CYXOCHALASINS FROM THE FUNGUS *XYLARIA*

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Graduate Studies, Addis Ababa University

In Partial Fulfilment for the Degree of Master of

Science in Chemistry

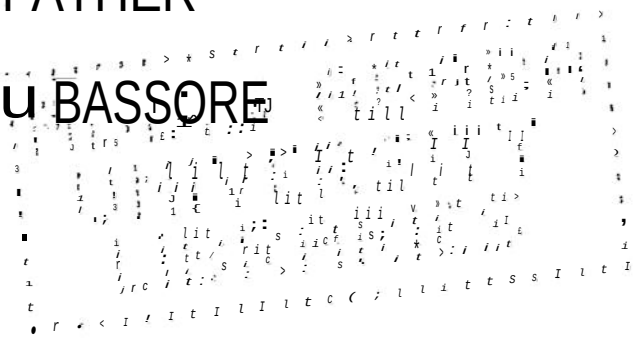
By

Solomon Mitiku

Addis Ababa, June 1994.

To MY FATHER

ATO Mitiku BASSORE



ACKNOWLEDGMENTS

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ABSTRACT

Three cytotoxic cytochalasins were isolated and characterized from *Xylaria* sp. (LEP-12) cultured on a sterile moist rice. The fungus grown on the above medium for 14 days at room temperature produced three cytochalasins, identified by spectroscopic methods and chemical derivatizations as cytochalasin Q (3), 19,20-epoxycytochalasin Q (4) and 19,20-epoxydeacetylcytochalasin Q (5). However only compounds 3 and 4 could be isolated when the fungus was allowed to grow in the medium for 18 days. This is the first report of cytochalasin Q from the genus *Xylaria*. All three cytochalasins were lethal to brine shrimp with LC_{50} value of 5, 5 and 2 μg/ml for compounds 3, 4 and 5, respectively.

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1. INTRODUCTION

1.1 Secondary metabolites of firngi

The fungi are able, in common with the higher plants and the bacteria, to produce secondary metabolites. In many cases secondary metabolites have no known role in the internal economy of the producing organism whereas primary metabolites play a well-defined physiological role in the producing organism. Williams [1] has recently suggested that secondary metabolites serve the producing organism by improving its survival fitness.

The history of secondary metabolites began in 1896, when Gasio published details of his chemical encounter, with what was later shown to be mycophenolic acid (2]. Subsequent significant milestones were the classic 1912 paper by Alsberg and Black on the extractive of mould-infested maize and the discovery of penicillin from *Penicillium chrosogenum* by Alexander Flemming in 1929 [2).

Later on a number of secondary metabolites possessing interesting biological properties such as antibacterial, antifungal, cytostatic activities and toxicities were isolated from microorganisms. Some notable examples include: belomycins, isolated from *Streptomyces verticillus* [3] and Mitomycin C, from *Streptomyces caespitonis* [4] which are used clinically in the treatment of malignancies of the breast, lung, colon, and stomach.

Turner [5] has classified the secondary metabolites of fungi, characterized by their enormous variety of chemical structures, according to biosynthetic origin rather than structural type. Terpenes and steroids are the most common secondary metabolites of basidiomycetes. Cytochalasins are commonly produced by fungi belonging to the Ascomycotina and/or their anamorphic stages.

The production of fungal secondary metabolites can be achieved in liquid culture. Recently solid cultures, i.e., cultures grown on surface of solid media are finding increasing use. In solid cultures, a complete development cycle always takes place unless steps are taken to inhibit that cycle at specific points [2].

1.2 Cytochalasins

The cytochalasins are a rapidly growing group of toxic fungal metabolites, first isolated in mid-1960s. Since then this class of compounds has attracted the attention of chemists and biologists. The first two members of this group of compounds were isolated and characterized independently in Basel, Switzerland [6] and at Imperial Chemical Industries in Great Britain [7]. The compounds isolated from the culture filtrates of *Phoma* sp. were named phomin and dehydrophomin [6] while those obtained from the culture filtrates of *Helminthosporium dematioideum* were called cytochalasins A and B [7]. A direct comparison of the compounds isolated by the two groups showed phomin to be identical with cytochalasin A, and dehydrophomin with cytochalasin B.

Later on these compounds were identified as metabolites of a number of fungi belonging to the Ascomycotina and/or their anamorphic stages. The compounds have been assigned different trivial names given by the different laboratories which named them after the different fungal sources. It is interesting to note that to date cytochalasins have not been found in bacteria or plants [8].

The name "cytochalasin" refers to the biological activity of the group, and is derived from the Greek words *cytos*, meaning cell, and *chalasis*, meaning relaxation. The name cytochalasin reflects the novel effects of cytochalasins on mammalian cells, has been adopted for the basic skeleton of the class of compounds. Structurally the cytochalasins bear phenylalanine or tryptophan moiety linked to a perhydroisoindole moiety, to which a macrocyclic ring is fused. The latter is CVC_{18} polyketide ring system containing a carbocyclic, a lactone, a cyclic carbonate or formyl moiety [13,14]. Zygosporin A isolated from cultures of *Zygosporium massonii* [9], proved to be identical to cytochalasin D from *Metarrhizium anisopliae* [10]. This was the beginning of the discovery of zygosporins [11].

Cytotoxic metabolites isolated from the strains of *Chaetomium globosum* and C. *cochliodes* led to the discovery of chaetoglobosins, a novel group of cytochalasans containing an indolyl group instead of the phenyl group in other cytochalasans. The aspochalasins, isolated from *Aspergillus microcysticus*, contain an isopropyl group in the same position (C-10) [12].

As some of the trivial names overlapped, a systematic nomenclature summarized below was proposed by Binder [15] which is now generally accepted.

(1) The skeleton of the perhydroisoindole unit including the macrocycle and the C-substituents of the isoindole system, except for the phenyl and the indol-3-yl rings, is termed cytochalasan. The number of atoms in the macrocycle, including the atoms common to it and the isoindole ring, is indicated by a number in a square brackets preceding the name cytochalasan.

(2) The absolute configuration of the isoindole system is established and doesn't require specification. For the substituents in the macrocycle, chirality may be designated using the symbols (R) and (S).

(3) The clockwise numbering system indicated in the formulae I to VI (Fig 1) is used to designate the positions of the substituent on the macrocycle and of additional functional groups attached to the isoindole system.

(4) Oxygen atoms which are part of the macrocycle are designated by the prefix oxa-with the appropriate locant.

(5) The prefixes (E) and (Z) indicate, respectively, *irons* and *cis* geometry of olefinic double bonds. This system of numbering allows the assignment of the same locants to that part of the molecule which is common to the hitherto known metabolites. Six types of naturally occurring basic skeletons are known so far and their structures are shown in Figure 1. The individual compounds will be characterized in accordance with this classification.

(I) [11]cytochalasan

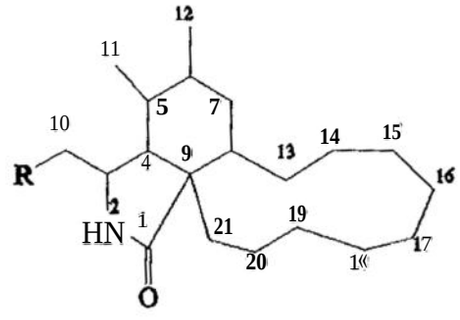
(II) [13]cytochaIasan

(III) 24-oxa-[14]cytochalasan

(IV) 2,23-dioxa-[13]cytochalasan.

(V) 25-oxa-[15]cytochalasan

(VI) 25,26-dioxa-[16]cytochalasan



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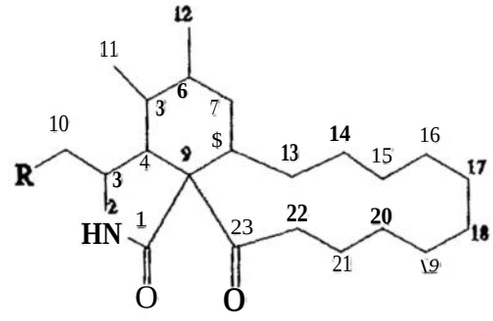

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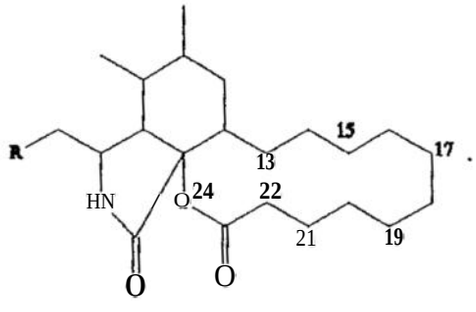

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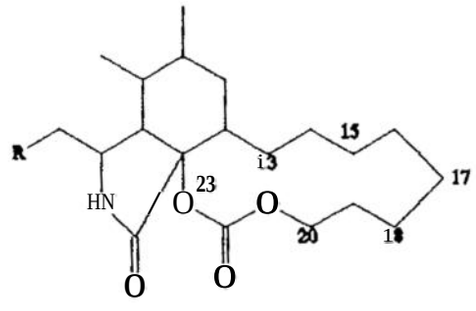

