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BIOACTIVE METABOLITES FROM FUSARIUM SP. ISOLATED  
FROM AN ALOE AND COLLETOTRICUM COFFEANUM ISOLATED  
FROM A COFFEE PLANT

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by  
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## ABSTRACT

Bioactive metabolites from Fusarium sp. isolated from an aloe and Colletotrichum, Coffeanum. isolated from a coffee plant.

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The Ethylacetate extracts of the fermentation culture broths of C. coffeanum and Fusarium sp. gave a phytotoxic and antibiotic compound identified as Brefeldin A and an uncharacterized antibiotic compound respectively. Brefeldin A has not hitherto been isolated from the genus Colletotrichum. The structure was elucidated by spectroscopic means and comparison with the literature. C. coffeanum is a fungus that causes coffee berry disease and has not been chemically studied before.

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## 1. INTRODUCTION

### 1.1. General

The fungi, along with the bacteria, are members of the Thallophyta, a somewhat artificial division of the plant kingdom, comprising organisms with no true roots, stems or leaves. Some authorities regard fungi and some other heterotrophic thallophytes as neither plants nor animals and group them in a separate kingdom<sup>1</sup>.

Fungi have no chlorophyll, reproduce by sexual or asexual spores and possess a mycellium. The classification of the fungi rests up on the type of spores which are formed together with the nature of the mycellium. Accordingly, fungi are grouped under four classes<sup>2</sup>.

The phycomycetes are the most primitive class of fungi, the lower forms being mainly aquatic with simple, often unicellular thalli. If present mycellium is aseptate. The class includes parasites of plants (Pythium, Phytophthera) and of man (Abaidia, Mucor).

The Ascomycetes derive their name from the aacus, the sack like vesel containing the sexual spores. The class includes at one extreme the unicellular yeasts and at the other extreme species with large fruiting bodies some of which, e.g. truffles are edible. Their mycellium is septate and the sexual spore are conidia, borne on condiphores, Many

.../

Ascomycetes are parasitic on plants e.g. Claviceps Purpurea.

The Basidiomycetes, the highest class of fungi, bear their spores on basidia. The mycellium is often perennial.

Basidiomycetes are responsible for the rust (Order Uredinales) and smuts (Order Ustilaginales) of cereals and brown and white rot of wood.

The Fungi Imperfecti, are those fungi for which no sexual (Perfect) stage has been observed. They form asexual spores on conidiophores. The most common genera are the Penicillia, Aspergilli, Colletotrichum and some Fusaria. Many species allocated to this class are known to be conidial stages of perfect fungi. Examples are Fusarium moniliforme which is the conidial state of Gibberella fujikuroi, and Helminthosporium sativum, the conidial stage of Cochliobolus sativus.

According to Ainsworth and Susan<sup>3</sup> (1968), there are about 4,300 valid genera and about 100,000 species living as parasites or saprophytes.

The Fungi, like other organisms and higher plants, are able to produce secondary metabolites. The reason why fungi produce secondary metabolites has been a topic of lively debate over the years and various suggestions were put forward. It is possible that secondary metabolites could serve as reserve foodstuffs accumulated at times of abundant carbohydrate<sup>4</sup> or assist in defence mechanism in

the organisms' natural environment<sup>5</sup> or provide a pathway for the removal of intermediates which otherwise would accumulate and produce stress on the primary metabolism<sup>6</sup>.

The great majority of fatalities from fungal poisoning have been caused by the amanita toxins in mushrooms<sup>3</sup>, ergot alkaloids and other mycotoxins in grains and foods<sup>7</sup>, numerous phytotoxins have been investigated in greater or lesser detail including altenaric acid from Altenaria Solani; colletoin from Colletotrichum fuscum; Colletotrochin, colletol, Colletochlorin and colletalol from C. capsici; diaporthin from Endothia parasitica and the trichothecanes and trichothecenes, fusaric acid, lycomarasmine, dihydroscirpenol and other varieties of toxins from many species of the genus Fusarium<sup>8</sup>.

The search for bioactive metabolites of fungi attracted greater attention with the discovery of the antibiotic penicillin from Penicillium chrysogenum by Alexander Fleming in 1929. Thereafter, the discovery of new and better antibiotics like cephalosporins from the genus Cephalosporium, streptomycins from the genus Streptomyces and macrolide antibiotics from many other fungi emerged.

Most antibiotics are derived from the actinomycetes of which the most important ones are aminoglycosides, tetracyclins, erythromycins, streptomycins, nystatin and other polyenes<sup>9</sup>:

The different varieties of metabolites that are produced by fungi are compiled in two volumes by Turner<sup>1,8</sup>. Most of these metabolites are "Secondary metabolites", so called because they play no obvious role in the economy of the organisms which produce them.

The primary metabolism of an organism is summation of an interrelated series of enzyme catalyzed reactions which provide the necessary energy of the organism. Whereas primary metabolism is basically the same for all living systems, secondary metabolism is restricted to the lower forms of life and is species, often strain specific<sup>1</sup>.

A few fungal metabolites are derived directly from glucose. The glucose is converted to pyruvate by two major pathways, the Embden-Meyerhof and the pentose phosphate. The latter route makes available pentoses important in nucleoside biosynthesis and a tetrose which can react with phosphoenolpyruvate to give shikimic acid which is the intermediate for the aromatic amino acid and also for many aromatic secondary metabolites. The triose is also a precursor of serine which is converted

to glycine by loss of one carbon atom and enters the  $C_1$ -pool.

Proceeding along the carbon pathway, the triose is converted first to pyruvate and then to acetyl Coenzyme A (acetyl CoA), the most important single intermediate in fungal secondary metabolism. Carboxylation of acetyl CoA gives malonyl CoA and linear condensation of many malonyl CoA leads to the polyketides or the fatty acids, which can in turn give many secondary metabolites.

Alternatively, condensation of three acetyl CoA gives mevalonic acid, the key intermediate in terpene and steroid biosynthesis. Finally, by condensation of acetyl CoA with oxaloacetate, the carbon from glucose enters the tri-carboxylic acid cycle which serves to complete the oxidation of glucose and which is the source of the carbon skeletons of many amino acids. Nitrogen containing metabolites are usually derived from the metabolism of amino acid, and from the biosynthesis of purines and pyrimidines<sup>10</sup>.

The most important aspect of the study of fungi is their identification and cultivation. To do a series of reproducible experiments on a fungus, one must ensure getting a continuous source of one and the same organism by keeping the organism alive, free from contamination and maintaining the biochemical property which is being studied. This is because fungi are amenable to strain change, hence to change in biochemical property with time and condition.

Suitable methods and techniques of cultivation must be followed with an appropriate media containing the necessary nutrients. Periodic transfer to a fresh medium in a sterile condition is indispensable. For most experiments, fungi are grown in liquid media but for storage purposes solid media are more convenient i.e. agar media which is allowed to solidify in a slopping test tube or culture tube.

The great advantage of the fungi as sources of secondary metabolites is their ability to produce the compounds in aqueous media. As a result, fungal metabolites of diverse type are conveniently available in the laboratory for chemical, biological and biochemical studies, and a few are manufactured on a commercial scale<sup>1</sup>.

#### 1.2 The genus Colletotrichum

The genus Colletotrichum belongs to the class Fungi Imperfecti, family Melanconiaceae. The conidial fructification is an acervulus (Pseudoparenchymatous aggregation of hyphae)<sup>11</sup>. 20 species have been distinguished<sup>12</sup>, some of which are serious plant pathogens.

The pathogenic species include, C. dematium which causes black rot of legumes, C. gossypi which causes pink ball rot of cotton, C. graminicola that causes wilt and rot of grasses and cereals, C. lindemuthianum

which causes anthracnose of phaseolus beans, C. lini that cause anthracnose and canker of flax<sup>13</sup>.

Eventhough pathogenicity of field crops of some of the species is studied, not much chemical investigation have been made and not many compounds have been isolated from this genus.

#### 1.2.1. The coffee fungus C. coffeanum

The coffee berry disease(CBD) is a very severe and trouble some disease especially in East Africa. The disease is caused by a special strain of the imperfect form of the anthracnose organism C. coffeanum var. Virulans Noak, which is the imperfect stage of Glomerella cingulata, From the imperfect stage there has arisen a mutant of special parasitic adaption and cultural character. This mutant strain was first isolated and described by pathologists in East Africa and the Congo<sup>14</sup>.

CBD was first discovered in Ethiopia in 1971 and it has been a problem to the producers. Since then, the search for disease resistant cultivars drew momentum mainly by breeding. Susceptibility is a dominant character and created a problem in the breeding trial. Eventhough resistant varieties have been discovered<sup>15</sup> by breeding, no chemical

investigations have been made on the cultivation and isolation of toxins from C. coffeanum. Therefore, one of the aims of this project was to generate data on the bioactive metabolites of the fungus.

