% ethyl acetate/hexane), 0.86 (5% methanol/chloroform)

m.p. = 97.0-97.5 ° C; $[\alpha]_D^{26.8^\circ} = +9.49^\circ$ (c 0.527, chloroform)

MS = m/z 292.174 (M)⁺, calcd. for $C_{17}H_{24}O_4$ 292.168;

¹H NMR δ (ppm) 0.74 (3H, H-15, s), 0.80 (3H, H-14, s), 1.0-2.0 (6H, H-7, H-4, H-8, m), 1.71 (3H, H-16, br.s.), 2.11 (3H, Ac-CH₃), 2.84, 3.07 (2H, H-13, $J_{A,B} = 4.0$ Hz, dd), 3.73 (1H, H-2, $J_{2,3} = 4.6$ Hz, d), 3.96 (1H, H-11, $J_{11,10} = 5.5$ Hz, d), 5.15 (1H, H-3, $J_{3,4} = 10.0$ Hz; $J_{3,2} = 4.6$ Hz, m), 5.44 (1H, H-10, $J_{10,11} = 5.5$ Hz; $J_{10,16} = 1.5$ Hz, m).

¹³C NMR Table 13, p. 94

IR max. cm^{-1} (KBr pellet)

1748 C=O (acetate)

1250 C-O

ii & iii) ISOLATION OF 3-AcDON (40) and
3-Ac-7-deoxyDON (42)

The further purification of fraction 34-43, i.e. from mixture A-2, was accomplished by rechromatographing the material (30.6 mg) using the Chromatotron and a solvent system of 20% ethyl acetate in hexane. In this case, the separation of the bands could be monitored by ultra violet light. The appropriate fractions were combined resulting in the isolation of two crystalline materials, 3-AcDON (40), and 3-Ac-7-deoxyDON (42), which upon recrystallization from ethyl acetate and hexane yielded 10.0 mg and 11.0 mg respectively.

3-AcDON (40) - Spectroscopic Data

R_f = 0.46 (75% ethyl acetate/hexane), 0.61 (5% methanol/chloroform)

m.p. = 186.5-187.0 °C; $[\alpha]_D^{26.8} = +21.74^\circ$ (c 0.46, chloroform)

MS m/z 338.124 (M)⁺, calcd. for C₁₇H₂₂O₇ 338.137

¹H NMR δ (ppm) 1.11 (3H, H-14, s) 1.85 (3H, H-16, J_{16,10} = 1.1 Hz, d) 2.11 (3H, Ac-CH₃, s) 2.15, 2.35 (2H, H-4, J_{A,B} = 15.1 Hz; J_{4,3} = 4.5 Hz, 11.2 Hz, m) 3.08, 3.14 (2H, H-13, J_{A,B} = 4.2 Hz, dd) 3.81 (OH) 3.83, 3.73 (2H, H-15, J_{A,B} = 11.7 Hz, dd) 3.87 (1H, H-2, J_{2,3} = 4.5 Hz, d) 4.66 (1H, H-11, J_{11,10} = 5.8 Hz, d) 4.78 (1H, H-7, J_{7,6H} = 2.0 Hz, d) 5.17 (1H, H-3, J_{3,4} = 11.2 Hz, 4.5 Hz; J_{3,2} = 4.5 Hz, m) 6.56 (1H, H-10, J_{10,11} = 1.6 Hz, dd)

¹³C NMR Table 11, p. 85

IR max cm⁻¹ (KBr pellet)
 3465 OH
 1745 C=O (acetate group)
 1685 C=O (in conjugation with C=C)
 1245 C-O

U.V. max = 219 nm, A = 0.667, c = 8.162 x 10⁻⁵ mol/L
 (in ethanol), ε = 8172

3-Ac-7-deoxyDON (42) - Spectroscopic Data

R_f = 0.43 (75% ethyl acetate/hexane), 0.62 (5% methanol/
 chloroform)

m.p. = 202.0-202.5 °C; [α]_D^{26.8°C} = +41.67° (c 0.19, chloroform)

MS m/z 322.146 (M)⁺, calcd. for C₁₇H₂₂O₆ 322.142

¹H NMR δ (ppm) 0.83 (3H, H-14, s) 1.80 (3H, H-16, J_{16,10} =
 1.4 Hz, br. s.) 2.12 (3H, Ac-CH₃, s) 2.17, 2.40 (2H, H-4,
 J_{A,B} = 14.7 Hz; J_{4,3} = 4.1 Hz, m) 2.42, 2.86 (2H, H-7, J_{A,B} =
 16.8 Hz, m) 2.86, 3.09 (2H, H-13, J_{A,B} = 3.9 Hz, dd) 3.64 (2H,
 H-15, s) 3.81 (1H, H-2, J_{2,3} = 4.6 Hz, d) 4.52 (1H, H-11,
 J_{11,10} = 5.7 Hz, d) 5.18 (1H, H-3, J_{3,2} = 4.6 Hz; J_{3,4} = 10.2
 Hz, 4.4 Hz, m) 6.55 (1H, H-10, J_{10,11} = 5.7 Hz; J_{10,16} = 1.4
 Hz, m)

¹³C NMR Table 11, p. 85.

IR max cm⁻¹ (thin film on NaCl cell)
 3475 OH
 1745 C=O (acetate group)
 1680 C=O (conjugated with double bond)
 1245 C-O

U.V. max = 225 nm, A = 0.604, c = 6.829 x 10⁻⁵ mol/L
 (ethanol), ε = 8844

iv) Isolation of 8-Hydroxycalonectrin (52)

The initial purification of fraction 44-60, from the mixture, A-2, was accomplished by recrystallizing this material (53 mg) from ethyl acetate and hexane to yield 29 mg of yellow, white powder. By tlc examination, this appeared to be composed of two products, one of which was determined to be 3,15-diacetyl-7,8-dihydroxydeoxyinivalenol (DDD) (50), also known as 7,8-dihydroxycalonectrin.

The purification of this unknown crystalline material necessitated the use of an analytical plate (0.25 mm thickness) and a solvent system of 5% methanol in chloroform. An overlapping band was isolated by grinding the silica gel to a fine powder and washing with ethyl acetate. The overlapping nature of this band required a second identical purification, which this time resulted in the isolation of a crystalline material (4.3 mg) which was not subjected to further purification.

8-Hydroxycalonectrin (52) - Spectroscopic Data

R_f = 0.37 (75% ethyl acetate/hexane), 0.65 (5% methanol/chloroform)

m.p. = 168.0-170.0 °C; $[\alpha]_D^{25} -6.67^\circ$ (c 0.15, chloroform)

MS m/z 306.145 (-AcOH) (M)⁺, calcd. for $C_{17}H_{22}O_5$ 306.147

1H NMR δ (ppm) 0.83 (3H, H-14, s) 1.85 (3H, H-16, $J_{16,10} = 0.56$ Hz, br. s.) 1.90-2.30 (4H, H-7, H-4, m: 2.25, $J_{AB} = 14.6$ Hz; $J = 5.7$ Hz; 2.21, $J = 10.1$ Hz) 2.03 (3H, Ac-CH₃, s) 2.11 (3H, Ac-CH₃, s) 2.89, 3.08 (2H, H-13, $J_{AB} = 4.0$ Hz, dd) 3.73 (1H, H-2, $J_{2,3} = 4.6$ Hz, d) 4.14 (2H, H-15, dd) 4.14 (2H, H-11, H-8, unresolved multiplet) 5.15 (1H, H-3, $J_{3,2} = 5.0$ Hz; $J_{3,4} = 10.0$ Hz, 5.1 Hz, m) 5.58 (1H, H-10, $J_{10,11} = 5.7$ Hz; $J_{10,16} = 1.1$ Hz, dd)

^{13}C NMR Table 15, p. 114

IR	max	cm ⁻¹	(thin film on NaCl cell)
		3500	OH
		1740	(broad) C=O (acetate group)
		1245	C-O (broad)

Mixture A-3

A sample dissolved in ethyl acetate (164 mg of A-3) was injected onto the Chromatotron and eluted with 50% ethyl acetate in hexane. The appropriate fractions were combined to yield five major samples (one through five) of varying purity.