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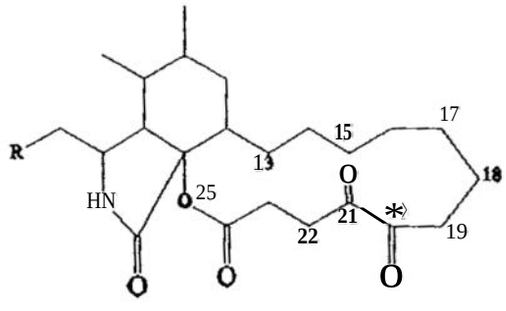
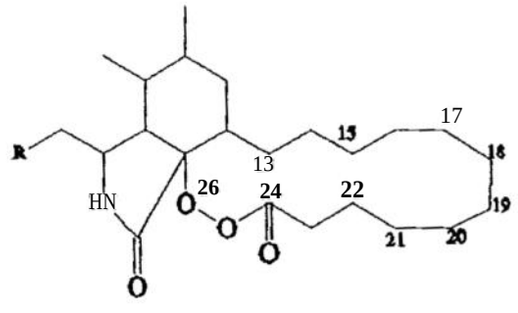


V



VI

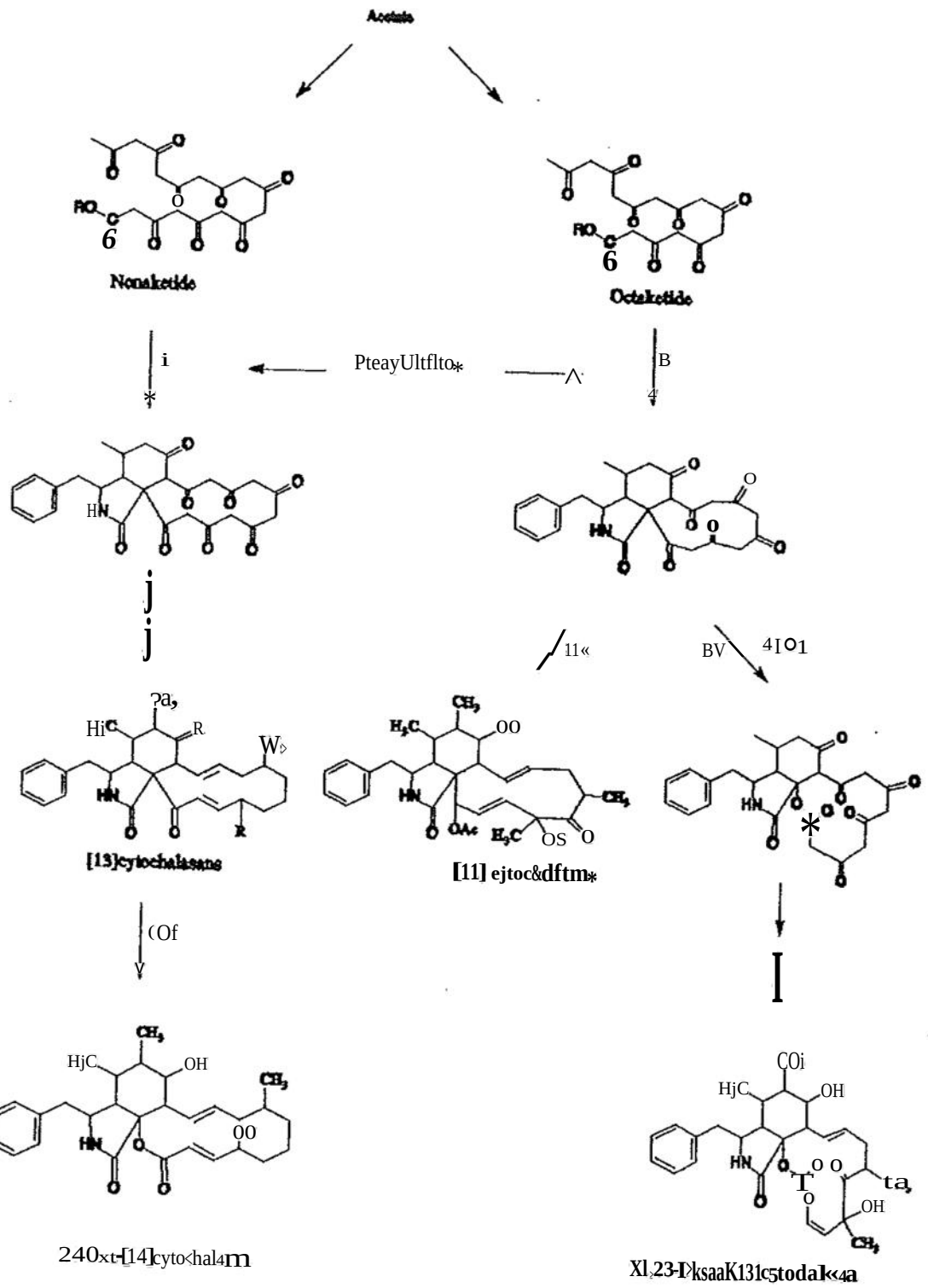
Figure 1. Six types of basic skeletons of cytochalasans. I- R = Phenyl, indol-3-yl or isopropyl; II - R = Phenyl or indol-3-yl; III - VI = R = Phenyl.

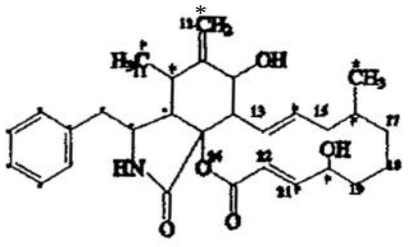
1.2.1 Biogenesis.

Since the isolation and structure elucidation of the first two cytochalasans, cytochalasin A and B in 1966, the number of members of this class of microbial metabolites has risen to 55. Nevertheless, studies on the biosynthesis of cytochalasans have until now been reported only for cytochalasin B [16,17,18 and 19] (a phenyl-24-oxa-[14]cytochalasan), cytochalasin D [18,20,21 and 22] (a phenyl-[11]eytochalasan), 19-O acetylchaetoglobosin A and chaetoglobosin A (3-indolyl [13]cytochalasans) [23].

Cytochalasin B (1) is believed to originate biogenetically from one molecule of phenylalanine (including the carboxylic group), two units of methionine, and nine acetate units or one acetate unit and eight malonate units [17, 24]. A nonaketide is probably first formed from the acetate or malonate units, and this, attached by an amide linkage to phenylalanine, could then be fitted together to form a carbocyclic precursor, [13]cytochalasan [19, 24]. The lactone ring of cytochalasin B would be formed only later from the large carbocyclic ring by insertion of an oxygen atom in a Baeyer-Villiger oxidation [19]. Scheme 1 summarizes the biogenesis of cytochalasins postulated by Tamm *et al*. [19, 24].

^{13}C - and JH - labelling studies of cytochalasin D (2) [20] demonstrated that it arises from one molecule of phenylalanine, three of methionine, and nine of acetate (Scheme 1).


Acetate
Nonaketide
Octaketide
PteayUltflto*
[13]cytochalasans
[11] ejtoc&dftm*
240xt-[14]cyto<hal4m
Xl,23-I>ksaaK131c5todak«4a



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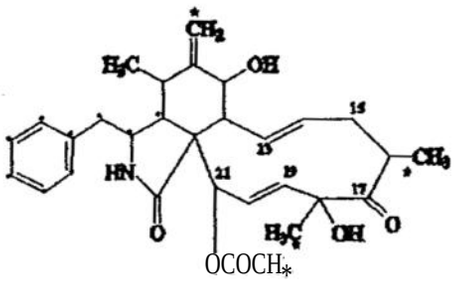


1

• Fkecyiahfek-*

* **Mechionine**

t **Dctaadaed by fc1- acetate feeding cjqxrisvent,**

Scheme 1. Biosynthetic proposals.

Based on the great structural similarity of the first four basic types of cytochalasans, i.e., the [13]cytochalasans (II), 24-oxa-]14]cytochalasans (in), [ll]cytochalasans (I), and 21,23-dioxa-[13]cytochalasans (IV) Tamm *et al.* [19, 24] have postulated a common biogenetic scheme for these classes of cytochalasans, which is shown in Scheme 1.

The fundamental units of the biogenesis of the cytochalasans are the primary metabolites phenylalanine and a nonaketide or octaketide. Phenylalanine is formed from shikimic acid, and is thus derived ultimately from sugars; the nonaketide or octaketide is formed from acetate units.

The linkage of phenylalanine to the nonaketide leads by path I to the [13]cytochalasans and to the 24-oxa-[14]cytochalasans, while linkage to the octaketide leads by path II to the [11]cytochalasans and to the 21,23-dioxa-[13]cytochalasans. The oxidative insertion of oxygen atoms could occur in path I and path lib, with the formation of lactone group on the one hand and the carbonic diester group on the other. Generally cytochalasans possessing a macrocyclic lactone system, as represented by cytochalasin B,

are obtained from a carbocyclic precursor by a Baeyer-Villiger type oxidation [16]. The third type of large ring, a cyclic carbonate group, as represented by cytochalasin E [25] might also arise from a carbocyclic precursor, the carbonate group resulting from a second Baeyer-Villiger-type insertion of an oxygen atom in to a lactone ring [11].

1.2.2 Biological activity of cytochalasins

Observations that culture filtrates of certain fungi produced morphological changes in other fungi and peculiar effects in animal cell cultures led to the discovery of the first members of cytochalasins [11].

