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Mycotoxins from *Fusarium* species: detection, determination and variety

John W. ApSimon[1], Barbara A. Blackwell[2], Lisa Blais[1], David A. Fielder[2], Roy Greenhalgh[2], Gertrude Kasitu[1], J. David Miller[2], and Marc Savard[2].

[1]Ottawa-Carleton Chemical Institute, Department of Chemistry, Carleton University, Ottawa, Ontario, Canada K1S 5B6
[2]Plant Research Centre, Agriculture Canada, Ottawa, Ontario, Canada K1A 0C6

Abstract - The infestation of many crops by fungi of the Fusarium genus has extensive economic consequences. This lecture will present an update of the progress achieved by the Canadian program aimed at an understanding of factors leading to the production of toxic secondary metabolites in nature and to the possible control of these materials. This brief report will concentrate on the biosynthetic diversity of common Fusarium species producing isoprenoids with biological activity.

INTRODUCTION

The pathogenic nature of certain species of fungi to plants has been observed virtually since the beginning of agriculture. These plant pathogens often produce metabolites when they are ingested. Several examples in recent history exemplify this property. In 1960, Turkey 'X' disease killed 100,000 turkeys, 14,000 ducklings, and thousands of partridge and pheasant poults in England. In the mid 1930's and late 1970's there were outbreaks of a sickness in horses called equine leuco-encephalomalacia (ELEM) in the United States (ref. 1) and alimentary toxic aleukia has been responsible for the distress and death of thousands of people since it was first recorded in the 19th. century (ref. 2). Although these syndromes are all very different, they have one thing in common; they are all caused by mycotoxins.

Mycotoxins are metabolites that are produced by fungi growing on cereals, nuts, soybeans and several other crops including apples. The Turkey 'X' disease outbreak in England was traced to contaminated nuts from Brazil and led to the discovery of aflatoxins produced primarily by Aspergillus flavus Link and A. parasiticus Speare. Equine leucoencephalomalacia is doubtless caused by toxins from Fusarium moniliforme and alimentary toxic aleukia is thought to be caused by the trichothecene mycotoxins. Since mycotoxin producing fungi grow on some of the staple foods of both humans and animals, both populations are affected by them. Also, products such as eggs, milk, diary products and meat can be contaminated through the ingestion of feed containing mycotoxins (ref. 2).

Toxigenic Fusarium species are now recognized to be a major agricultural problem. The extensive research carried out over the past two decades has revealed a large number of toxic Fusarium secondary metabolites and their ubiquitous nature. These metabolites include butenolide, moniliformin, zearalenone, enniatins, fusarin-C and the trichothecenes (ref. 3). The latter compounds, especially deoxynivalenol (DON) and nivalenol (NIV), have been reported as contaminants of cereals world-wide in China, Japan, Korea, Russia, Poland, Germany, the UK, France, Australia, Canada and the US (ref. 4) and represent health hazards to both animals and humans.

In a study published in 1986 (ref. 5) Duthie and his colleagues examined 153 samples of winter wheat seed from Eastern Canada and identified fourteen species of Fusarium (Table 1). Of these F.graminearum, F. culmorum, and to a lesser extent F. avenaceum have been implicated in seedling blight, head blight and root and crown rot in wheat.)

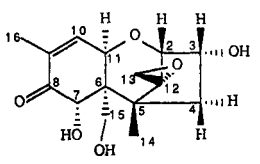
A maximum permissible concentration of 0.3 mg/kg in final products derived from uncleaned Ontario soft white winter wheat and destined for human consumption was introduced in 1982 by Health and Welfare Canada. This level has since been increased to 2 mg/kg in uncleaned wheat, based on estimated losses during further processing and revised toxicological data.