¹³C NMR Table 21, p. 141

IR max cm⁻¹ (thin film on NaCl cell)
 3420 OH
 1725 } Band at 1725 cm⁻¹ more intense and
 1743 } broader than the band at 1743 cm⁻¹
 1380 & Geminal dimethyls
 1370

Calonectrin (47) - Spectroscopic Data

R_f = 0.69 (75% ethyl acetate/hexane), 0.86 (5% methanol/chloroform)

m.p. = 75.5-78.5 °C; [α]_D^{23.8} = +7.97° (c 0.121, chloroform)

MS m/z 290.149 (M)⁺, calcd. for C₁₇H₂₂O₄ 290.152 (-AcOH)

¹H NMR δ (ppm) 0.81 (3H, H-14, s) 1.70 (3H, H-16, br.s.)
 2.03 (3H, Ac-CH₃, s) 2.10 (3H, Ac-CH₃, s) 1.78-2.19 (6H, H-4,
 H-7, H-8; J_{4,3} = 10.0 Hz, m) 2.84, 3.08 (2H, H-13, J_{A,B} = 4.0
 Hz, dd) 3.74 (1H, H-2, J_{2,3} = 4.6 Hz, d) 3.82, 4.06 (2H,
 H-15, J_{A,B} = 12.2 Hz, dd) 4.00 (1H, H-11, J_{11,10} = 5.6 Hz, dd)
 5.15 (1H, H-3, J_{3,2} = 5.0 Hz; J_{3,4} = 9.6 Hz, 7.4 Hz, m) 5.45
 (1H, H-10, J_{10,11} = 5.4 Hz; J_{10,16} = 1.3 Hz, m)

¹³C NMR Table 15, p. 114

IR max cm⁻¹ (Thin film on NaCl cell)
 1745 C=O (Acetate group) broad band
 1245 C-O broad band

x) Isolation of 3,15-diAcDON (41)

Sample number 16 (112.4 mg) was purified using the Chromatotron and a solvent system of chloroform which was changed after eluting the plate with 500 mL to 2% methanol

v. Isolation of DDD 50

Sample three 50 mg from the mixture A-3 was purified in a similar manner to that of culmorin which resulted in the isolation of 47 mg of a white precipitate which was not recrystallized.

DDD (50) - Spectroscopic Data

R_f = 0.33 (5% ethyl acetate/hexane), 0.58 (5% methanol; chloroform)

m.p. = 190.5-191.5 °C; $[\alpha]_D^{25.8} = +7.13^\circ$ (c 1.122, chloroform)

MS. m/z 382.179 (M)⁺, calcd. for $C_{19}H_{26}O_8$ 382.163

¹H NMR δ (ppm) 1.12 (3H, H-14, s) 1.86 (3H, H-16, $J_{16,10} = 1.0$ Hz, br.s.) 2.02 (3H, Ac-CH₃, s) 2.10 (3H, Ac-CH₃, s) 2.13 2.34 (2H, H-4, $J_{4,3} = 11.1$ Hz, 4.6 Hz; $J_{AB} = 15.0$ Hz, m) 2.76 (1H, 8-OH, $J_{8,OH} = 8.0$ Hz, d) 3.00 (1H, 7-OH, $J_{7,OH} = 9.7$ Hz, d) 3.09, 3.20 (2H, H-13, $J_{AB} = 4.3$ Hz, dd) 3.77 (1H, H-2, $J_{2,3} = 4.3$ Hz, dd) 3.77 (1H, H-2, $J_{2,3} = 4.4$ Hz, d) 5.99 (1H, H-8, $J_{8,7} = 5.8$ Hz; $J_{8,OH} = 7.5$ Hz, m) 4.14, 4.44 (2H, H-15, $J_{AB} = 12.3$ Hz, dd) 4.32 (1H, H-11, $J_{11,10} = 5.8$ Hz, d) 4.50 (1H, H-7, $J_{7,OH} = 9.6$ Hz; $J_{7,8} = 5.4$ Hz, m) 5.15 (1H, H-3, $J_{3,2} = 4.4$ Hz; $J_{3,4} = 4.5$ Hz, 11.1 Hz, m) 5.58 (1H, H-10, $J_{10,11} = 5.8$ Hz; $J_{10,16} = 0.9$ Hz, m)

¹³C NMR Table 15, p. 114

IR	max	cm ⁻¹	(KBr pellet)
		3540	OH
		1735	C=O (acetate group)
		1715	C=O (acetate group)
		1252	C-O
		1265	C-O

¹H NMR of DDD (50) run in deuterated methanol indicated that the multiplets at 3.99 and 4.30 ppm collapsed to two doublets at 3.84 and 4.44 ppm respectively ($J = 5.3$ and 5.9 Hz). Also a disappearance of the two doublets at 2.76 and 3.00 ppm was noted.

3-2 Method 2:

Medium pressure liquid chromatography (MPLC) was used as an alternative method of purifying fraction A. A one meter long column with an inside diameter of 2.5 cm (Beckman supplies) was packed with approximately 220 g of LiChroprep silica gel (BDH chemicals). The solvent was pumped through the column using a FMI laboratory pump, at a rate of approximately 5 mL/min. The column was initially rinsed with ethyl acetate. The polarity of the solvent was gradually decreased until a solution of 10% ethyl acetate in hexane was being pumped through the column.

Fraction A (4.08 g) was dissolved in a minimum amount of ethyl acetate and injected onto the column. The column was eluted with the following solvents and approximately 18 mL fractions were collected:

- i) 10% ethyl acetate/hexane (1.3 L) Fractions 1-69
- ii) 20% ethyl acetate/hexane (1.3 L) Fractions 70-142
- iii) 30% ethyl acetate/hexane (5.8 L) Fractions 143-460
- iv) The concentration of ethyl acetate in hexane was gradually increased to 100% ethyl acetate for Fractions 461-690.

The appropriate fractions were combined resulting in the isolation of 50 samples of varying purity.

vii. Isolation of Sambucosin (53)

By tlc examination, sample number 11 appeared to be composed of mainly one compound. The further purification of this material (47.1 mg) was accomplished by recrystallizing it from ethyl acetate and hexane which resulted in the isolation of 20 mg of a white crystalline material known as sambucosin (53).