All cytochalasins inhibit cytoplasmic cleavage in mammalian cell cultures, but differ in the intensity of their action [24]. HIV-1 protease has been identified as a potential therapeutic target to block the formation of various infectious particles [27]. Recently 18-deoxycytochalasin H (**7**), an isolate of *Hypoxylon fragiforme*, was observed to have HIV-1 protease inhibitory activity [28]. However 19,20-epoxycytochalasin Q (**8**) did not give rise to positive result [29]. In the presence of cytochalasin B (1 μg/ml), normal mitosis occurs in mammalian cells, but cytokinesis, the cleavage of the cell plasma, is blocked [24].

Another general effect of the cytochalasins is their inhibition of a wide variety of cell movements [30]. It has been shown that the primary site of action of cytochalasins on cell motility processes is actin [31]. Cytochalasin B was found to inhibit both the rate of actin polymerization and the interaction of actin filaments in solution. The polymerization rate was reduced by slowing down the addition of actin monomer to the "barbed" end of the filaments, to which monomers are normally added more rapidly; little effect of monomer addition was observed at the slow "pointed" end of the filaments [32]. The study of antimicrobial properties of cytochalasins by different groups indicated cytochalasin A to possess bactericidal activity [33, 34]. Cytochalasins A and D have antifungal properties,

Cytochalasin B shows neither antibacterial nor antifungal activity [11].

The study of the role of the cytoskeleton in germination and nuclear translocation showed cytochalasin D inhibited germ tube elongation suggesting that actin microfilaments are probably involved in this developmental process. Actin microfilaments are primarily responsible for complete germination, specifically germ tube elongation [35]. Some other cytochalasins are also known to be plant growth inhibitors, Cytochalasin H inhibits floral development in tobacco [36]. Chaetoglobosin K, epoxycytochalasin H and epoxydeacetylcytochalasin H were found to inhibit the growth of wheat coleoptiles at concentrations of 10^{-7} [37], 10^{-5} [38] and 10^{-5} M [38], respectively. Cytochalasin F shows significant activity on tomato seedling [39].

Cole and Cox [40] presented compiled data on the toxicity of cytochalasans. LD_{50} values (toxicity) and ED_{50} values (cytotoxicity) of selected cytochalasans are given in Table 1. Because of the strong anti-endemic abscess-inhibiting properties of cytochalasin D in rats, Japanese workers have patented the production of this compound from *Zygosporium masoni* [41] and its use in therapy. Application in human medicine doesn't appear to be ruled out [24].

Table 1. Toxicity and cytotoxicity of cytochalasans. (Based on data compiled by Cole and Cox. [40]).

Cytochalasan	LD_{50} (mg kg^{-1})	ED^{a}_{50} (μg ml^{-1})
Cytochalasin A		3.2
Cytochalasin B		1-2.5
Cytochalasin E	9.1 (rats, orally)	
	2.6 (rats, intraperitoneally)	
Cytochalasin H	12.5 (cockerels, orally)	
Chaetoglobosin A	6.5 (male mice, same route)	3.2-10
Chaetoglobosin B		3.2-10
Chaetoglobosin C		10-32
Chaetoglobosin D		3.2-10
Chaetoglobosin E		3.2-10
Chaetoglobosin F		10
Chaetoglobosin G		*ca* 3
Chaetoglobosin J		*ca* 3
Deoxaphomin		0.29[b]

[a]In HeLa cells.[b]In P-185 mastocyte cells.

1.3 The genus *Xylaria*

Xylaria Hill ex Schrank, family Xylariaceae, contains a large number of species most of them inhabiting tropical and subtropical regions. Those growing on wood cause white rots, i.e. they degrade both lignin and cellulose [42]. They are mostly saprobic or weakly parasitic on woody plants. Their stromata are usually *epixylous* (Gr. *epi* = upon + *xylon* = wood) but some species produce them on sawdust, leaf mould, dung or in the soil. They vary greatly in shape, size and form [43].

1.3.1 Prior work on *Xylaria*

Hexenyl (methyl) succinic acid has been isolated from the culture medium of a number of *Xylaria* species [44]. There was no evidence of any significant additional metabolites in the culture media of these species, until the isolation of a unique polysubstituted C_{22} fatty acid from the fungus *Xylaria cubensis* reported by Edwards in 1991 [45]. Cytochalasin D (3) is the first compound of the group, isolated from the genus *Xylaria* [45]. Recently two new cytochalasins, 19,20-epoxycytochalasin Q (4) and its deacetyl derivative (5) have been reported by our group from *Xylaria obovata* [29].

1.3.2 The objective of this study

The objective of the present study is to investigate bioactive fungal secondary metabolites from the fungus *Xylaria* sp. (LEP-12), belong to the corniformis-complex. To our knowledge the fungus has not yet been subjected to phytochemical or pharmacological study. The present study covers bioassay guided isolation of active components from different stages of growth of the fungus on sterile moist rice.

2. RESULTS AND DISCUSSION

2.1 Isolation of Cytochalasins.

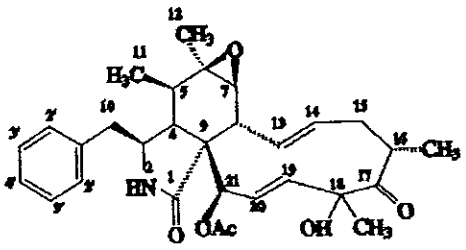
The fungus *Xylaria* sp. (LEP-12) was grown in a stationary culture on 250 g sterile moist rice, placed in a 2-l Erlenmeyer flask. Both medium and mycelium were dried in an oven at 40°. The dried material was ground and extracted with $CHCl_3$. The yield of the extract depended on how long the fungus was allowed to grow on rice. Thus a 14-day fermentation yielded 1.5 g of crude extract while 18-day fermentation resulted in 4.5 g and the continuation of the fungal growth for 30 and 40 days resulted in 3 and 5 g of crude extract, respectively.

In all cases the crude extracts were lethal to brine shrimp (*Artemia salina*) indicating the presence of cytotoxic principles [46]. The extracts were purified through a combination of column chromatography and PTLC, as described in detail in the Experimental Section, to yield the cytochalasins **3**, **4** and **5**. The elucidation of the structures of these compounds was based on spectroscopic means as well as chemical transformations.

2.2 Structure elucidation

2.2.1 Cytochalasin Q (3).

Compound **3** had the composition $C_{30}H_{37}NO_6$ as determined by LREIMS and ^{13}C NMR spectroscopy. The IR spectrum indicated the occurrence of amide (1700 cm^{-1}) and ester carbonyl (1740 cm^{-1}) functions. The ^1H NMR spectrum in pyridine-d_5 disclosed the presence of amide NH (δ 9.44, s, 1H, exchangeable with D_2O), 5 aromatic protons at δ 7.2-7.4, m), an acetyl methyl group (δ 2.34, s, 3H), four methyl groups (δ 0.73, 1.04, 1.18 and 1.53) and four olefinic protons (Table 2).



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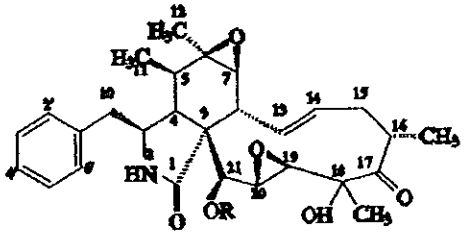
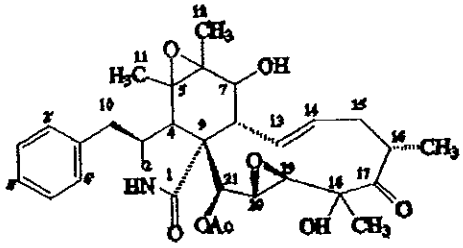


4 R = Ac
5 R = H



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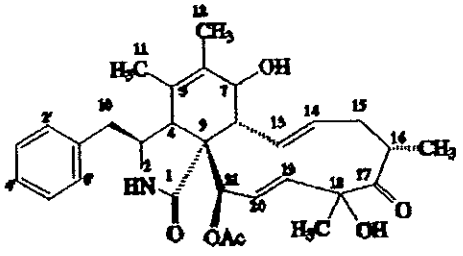

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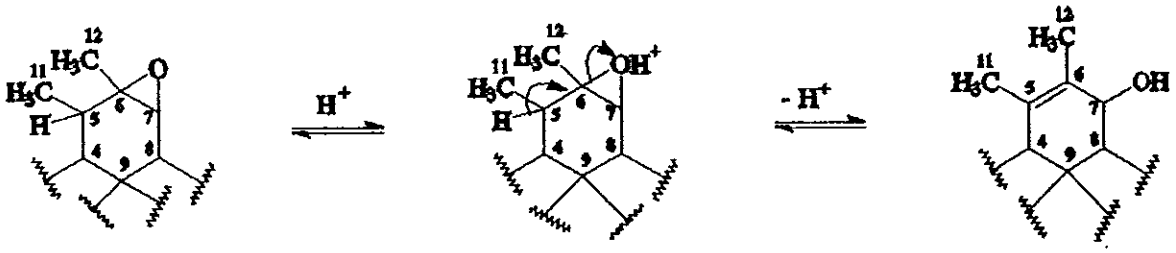
The ^{13}C NMR spectrum, analyzed with the aid of DEPT, showed presence of 7 quaternary carbons (3 carbonyl: amide, ester and ketone, 2 oxygenated carbons, 1 aromatic carbon and 1 aliphatic carbon), 16 methines, 2 methylenes and 5 methyl carbons. Of the 16 methine carbons, 9 were in the aromatic/olefinic regions assignable to 5 aromatic and 4 olefinic carbons based on the results of 2D ^{13}C-^{1}H correlation spectroscopy (HETCOR). Assignment of the aromatic carbons (by HETCOR) leaves the signals at δ 133.0 (C-20), 132.1 (C-13), 131.9 (C-14) and 128.5 (C-19) which are attributed to two 1,2- disubstituted double bonds. The spectrum also indicated the presence of 4 oxygenated carbons (δ 57.2 (s), 63.1 (d), 76.1 (d) and 78.7 (s)) in addition to the 3 carbonyl carbons (δ 210.8 (s) (ketone), 175.44 (s) (amide) and 170.6 (s) (ester)).