TABLE 1. Percentages of winter wheat seed samples with one or more seeds infested by species of Fusarium

Species	Ontario[1] 1983	Ontario[1] 1984	Ontario 1983	Ontario 1984	Quebec 1983	Maritimes 1984	All Samples
F. avenaceum	13	25	38	25	98	86	48.8
F. graminearum	27	41	36	71	64	100	51.2
F. culmorum	0	3	17	25	19	0	13.6
F. sambucinum	7	0	2	0	43	0	12.3
F. poae	60	72	57	63	93	100	72.2
F. sporotrichioides	47	44	55	79	100	57	67.3
F. tricinctum	0	3	0	8	24	0	8.0
F. acuminatum	0	22	10	54	64	0	25.3
F. equiseti	13	38	55	88	88	14	59.3
F. semitectum	0	0	0	4	0	0	0.6
F. oxysporum	7	38	31	75	29	14	35.2
F. solani	7	3	0	8	5	0	3.7
F. moniliforme	7	38	26	13	21	43	24.1
F. merismoides	7	0	2	0	0	0	1.2
Fusarium species(>1)	73	100	88	100	100	100	94.4
Number of samples	15	32	4	25	32	7	153.0

[1]CSGA = Canadian Seed Growers' Association; other from Research Stations Maritimes = New Brunswick and Prince Edward Island; 50 seeds per sample were examined in 1983, 100 seeds in 1984.

DON was first isolated from contaminated barley in Japan (ref. 9), and later it was found in the U.S.A. in corn that had caused emesis (ref.10). The identification of DON as the causative agent responsible for feed refusal in swine created a demand for material in order to carry out animal feeding trials and toxicological studies. Several procedures have been employed to achieve this goal. They include the infection of field corn using toothpicks inoculated with Fusarium graminearum (refs. 5,6) and the use of cultures on solid matrices such as rice and cracked corn (refs.7,8). These methods, however, require extensive clean-up of the crude extract due to the large amounts of co-extractant present. On the other hand, the use of liquid cultures offers an advantage for the production of secondary metabolites, in that the crude extract is more easily purified and the procedures may be carried out on a large scale. In liquid cultures, Fusarium fungi predominantly produce acetylated trichothecenes, which, in the case of Fusarium graminearum and F. culmorum, is an advantage since 3-acetyldeoxynivalenol (ADON) is more readily purified than DON.



Deoxynivalenol

Fig. 1. Structure of DON 1

TABLE 2. Economic and health impact of mycotoxin contamination in Canada

	$ Millions/Yr
Losses to grain and animal production	176.060
Multiplier effect on other industries	352.120
Chemical analyses	2.000
Human illness and medical treatment	1.000
Regulatory expenses (AgCan, H&W Can)	0.150
Research	1.500
Total estimated loss	$ 533.830 M/Yr

The conditions for liquid cultures of Fusarium roseum (ATCC 28114) (now reclassified as F. graminearum) (ref. 12) to produce ADON were established in our laboratories (ref. 11), and the process scaled-up initially to 70 litre batches in still culture (ref. 12). It was later found the F. culmorum (CMI 14764) produced higher yields of 3-acetyl-DON in stirred jar fermentors and this species has been adopted in current work for batches of up to 150 litres. The kinetics of the large scale process with F. culmorum are shown in Figure 2. In the initial phase (0-36 hr), rapid growth occurs and the nitrogen content (NH_4), is reduced to zero. During this period butenolide 2 is produced, but disappears as the fermentation proceeds. The NH_4 level is the nutrient limitation required to induce trichothecene production in this strain. Following the disappearance of NH_4, the formation of ADON commenced, reaching a maximum (710 mg/L) after 7 days at 28°C. In addition to ADON 1, several minor metabolites including culmorin 4, dihydroxycalonectrin (DHCAL) and sambucinol 3 (Fig. 3) were produced in amounts varying from 20 to 200 mg/L.

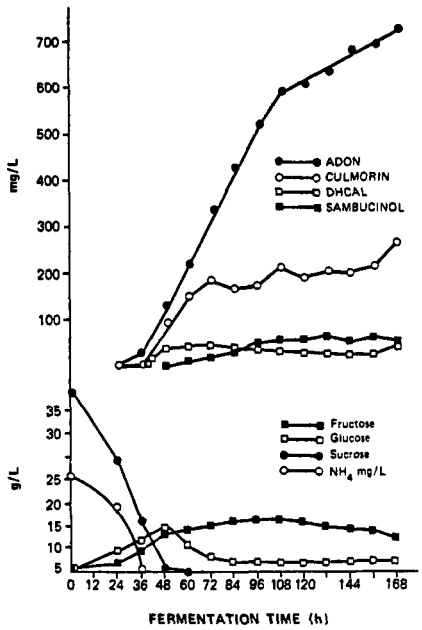


Fig. 2. Mycotoxin production and nutrient supply in a 10 L stirred jar fermentation of F. culmorum HLX 1503