Besides, one objective in this project was to observe the biological activity of the metabolites of the fungus on other micro organisms and the effect on seed germination. The latter ought to be done since the effect of the fungus is that it mummifies the seeds and berries of the coffee plant.

### 1.3 The genus Fusarium

The genus Fusarium belongs to the class Fungi imperfecti, family Hyphomycetae<sup>11</sup>. The members of this genus are plant pathogens causing wilt, blackrot of stems and roots of plants. Some species are saprophytic on plant substrata in soil, on dung, etc. Their conidial states are phialidic and have brightly colored perithecia.

In this genus 32 species have been identified most of which are parasites and cause chronic disease of plants<sup>12</sup>. Some of the parasitic species that have been studied by pathologists are; F. avenaceum that causes dry rot of potato, F. culmorum associated

with foot rot and ear blight of cereals, F. equiseti that causes brown rot of cereals, F. oxysporum that causes root rot of strawberry and wilt of dwarf and runner beans. F. pelagronii that causes stem rot of pelargonium, F. solani that causes rot of apples, pea, potato and damping-off of mushrooms<sup>12</sup>.

The aloes are typical Xerophytic plants with fleshy leaves usually having spines at the margins. The word aloe is derived from Arabic word alloseh or the Hebrew halal, meaning a shining, bitter substance<sup>16</sup>.

The gel and dried leaf exudates of Aloe species have been used medicinally in ancient times<sup>17</sup>. These species have wide medicinal use in the developed and underdeveloped societies. The bitter leaf exudates or latex of some Aloe species are sources of the laxative aloe drug and are also important items in the cosmetic industry. The drug aloe is one of the best stimulating purgatives<sup>14</sup>. The well recognized varieties of aloe that have significant medicinal importance are; Curcao, Barbados, Cape and Socotrine aloes<sup>16</sup>.

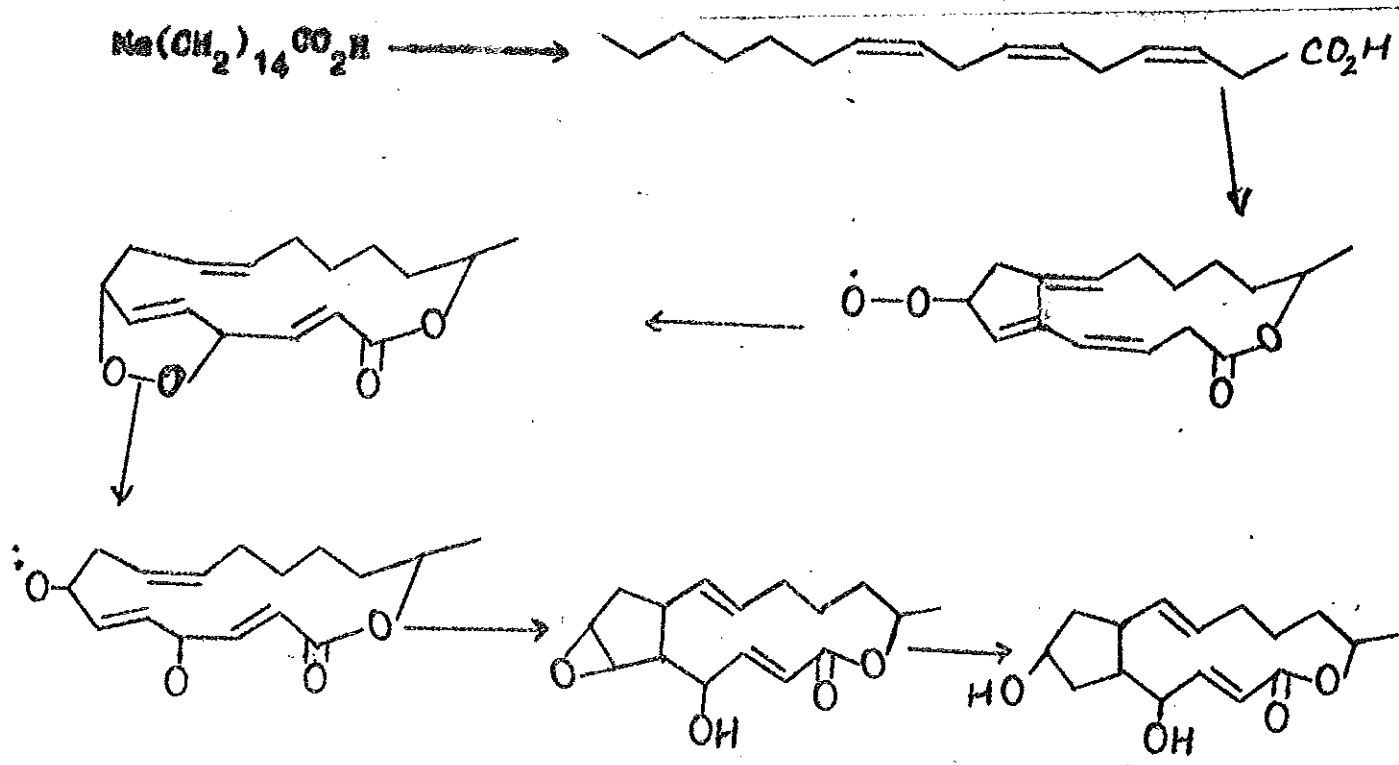
Due to their industrial and commercial importance, the aloes are widely cultivated and the need to study aloes chemically arises due to the facts mentioned above.

The aloes are often attacked by fungi. It was earlier noted that the fungus Uromyces aloes (Cooke) Magn., causes aloe leaf rust. Some species of aloe (Unidentified) were attacked by the U. aloes<sup>12</sup>. Teleutospores were also observed on Aloe glauca. while collecting different Aloe species for chemotaxonomic studies, an aloe plant infected with a fungus was observed and having looked at the medicinal values of the plant, we decided to undertake this particular research. Eventhough very few fungi have been isolated from infected aloe plants, no chemical investigations have been made concerning the metabolites produced by the fungi. Another driving motive to pursue the investigation was the antimicrobial effect of the crude extract of the fungus. Therefore, it is worth investigating this particular fungus chemically and biologically.

#### 1.4 The biosynthesis of main metabolites of the genera Colletotrichum and Fusarium.

##### 1.4.1. Biosynthesis of Brefeldin A (1)

The similarity between the structures of brefeldin A and the fatty acid derived prostaglandins led to the suggestion that the former may also be derived from a fatty acid. This speculation was supported by the specific incorporation of 9-<sup>14</sup>C palmitate into brefeldin A<sup>19</sup>.



Scheme 1. The biosynthesis of Brefeldin A(1).

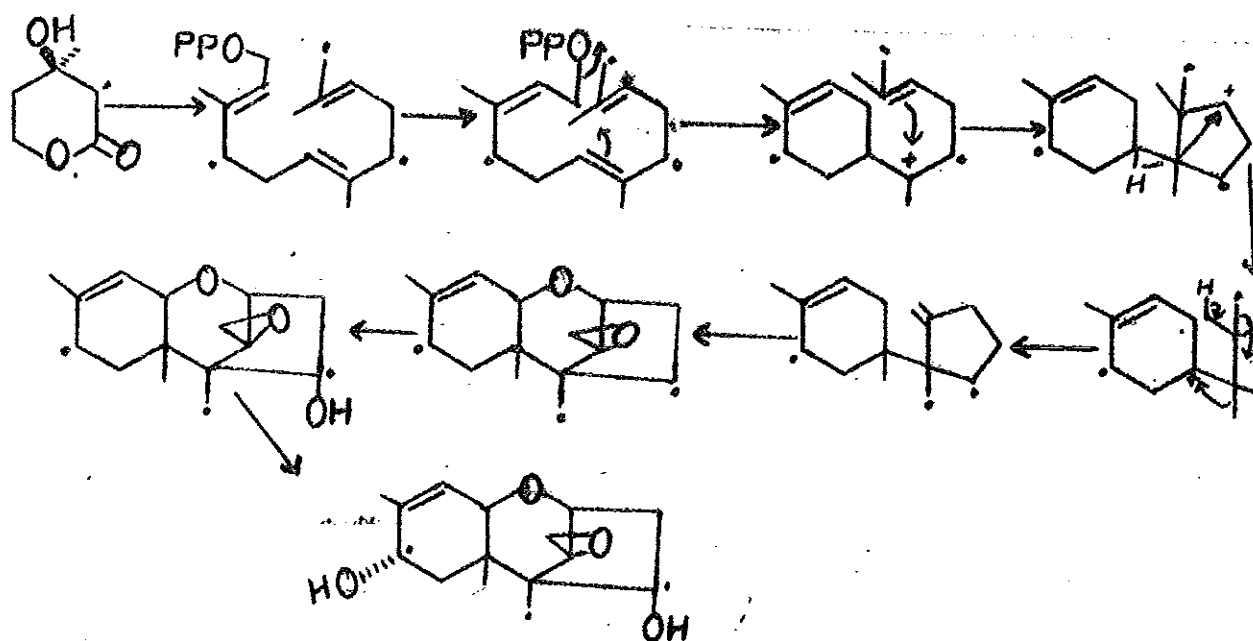
1.4.2. The biosynthesis of the Trichothecanes

The largest class of fungal sesquiterpenes of the genus Musarium is based on the trichothecane skeleton. All the known examples have a 12,13-epoxide ring, a 9,10-double bond and one or more -OH or CH<sub>3</sub>CO- groups(see list). Much of the interest in these compounds has arisen from their likely involvement in mycotoxineses of farm animals<sup>8</sup>.