The occurrence of the 12-Me as a singlet and the 11-Me as a doublet in the proton NMR spectrum suggested an epoxide linkage across the 6,7-positions. This is

supported by the occurrence of two oxygenated quaternary carbon signals in the ^{13}C NMR spectrum, at 77.7 and 56.9 (C-18 and C-6), and two oxygenated methine carbons at 75.8 and 62.5 (C-21 and C-7). Chemical proof of the location of the epoxide across the 6,7-positions was obtained by treating compound 3 with a trace of mineral acid; this resulted in a mixture whose ^{1}H NMR spectrum showed the disappearance of the high-field doublet indicative of isomerization at C-5 as shown in Scheme 2. In this property it showed a close similarity with other cytochalasin 6,7-epoxides.



3(4)

The above presented data suggested a cytochalasin-type structure for 3 with a 3-benzylperhydroisoindolyl residue attached to a macrocyclic ring and containing a carbonyl group, two double bonds, one oxirane ring and an acetate group. Based on an analogy with known cytochalasins, the carbonyl and an acetate functions were placed at C-17 and C-21, respectively.

The olefinic region of the ^{1}H NMR spectrum of 3 displayed four signals of 1H each at δ 5.44 (ddd, J = 15.6, 10.4 and 5.6 Hz), 5.60 (dd, J = 16.0 and 2.4 Hz), 6.44 (dd, J = 15.6 and 10.1 Hz) and 6.83 (dd, J = 15.6 and 2.4 Hz) which suggested

that the two double bonds are *trans*-1,2-disubstituted. These double bonds will have to be placed in the macrocyclic ring system and are located most likely in the biogenetically favourable 13,14 and 19,20 positions.

Comparison of ^{1}H and ^{13}C NMR data of compound 3 with those of cytochalasins E [47], R [48], K [49], L [49], M [49], Q [48] and 19,20-epoxycytochalasin Q [29] showed agreement with cytochalasin Q, reported by Edwards *et al.*[48]. Edwards *et al.* reported ^{13}C NMR spectral data, only for 29 carbons, without assignments. We have therefore performed detailed ^{13}C NMR studies and have been able to make proper assignments of the carbon resonances of cytochalasin Q. Analysis of HMBC spectrum (Figure 2) indicated that the singlet at δ 57.2, in the ^{13}C NMR spectrum correlated with H-11, thus confirming its assignment to the oxygenated carbon (C-6). Assignment of the doublets present at 130.0 and 128.9 in the ^{13}C NMR spectrum either to C-2'& 6' or C-3'& 5' was resolved with the help of the HMBC spectrum, i.e., the doublet at 130.0 correlated with both protons at C-10 confirming the assignment to C-2'& 6'. Some of the observed connectivities are presented in Figure 3.

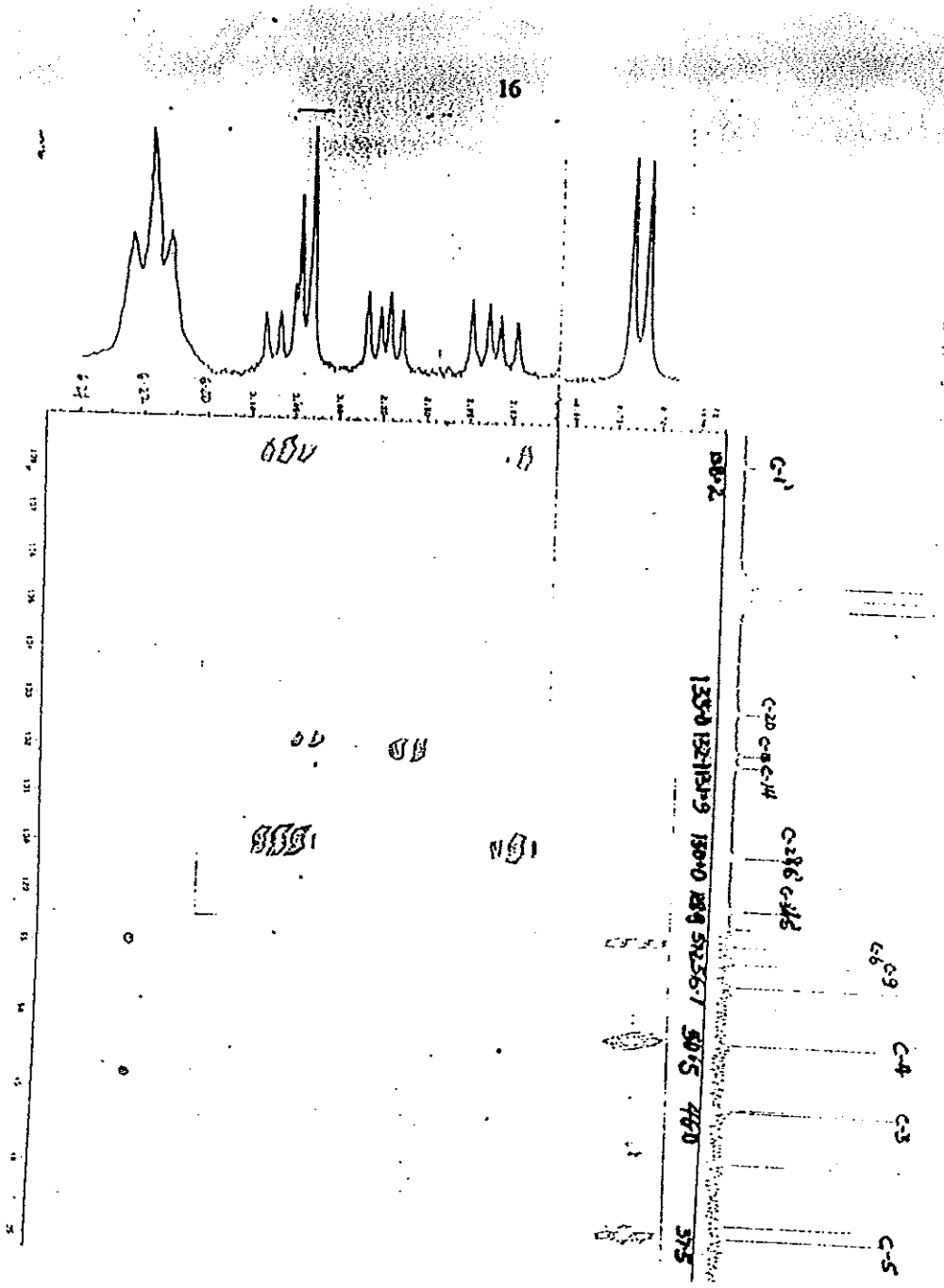


Figure 2. Some regions of HMBC spectrum of compound 3.

This allowed the establishment of compound **3** as (13E,19E)-21-acetoxy-6,7-epoxy-18-hydroxy-16,18-dimethyl-10-phenyl[11]cytochalasa-13,19-diene-1,17-dione or cytochalasin Q.

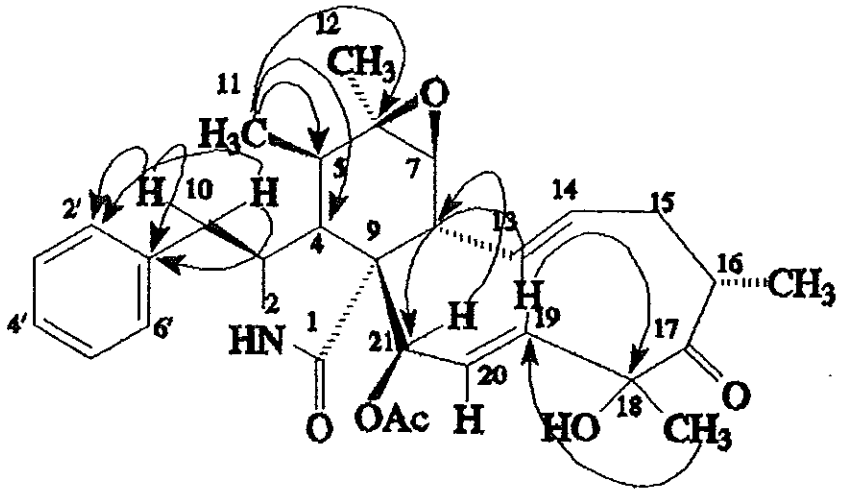


Figure 3. Some selected HMBC correlations of **3**.

Treatment of compound **3** with *m*-chloroperbenzoic acid resulted in two products. The minor and more polar product was identical on TLC with a compound obtained from the treatment of 19,20-epoxycytochalasin Q (**4**) with *m*-chloroperbenzoic acid. ^{1}H NMR spectral data suggested this compound to be the diepoxide (**6**) in which the double bond 13(14) remained intact. This in agreement with the findings by Edwards *et al.* [48] on the ease and site of epoxidation cytochalasin C (**7**) with m-chloroperbenzoic acid in which the order of reactivity was 5(6) > 19(20) > 13(14).