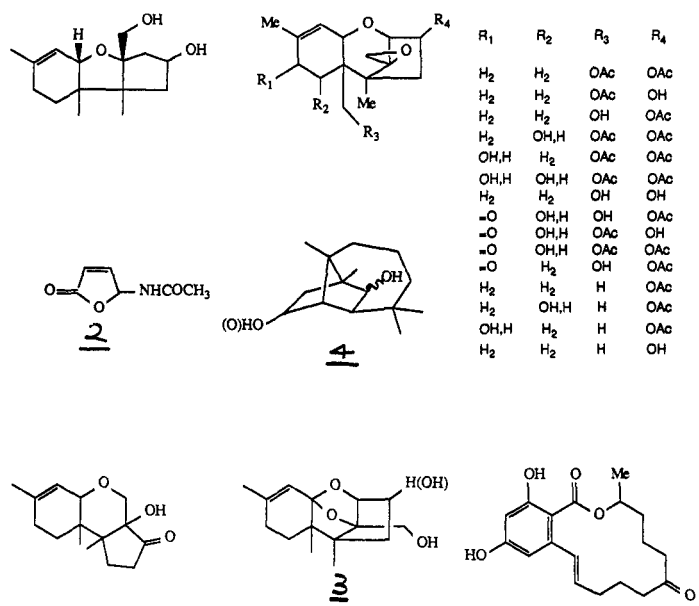


Fig. 3. Some secondary metabolites isolated from the fermentation of Fusarium culmorum and F. graminearum

The Canadian Mycotoxin Program is based on a systematic study of toxigenic Fusaria species with possible impacts on the ariculture industry with the goal of identifying toxic materials produced by these fungi that pose threats to human and animal safety and to develop technology to facilitate the breeding of Fusarium-resistant germplasm of wheat and corn in an attempt to eliminate this problem.

It has been calculated that the cumulative costs of mycotoxin effects in Canada alone is in the order of $500 Million as outlined in Table 2 (ref. 6).

An idea of the variety of secondary metabolites produced may be seen from Figure 4 which displays a GC/MS plot of a crude fungal extract of Fusarium graminearum.

This profile of secondary metabolites varies with the strain of fungus. Thus, the toxigenic potential of strains isolated from different locations varies enormously. In a study recently completed in the Agriculture Canada laboratories in Ottawa in collaboration with a group from the Jiangsu Academy of Agricultural Sciences, Miller and co-workers (ref. 13) have surveyed the mycotoxins produced by 43 strains of three different species (F. crookwellense, F. culmorum and F. graminearum) finding some remarkable differences even between strains of close geographic origins.

One of the most interesting findings in this study of these species is the variability in toxigenic potential from strains isolated from different locations (ref. 14). This variability in the production of mycotoxins by different strains of the same species has major toxicological implications.

There is a need for the investigation of metabolites that occur in minor amounts in these fungi as well as a need to provide adequate amounts of the inactive species for synergistic studies. This has been a major focus of the chemical aspects of this program. In doing so many new compounds have been isolated that are structurally significant as well as being of great biosynthetic interest. Some of these observations are now presented.