The incorporation of [<sup>1-13</sup>C]acetate confirms the broad derivation of trichodermin via farnesol. Thus, it has been shown that prior to cyclization trans, trans- farnesyl pyrophosphate isomerizes to cis, trans-farnesyl pyrophosphate with loss

of a mevalonoid 5-pro-S- hydrogen atom; the 5-pro-R-hydrogen at C-1 of the farnesyl pyrophosphate is retained at C-11 of the trichothecanes<sup>8</sup>.

Existing knowledge of the trichothecanes can thus be summarized as in scheme 3.



Scheme 3 The biosynthesis of trichothecanes

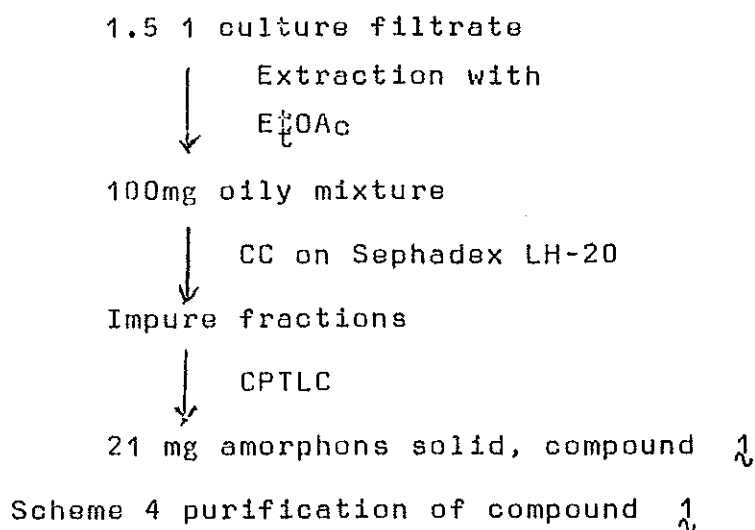
## RESULTS AND DISCUSSIONS

2.1. A phytotoxic and antifungal metabolite from the coffee fungus C. coffeanum

During the screening work, the ethyl acetate extract of the culture broth in diluted sweet wort showed phytotoxic and antifungal activity. In submerged culture of sweet wort medium, C. coffeanum produced a major compound which has biological activity.

## 2.1.1. Fermentation and isolation

The fungus grown in sweet wort media, as indicated in the experimental for 10 days in shake culture, was extracted with EtOAc. The crude extract 100mg obtained after evaporation was applied on a column packed with sephadex LH-20 and eluted with  $\text{CHCl}_3/\text{MeOH}$  (1:1). Fractions containing the active spot were mixed based on TLC and further applied on CPTLC and eluted with the same solvent system to get the bioactive compound<sup>1</sup>.



## 2.1.2. Phytotoxicity of compound 1

The germination inhibitory effect of compound 1 was tested on Eragrostis teff, Brassica napus, salad seeds and lentiles. Germination of the test seeds was strongly inhibited by the compound in the case of E. teff, B. napus and salad seeds upto a concentration level of 20µg/disc of the compound. In the case of lentiles root and shoot elongation retardation was observed when compared with the control groups.

Table 3. Effect of compound 1 on seed germination

Test seeds	Concentration of compound in µg/disc			
	200	100	50	20
<u>Eragrostis teff</u>	-	-	-	-
<u>Brassica napus</u>	-	-	-	-
Salad seeds	-	-	-	-
Lentiles	+	+	++	++

Key: - no germination

+ about 50% reduction in growth

++ growth same as control

## 2.1.3. Antibiotic effect of compound 1

When tested by the agar diffusion assay, compound 1 was active against the fungus C. albicans up to a concentration level of 10µg/disc and it was also active against the gram positive bacteria B. subtilis at a concentration level of 200 µg/disc.

Table 2. Antibiotic activity of compound 1

Test organism	Concentration in µg/disc				
	200	100	50	20	10
<u>C. albicans</u>	++	++	++	+	+
<u>B. subtilis</u>	+	-	-	-	-

Key: ++ strongly active

+ active

- inactive

## 2.1.4. Structure elucidation of compound 1

Compound 1 was obtained as white needles from MeOH, mp 202 - 204<sup>o</sup>. Its uv spectrum showed a maximum at 205 nm and a shoulder at 250 nm. The IR spectrum showed a broad

peak at  $3450\text{ cm}^{-1}$  indicating the presence of a C=O group. The  $^1\text{H}$  NMR showed a double-doublet with a coupling constant ( $16\text{ Hz}$ ), characteristic of trans-coupled olefinic protons centered at 5.82 and 7.42 ppm; and another double-doublet with  $J = 16\text{ Hz}$  for two olefinic protons appeared at 5.2 and 5.68 ppm.

A doublet appeared at 1.23 ppm assigned for a methyl group attached to a methine carbon. The multiplet centered at 2ppm indicated the presence of many methylene protons. The  $^{13}\text{C}$  NMR showed 16 carbon signals assigned for  $\text{SP}^2$  and 11  $\text{SP}^3$  carbon atoms. The suggested type of carbon atoms are, three oxygenated methine carbons, two non oxygenated carbon atoms, one carbonyl carbon (lactone or ester), two vinyl groups, five methylene carbons and one methyl carbon.

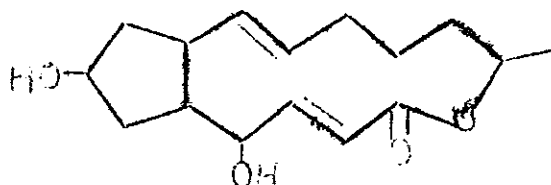
Table 3.  $^{13}\text{C}$  NMR data for compound 1

Carbon No.	$\delta$ value	carbon No.	$\delta$ value
1	167.7	9	52.6
2	117.9	10	131.1
3	154	11	137.5
4	76.4	12	34.9
5	46.5	13	27.3
6	45.2	14	32.5
7	72.7	15	72.5
8	41.5	16	20.9

Table 4. H NMR data of compound 1 (Integration, Multiplicity, J)

Proton No.	$\delta$ value	Proton No.	$\delta$ value
2	5.82(1H,dd,16)	10	5.35(1H,dd,12)
3	7.42(1H,dd,16)	11	5.68(1H,dd,12)
4	4.2(1H,m)	15	4.85(1H,m)
7	4.2 (1H,m)	16	1.23(3H,d,8)

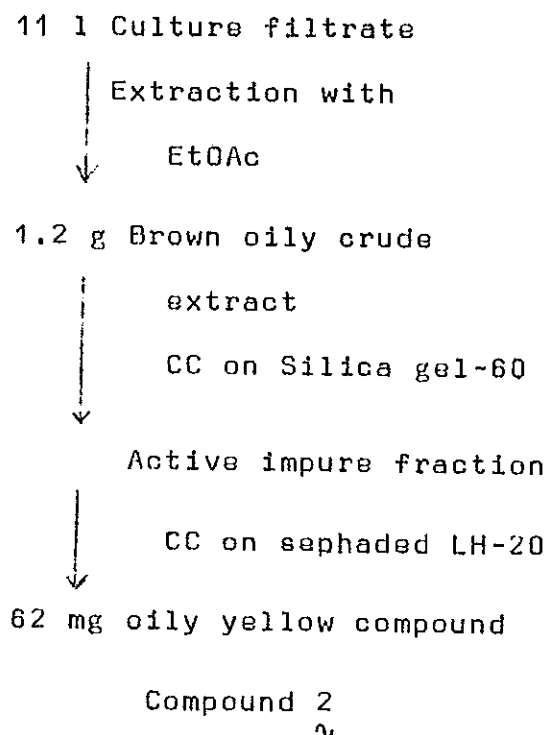
Based on the above spectroscopic data and comparison with the literature, the following structure was suggested for compound  $\underline{1}$ .