This is the first report on the occurrence of cytochalasin Q in the genus *Xylaria*. The ^{13}C NMR data of the compound isolated from *Hypoxylon terricola* [48] has been reported recently. We have performed detailed ^{13}C NMR studies and have generated new data that assisted in the establishment of the structure unequivocally.

2.2.2 19,20-Epoxycytochalasin Q (4)

The IR spectrum indicated amide and ester carbonyl (1699 and 1751 cm^{-1}, respectively) functions.

The ^1H NMR spectrum showed acetate methyl at δ 2.18 s (3H) and four other methyl signals, of which two appeared as doublets. The 13,14- olefinic protons occur in their expected low field positions as in cytochalasin Q (3). However, the 19,20-protons occur at δ 3.72 and 4.31, respectively. The spectrum also displayed 5 aromatic H at δ 7.2-7.4, m, two benzylic protons at δ 2.87 and 3.1 as well as an amide NH δ 9.58 (exchangeable with D_2O) (Table 2).

The ^{13}C NMR spectrum, analyzed with the aid of DEPT, showed 7 quaternary carbons which were attributable to 3 carbonyls (amide, ester and ketone), 2 oxygenated carbons, 1 aromatic carbon and 1 aliphatic carbon. In addition 16 methines, 2 methylenes and 5 methyl carbons were observed (Table 3). Out of the 16 methine carbons 7 were in aromatic/ olefinic regions and these may be assigned to 5 aromatic and 2 olefinic carbons. The spectrum also indicated the presence of 6 oxygenated methine carbons at δ 52.7 (d), 57.0 (s), 59.6 (d), 62.3 (d), 72.7 (d) and 76.2 (s).

The occurrence of the 12-Me as a singlet and the 11-Me as a doublet in the proton NMR spectrum suggested an epoxide linkage across the 6,7-positions. Chemical proof of the location of the epoxide across the 6,7- positions was also obtained by the same method as used for compound **3**.

Compound **4** is isomeric with cytochalasins Q (**3**) and R [48]. The compound is different from cytochalasins Q because it has one more oxygen only one double bond in the macrocyclic ring system as in cytochalasin R. The 19,20-protons occur at much higher field than cytochalasin Q and their coupling constant is only 2 Hz, which is typical of a *trans* epoxide. Additional support for the diepoxide structure was obtained

from the ^{13}C NMR spectrum showed only eight methine and quaternary unsaturated carbons in the range 120-140 and two additional methine carbons at δ 50-65 compared with the spectrum of cytochalasin Q. Cytochalasin R contains 1,2-*trans*-disubstituted double bond at 19,20 and a *trans*-epoxide at 13,14 positions [31]. The R_f of 19,20-epoxycytochalasin Q (**4**) is different from cytochalasin R [29]. Co-TLC of compound **4** and authentic 19,20-epoxycytochalasin Q showed the two compounds to be identical.

The 13,14-olefinic protons occur at δ 5.83 (ddd, J= 15.6, 9.6, and 5.6 Hz) and 6.65 (dd, J= 15.6 and 10.0), suggestive of a *trans*-1,2-disubstituted double bond. Based on the biogenetic arguments the epoxides should be placed at 6,7 and 19,20 positions and the double bond position and the double bond will have to be placed in the macrocyclic ring system at 13,14 positions. From the foregoing the structure of the cytochalasin was identified as (13E)-21-acetoxy-6,7,19,20-diepoxy-18-hydroxy-16,18-dimethyl-10-phenyl[11]cytochalasa-13-ene-1,17-dione or 19,20-epoxycytochalasin Q (**4**). Further confirmation of the identity of compound **4** was derived from the fact that its mixed melting point with authentic 19,20-epoxycytochalasin Q was undepressed. The ^{13}C NMR and ^{1}H NMR data of **4** were in good agreement with that reported for 19,20-epoxycytochalasin Q [29].

2.2.3 19,20-epoxydeacetylcytochalasin Q (5)

The IR spectrum of **5** lacked the absorption signal at 1751 cm^{-1}, as compared with the compound **4**, indicating the absence of ester carbonyl in **5**.

Its ^{1}H NMR spectrum was almost superimposable with that of **4** except for the absence of resonance due to acetate methyl at δ 2.18 s and up-field shift of the signal due to H-21. These suggested that the compound may be the deacetyl analog of **4**, which was supported by the ^{13}C NMR spectral data (Table 3). Further structural

evidence was achieved by comparing its ^{1}H NMR (90 MHz) and ^{13}C NMR with that reported for 19,20-epoxydeacetylcytochalasin Q [29].

The foregoing suggested compound **5** is 6,7,19,20-diepoxy-18,21-dihydroxy-16,18-dimethyl-10-phenyl[11]cytochalasa-13-ene-1,17-dione or 19,20-epoxydeacetylcytochalasin Q (**5**). Additional confirmation of the identity was derived from co-TLC and mixed melting point determination with an authentic sample of 19,20-epoxydeacetylcytochalasin Q.

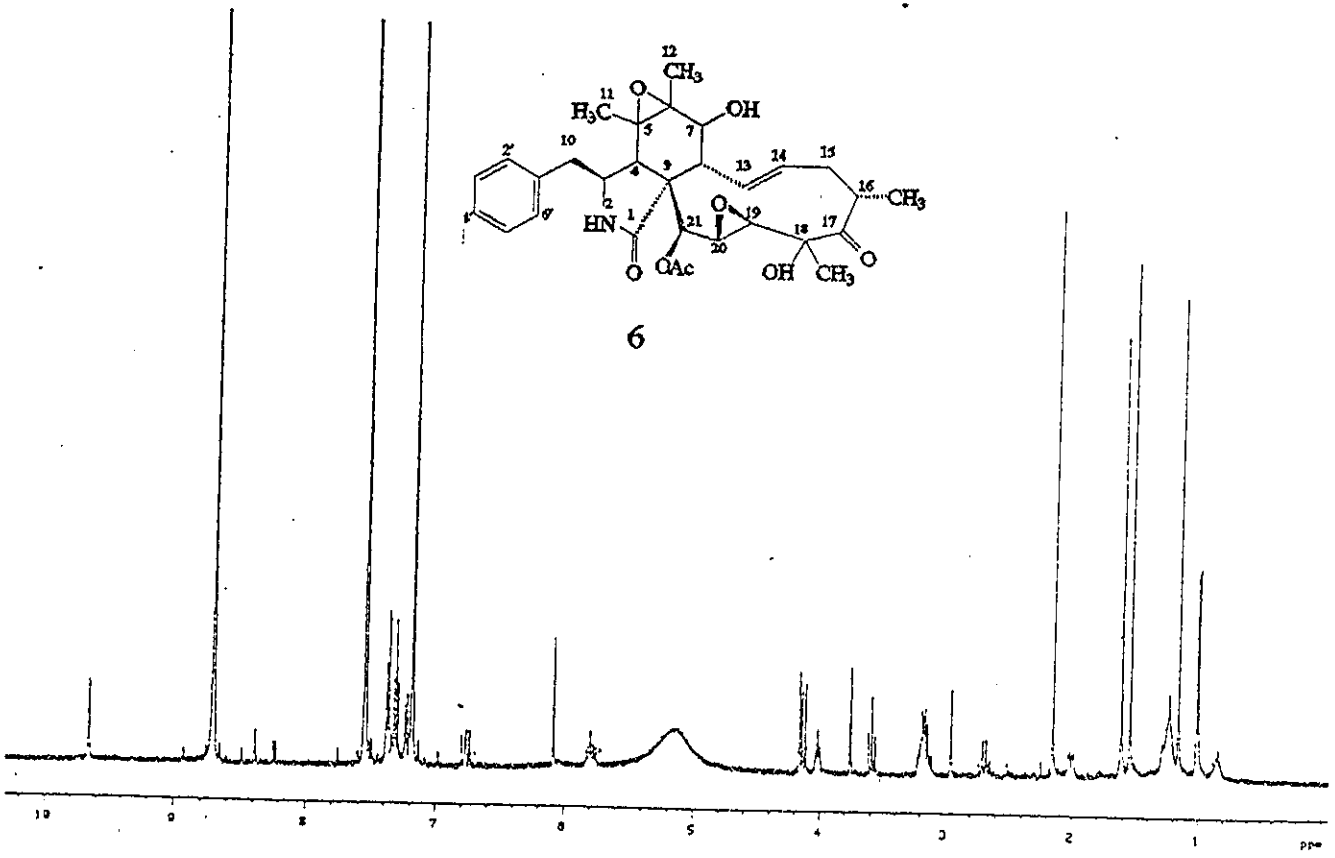
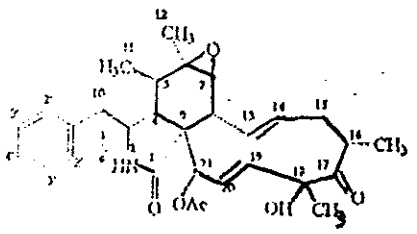


Figure 4. ^{1}H NMR spectrum of compound 6 (400 MHz, pyridine-d_5).