Sambucosin (53) - Spectroscopic Data

R_f = 0.71 (75% ethyl acetate/hexane), 0.64 (5% methanol/chloroform)

m.p. = 243.0-243.5 °C; $[\alpha]_D^{25}$ = +120.0 (c 0.10 chloroform)

MS m/z 250.160 (M)⁺, calcd. for C₁₅H₂₂O₃ 250.157

¹H NMR δ (ppm) 0.63 (3H, H-14, s) 1.13 (3H, H-15, s) 1.43 (2H, H-7, J_{7,8} = 2.3 Hz, 5.4 Hz; J_{AB} = 13.3 Hz, m) 1.64 (3H, H-16, br. s.) 1.74 (1H, H-4, J_{4,3} = 7.9 Hz, 11.7 Hz; J_{AB} = 13.8 Hz, ddd) 1.8-1.9 (2H, H-8, broad band) 1.99 (1H, H-4, J_{4,3} = 3.2 Hz, 10.2 Hz; J_{AB} = 13.6 Hz, ddd) 2.26 (1H, H-3, J_{3,4} = 7.9 Hz, 10.0 Hz; J_{AB} = 19.9 Hz, ddd) 2.56 (1H, H-3, J_{3,4} = 3.0 Hz, 11.8 Hz; J_{AB} = 19.95 Hz, ddd) 3.42 (1H, H-13, J_{AB} = 11.2 Hz, d) 4.18 (1H, H-13, J_{AB} = 11.2 Hz, d) 3.94 (1H, H-11, br. s.) 5.18 (1H, H-10, br. s.)

¹³C NMR Table 17, p. 122

IR	$\nu_{\max}$	cm ⁻¹	(KBr pellet)
		3380	OH (sharp)
		1735	C=O (5-membered ring)

viii & ix) Isolation of Culmorone (59) and Calonectrin (47)

Sample number 13 (167.2 mg) was purified using the Chromatotron and a solvent system of chloroform which was changed after eluting the plate with 500 mL to 5% methanol in chloroform. The appropriate fractions were combined resulting in the isolation of two crystalline materials, culmorone (59) and calonectrin (47). Culmorone (59) was recrystallized from ethyl acetate and petroleum ether to yield 12.0 mg of white crystals while calonectrin (47) was recrystallized from ether and petroleum ether to yield 8.6 mg of a crystalline solid.

Culmorone (59) - Spectroscopic Data

R_f = 0.67 (75% ethyl acetate/hexane), 0.64 (5% methanol/chloroform)

m.p. = 116.0-119.0 °C; $[\alpha]_D^{23.6} = -16.5^\circ$ (c 0.121, chloroform)

MS m/z 236.178 (M)⁺, calcd. for C₁₅H₂₄O₂ 236.178

¹H NMR δ (ppm). 0.88 (3H, H-13, s) 0.95 (3H, H-14/15, s) 0.98 (3H, H-15/14, s) 1.01 (3H, H-12, s) 1.24 (1H, H-7, J_{7,8} = 4.8 Hz, d) 1.38-1.49 (6H, H-3, H-4, H-5, m) 1.71 (1H, 8-OH, br. s.) 1.82 (1H, H-10 (β), J_{A,B} = 18.5 Hz, J = 1.5 Hz, d) 2.33 (1H, H-1, br. s.) 2.59 (1H, H-10, (α), J_{A,B} = 18.5 Hz, d) 4.03 (1H, H-8, br. s.)

^{13}C NMR Table 21, p. 141

IR	max	cm^{-1}	(thin film on NaCl cell)
		3420	OH
		1725	{ Band at 1725 cm^{-1} more intense and
		1743	
		1380 &	Geminal dimethyls
		1370	

Calonectrin (47) - Spectroscopic Data

R_f = 0.69 (75% ethyl acetate/hexane), 0.86 (5% methanol/chloroform)

m.p. = 75.5-78.5 $^{\circ}\text{C}$; $[\alpha]_D^{23.8} = +7.97^{\circ}$ (c 0.121, chloroform)

MS m/z 290.149 (M) $^{+}$, calcd. for $\text{C}_{17}\text{H}_{22}\text{O}_4$ 290.152 (-AcOH)

^1H NMR δ (ppm) 0.81 (3H, H-14, s) 1.70 (3H, H-16, br.s.)
 2.03 (3H, Ac-CH₃, s) 2.10 (3H, Ac-CH₃, s) 1.78-2.19 (6H, H-4, H-7, H-8; $J_{4,3} = 10.0\text{ Hz}$, m) 2.84, 3.08 (2H, H-13, $J_{A,B} = 4.0\text{ Hz}$, dd) 3.74 (1H, H-2, $J_{2,3} = 4.6\text{ Hz}$, d) 3.82, 4.06 (2H, H-15, $J_{A,B} = 12.2\text{ Hz}$, dd) 4.00 (1H, H-11, $J_{11,10} = 5.6\text{ Hz}$, dd) 5.15 (1H, H-3, $J_{3,2} = 5.0\text{ Hz}$; $J_{3,4} = 9.6\text{ Hz}$, 7.4 Hz, m) 5.45 (1H, H-10, $J_{10,11} = 5.4\text{ Hz}$; $J_{10,16} = 1.3\text{ Hz}$, m)

^{13}C NMR Table 15, p. 114.

IR	max	cm^{-1}	(Thin film on NaCl cell)
		1745	C=O (Acetate group) broad band
		1245	C-O broad band

x) Isolation of 3,15-diAcDON (41)

Sample number 16 (112.4 mg) was purified using the Chromatotron and a solvent system of chloroform which was changed after eluting the plate with 500 mL to 2% methanol.

in chloroform. The appropriate fractions were combined to give samples of varying purity. One of these samples was further purified by recrystallization from ether and petroleum ether to yield 7.2 mg of a white crystalline material known as 3,15-diAcDON (41).

3,15-diAcDON (41) - Spectroscopic Data

R_f = 0.63 (75% ethyl acetate/hexane), 0.84 (5% methanol/chloroform)

m.p. = 117.0-118.5 °C; $[\alpha]_D^{21.3} = +51.0^\circ$ (c 0.98, chloroform)

MS m/z 380.148 (M)⁺, calcd. C₁₉H₂₄O₈ 380.147

¹H NMR δ (ppm) 1.09 (3H, H-14, s) 1.88 (3H, Ac-CH₃, s) 1.89 (3H, H-16, J_{16,10} = 0.8 Hz, d) 2.13 (3H, Ac-CH₃, s) 2.14-2.28 (2H, H-4, J_{4,3} = 4.7 Hz, 11.0 Hz, m) 3.10, 3.15 (2H, H-13, J_{A,B} = 4.3 Hz, dd) 3.76 (1H, 7-OH, J_{OH,7} = 2.0 Hz, d) 3.91 (1H, H-2, J_{2,3} = 4.5 Hz, d) 4.22, 4.28 (2H, H-15, J_{A,B} = 12.0 Hz, dd) 4.70 (1H, H-11, J_{11,10} = 5.8 Hz, d) 4.81 (1H, H-7, J_{7,OH} = 2.0 Hz, d) 5.22 (1H, H-3, J_{3,2} = 4.6 Hz; J_{3,4} = 10.9 Hz, 4.6 Hz, m) 6.57 (1H, H-10, J_{10,11} = 5.8 Hz; J_{10,16} = 1.6 Hz, dd)

¹³C NMR Table 11, p. 85

IR max cm⁻¹ (Thin film on NaCl cell)
 3455 OH
 1745 C=O (Acetate) (Broad band)
 1685 C=O (In conjugation with a double bond)
 1240 C-O (Broad band)

UV λ max = 220, A = 0.680, c = 9.470 x 10⁻⁵ mol/L (ethanol) ϵ = 7181

x1) Isolation of 15-Deacetylcalonectrin: (49)

Sample number 20 (59.2 mg) was purified using the Chromatotron and a solvent system of 15% ethyl acetate in hexane. The fractions were combined and the resulting product was recrystallized from ethyl acetate and hexane to give colourless crystals (13.5 mg).