Brefeldin A ( $\underline{1}$ )

Brefeldin A ( $\underline{1}$ ) has not hitherto been isolated from the genus Colletotrichum but, it has been isolated from Phyllostica medicaginis, Ascochyta imperfecta, Penicillium janthinellum, Penicillium cremeogriseum, P. brefeldianum and Curvaularia lunata<sup>8</sup>.

The structure of the epimer was ruled out in that the mp of compound  $\underline{1}$  was found to be (202-204<sup>0</sup>) very close to the literature value of brefeldin A i.e. (204-206<sup>0</sup>) but the mp of 7-epi brefeldin A is 124-125<sup>0</sup><sub>c</sub><sup>55</sup>.



Scheme 5 Purification of compound 2  
~

#### 2.2.2. Antibiotic activity of compound 2 ~

The crude extract and compound 2 showed anti-biotic activity. By the agar diffusion assay during screening against the gram positive bacteria B. subtilis and S. aureus upto a concentration level of 50 µg/disc of the compound. The compound was also found to be fairly active against the same bacteria up to a concentration level of 10 µg/disc. Besides, by the same assay, the compound was moderately active against the fungus C. albicans up to concentration of 50 µg/disc of the active compound.

Table 5 Antibiotic effect of compound  $\underline{2}$ 

Test Organism	Concentration in $\mu\text{g}/\text{disc}$				
	200	100	50	20	10
<u>S. aureus</u>	++	++	++	+	+
<u>B. subtilis</u>	++	++	++	+	+
<u>C. albicans</u>	+	+	+	-	-

Key: ++ Strongly active

+ Weakly active

- inactive

### 2.2.3. Structure elucidation of compound $\underline{2}$

Compound  $\underline{2}$  is an oily compound that fluoresces at 254 nm under uv light. Its uv spectrum showed a maximum at 201 nm (intense) and at 285-nm (shoulder). The IR spectrum showed absorption bands at  $3450\text{ cm}^{-1}$  (broad) assignable for one or more - OH groups, at  $1740\text{ cm}^{-1}$  and  $1690\text{ cm}^{-1}$  indicating the presence of two carbonyl groups. The  $^{13}\text{C}$  NMR showed 16 carbon signals; the two signals at 170 and 169 ppm are assignable for two carbonyl carbons which supports the IR data, only one methylene carbon is found as observed from

the DEPT spectrum appearing at 25.5 ppm, the signals at 10.5, 15.9, 18.3, 19.3 and 19.9 ppm are assignable to methyl carbon signals, the signals at 75.6, 62.8 and 61.1 ppm have been assigned for three oxygenated methine carbons and the signals at 34, 32, 4, 32.1 29.9 and 27.9 could be methine carbons which are non oxygenated.

The 90MHz  $^1\text{H}$  NMR spectrum was not well resolved. From the data that we have at hand, it has become very much difficult to suggest a structure for this compound.

## 3. EXPERIMENTAL

## 3.1. Chemicals, Solvents, Reagents and Media

Acetone (M&B), Ethylacetate (Riedel-de Haen), Chloroform (WinLAB), Methanol (CODEX), Benzene (Riedel-de Haen), Deuterated methanol and Chloroform (Merck), Filter paper discs (6 and 13 mm diameter), Silica gel-60 (FLUKA Merck), Sephadex LH-20 (Pharmacia), TLC sheet Silica gel-60 F<sub>254</sub> (Kiesegel), Unhoped Sweet Wort, malt extract (merck), agar (Difco), Potatodextrose agar (Oxoid), Sodiamsulfate (BDH).

Table 6. Solvent Systems

No.	Solvent System	Ratio
1	Benzene/EtOAc	1:1
2	" "	45:55
3	CHCl <sub>3</sub> /MeOH	95:5
4	" "	9:1
5	" "	7:3

Apparatus:- Melting points were determined on Kofler Block apparatus. UV spectra (MeOH) were recorded on a BECKMAN Du-65 Spectrophotometer. IR spectra (KB<sub>r</sub>) were run on a Perkin Elmer 727B Spectrophotometer.

$^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were run using FT Joel FX 90Q instrument at 90  $\text{MH}_z$  and 22.5 $\text{MH}_z$  respectively in  $\text{CDCl}_3$  and  $\text{MeOH-d}_4$ . Chemical shifts are given in  $\delta$ -values using TMS as internal standard.

### 3.2 Collection and Identification

#### 3.2.1. The coffee fungus

The CBD causing fungus, C. coffeanum, pure cultures were obtained from the IAR- Jimma Research Center on December, 1989. Identification of the fungus and its isolation was done by the experts of the same institute. The pure cultures were obtained on test tube agar slants. The fungal culture specimen is available in slants kept in the refrigerator at 4 $^{\circ}\text{C}$ .

#### 3.2.2. The aloe fungus

The aloe fungus was isolated from an infected aloe plant, A. macrocarpa (identification by Dr. Sebsebe, Department of Biology, AAU voucher no. 048129) collected from Debresina area on December 1989. Isolation of the fungus and identification was done by Dr. Dawit Abate of the Department of Biology, AAU. The fungus was identified as a member of the genus Fusarium. Pure cultures were maintained on agar slants.

Both fungi were serially transferred to fresh agar slants when necessary and covered with parafilm to minimize contamination and drying.

### 3.3 Cultivation and Extraction

#### 3.3.1. Cultivation and Extraction of the coffee fungus

The coffee fungus (C. coffeanum), produces a black colony when grown on different media. It grows very fast on potato dextrose agar, wort agar and commercial agar and covers the petridish (9cm) in 5 days.

The fungus was grown first on 3% malt extract agar (MEA) in petridishes, cut into pieces under sterile conditions and transferred into twenty 250 ml capacity flasks each containing 100 ml of 3x diluted sweet wort. The flasks were placed on the shaker and shaken at 100 rpm at room temperature for 10 days. Then, the culture broth was filtered with cotton to get 1.2 l of filtrate.

The culture filtrate was extracted with 1l EtOAc (2 x 500 ml) dehydrated with  $\text{Na}_2\text{SO}_4$  and evaporation of the solvent under reduced pressure gave a creamlike viscous crude extract.

### 3.3.2. The aloe fungus, cultivation and extraction

The aloe fungus when grown on 3% MEA produces an orange colony with time. It grows very fast on MEA, commercial agar and sweet wort. It grows very fast to cover the petridish (9cm) within 6 days.

The fungus was grown in petridishes containing 3% MEA, cut into pieces and transferred into 250 ml flasks each containing 100 ml 3X diluted sweet wort. The flasks were shaken for 4 days on the shaker (100 rpm) and this was used for inoculum. Large Erlenmeyer flasks (2-5l) were used for large scale submerged cultivation. A total of 15 l diluted sweet wort media was used for the cultivation and the fungus was let to grow for 10 days in the stationary culture at room temperature after inoculation.

After 10 days, the culture broth was filtered using cotton which gave 11 l of culture filtrate. The filtrate was extracted with 5 l of EtOAc, dehydrated with  $\text{Na}_2\text{SO}_4$  and evaporated to dryness.

## 3.4 Isolation and Biological and Chemical analysis

### 3.4.1. The coffee fungus extract

The coffee fungus crude extract when developed

on silica gel TLC using different solvent systems showed a major spot fluorescing at 254 nm under UV light and a minor spot (366 nm).

The crude extract was applied on a column packed with Sephadex LH-20 and eluted with  $\text{CHCl}_3/\text{MeOH}$  (1:1). Four fractions were collected of which the first two contained the minor oily substance and the latter contained the major component. Evaporation and purification by CPTLC using the same solvent system gave a crystalline substance when dry.

#### 3.4.2. The aloe fungus extract

The aloe fungus crude extract when developed on silica gel TLC and developed with Benzene/EtOAc (1:1) showed many spots when viewed under UV light. Identification of active spot was done by bioautography.

The crude extract was absorbed on silica gel-60 and applied on a column packed with the same silica type. Elution was done with Benzene/EtOAc (45:55) i.e. Fr 1-10 and then with  $\text{CHCl}_3/\text{MeOH}$  (7:3) Fr 11-13. By agar diffusion Fr 3 was found to contain the active substance. This fraction was applied on a column packed with Sephadex LH-20 and eluted with  $\text{CHCl}_3/\text{MeOH}$ (1:1)

to get an oily substance.