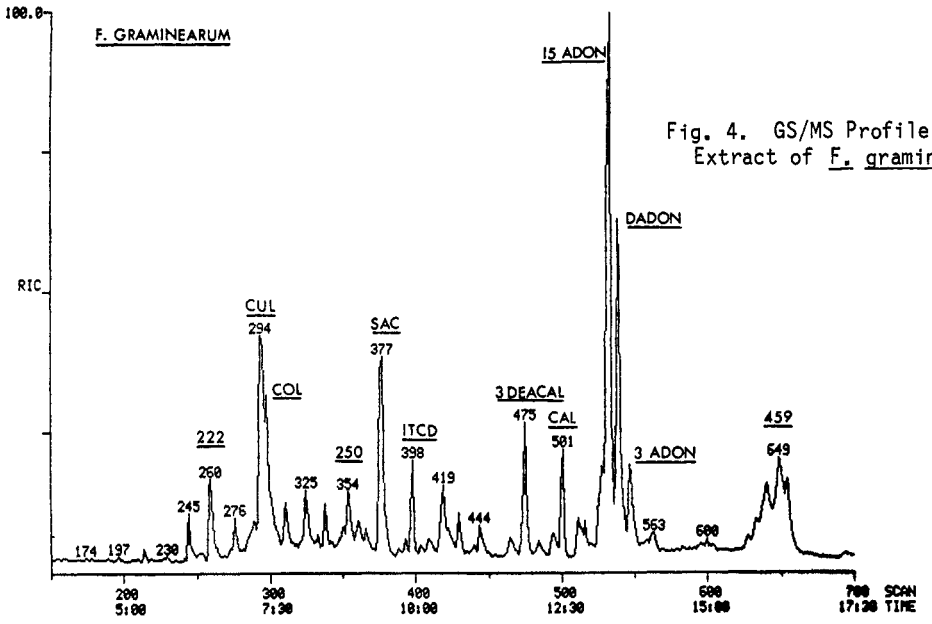


Fig. 4. GS/MS Profile of Crude Fungal Extract of F. graminearum

MODIFIED TRICHODIENOIDS

The trichothecene mycotoxins are well described and their biosynthesis from trichodiene 5 well established. However, it is interesting to examine the structures of other materials identified during our work and that described by others. Figure 5 shows structures identified so far from various Fusarium species. These compounds reveal the versatility of the trichodiene cycloses present in these species.

The exact precursor to the cyclisation routes is uncertain but recent work by McCormick (ref. 15) using mutants of F. sporotrichioides has enabled the isolation of oxygenated trichodienes which are at least chemically reasonable precursors to the compounds shown (Fig. 6).

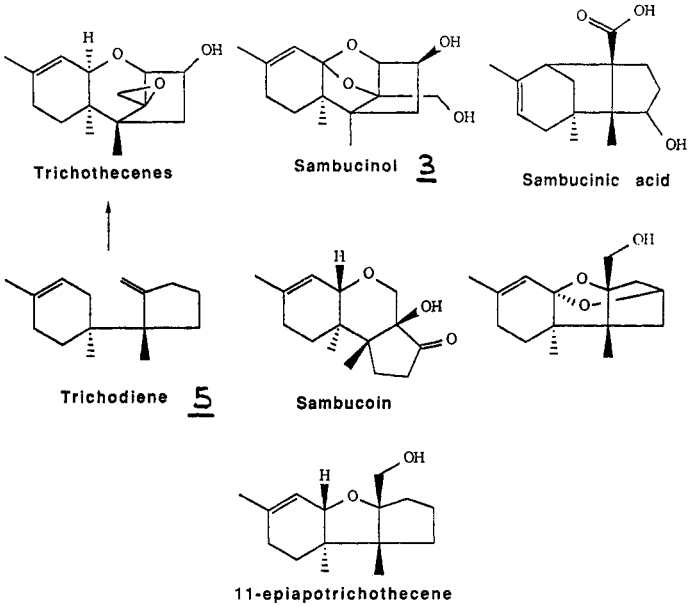


Fig. 5. Trichodienoids from Fusarium species

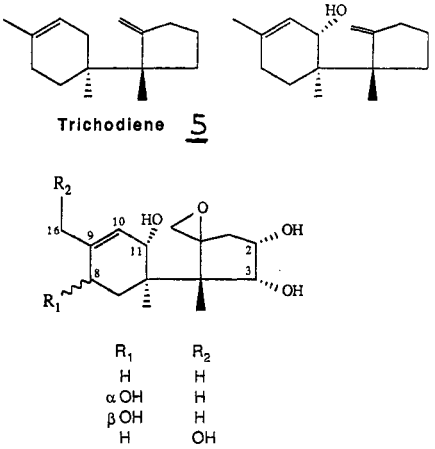


Fig. 6 Modified trichothecenes accumulated by F. sporotrichioides mutants.

Sambucinol 3 represents an interesting case; Mohr and Tann (ref. 16) in their first description of the compound suggested two possible species that are doubtless derived from trichodiene biosynthetic routes to this skeleton 16 (Fig. 7). From a chemical point of view the ketal formation route via the unsaturated ketone 6 is most attractive. Some preliminary computational work from this laboratory supports this view (ref. 17).