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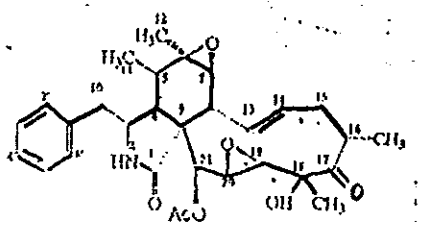

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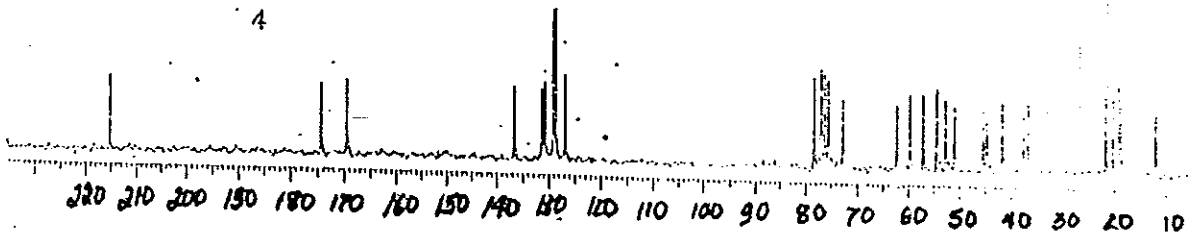

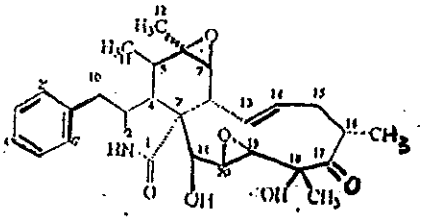

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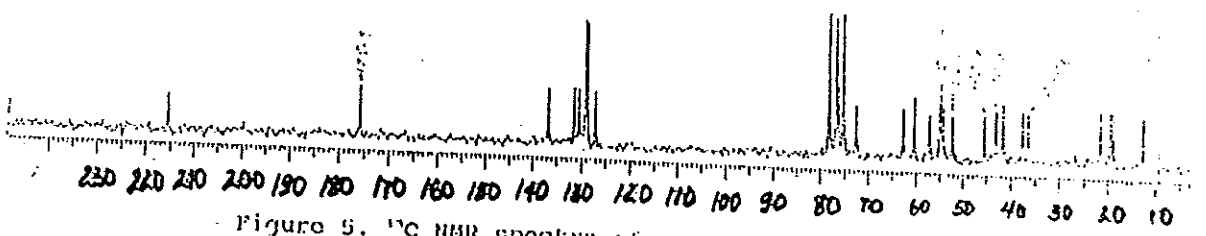
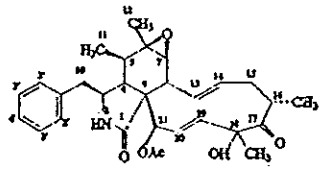


Figure 5. ^{13}C NMR spectra of compounds: 3, 4 and 5.



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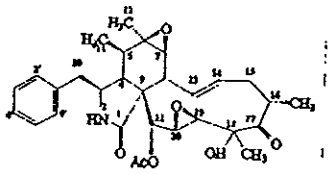

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Figure 6. ^{1}H NMR spectra of compounds 3 and 4

Table 2. ^{1}H NMR data for cytochalasins 3-5.[a]

H	3[b]	4[b]	5[c]
3	3.9 brt (7.2)	3.89 brt (7.2)	3.9 brt
4	2.42 dd (6.0,2.0)	2.52 dd (5.6,1.6)	2.2 m
5	1.91-2.00 dq (7.2,6.0)	1.89 dq (6.8,6.0)	1.9 m
7	3.04 d (5.6)	3.02 d (5.6)	3.05 d (5.0)
8	2.95 dd (9.9,5.7)	2.76 m	2.75 d
10	3.06 dd (13.2,6.6) 2.82 dd (13.2,7.9)	3.1 dd (13.2,6.0) 2.87 dd (13.2,7.6)	2.8 d 3.0 d
11	0.73 d (7.2)	0.64 d (7.2)	0.8 d (8.0)
12	1.18 s	1.17 s	1.2 s
13	6.44 dd (15.6,10.1)	6.65 dd (15.6,10.0)	6.6 dd
14	5.44 ddd (15.6,10.4,5.6)	5.83 ddd (15.6,9.6,5.6)	5.85 m
15	1.90-2.05 m 2.6-2.74 m	2.01 m 2.75 m	1.97 m 2.75 m
16	2.6-2.74 m	3.24 m	3.21 m
19	5.6 dd (16.0,2.4)	3.72 d (2.0)	3.98 d (2.0)
20	6.83 dd (15.6,2.4)	4.31 dd	4.27 brs
21	6.22 dd (2.2,2.4)	6.19 brs	4.97 brs
22	1.04 d (6.8)	1.07 d (6.4)	1.1 d (7.0)
23	1.54 s	1.64 s	1.6 s
NH	9.44 s	9.58 s	9.25 s
OAC	2.34 s	2.18 s	.....
2', 6'			
3', 5'	7.2-7.4 m	7.23-7.4 m	7.20 m
4'			

[a] Assignment by comparison with the [29] and [48] and from HETCOR spectra in the case of compound **3**. Coupling constants (Hz) in parenthesis.
[b] & [c] In pyridine-d$_5$ at 400 and 90 MHz, respectively.

Table 3. ^{13}C NMR spectral data of 3-5[a]

C	3[a,c,e]	4[b,d]	5[b,d]
1	175.4 (s)	174.3 (s)	175.9 (s)
3	54.50 (d)	54.3 (d)	54.9 (d)
4	50.5 (d)	50.7 (d)	52.4 (d)
5	37.3 (d)	36.5 (d)	36.6 (d)
6	57.2 (s)	57.0 (s)	57.3 (s)
7	63.1 (d)	62.3 (d)	62.9 (d)
8	46.0 (d)	44.6 (d)	43.2 (d)
9	56.1 (s)	54.5 (s)	54.9 (s)
10	46.2 (t)	45.3 (t)	45.7 (t)
11	12.5 (q)	12.1 (q)	12.6 (q)
12	19.6 (q)	19.2 (q)	19.6 (q)
13	132.1 (d)	131.4 (d)	131.62 (d)
14	131.9 (d)	130.7 (d)	130.7 (d)
15	38.3 (t)	37.7 (t)	37.7 (t)
16	42.5 (d)	41.6 (d)	41.8 (d)
17	210.8 (s)	215.2 (s)	215.5 (s)
18	78.7 (s)	76.2 (s)	76.4 (s)
19	128.5 (d)	59.6 (d)	60.5 (d)
20	133.0 (d)	52.7 (d)	55.2 (d)
21	76.74 (s)	72.7 (s)	72.8 (s)
22	19.39 (q)	18.8 (q)	18.9 (q)
23	24.6 (q)	21.7 (q)	21.8 (q)
1'	138.26 (s)	136.6 (s)	136.8 (s)
2', 6'	130.0 (d)	129.3 (d)	129.3 (d)
3', 5'	128.9 (d)	128.6 (d)	128.9 (d)
4'	127.0 (d)	126.7 (d)	127.1 (d)
CH_3CO	20.5 (q)	20.2 (q)	.....
CH_3CO	170.6 (s)	169.5 (s)	.....

[a] & [b] In pyridine-d_5 and $CDCl_3$ at 100.57 MHz and 22.5 MHz, respectively; multiplicity from DEPT experiment.
[c] Assignment based on ^1H-^{13}COSY (HETCOR) data.
[d] Assignment by comparison with ref. 29.
[e] Assignment supported by HMBC data.

2.3 Biogenetic relationship of cytochalasins of *Xylaria* sp. (LEP-12).

The occurrence of cytochalasin Q (3), 19,20-epoxycytochalasin Q (4) and 19,20-epoxydeacetylcytochalasin Q (5) as co-metabolites of the same fungus raise interesting questions. Is cytochalasin Q the precursor of 19,20-epoxycytochalasin Q? Is 19,20-epoxycytochalasin Q formed from 19,20-epoxydeacetylcytochalasin Q?

Epoxidation of double bonds is a common biosynthetic reaction encountered in secondary metabolites and therefore 4 may arise from cytochalasin Q (3) as a result of biological epoxidation.

In the early growth stage (14 days) of the fungus, 19,20-epoxydeacetylcytochalasin Q (5) is the major metabolite. However it was not isolated from the same fungus grown for four days longer in the same medium. This and the biosynthetic studies by Tamm *et al.* [24] may indicate 19,20-epoxycytochalasin Q could be formed from its deacetyl analog in *Xylaria* sp. (LEP-12) under the growing conditions shown in the Experimental section.

2.4 Biological activities of the isolated cytochalasins

The biological processes affected by the group of cytochalasins, include toxicity to animals, effects on morphogenic properties in embryonic tissues, inhibitory effect on specific tissues or organs in culture and toxicity to bacteria, algae, fungi and protozoas [24, 34]. Some cytochalasins are known also for their phytotoxic activity. The detail on the biological activity of the group is discussed in the Introduction.