#### 3.4.3. Bioautography

The fungal extracts were applied on 20 x 10 silica gel plates and developed with the solvent system that gives good separation, when the chromatograms were dry, the spots were detected by UV light, circled with pencil and cut out. The cut out pieces were placed up side down on agar media seeded with fungal spores or bacteria. After incubation at an appropriate temperature for 24 to 72 hrs, activity and inhibitory effects were recorded.

#### 3.4.4. Phytotoxicity Test

Filter paper discs (13mm) on which different concentrations of the test substance have been absorbed were placed in small vials and moistened with distilled and sterile water (150 $\mu$ l each). Test seeds were washed with 1%  $H_gCl_2$  solution for 1 minute and immediately rinsed with the sterile and distilled water. Then, the seeds were placed (3-5) on each filter paper discs and the vials were kept in a moistened glass chamber covered with glass. The whole set was incubated for 2 days in the dark at 24 $^{\circ}$ c and for 4 days in the light at room temperature. The seed germination and growth of shoot and root was recorded and compared with the control after 48,96 and 144 hr. The controls were in duplicate.

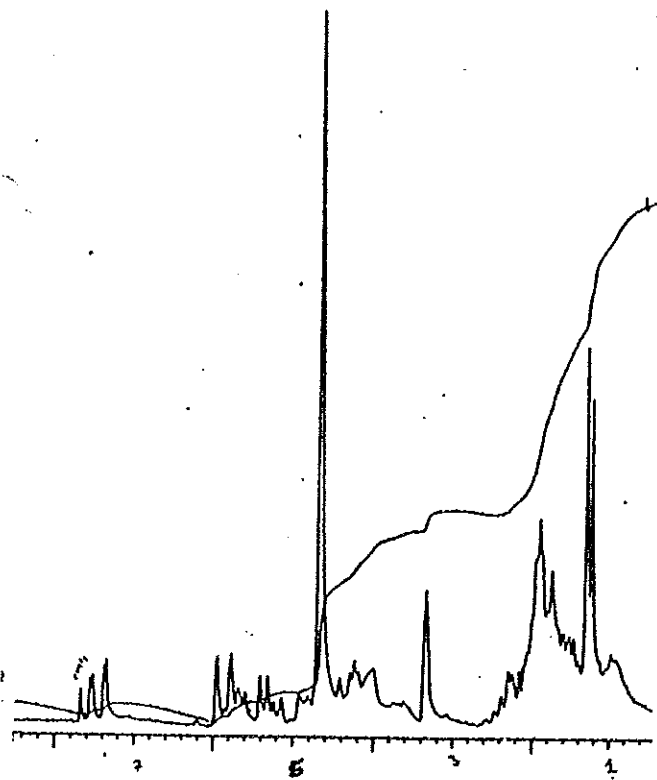
## 3.4.5. Antibiotic Test

The antifungal and antibacterial activity were tested by the agar diffusion assay. Different amounts of test substances were absorbed on 6 mm antibiotic assay discs and the test discs containing the sample when dry were put on agar media seeded with test organism. Inhibition zones were recorded after 24-72 hr of incubation at 27<sup>o</sup>c for bacteria and 37<sup>o</sup>c for fungi.

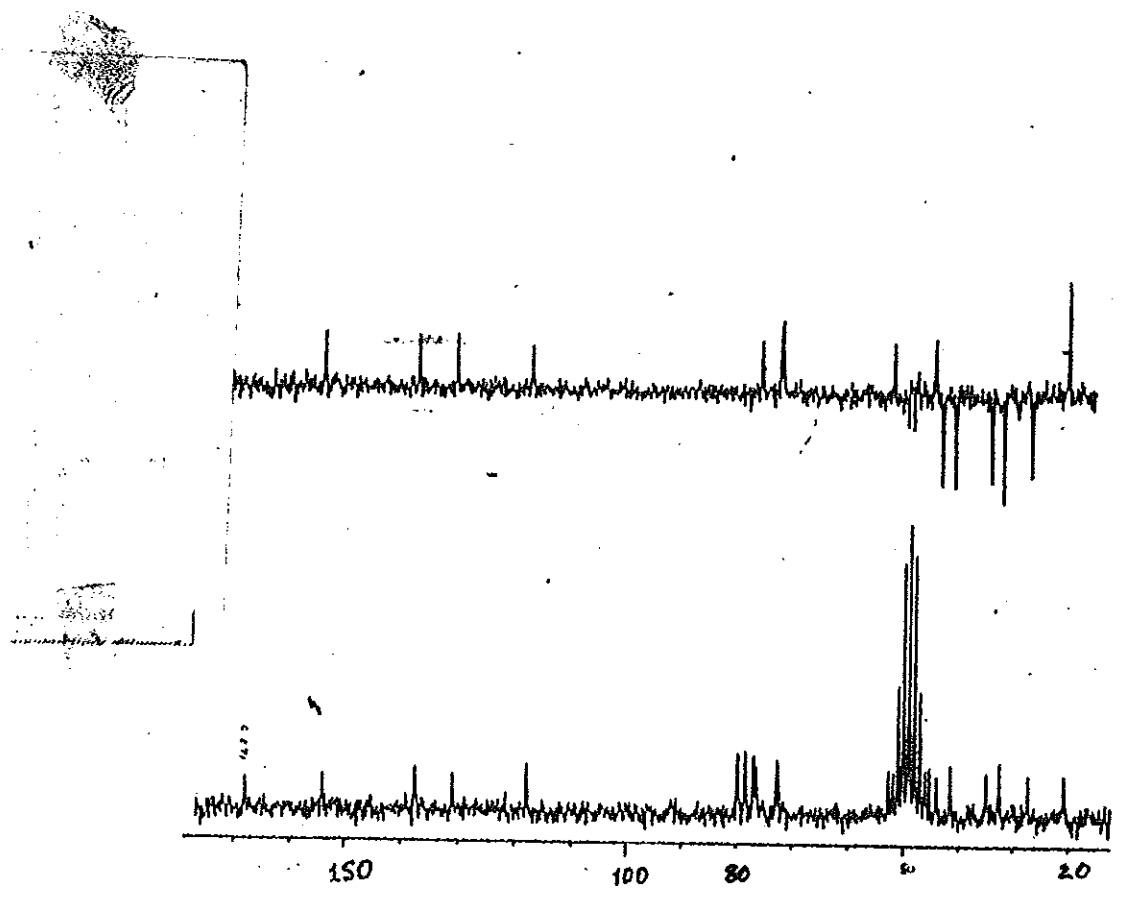
## 3.5 Physicochemical data

Brefeldin A (1). White needles from MeOH, R = 0.67 in system 4, mp 202-204<sup>o</sup>c; uv fluorescence at 254 nm, purple IR  $\nu_{\text{max}}^{\text{KBr}} \text{ cm}^{-1}$ : 3450 (OH), 1740 (C=O), 1260 (C-O-C); UV  $\lambda_{\text{max}}^{\text{MeOH}}$  nm: 205, 250 (sh); <sup>1</sup>H NMR (CD<sub>3</sub>OD/CDCl<sub>3</sub>): 5.82 (1H, dd, J = 16 Hz), 7.42 (1H, dd, J = 16 Hz), 4.2 (1H, m), 5.35 (1H, dd, J = 12 Hz), 5.68 (1H, dd, J = 12 Hz), 4.85 (1H, m), 1.23 (3H, d, J = 8 Hz) <sup>13</sup>C NMR (CD<sub>3</sub>OD/CDCl<sub>3</sub>) See table 5.