Examination of the plethora of compounds formed by the Fusarium species studied in our laboratories reveals the scope of the oxygenation routes and the sites of oxygenation are closely related to the species being examined, thus F. graminearum and F. culmorum are not observed to provide oxygenation at the trichothecene C-4 position, whereas F. sporotrichioides produce C-3,4 dioxygenated species.

Returning to the trichodiene cyclisation process, an interesting experiment has recently been completed by Savard and Greenhalgh (ref. 18) where the stereochemical sensitivity of the cyclase system has been probed by use of the trichodiene stereoisomer bazzanene 7 which on feeding to cultures of F. culmorum provides analogs of the apotrichothecene system such as 8 demonstrating the adaptability of the oxacyclases to unnatural substrates (Fig. 8).

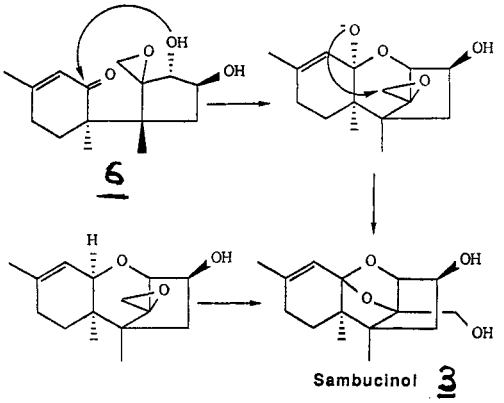


Fig. 7. Possible routes for the formation of sambucinol

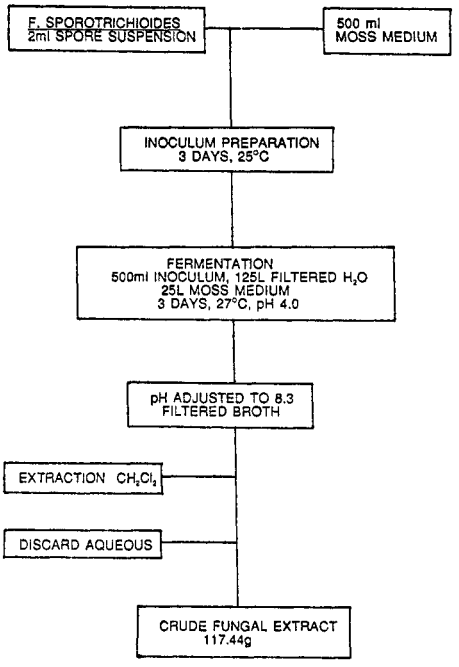


Fig. 9. Fermentation procedure of F. sporotrichioides DOAM 165006

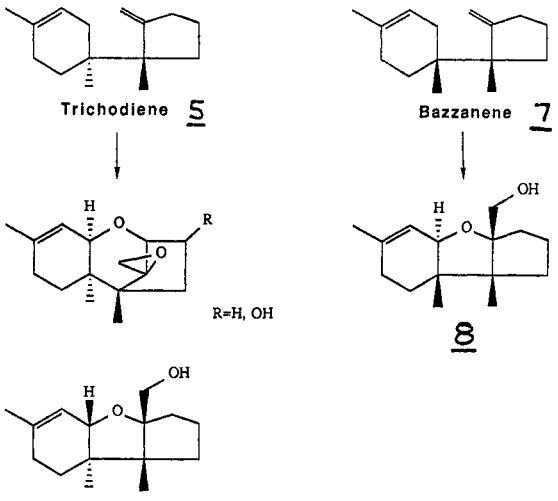


Fig. 8. Cyclization of Trichodiene and Bazzanene

FUSARIUM SPOROTRICHIOIDES

F. sporotrichioides is a soil microorganism occurring on a variety of plants, mainly cereal grains and grasses. Unlike other species of Fusarium, which proliferate in warm climates, this species thrives in cool temperature regions of the world such as Canada, the northern USA, Japan, northern Europe and the USSR. Infestation of grain by the organism usually occurs when it is left in the fields in autumn under wet conditions or overwintered under snow (refs. 4,19).