Screening test for the biological activities of the crude extract of the fungus grown on the sterile moist rice showed toxicity to larvae of brine shrimp and retardation of growth in *teff* seedling. Bioassay guided fractionations yielded toxic compounds **3, 4 & 5**. The three isolated cytochalasins and the chemically transformed compound were lethal to

brine shrimp (*Artemia salina*) with the LC_{50} of 5, 5, 2 and 10 μg/ml for compound **3**, **4**, **5** and **6**, respectively. This corresponds to cytotoxicity to cell lines (9KB) with the ED_{50} values of about one tenth LC_{50} values [50] in the brine shrimp test i.e. 0.5, 0.5, 0.2 and 1 μg/ml, in the same order above. Compound **5** appears to be slightly more active than 19,20-epoxycytochalasin Q. The phytotoxicity test of the compounds showed retardation in the growth of *teff* with 100 μg/ml.

Different groups have studied the relationship of structure of cytochalasans with cytotoxicity.

The effect of the substituents at C_{10}, the cellular events and the *in vitro* effects, were in the same order in the phenyl and indolyl series. When the group at C-10 was replaced by an isopropyl group the same effects were observed though the *in vitro* was quite weak [51]. Considering the results obtained by the Shionogi group [52], a conclusion can be drawn that the substituents at C_{10} influence the magnitude of the effects but a specific group is not required as a binding site.

The effects of the pairs of compounds, in which the structures of the whole molecules are the same except for the functionalities at C_5, C_6, and C_7 exhibited no obvious differences. However in the case of compounds having a hydroxyl group at C_7, acetylation of the group clearly rendered a decrease of the activity [53].

The macrocyclic ring starting at C_8 and running to C_9 varies in the size of the ring and the functionalities. However no definite functional group in this part of the molecule has a role as a binding site with the contractile protein. It is therefore probable that the region from C_{13} to C_{16} common to almost all cytochalasans acts as an anchor in a hydrophobic domain [51].

As a whole, the perhydroisoindolone, the macrocyclic ring and the hydrophobic region from C_{13} to C_{16} are assumed to be essential for the effects [51].

3. EXPERIMENTAL

3.1 Instruments and materials. Mps were determined on a Koefler hot stage apparatus and are uncorrected, FT-IR spectra were measured using KBr discs on a Perkin-Elmer spectrophotometer model 1600, UV spectra were obtained in MeOH with the Spectronic 1001 plus and optical rotations on a Perkin-Elmer 241 polarimeter. ^{1}H NMR spectra of compounds **3**, **4** and **6** were recorded on a Varian Unity 400 spectrometer at 400 MHz and of **5** on a Jeol-FX 90 Q at 90 MHz. ^{13}C NMR spectra of compound **3** were recorded in pyridine-d_5 on a Varian Unity 400 spectrometer at 100.57 MHz and those of compounds **4** and **5** in $CDCl_3$ on a Jeol- FX 90 Q spectrometer at 22.5 MHz, with TMS as internal standard in both cases. 2D ^{1}H- ^{13}C HETCOR and HMBC experiments were performed on the Varian Unity 400 spectrometer. Chemical shifts are given in ppm relative to TMS at 0 ppm. Mass spectra was taken on a VG 707 E-HF instrument. TLC was done on silica gel 60 F_{254} 0.2 mm thick layer on aluminium sheets (Merck). Preparative TLC (PTLC) were run on silica gel 60 $PF_{254+366}$ (Merck), 2mm glass plates. Flash chromatography was performed on silica gel 60 (230-400 mesh ASTM) (Merck). Components on TLC were visualized by spraying with 50% ethanolic H_2SO_4 and heating at 100-110° and viewing under UV light at 366 nm.

3.2 Fungal material. Fruit bodies of the fungus *Xylaria* sp. (LEP-12) were collected from Lepis forest in southern Shoa province, Ethiopia. A voucher specimen of the fruit body is deposited under the cipher LEP-12 at the National Herbarium, Addis Ababa University (Fungal Culture Collections of Dr Dawit Abate).

The fungus was identified by Thomas Læssoee, Royal Botanic Gardens, Kew, England, as *Xylaria cf.* curta, spores typical or perhaps slightly on the large side, stromata a bit more irregular than in typical specimens. A complexarea with in the *X. corniformis* complex.

3.3 Cultivation of Xylaria sp. (LEP-12). To each of two 2 l Erlenmeyer flasks were added 250 g of rice and 150 ml of distilled water. The rice was allowed to soak for 3 hrs. and then sterilized at 121° for 30 min. About 100 ml of *Xylaria* sp. inoculum, grown for 6 days in YMG (yeast extract, 4 g; malt extract, 10 g; glucose, 4 g; tap water 1 l) medium, was added to the sterile moist rice. Fourteen and 18 days after inoculation of each of the two flasks, the mouldy rice was ground with a mortar and pestle.

3.4 Extraction and isolation of compounds. The ground mouldy rice was dried at 40° (overnight) and soaked (24 h) in a total volume of 1.5 litres of $CHCl_3$. It was then filtered and the filtrate dried over anhydrous Na_2SO_4 and evaporated under reduced pressure.

3.4.1 Isolation of cytochalasins from Xylaria sp. (LEP-12) grown for 14 days. The residual gummy solid (1.5 g) obtained by the above method of extraction was applied to a flash column of SiO_2 (2 x 30 cm) in petrol-acetone (5:1) and elution was carried out using petrol-acetone mixtures of increasing polarities. A total of 29 fractions each 100 ml were collected and analyzed by TLC. The first four fractions eluted with petrol-acetone (5:1) gave a gum which was discarded.

Cytochalasin Q (3). Fractions 5 & 6 eluted with solvent petrol-acetone (4:1) yielded cytochalasin Q (3) (64 mg) after further purification by PTLC (petrol- acetone 3:2) and upon crystallization from petrol-acetone (1:1)

19,20-Epoxycytochalasin Q (4). Fractions 7-11 eluted with solvent petrol-acetone (4:1) gave mixed cytochalasins, namely, 19,20 epoxycytochalasin Q (4) and its deacetyl derivative, 19,20 epoxydeacetylcytochalasin Q (5) which was further purified on SiO_2 plates using petrol-acetone (3:2) to afford 190 mg of 4 and 30 mg of 5.

19,20-Epoxydeacetylcytochalasin Q (5). Fractions 12-14 eluted with petrol-acetone (4:1) gave pure 19,20-epoxydeacetylcytochalasin Q (5) (219 mg) which was the major product of this batch of growth.

3.4.2 Isolation of cytochalasins from Xylaria sp. (LEP-12) grown for 18 days. By the same extraction method used above 4.5 g residual gummy solid was obtained. It was applied to a flash column of SiO_2 (3 x 30 cm) in petrol-acetone (5:1) and elution was carried on using petrol-acetone mixtures of increasing polarities. A total of 19 fractions each 200 ml were collected and analyzed by TLC.

Fractions 1-3 eluted with petrol: acetone (5:1) gave a yellow gum.

Fraction 7 eluted with petrol: acetone (4:1) gave 219.0 mg of pure cytochalasin Q (3).

Fractions 9-13 eluted with petrol:acetone (4:1) afforded 527 mg of pure 19,20 epoxycytochalasin Q (4).

3.5 Epoxidation of cytochalasin Q (3) with m-chloroperbenzoic acid.

A mixture of *m*-chloroperbenzoic acid (105 mg) and cytochalasin Q (40 mg) in

dichloromethane (3 ml) was set aside. After 36 h (TLC control) excess CH_2Cl_2 was added and the solution was washed with saturated aqueous $NaHCO_3$, dried and evaporated and the residue product was purified by PTLC in petrol-acetone (3:2) to obtain the diepoxide (**6**, 5 mg) and other products.

3.6 Epoxidation of the authentic sample 19,20-epoxycytochalasin Q (4) and compound 4, isolated from the fungus Xylaria sp. (LEP-12) with m-chloroperbenzoic acid.

A mixture of *m*-chlorperbenzoic acid (100 mg) and 19,20-epoxycytochalasin Q (20 mg) in dichlorometane (3 ml) was set aside. After 24 h (TLC control) excess CH_2Cl_2 was added and the solution was washed with saturated aqueous $NaHCO_3$, dried and evaporated and the residue product was purified by PTLC in petrol-acetone (3:2) to obtain the diepoxide (**6**, 6 mg) and other products. Following the same procedure above compound **4**, isolated from *Xylaria* sp. (LEP-12) yielded the same diepoxide (**6**).

3.7 Physicochemical data.

Cytochalasin Q (3).

White crystalline solid from petrol-acetone, m.p. 148- 152° (Lit. [48] m.p. 145-147°).

IR ν_{max} (KBr): 1227, 1373, 1455, 1701, 1743, 2971, and 3400 cm^{-1}.

UV λ_{max}: End absorption.

$[\alpha]_D$: -104° (c = 0.45, $CHCl_3$)

R_f (TLC):0.55 (petrol:acetone; 3:2)

^{13}C NMR: see Table 3.

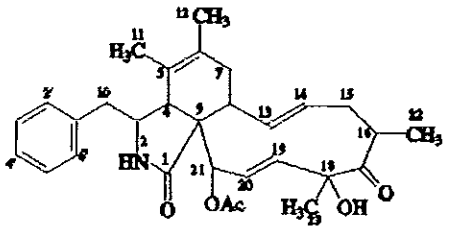
^{1}H NMR: see Table 2.

Appendix 1. Natural [11]Cytochalasans.