Compound 2: Brown oily compound, obtained from Fr 3 eluted with Benzene/EtOAc (45:55); R<sub>f</sub> = 0.6 in solvent system 1; Visible under short wave UV light, IR  $\nu_{\text{max}}^{\text{KBr}} \text{ cm}^{-1}$ , 3450 (OH), 1740, 1690 (C=O); UV  $\lambda_{\text{max}}^{\text{MeOH}}$  nm: 201, 285 (sh) <sup>1</sup>H NMR difficult to interpret; <sup>13</sup>C NMR (CDCl<sub>3</sub>): 170, 169, 75.6, 62.8, 61.1, 34, 32.4, 32.1, 29.9, 27.9, 25.5, 19.9, 19.3, 18.3, 15.9, 10.5



90 MHz  $^1\text{H}$  NMR of compound 48 in  $\text{CDCl}_3/\text{MeOH-d}_4$



22.5 MHz  $^{13}\text{C}$  NMR of compound 48 in  $\text{CDCl}_3/\text{MeOH-d}_4$

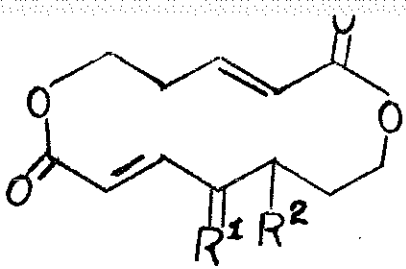
Appendix 1. Compounds isolated from the genus Colletotrichum

St. No.	Name of Compound	Molecular formula	Source	Reference
1 ~	Colletoketol	$C_{12}H_{14}O_6$	<u>C. capsici</u>	20
2 ~	Colletol	$C_{12}H_{16}O_5$	"	"
3 ~	Colletalol	$C_{12}H_{16}O_5$	"	"
4 ~	Cholesterol	$C_{27}H_{46}O$	<u>C. demantium</u>	8
5 ~	Aspergillomarasmine A	$C_{10}H_{17}N_3O_8$	<u>C. gleosporides</u>	
6 ~	2-Pyruvoylaminobenzamide	$C_{10}H_{10}N_2O_3$	<u>C. lagenarium</u>	21
7 ~	Colletochlorin	$C_{18}H_{25}C_{10}O_5$	<u>C. nicotinae</u>	22
8 ~	Colletochlorin C	$C_{18}H_{23}C_{10}O_5$	"	"
9 ~	Colletorin A	$C_{18}H_{26}O_5$	"	"
10 ~	Colletorin C	$C_{18}H_{24}O_5$	"	"
11 ~	Colletochlorin D	$C_{13}H_{15}O_3Cl$	"	23
12 ~	Colletopyrone	$C_{15}H_{16}O_6$	"	24
13 ~	Colletrichin	$C_{28}H_{42}O_7$	"	25
14 ~	Colletotrichin B	$C_{29}H_{42}O_8$	"	"
15 ~	Colletotrichin C	$C_{28}H_{42}O_7$		

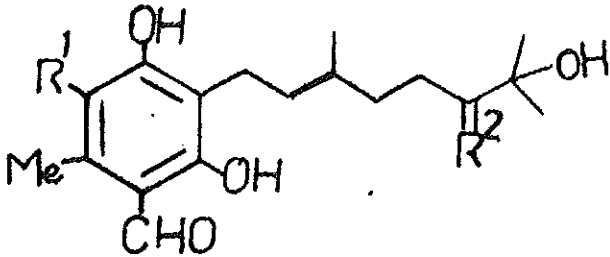
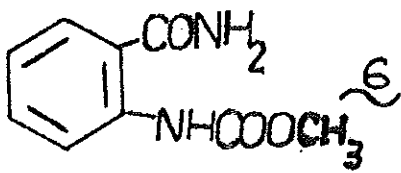
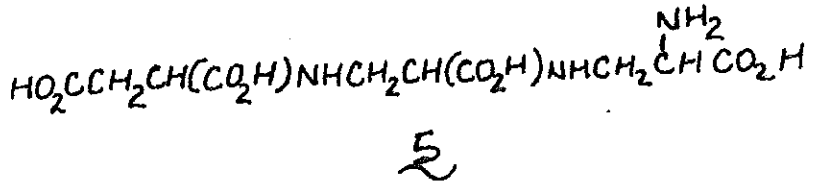
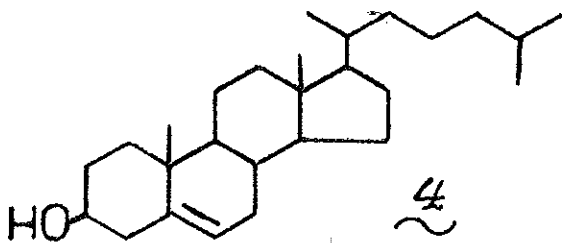
Appendix 2. Compounds isolated from the genus Fusarium

St. No.	Name of Compound	Molecular formula	Source	Reference
16 ~	Antibiotic LL-Z 1272	C H O Cl 21 27 3	<u>Fusarium</u> sp	26
17 ~	" LL-Z 1272	C H O 21 28 3	"	"
18 ~	Fusamarine	C H O 18 26 3	"	27
19 ~	Vomitoxin	C H O 15 20 6	"	28
20 ~	7-hydroxydiacetoxysirpenol	C H O 19 26 8	"	29
21 ~	Neosolaniol	C H O 19 26 8	<u>F. lateritium</u>	30
22 ~	T-2 tetraol	C H O 15 22 6	<u>F. poae</u>	31
23 ~	Acetyl T-2 toxin	C H O 26 35 10	"	32
24 ~	4-acetoxy-scirpendiol	C H O 17 24 6	<u>F. roseum</u>	33
25 ~	Neosolaniolmonoacetate	C H O 21 29 9	<u>F. trincinctum</u>	34
26 ~	Moniliformin	C HO Na 4 3	<u>F. acuminatum</u>	35
27 ~	Bostrycoidin	C H O N 13 12 5	<u>F. bostrucoidin</u>	36
28 ~	O-methylbostrycoidin	C H O N 14 14 5	<u>F. moniliforme</u>	37
29 ~	Rubrofusarin	C H O 15 12 5	<u>F. culmorum</u>	38
30 ~	Anhydrofusarubin	C H O 13 12 6	<u>F. solani</u>	39
31 ~	6-ethyl-dihydrofusarubin	C H O 15 18 7	"	

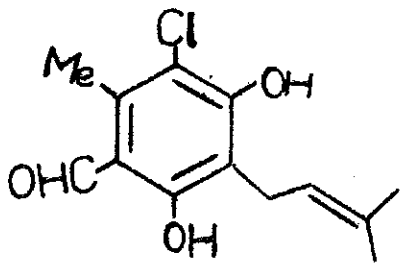
St. No.	Name of compound	Molecular formula	Source	Reference
32 ~	Aurofusarin	C <sub>30</sub> H <sub>18</sub> O <sub>12</sub>	<u>F. graminearum</u>	41
33 ~	Javanicin	C <sub>15</sub> H <sub>14</sub> O <sub>6</sub>	<u>F. javanicum</u>	42
34 ~	O-methylsolaniol	C <sub>16</sub> H <sub>18</sub> O <sub>6</sub>	<u>F. moniliforme</u>	37
35 ~	Mycogenin	C <sub>20</sub> H <sub>14</sub> O <sub>8</sub>	<u>F. oxysporum</u>	43
36 ~	Oxysporone	C <sub>7</sub> H <sub>8</sub> O <sub>4</sub>	"	44
37 ~	Zearalenone	C <sub>18</sub> H <sub>22</sub> O <sub>5</sub>	<u>F. graminearum</u>	45
38 ~	Fusaric acid	C <sub>10</sub> H <sub>13</sub> O <sub>2</sub> N	<u>F. bulbigenum</u>	
39 ~	2-Acetylguinaxoline-4 (3H)-one	C <sub>10</sub> H <sub>8</sub> O <sub>2</sub> N <sub>2</sub>	<u>F. culmorum</u>	46
40 ~	L-prolylglycyl	C <sub>7</sub> H <sub>10</sub> O <sub>2</sub> N <sub>2</sub>	<u>F. nivale</u>	47
41 ~	Prolyltyrosylan hydride	C <sub>14</sub> H <sub>17</sub> O <sub>3</sub> N <sub>2</sub>	"	48
42 ~	Equisetin	C <sub>17</sub> H <sub>23</sub> O <sub>4</sub> N	<u>F. equiseti</u>	49
43 ~	Fusariocin C	C <sub>27</sub> H <sub>28</sub> O <sub>6</sub>	<u>F. moniliforme</u>	50
44 ~	(-)-Kaurenal	C <sub>19</sub> H <sub>28</sub> O	"	51
45 ~	Campesterol	C <sub>28</sub> H <sub>47</sub> O	<u>F. sporotrichiodes</u>	52
46 ~	Poefusarin	C <sub>24</sub> H <sub>30</sub> O <sub>4</sub>	<u>F. poae</u>	53
47 ~	Fusarinine	C <sub>11</sub> H <sub>20</sub> O <sub>5</sub>	<u>F. roseum</u>	54



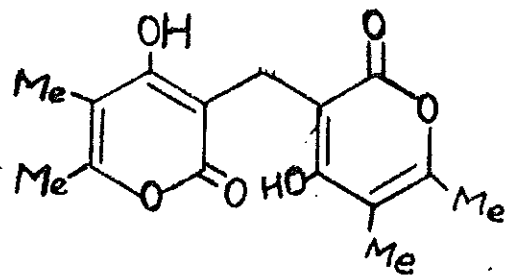
R <sup>1</sup>	R <sup>2</sup>	
O	OH	<u>1</u>
H <sub>2</sub>	OH	<u>2</u>
H, OH	H	<u>3</u>