15-Deacetylcalonectrin (49) - Spectroscopic Data:

R_f = 0.57 (75% ethyl acetate/hexane), 0.69 (5% methanol/chloroform);

m.p. = 184.0-184.5 °C; $[\alpha]_D^{25.0} = +10.0^\circ$ (c 0.100, chloroform);

MS m/z 308.167 (M^+), calcd. for $C_{17}H_{24}O_5$ 308.162;

1H NMR δ (ppm) 0.90 (3H, H-14, s) 1.71 (3H, H-16, $J_{16,10} = 1.5$ Hz, br.s.) 2.09 (3H, Ac-CH₃, s) 1.73-2.30 (6H, H-7, H-8, H-4, m) 2.84, 3.07 (2H, H-13, $J_{A,B} = 4.0$ Hz, dd) 3.47, 3.66 (2H, H-15, $J_{A,B} = 11.6$ Hz; $J_{15,OH} = 4.5$ Hz; dd) 3.72 (1H, H-2, $J_{2,3} = 4.7$ Hz, d) 3.96 (1H, H-11, $J_{11,10} = 5.3$ Hz, d) 5.13 (1H, H-3, $J_{3,2} = 4.6$ Hz; $J_{3,4} = 10.0$ Hz, 5.0 Hz, m) 5.46 (1H, H-10, $J_{10,16} = 1.5$ Hz; $J_{10,11} = 5.3$ Hz, dd)

^{13}C NMR Table 15, p. 114

IR ν_{max} cm^{-1} (Thin film on NaCl cell)

3516	OH (sharp)
1748	C=O (acetate)
1240	C-O

xii) Isolation of 7-Hydroxyisotrichodermin (45)

Sample number 24 (135.9 mg) was purified using the Chromatotron and a solvent system of chloroform which was changed after eluting the plate with 500 mLs to 2% methanol in chloroform. The appropriate fractions were combined to yield a solid material which upon recrystallization from ether and petroleum ether (high boiling, 65-110°C) gave 9.5 mg of a crystalline solid known as 7-hydroxyisotrichodermin (45).

7-Hydroxyisotrichodermin (45) - Spectroscopic Data:

R_f = 0.55 (75% ethyl acetate/hexane), 0.65 (5% methanol/chloroform);

m.p. = 154.0-155.5°C; $[\alpha]_D^{23.5} = -10.5^\circ$ (c 0.095, chloroform);

MS m/z 308.163 (M)⁺, calcd. for C₁₇H₂₄O₅ 308.162;

¹H NMR δ (ppm) 0.82 (3H, H-15, s) 1.04 (3H, H-14, s) 1.09 (1H, 7-OH, $J_{OH,7} = 6.3$ Hz, d) 1.70 (3H, H-16, br.s.) 2.10 (3H, Ac-CH₃, s) 2.06 (2H, H-4, $J = 2.7$ Hz) 1.9, 2.19 (2H, H-8, $J_{A,B} = 17.2$ Hz; $J_{8,7} = 5.9$ Hz, 2 dd) 3.08, 3.10 (2H, H-13, $J_{A,B} = 4.4$ Hz, dd) 3.76 (1H, H-2, $J_{2,3} = 4.5$ Hz, d) 4.10 (1H, H-11, $J_{11,10} = 5.5$ Hz, d) 4.46 (1H, H-7, $J_{7,OH} = 6.0$ Hz; $J_{7,8} = 10.1$ Hz, dt) 5.16 (1H, H-3, $J_{3,2} = 4.5$ Hz; $J_{3,4} = 6.7$ Hz, 9.2 Hz, m) 5.40 (1H, H-10, $J_{10,11} = 5.5$ Hz; $J_{10,16} = 1.2$ Hz, dd)

¹³C NMR Table 13, p. 94

IR	max	cm ⁻¹
	3480	OH
	1743	C=O (Acetate)
	1245	C-O

xiii & xiv: Isolation of 7-Hydroxycalonectrin (51)
and 8-Hydroxyisotrichodermin (46).

Sample number 25 (356.3 mg) was purified using the Chromatotron and a solvent system of chloroform which was changed after eluting the plate with 500 mLs to 5% methanol in chloroform. The appropriate fractions were combined to give five samples of varying purity, one of which was further purified using a preparative tlc plate (silica gel thickness = 2 mm) with a solvent system of 7% methanol in chloroform. Two bands were removed from the plate and the resulting material was recrystallized, in both cases from ethyl acetate and hexane to yield 1.5mg of a whitish faintly yellow crystalline material (51) and 9.3 mg of a whitish crystalline material (46).

7-Hydroxycalonectrin (51) - Spectroscopic Data:

R_f = 0.50 (75% ethyl acetate/hexane), 0.75 (5% methanol/chloroform);

m.p. = 174.0-175.0°C; $[\alpha]_D^{23.2} = -14.3^\circ$ (c 0.14, chloroform);

MS m/z 306.148 (-AcOH) (M)⁺ calcd. for C₁₇H₂₂O₅ 306.147;

¹H NMR δ (ppm) 1.14 (3H, H-14, s) 1.14 (1H, 7-OH, d) 1.72 (3H, H-16, br.s.) 2.08 (3H, Ac-CH₃, s) 2.09 (3H, Ac-CH₃, s) 1.97-2.19 (2H, H-4, partly unresolved, J_{4,3} = 9.8 Hz, m) 2.1, 2.41 (2H, H-8, J_{A,B} = 17.6 Hz; J = 5.8 Hz, dd) 3.09, 3.23 (2H, H-13, J_{A,B} = 4.4 Hz, d) 3.78 (1H, H-2, J_{2,3} = 4.4 Hz, d) 4.11 (1H, H-11, J_{11,10} = 5.4 Hz, d) 4.05, 4.31 (2H, H-15, J_{A,B} = 12.2 Hz; J = 1.1 Hz, dd) 4.55 (1H, H-7 J_{7,OH} = 5.5 Hz; J_{7,8} = 11.2 Hz, m) 5.17 (1H, H-3, J_{2,3} = 4.5 Hz; J_{3,4} = 10.0 Hz, 8.0 Hz, m) 5.38 (1H, H-10, J_{10,11} = 5.4 Hz; J_{10,16} = 1.3 Hz, d)

¹³C NMR Table 15, p. 114

IR	max	cm ⁻¹	
	3515		OH
	1740		C=O (Acetate) Broad band
	1245		C-O Broad band

8-Hydroxyisotrichodermin (46) - Spectroscopic Data:

R_f = 0.44 (75% ethyl acetate/hexane), 0.64 (5% methanol/chloroform);

m.p. = 153.0-155.5°C; $[\alpha]_D^{23.3} = -18.2^\circ$ (c 0.11, chloroform);

MS m/z 308.166 (M)⁺, calcd. for C₁₇H₂₄O₅ (308.162);