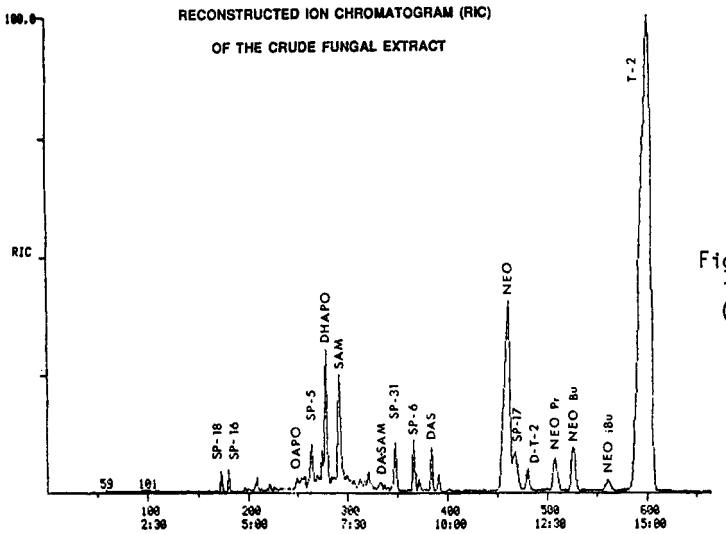


Fig. 10. RIC of crude fungal extract from Fusarium sporotrichioides (DOAM 165006)

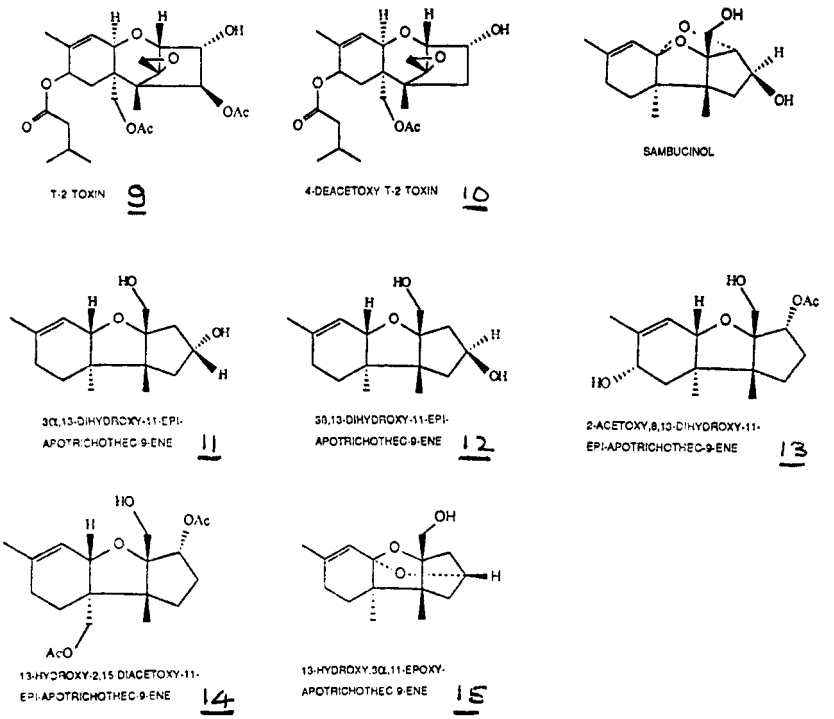


Fig. 11 Some metabolites of F.sporotrichioides

The quantity and type of trichothecenes and other secondary metabolites seem to vary between strains of F. sporotrichioides. However in many strains that have been widely studied, T-2 toxin, 9 is the most abundant and the most toxic of the trichothecenes produced. Crude extracts containing T-2 toxin isolated from Fusarium cultures produce a wide range of symptoms associated with trichothecene poisoning in experimental animals. As a powerful dermal toxin T-2 also causes localized irritation, inflammation and histopathological lesions.

We have performed large scale fermentations of this species (Fig. 9) in order to examine the variety of metabolites produced. A reconstructed ion chromatograph of the crude fungal extract (Fig. 10) shows the great variety of materials obtainable by this route. To date, this work has produced a number of new trichothecene-derived compounds 10 to 15 (Fig. 11)(ref. 20).