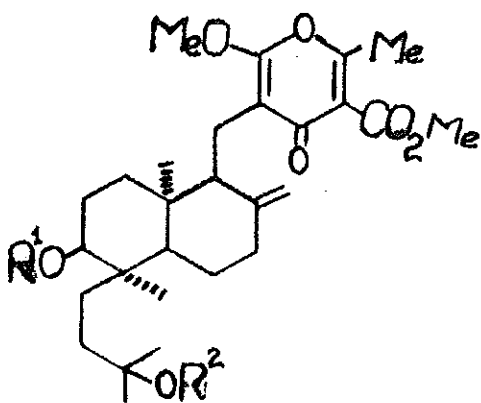
R <sup>1</sup>	R <sup>2</sup>	
Cl	H, OH	<u>7</u>
Cl	O	<u>8</u>
H	H, OH	<u>9</u>
H	O	<u>10</u>



11

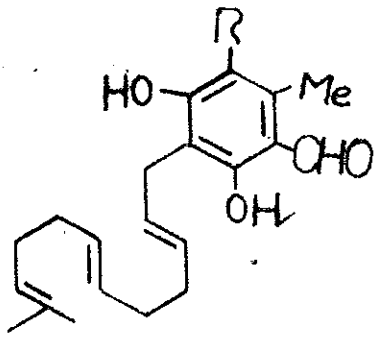


12



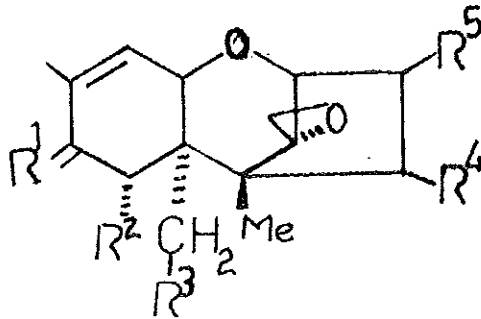
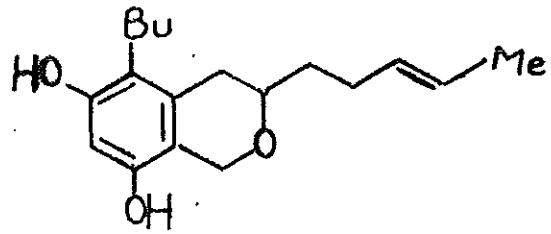
R <sup>1</sup>	R <sup>2</sup>	
H	H	<u>13</u>
CHO	H	<u>14</u>
H	CHO	<u>15</u>

Compounds Isolated from the genus Fusarium.

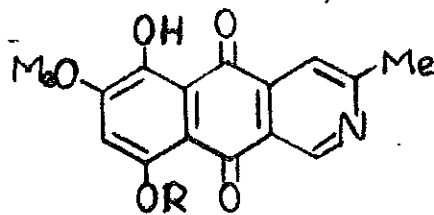
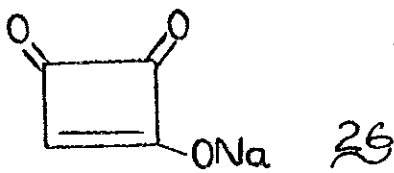


R = Cl 16

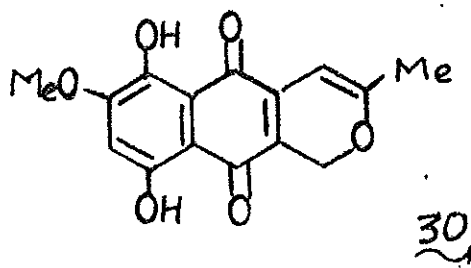
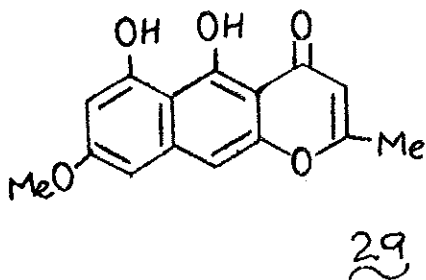
R = H 17



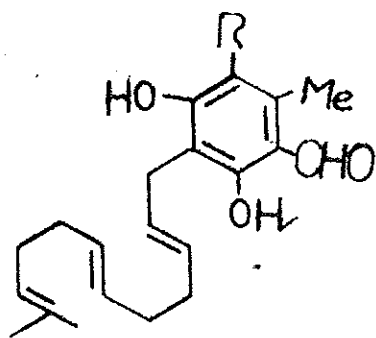
R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	R <sup>4</sup>	R <sup>5</sup>	
O	OH	OH	H	OH	<u>19</u>
H <sub>2</sub>	OH	OAc	OAc	OH	<u>20</u>
OH	H	OH	OH	OH	<u>21</u>
OH	H	OAc	OAc	OH	<u>22</u>
CO <sub>2</sub> CH <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	H	OAc	OAc	OAc	<u>23</u>
H <sub>2</sub>	H	OH	OAc	OH	<u>24</u>
OAc	H	OAc	OAc	OH	<u>25</u>



R = H 27  
R = Me 28

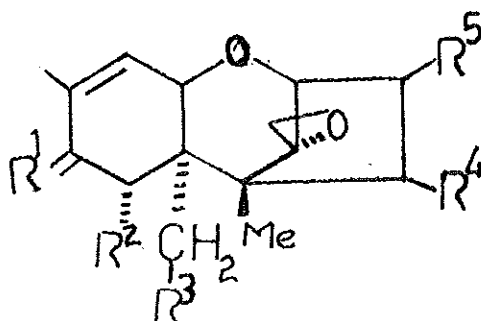
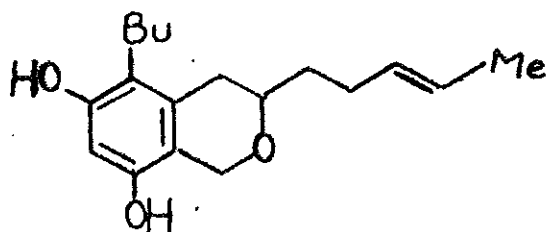


Compounds isolated from the genus Fusarium.

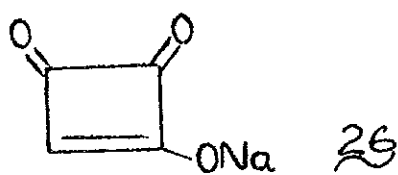


R = Cl     16

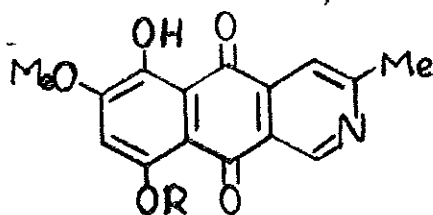
R = H     17



R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	R <sup>4</sup>	R <sup>5</sup>	
O	OH	OH	H	OH	19
H <sub>2</sub>	OH	OAc	OAc	OH	20
OH	H	OH	OH	OH	22
OH	H	OAc	OAc	OH	21
CO <sub>2</sub> CH <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	H	OAc	OAc	OAc	23
H <sub>2</sub>	H	OH	OAc	OH	24
OAc	H	OAc	OAc	OH	25

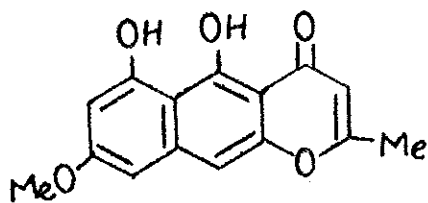


26

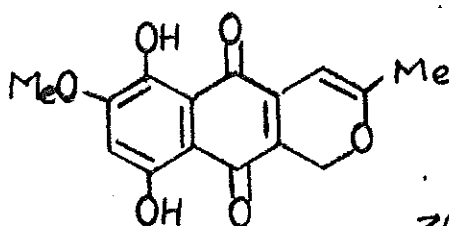


R = H     27

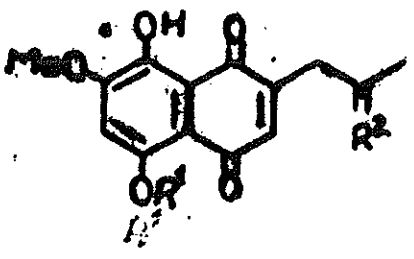
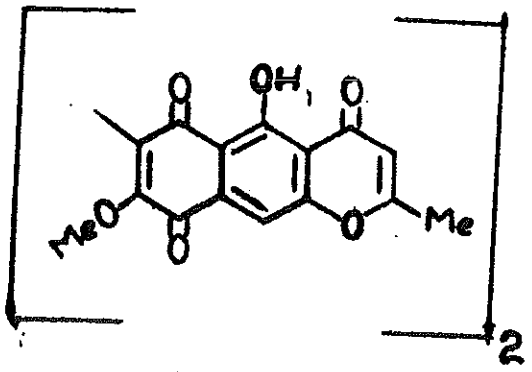
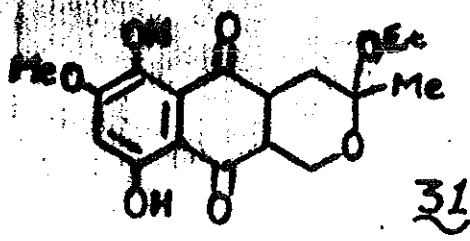
R = Me     28