^1H NMR δ (ppm) 0.77 (3H, H-14, s) 0.98 (3H, H-15, s) 1.69 (1H, H-7, J = 1.4; $J_{A,B}$ = 14.3 Hz, dd) 1.86 (3H, H-16, br.s.) 2.12 (3H, $\text{CH}_3\text{-Ac}$, s) 2.07-2.11 (2H, H-4, $J_{4,3}$ = 9.6 Hz, 5.8 Hz, dd) 2.87, 3.07 (2H, H-13, $J_{A,B}$ = 4.0 Hz, dd) 3.72 (1H, H-2, $J_{2,3}$ = 4.6 Hz, d) 4.04 (1H, H-11, $J_{11,10}$ = 5.7 Hz, d) 4.11 (1H, H-8, $J_{8,7}$ = 5.4 Hz, t) 5.16 (1H, H-3, $J_{3,2}$ = 4.5 Hz; $J_{3,4}$ = 5.5 Hz, 9.8 Hz, m) 5.59 (1H, H-10, $J_{10,11}$ = 5.9 Hz, d)

^{13}C NMR Table 13, p. 94

IR	max	cm^{-1}
	3480	OH
	1740	C=O (Acetate)
	1245	C-O (Broad)

xv) Isolation of "Apotrichothecene" (54)

An attempt at purifying sample number 48 (43.9 mg) solely by recrystallization from ethyl acetate and hexane was undertaken. Several crystals (0.6 mg) were removed from the mother liquor. A capillary G.C. trace of this material indicated two major compounds in an 11:1 ratio in addition to minor impurities.

"Apotrichothecene" (54) - Spectroscopic Data

$R_f = 0.08$ (60% ethyl acetate/hexane)

MS m/z 253 ($M + H$)⁺

¹H NMR δ (ppm) 0.93 (3H, H-14, s) 1.00 (3H, H-15, s) 1.35 (1H, H-7, J = 2.9 Hz, 5.9 Hz, q) 1.41 (1H, H-7, J = 2.9 Hz, 5.4 Hz, q) 1.50-1.57 (1H, H-2, unresolved m) 1.64 (3H, H-16, J_{16,10} = 1.2 Hz, t) 1.60-1.78 (1H, H-4, J = 2.0 Hz, 11.9 Hz, 18.8 Hz, octet) 1.98-2.03 (2H, H-8, J = 2.1 Hz, br. s.) 2.13 (1H, H-4, J = 10.1 Hz, 13.0 Hz, dd) 2.60 (1H, H-2, J = 1.6 Hz, 12.2 Hz, dq), 3.17 (1H, H-13, J_{A,B} = 10.7 Hz, br. d.) 3.71 (1H, H-13, J = 2.1 Hz; J_{A,B} = 11.4 Hz, dd) 4.12 (1H, H-11, J = 2.1 Hz, br. s.) 4.29 (1H, H-3, unresolved multiplet), 5.52 (1H, H-10, J = 1.6 Hz, 3.2 Hz, br. s.)

¹³C NMR Table 19, p. 128

"Apotrichothecene" (54) - Spectroscopic Data

$R_f = 0.08$ (60% ethyl acetate/hexane)

MS m/z 253 ($M + H$)⁺

¹H NMR δ (ppm) 0.93 (3H, H-14, s) 1.00 (3H, H-15, s) 1.35 (1H, H-7, J = 2.9 Hz, 5.9 Hz, q) 1.41 (1H, H-7, J = 2.9 Hz, 5.4 Hz, q) 1.50-1.57 (1H, H-2, unresolved m) 1.64 (3H, H-16, J_{16,10} = 1.2 Hz, t) 1.60-1.78 (1H, H-4, J = 2.0 Hz, 11.9 Hz, 18.8 Hz, octet) 1.98-2.03 (2H, H-8, J = 2.1 Hz, br. s.) 2.13 (1H, H-4, J = 10.1 Hz, 13.0 Hz, dd) 2.60 (1H, H-2, J = 1.6 Hz, 12.2 Hz, dq), 3.17 (1H, H-13, J_{A,B} = 10.7 Hz, br. d.) 3.71 (1H, H-13, J = 2.1 Hz; J_{A,B} = 11.4 Hz, dd) 4.12 (1H, H-11, J = 2.1 Hz, br. s.) 4.29 (1H, H-3, unresolved multiplet), 5.52 (1H, H-10, J = 1.6 Hz, 3.2 Hz, br. s.)

¹³C NMR Table 19, p. 128

supernatant liquid was decanted and the precipitate was washed several times with dichloromethane. The combined dichloromethane extracts were washed with a 5% solution of HCl (aqueous) three times, a saturated sodium bicarbonate solution (aqueous) three times, and finally with water, three times. The organic layer was then dried over anhydrous magnesium sulfate, filtered and the solvent removed under reduced pressure to yield a faintly yellow oily solid (26.4 mg, $R_f = 0.73$ in 50% ethyl acetate/hexane), barely visible by tlc (UV and charring at 100-200°C, after spraying with a 1% solution of ceric ammonium nitrate in a 50% solution of sulfuric acid). No starting material remained by tlc analysis.

¹H NMR data of Culmorone Diketone (62)

0.97	(3H)	s
1.00	(3H)	s
1.05	(3H)	s
1.21	(3H)	s
1.25-1.70	(6H) H-3, H-4, H-5	br. band
1.80	(1H) H-1/H-7	s
1.91	$J_{AB} = 18.7$ Hz (1H)	H-10 d
2.26	$J_{AB} = 18.6$ Hz (1H)	H-10 d
2.69	(1H) H-1/H-7	s

3-4 Oxidation of DDD (50) to 3,15-diAcDON (41):

Manganese dioxide was activated (104) by azeotropic distillation with benzene to remove any occluded water.

DDD (50) (5.4 mg, 0.014 mmol) was weighed into a screw cap one dram vial. To this was added 0.6mLs of ethyl acetate followed by 57.5 mg of MnO_2 . The sample was flushed with argon and stirred (magnetically) at room temperature. Further additions of 67.2 mg and 50.1 mg of MnO_2 were made at 24 and 48 hours respectively. Stirring was continued for an additional 24 hours, following this last addition. The MnO_2 was removed by filtering through a "Sep Pac Silica Cartridge" (Waters Associates) and rinsing the cartridge

with ethyl acetate. The solvent was removed under reduced pressure and the crude product was purified using a silica gel analytical plate (0.25 mm thickness) with a solvent system of 50% ethyl acetate in hexane to give 2.8 mg of crystalline material (52.2% yield). Starting material still remained. The ^1H NMR was identical with that for 3,15-diAcDON (41).