Of particular interest are the compounds marked SP-16 and SP-18 on the chromatogram. It turns out that these are novel sesquiterpenes, 16, 17 and the analog 18 has also been detected very recently (Fig. 12). Biosynthetically, these materials represent a novel isoprene skeletal formation route in which oxygen is involved. To the best of our knowledge, this is a new mode of isoprenoid skeletal construction. Interestingly, these compounds appear to occur in other species of Fusarium even when the isoprenoid pathway does not produce other compounds. Another series of versatile isoprenoids that appear to occur is based on the culmorin skeleton 4. To date four analogs have been identified from a variety of species (Fig. 13) and several others are in hand although the final structures are yet to be determined.

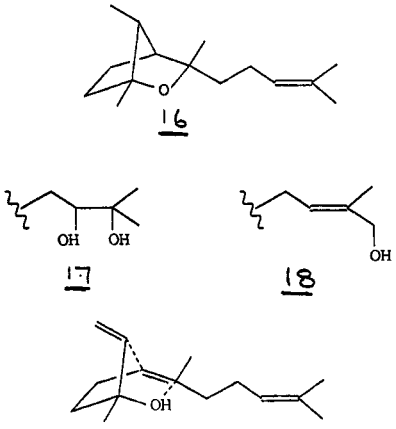
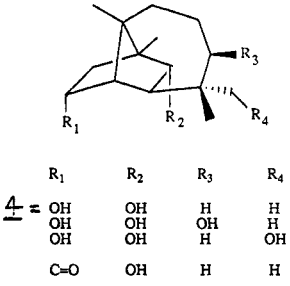


Fig 12. Novel sesquiterpenes from Fusarium



	R_1	R_2	R_3	R_4
4 =	OH	OH	H	H
	OH	OH	OH	H
	OH	OH	H	OH
	C=O	OH	H	H

Fig. 13 Culmorin Derivatives isolated from F. culmorum, F. compactum, F. crookwellense, F. sporotrichioides, F. graminearum

Finally it is of interest to report some recent observations on the toxic effects of deoxynivalenol 1. Although this material is a major toxic ingredient of infected crops, it does not appear to be the sole toxic agent (ref. 21) and much effort has been expended in our laboratories in attempts to identify other toxic materials. In a joint program with the United States Department of Agriculture laboratories, and Agriculture Canada it has been observed that a mixture of DON 1 with the sambucinol 3 or culumorin 4 leads to enhanced toxicity towards caterpillars (Table 3) (ref. 22).

These observations open the important question of the synergistic effects of all of the supposedly inactive metabolites produced by the toxigenic fungi described. Our ongoing work is exploring this problem.

I have attempted in the limited time available to give you a snapshot of some of the interesting and biologically active compounds available from Fusarium species of key interest to the Canadian mycotoxin program but with world-wide toxicological considerations.

TABLE 3. Interactions of Fusarium graminearum metabolites individually and in combinations at naturally occurring levels[a,b] after 7 days.

Compound[c]	*Heliothis zea*		*Spodoptera frugiperda*	
	Mortality (%)[d]	Wt. (mg)[d]	Mortality (%)	Wt. (mg)
Control	0.0	42.1 ± 3.9	0.0	43.8 ± 3.4
DON	5.6	19.6 ± 8.6	7.7	6.4 ± 0.4
CUL	2.6	66.9 ± 3.3	8.6	38.7 ± 3.2
DHCAL	2.7	50.7 ± 2.4	2.8	30.8 ± 2.7
SAM	2.6	59.6 ± 14.1	2.9	39.4 ± 2.5
DON + CUL	56.8*	12.6 ± 2.4*	23.1	4.3 ± 0.4
DON + DHCAL	61.5*	15.1 ± 1.9*	29.3*	4.8 ± 0.6*
DON + SAM	36.1*	21.4 ± 5.2*	2.9	3.7 ± 0.5
All	12.5	16.4 ± 7.1*	67.5*	4.5 ± 1.0*

[a] DON, 25 μg/g; CUL, 10 μg/g; DHCAL and SAM, 1 μg/g.
[b] Values for combinations of chemicals followed by an "*" indicate a significant interaction at $p < 0.05$ for chi square analysis (mortality) or factorial analysis (weights) (Anonymous, 1985).
[c] DON = deoxynivalenol; CUL = culmorin; DHCAL = 7,8-hydroxycalonectrin; SAM = sambucinol.
[d] Mortality is based on *ca* 40 insects per chemical/combination. Weights are means ± standard errors of survivors.

Acknowledgement

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