29

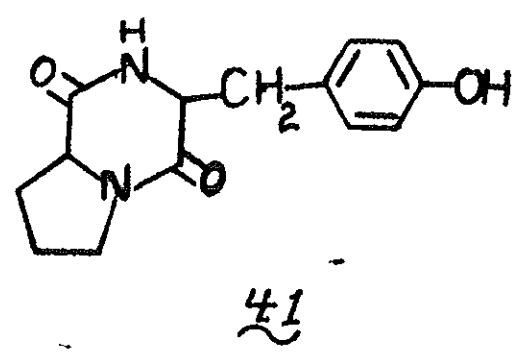
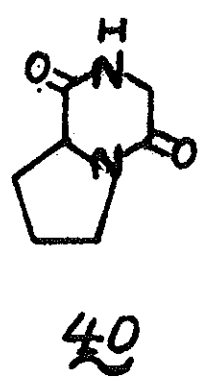
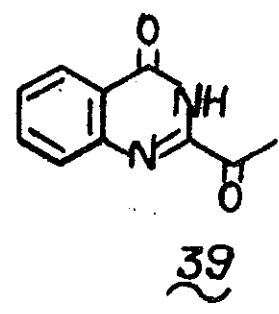
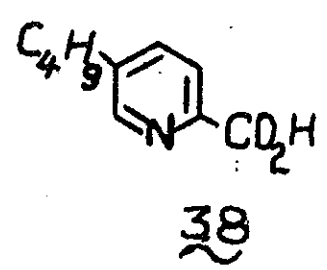
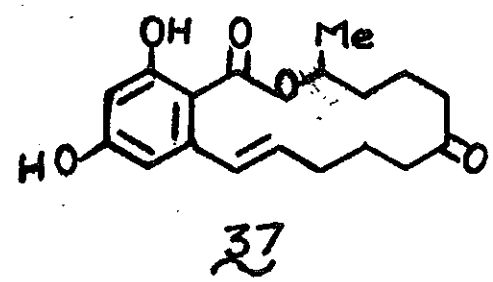
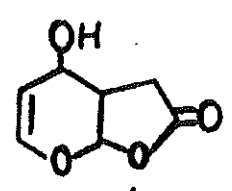
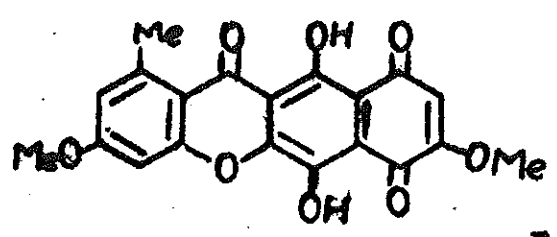


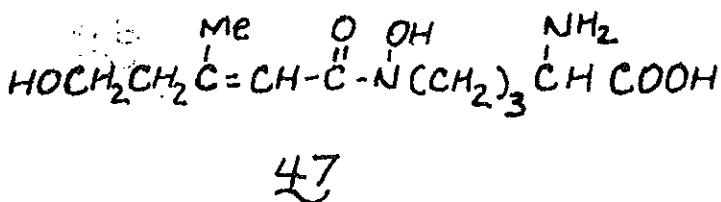
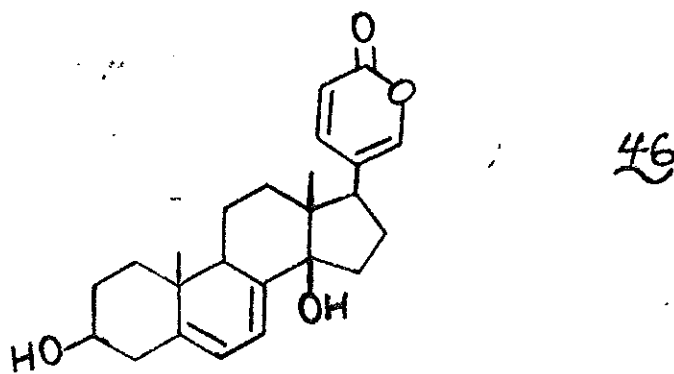
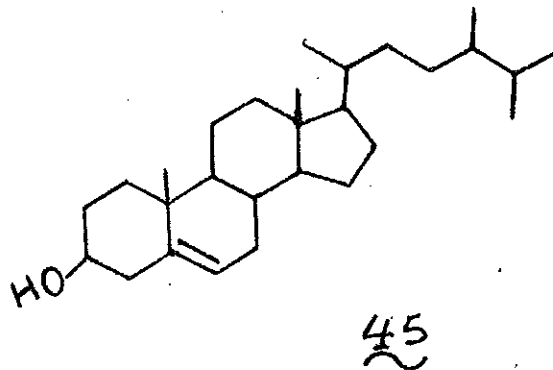
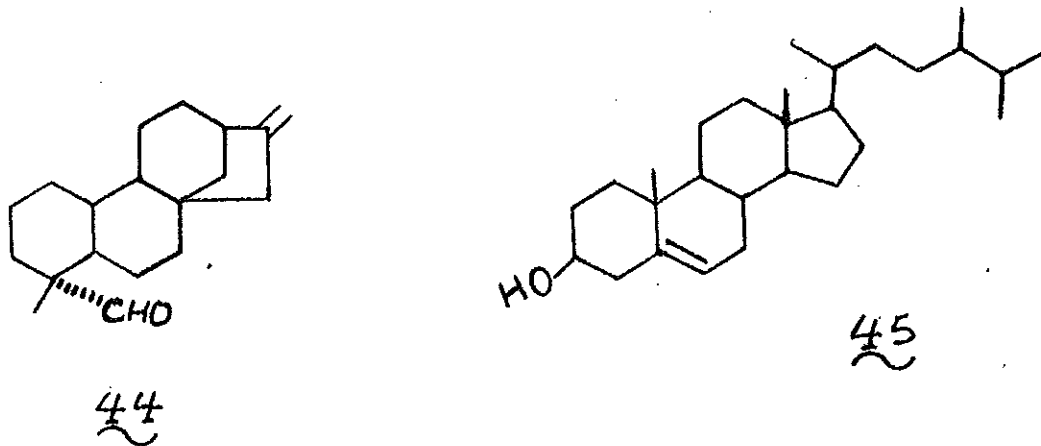
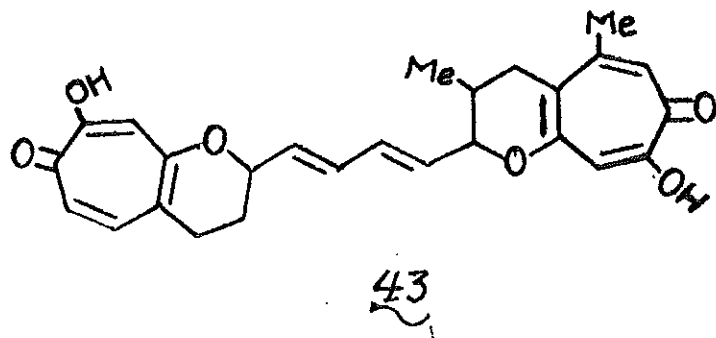
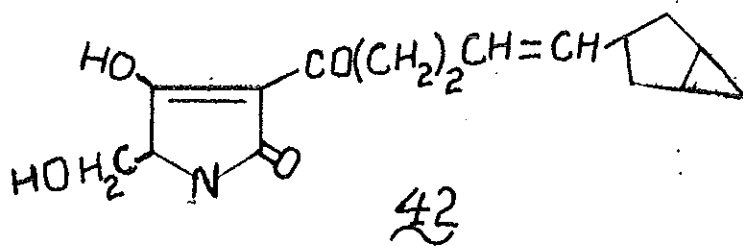
30



33  $R^1 = H, R^2 = O$

34  $R^1 = Me, R^2 = H, OH$





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DECLARATION

The thesis is my own original work and has not been presented for a degree in any other university.

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The thesis has been submitted for examination with my approval as a University Advisor.

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