3-5 Benzoylation of DDD (50):

a: Benzoyl chloride / Pyridine

To a 5 mL round bottom flask containing 20.1 mg of DDD (50) (5.26×10^{-2} mmol) fitted with a condensor and a stirring bar, under argon, was added 2 mLs of a stock solution of benzoyl chloride in pyridine (1.373×10^{-1} mmol/mL) resulting in a 2.6 times excess of benzoyl

chloride. The stock solution had been prepared by taking 0.1605 mL (1.373 mmol) of benzoyl chloride and making up to a volume of 10 mLs with pyridine. After refluxing for 48 hours the reaction was quenched by adding several mLs of methanol. The organic solvents were then removed under reduced pressure resulting in the appearance of a brownish solid. By tlc, the presence of benzoic acid, benzoyl chloride and pyridine as well as a new spot believed to correspond to the dibenzoylated DDD were noted. This residue was dissolved in ethyl acetate and washed with water (3 times), a saturated aqueous solution of sodium bicarbonate (3 times), and a saturated (aqueous) sodium chloride solution. The organic layer was dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure to yield a brownish oil. The purification of this oil was accomplished using a silica gel preparative plate (2 mm thickness)) and a solvent system of 50% ethyl acetate in hexane. The appropriate band was isolated and washed with ethyl acetate. After removal of the solvent under reduced pressure a faintly yellow oil resulted. Upon the addition of hexane, the oil became milky and a whitish solid appeared (9.6 mg) after removal of the hexane under reduced pressure ($R_f = 0.59$, 50% ethyl acetate/hexane). The ^1H NMR spectrum suggested the presence of a mixture of at

least two compounds. The FAB mass spectrum run in diethanolamine (DEA) contained a peak representing the dibenzoylated product, i.e. $M + H + DEA$. In order to further purify this 9.6 mg of material, an analytical HPLC was used (silica gel column, solvent system 15% ethyl acetate/hexane, UV detector set at 254 nm). Two peaks of unequal intensity were present in the material under these conditions. After repeated runs, 2.4 mg of a white precipitate had been collected. By FAB mass spectroscopy, the dibenzoylated sample had been isolated. 1H NMR spectroscopy also confirmed that a substantial purification had occurred as a result of the HPLC experiment.

1H NMR Data for Dibenzoylated DDD.

0.94	(3H)	H-14	s
1.81	(3H)	H-16	Br. s.
1.87	(3H)	acetate	s
2.14	(3H)	acetate	s
2.3-2.0	(2H)	H-4	multiplet
2.70, 3.14	(2H)	H-13	$J_{AB} = 3.5$ Hz dd
3.85	(1H)	H-2	$J_{2,3} = 4.5$ Hz
4.61	(1H)	H-11	$J_{11,10} = 5.7$ Hz

4.53, 4.70 (2H) H-15 $J_{AB} = 12.5$ Hz, dd
5.17 (1H) H-3 $J_{4,3} = 11.6, 4.0$ Hz, m
5.85 (1H) H-10 $J_{10,11} = 5.5$ Hz, d
6.01 (1H) H-7 $J_{7,8} = 5.1$ Hz, d
6.16 (1H) H-8 $J_{8,7} = 5.0$ Hz
7.2-8.1 (10H) benzoyl groups, m

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APPENDIX 1

Summary of the Trichothecenes Isolated *

* (Taken from reference 34 unless stated otherwise)

Fig. 2. The structures of the trichothecenes with simple hydroxyl or acyl substituents (class A). Stereochemistry of the double bonds (c=cis, t=trans, Z or E) and the chiral centers (S or R) is shown for some of the structures)

Name	R ¹	R ²	R ³	R ⁴	R ⁵
Trichothecene	H	H	H	H	H
Trichodermol	H	OH	H	H	H
Trichodermin	H	OAC	H	H	H
Dihydroxytrichothecene	H	OH	H	H	OH
Verrucarol	H	OH	OH	H	H
Diacetylverrucarol	H	OAC	OAC	H	H
Scirpentriol	OH	OH	OH	H	H
T-2 Tetraol	OH	OH	OH	H	OH
Pentahydroxyscirpene	OH	OH	OH	OH	OH
15-Acetoxyscirpendiol	OH	OH	OAC	H	H
Diacetoxyscirpenol	OH	OAC	OAC	H	H
4-Acetoxyscirpendiol	OH	OAC	OH	H	H
Calonectrin	OAC	H	OAC	H	H
15-Desacetylcalonectrin	OAC	H	OH	H	H
Triacetoxyscirpene	OAC	OAC	OAC	H	H
<i>F. scirpi</i> triacetate	OH	OAC	OAC	OH	OAC
7-Hydroxydiacetoxy-scirpenol	OH	OAC	OAC	OH	H

Name	R ¹	R ²	R ³	R ⁴	R ⁵
Neosolanol	OH	OAC	OAC	H	OH
7,8-Dihydroxydiacetoxyscirpenol	OH	OAC	OAC	OH	OH
8-Acetylnesosolanol	OH	OAC	OAC	H	OAC
4,8-Diacetoxo-3,14-dihydroxy-trichothecene (NT-1)	OH	OAC	OH	H	OAC
4-Acetoxo-3,8,15-trihydroxy-trichothecene (NT-2)	OH	OAC	OH	H	OH
HT-2 Toxin	OH	OH	OAC	H	OOCCCH ₂ CH(CH ₃) ₂
T-2 Toxin	OH	OAC	OAC	H	OOCCCH ₂ CH(CH ₃) ₂
Acetyl T-2 Toxin	OAC	OAC	OAC	H	OOCCCH ₂ CH(CH ₃) ₂
4-Desacetylnesosolanol	OH	OH	OAC	H	OH
3,4-Dihydroxy-15-acetoxo-8-(3-hydroxy-3-methylbutyryloxy) trichothecene	OH	OH	OAC	H	OOCCCH ₂ COH(CH ₃) ₂
3,4,15-Trihydroxy-8-(3-hydroxy-3-methylbutyryloxy) trichothecene	OH	OH	OH	H	OOCCCH ₂ COH(CH ₃) ₂
Trichoderma diene	H	OOCCCH=CHCH=CHCH ₂ CH ₃ (c) (t) (R) (R)	OH	H	OOCCCH ₂ COH(CH ₃) ₂
Trichoderma dieneol A	H	OOCCCH=CHCH=CHCH ₂ CH ₂ CH ₃ (c) (t) (S) (S)	H	H	H
Trichoderma dieneol B	H	OOCCCH=CHCH=CHCH ₂ CH ₂ CH ₃ (c) (t) (S) (R)	H	H	H
Trichoverrol A	H	OOCCCH=CHCH=CHCH ₂ CH ₂ CH ₃ (c) (t) (S) (S)	OH	H	H
Trichoverrol B	H	OOCCCH=CHCH=CHCH ₂ CH ₂ CH ₃ (c) (t) (S) (R)	OH	H	H
Trichoverrin A	H	OOCCCH=CHCH=CHCH ₂ CH ₂ CH ₃ (c) (t) (S) (S)	OOCCCH-C(CH ₃)CH ₂ CH ₂ OH (E)	H	H
Trichoverrin B	H	OOCCCH=CHCH=CHCH ₂ CH ₂ CH ₃ (c) (t) (S) (R)	OOCCCH-C(CH ₃)CH ₂ CH ₂ OH (E)	H	H

Fig. b. The structures of the 8-keto trichothecenes (class B)

Name	R ¹	R ²	R ³	R ⁴
Trichothecolone	H	OH	H	H
Trichothecin	H	OOCCH=CHCH ₃	H	H
4-Acetyltrichothecolone	H	OAC	H	H
Nivalenol	OH	OH	OH	OH
Fusarenon-X	OH	OAC	OH	OH
4,15-Diacetylnivalenol	OH	OAC	OAC	OH
4-Desoxynivalenol (vomitoxin)	OH	H	OH	OH
3-Acetyl-desoxynivalenol	OAC	H	OH	OH
7-Acetyl-desoxynivalenol	OH	H	OH	OAC
3,15-Diacetyl-desoxynivalenol	OAC	H	OAC	OH
4,7-Didesoxynivalenol	OH	H	OH	H
7-Desoxynivalenol	OH	OH	OH	H

Fig. c. The structures of the macrocyclic trichothecenes (class C). Stereochemistry of the double bonds (c=cis, t=trans, Z or E) and the chiral centers (S or R) is shown for some of the structures

Name	R ¹	R ²
Verrucaric A	$ \begin{array}{c} \text{O} \\ \\ -\text{CCHOHCH}(\text{CH}_3)\text{CH}_2\text{CH}_2\text{OCCH}=\text{CHCH}=\text{CHC}- \\ \text{(s)} \quad \text{(R)} \qquad \qquad \qquad \text{(t)} \quad \text{(c)} \end{array} $	H

Name	R ¹	R ²
Roridin J	$ \begin{array}{c} \text{O} \qquad \qquad \qquad \text{O} \\ \qquad \qquad \qquad \\ -\text{CCH}=\text{C}(\text{CH}_3)\text{CH}(\text{OH})\text{CH}(\text{CH}_3)\text{CH}=\text{CHCH}=\text{CHC}- \\ \text{(Z)} \qquad \qquad \qquad \quad \quad \\ \qquad \qquad \qquad \text{C}-\text{CH} \quad (\text{t}) \quad (\text{c}) \\ \qquad \qquad \qquad \\ \qquad \qquad \qquad \text{CH}_3 \end{array} $	H
Roridin K	$ \begin{array}{c} \text{O} \qquad \qquad \qquad \text{O} \\ \qquad \qquad \qquad \\ -\text{CCH}=\text{C}(\text{CH}_3)\text{CH}_2\text{CH}_2\text{OCH}(\text{CH}_3)\text{CH}=\text{CHCH}=\text{CHC}- \\ \text{(Z)} \qquad \qquad \qquad \quad \quad \\ \qquad \qquad \qquad \text{CH}(\text{OH}) \quad (\text{t}) \quad (\text{c}) \\ \qquad \qquad \qquad \\ \qquad \qquad \qquad \text{CH}_3 \end{array} $	OAc
Satratoxin F	$ \begin{array}{c} \text{O} \qquad \qquad \qquad \text{O} \\ \qquad \qquad \qquad \\ -\text{CCH}(\text{CH}_2\text{CH}_2\text{OCCH}(\text{CH}_3)\text{CH}=\text{CHCH}=\text{CHC}- \\ \quad \quad \quad \\ \text{O} \quad \text{CH} \quad \text{CH}(\text{OH}) \quad \\ \quad \quad \\ \text{OH} \quad \text{CH}_3 \end{array} $	H
Satratoxin G	$ \begin{array}{c} \text{O} \qquad \qquad \qquad \text{O} \\ \qquad \qquad \qquad \\ -\text{CCH}(\text{CH}_2\text{CH}_2\text{OCCH}(\text{CH}_3)\text{CH}=\text{CHCH}=\text{CHC}- \\ \quad \quad \quad \\ \text{O} \quad \text{CH} \quad \text{C}=\text{O} \quad \\ \quad \quad \\ \text{OH} \quad \text{CH}_3 \end{array} $	H
Satratoxin H	$ \begin{array}{c} \text{O} \qquad \qquad \qquad \text{O} \\ \qquad \qquad \qquad \\ -\text{CCH}=\text{CCH}_2\text{CH}_2\text{OCCH}(\text{CH}_3)\text{CH}=\text{CHCH}=\text{CHC}- \\ \quad \quad \quad \\ (\text{c}) \quad \text{CH} \quad \text{CH}(\text{OH}) \quad \\ \quad \quad \\ \text{OH} \quad \text{CH}_3 \end{array} $	H
Vertisporin	$ \begin{array}{c} \text{O} \qquad \qquad \qquad \text{O} \\ \qquad \qquad \qquad \\ -\text{CCH}=\text{CCH}_2\text{CH}_2\text{OCCH}_2\text{CH}_2\text{CH}=\text{CHC}- \\ \text{(Z)} \qquad \qquad \qquad \quad \quad \\ \qquad \qquad \qquad \text{C} \quad \text{CH} \quad \text{OH} \\ \quad \quad \\ \text{O}-\text{C} \quad \text{OH} \\ \\ \text{H} \end{array} $	H
Isororidin E	$ \begin{array}{c} \text{O} \qquad \qquad \qquad \text{O} \\ \qquad \qquad \qquad \\ -\text{CCH}=\text{C}(\text{CH}_3)\text{CH}_2\text{CH}_2\text{OCH}(\text{CH}_3)\text{CH}=\text{CHCH}=\text{CHC}- \\ \qquad \qquad \qquad \quad \quad \\ \qquad \qquad \qquad (\text{s}) \text{CH}(\text{OH}) \quad (\text{s}) \quad (\text{c}) \\ \qquad \qquad \qquad \\ \qquad \qquad \qquad \text{CH}_3 \end{array} $	H

Fig. 6. The structures of the 7,8-epoxy trichothecenes (class D)

Name	R ¹	R ²
Crotocot	H	OH
Crotocot	H	COCH=CHCH ₃

R¹-R²

Fig. 7. The structure of verrucaridin K, a macrocyclic trichodermediene

Name	R
Verrucaridin K	-CCH=CHCH(CH ₃)CH ₂ CH ₂ OCCH=CHCH=CHC(=O)-

Recently Isolated Trichothecenes as Reported in the Literature:

Myotoxin A (100)

16-Hydroxyroridin L-2 (98)

Revidin L-2 (99)

Trichoverritone(98)

(86)

Sambucinol (92)

Sambucoln (92)

CBD₂ (97)

APPENDIX 2

¹H NMR Spectra for the Isolated Mycotoxins

3-ACETYL DEOXYNIVALENOL

3, 15-DIACETYLDEOXYNIVALENOL

ISOTRICHODERMIN

7 HYDROXYISOTRICHODERMIN

8-HYDROXYISOTRICHODERMIN

CALONECTRIN

15-DEACYLCALONECTRIN

"APOTRICHOTHECENE"

APPENDIX 3

¹³C NMR Spectra for the Isolated Mycotoxins

3, 15 DIACETYLDEOXYNIVALENOL

3 Ac, 7 DoDON

ISOTRICHODERMIN

7-HYDROXYISOTRICHODERMIN

8-HYDROXYISOTRICHODERMIN

CALONECTRIN

7-HYDROXYCALONECTRIN

CULMORIN

CULMORIN

SAMBUCCOIN

"APOTRICHOTHECENE"

APPENDIX 4

2D $^1\text{H}/^1\text{H}$ and $^1\text{H}/^{13}\text{C}$ Spectra

7-HYDROXYISOTRICHODERMIN

¹³C/¹H CORRELATION SPECTRUM
CULMORONE

APPENDIX 5

Mass Spectra for the Isolated Mycotoxins

MASS SPECTRUM
08/09/83 1:46:00 + 15:50
SAMPLE: 3-ACDON (40)
COND.: DBS, 20M, 150 TO 240 @ 5 C MIN
ENHANCED (S 158 24 07)

DATA: 83MAT200 0300
CAL: PFKAU9 03

BASE M/E: 79
RIC: 120576.

MASS SPECTRUM
08/09/84 15:12:00 + 11:50
SAMPLE: 3,15-DIACDON (41)
COND.: SOLID PROBE
050 TO 060 AVERAGED - 024 TO 025 X1.00

DATA: 84MAT11 030
CAL: PFKAU7 03

BASE M/E: 43
RIC: 163320.

MASS SPECTRUM
08/03/83 2:20:00 + 15:22
SAMPLE: 3-Ac-7-deoxyDOM (42)
CONDOS.: DBS, 20M, 150 TO 240 @ 5 C/MIN
ENHANCED (S 150 24 BT)

DATA: 83MAT201 0363
CAL1: PFKALUS 83

BASE M/E: 109
RIC: 23984.

MASS SPECTRUM
11/03/83 15:06:00 + 6:06
SAMPLE: ISOTRICHODENDREN (43)
CONDOS.: DBS, 20M, 150 TO 240 @ 3 C/MIN
ENHANCED (S 150 24 BT)

DATA: 83MAT251 0183
CAL1: PFKMDS 83

BASE M/E: 124
RIC: 497664.

MASS SPECTRUM
08/03/04 15:22:00 + 2:28
SAMPLE: 7-HYDROXYISOTRICHODERMIN (45)
COND.: SOLID PROBE
670 - 832 X1.00

DATA: 84047612 070
CALI: PPK017 03

BASE M/E: 109
RIC: 235528.

MASS SPECTRUM
08/03/04 15:33:00 + 2:30
SAMPLE: 8-HYDROXYISOTRICHODERMIN (46)
COND.: SOLID PROBE
675 TO 875 AVERAGED

DATA: 8504120 075
CALI: PPK026 02

BASE M/E: 43
RIC: 12422.

MASS SPECTRUM
08/09/04 13:54:00 + 2:14
SAMPLE: CALONECTRIN (47)
COND.: SOLID PROBE
067 TO 088 AVERAGED - 027 TO 029 X1.00

DATA: 84WAT613 067
CALI: PFKAL7 03

BASE M/E: 290
RIC: 308224.

MASS SPECTRUM
04-11/04 13:08:00 + 1:42
SAMPLE: 15-DEACETYL CALONECTRIN (49)
COND.: SOLID PROBE
040 TO 043 AVERAGED - 06 TO 07 X1.00

DATA: 84WAT657 041
CALI: PFKAP11A 03

BASE M/E: 43
RIC: 494392.

MASS SPECTRUM
07/06/83 9:39:00 + 6:05
SAMPLE: 000 (50)
COND.: SOLID PROBE
#146 TO #147 AVERAGED - #167 TO #170 X1.00

DATA: 83MAT177 #146
CALI: PFKJUL6 #3

BASE M/E: 82
RIC: 782464.

MASS SPECTRUM
08/01/84 13:42:00 + 2:27
SAMPLE: 7-HYDROXYCALONECTRIN, (51)
COND.: SOLID PROBE

DATA: 84MAT599 #49
CALI: PFKAU1 #3

BASE M/E: 121
RIC: 1849348.

MASS SPECTRUM
 08/01/84 13:25:00 + 2:54
 SAMPLE: 8-HYDROXYCALONECTRIN (52)
 COND.: SOLID PROBE
 #58 TO #59 AVERAGED - #37 TO #38 X1.00

DATA: 84MAT590 #58
 CALI: PFKAU1 #3

BASE M/E: 121
 RIC: 284168.

MASS SPECTRUM
 09/07/83 14:27:00 + 2:24
 SAMPLE: SAMBUCCIN (53)
 COND.: SOLID PROBE
 #45 TO #52 AVERAGED - #88 TO #85 X1.00

DATA: 83MAT225 #48
 CALI: PFKSE7A #3

BASE M/E: 33
 RIC: 142592.

MASS SPECTRUM
12-18-84 12:44:00 + 0:55
SAMPLE: APOTRIQHOHECENE (54)
CONDOS: F-8-GLY

DATA: 84MAT0674 #5
CALI: PEGDE10 #2

BASE M/E: 251
RIC: 2390070.

MASS SPECTRUM
07-06/83 9:21:00 + 2:30
SAMPLE: CULMORIN (55)
CONDOS: SOLID PROBE
#50 - #5 X1.00

DATA: 83MAT176 #50
CALI: PFKJUL6 #3

BASE M/E: 95
RIC: 341504.

MASS SPECTRUM
05/25/04 9:31:00 + 2:00
SAMPLE: CULMORONE (59)
CONDOS.: SOLID PROBE
059 TO 061 AVERAGED - 019 TO 021 X1.00

DATA: 84MAT376 060
CALI: PFT01A22 03

BASE M/E: 110
RIC: 601000.

APPENDIX 6

Chart I - Summary of the Compounds Isolated

CHART 1

SUMMARY OF THE COMPOUNDS ISOLATED

3-AcBON (40)

$C_{17}H_{22}O_7$ M.W. = 338

M.P. = 186.5-187.0°C [α]_D^{26.8} = +21.74°

3,15-diAcBON (41)

$C_{19}H_{24}O_8$ M.W. = 380

M.P. = 117.0-118.5°C [α]_D^{21.3} = +51.0°

3-Ac-7-deoxyBON (42)

$C_{17}H_{22}O_6$ M.W. = 322

M.P. = 202.0-202.5°C [α]_D^{26.8} = +41.67°

Isotrichodermin (43)

$C_{17}H_{25}O_4$ M.W. = 292

M.P. = 97.0-97.5°C [α]_D^{26.8} = +9.49°

7-Hydroxyisotrichodermin (45)

$C_{17}H_{24}O_5$ M.W. = 308

M.P. = 154.0-155.5°C [α]_D^{23.6} = -10.5°

8-Hydroxyisotrichodermin (46)

$C_{17}H_{24}O_5$ M.W. = 308

M.P. = 153.0-155.5°C [α]_D^{21.3} = -18.2°

Calonelectrin (47)

$C_{19}H_{26}O_6$ M.W. = 350

M.P. = 75.5-78.5°C [α]_D^{23.6} = -7.97°

15-Deacetylcalonelectrin (49)

$C_{17}H_{24}O_5$ M.W. = 308

M.P. = 184.0-184.5°C [α]_D^{28.9} = +10.0°

BBO (50)

$C_{19}H_{26}O_8$ M.W. = 382

M.P. = 190.5-191.5°C [α]_D^{26.8} = +7.13°

7-Hydroxycalonelectrin (51)

$C_{19}H_{26}O_7$ M.W. = 366

M.P. = 174.0-175.0°C [α]_D^{23.2} = +14.3°

8-Hydroxycalonelectrin (52)

$C_{19}H_{26}O_7$ M.W. = 366

M.P. = 168.0-170.0°C [α]_D^{21.3} = -6.67°

Sambucosin (53)

$C_{15}H_{22}O_3$ M.W. = 250

M.P. = 243.0-243.5°C [α]_D^{21.3} = +120.0°

Culmarin (55)

$C_{15}H_{26}O_2$ M.W. = 238

M.P. = 179.5-180.0°C [α]_D^{26.8} = -12.22°

Culmarone (59)

$C_{15}H_{24}O_2$ M.W. = 236

M.P. = 176.0-179.0°C [α]_D^{23.6} = -16.5°

Apotrichothecene (54)

$C_{15}H_{24}O_3$ M.W. = 252

END

1	5	0	4	8	6
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FIN