Trivial name	Structural formula	Microorganism	Ref.
Cytochalasin C	7	Metarrhizium anisopliae	54, 7
Cytochalasin D	2	Metarrhizium anisopliae,	10, 54
		Xylaria cubensis	44
= zygosporin A		Zygosporium masonii, Z. mycophilum	55, 56
Cytochalasin G	8	Nigrosabulum sp.	57
Cytochalasin H	9	Phomopsis paspali	58, 59
= paspalin P-1		Hypoxylon fragiforme	28
= kodo-cytochalasin-1			
Epoxycytochalasin H	10	Phomopsis sojae	38
Epoxydeacetylcytochalasin H	11	Phomopsis sojae	38
18-Dehydroxycytochalasin H	12	Hypxylon fragiforme	28
Cytochalasin	14	Hypoxylon fragiforme	28
Cytochalasin	15	Hypoxylon fragiforme	28
Cytochalasin J	16	Phomopsis paspali	58, 59
= paspalin P-2			
= kodo-cytoochalasin-2			
Cytochalasin N	17	Hypoxylon terricola	48
Cytochalasin O	18	Hypoxylon terricola	48

Appendix 1 Contd.

Cytochalasin P	19	Hypoxylon terricola	48
Cytochalasin Q	3	Hypoxylon terricola	48
19,20-Epoxycytochalasin Q	4	Xylaria obovata	29
19,20-Epoxydeacetylcytochalasin Q	5	Xylaria obovata	29
Cytochalasin R	20	Hypoxylon terricola	48
Zygosporin D	21	Zygosporium masonii	60
Zygosporin E	22	Zygosporium masonii	60
Zygosporin F	23	Zygosporium masonii	60
Zygosprin G	24	Zygosporium masonii	60
Aspochalasin A	25	Aspergillus microcysticus	11
Aspochalasin B	26	Aspergillus microcysticus	11
Aspochalasin C	27	Aspergillus microcysticus	11
Aspochalasin D	28	Aspergillus microcysticus	11
Engleromycin	29	Englermyces goetzei	61



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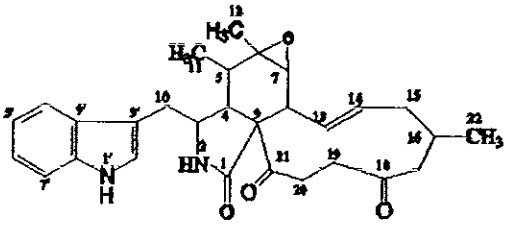

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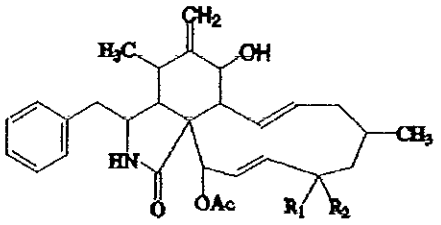
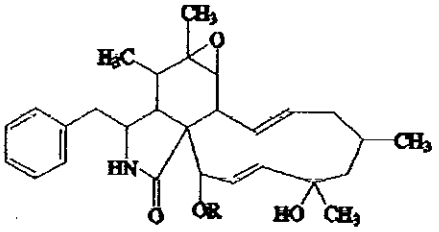


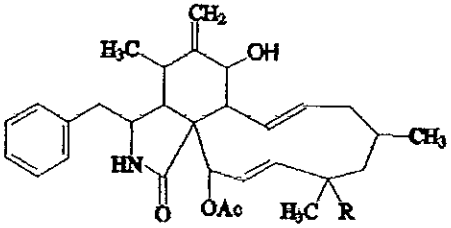
9 $R_1 = CH_3$ $R_2 = OH$

12 $R_1 = CH_3$ $R_2 = H$



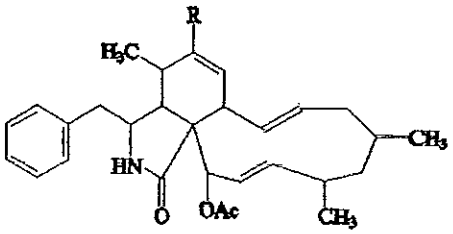
10 R = OAc

11 R = H



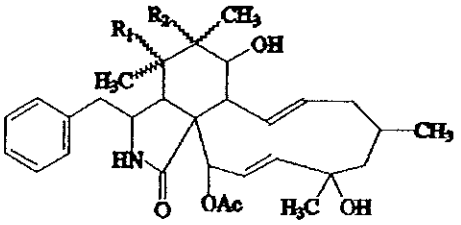
13 R = OH

16 R = H



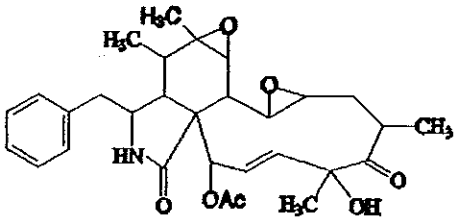
14 R = CH_3

15 R = CH_2OH



14 $R_1, R_2 = O$

18 and 19 $R_1 = H, R_2 = OH$



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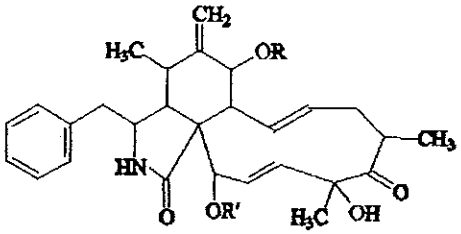
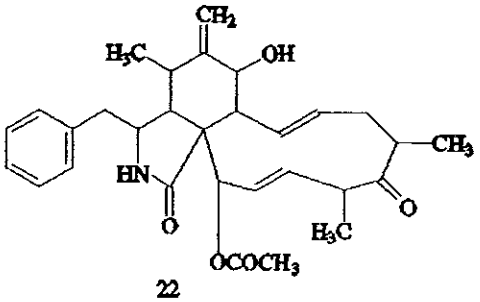


21 R = R' = H

23 R = R' = $COCH_3$



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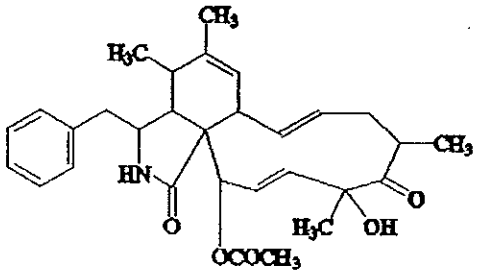

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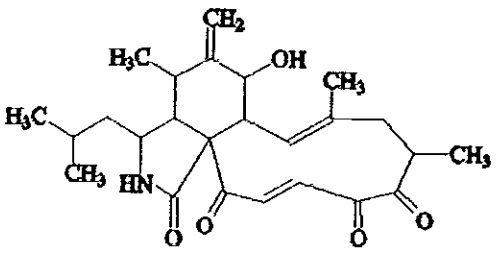

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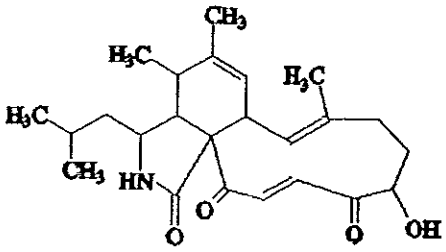

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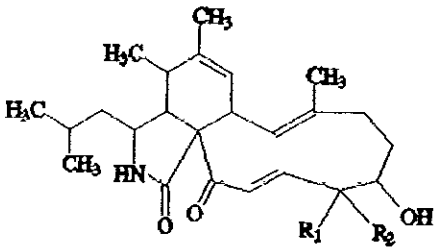
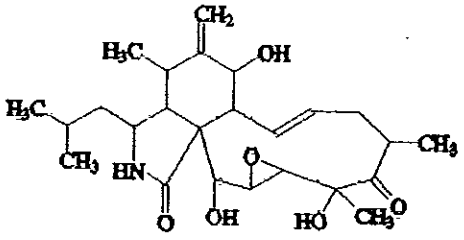


27 R_1 = H, R_2 = OH
28 R_1 = OH, R_2 = H



29

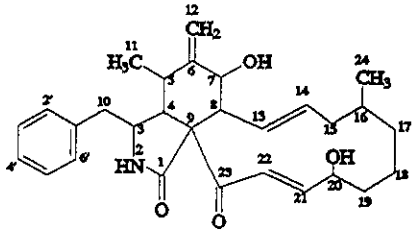
Figure 7. Structures of [11]cytochalasans

Appendix 2. Natural [13]cytochalasans.

Trivial name	Structural formula	Microorganism	Ref.
Deoxaphamin	**30**	*Phoma exigua*	62, 63
Proxiphomin	**31**	*Phoma exigua*	62, 64
Protophomin	**32**	*Phoma exigua*	64
Asochalasin	**33**	*Ascochyta heteromorpha*	65
Chaetoglobosin A	**35**	*Chaetomium globosum*	66
19-O-Acetylchaetoglobosin A	**36**	*Chaetomium globosum*	67
Chaetoglobosin B	**37**	*Chaetomium globosum*	66
19-O-Acetylchaetoglobosin B	**38**	*Chaetomium globosum*	67
Chaetoglobosin C	**39**	*Chaetomium globosum* *Penicillium aurantio-virens*	66, 68 69
Chaetoglobosin D	**40**	*Chaetomium globosum*	68, 74
19-O-Acetylchaetoglobosin D	**41**	*Chaetmium globosum*	67
Chaetoglobosin E	**42**	*Chaetomium globosum*	68
Chaetoglobosin F	**43**	*Chaetomium globosum*	68

Appendix 2 contd.

Chaetoglobosin G	**44**	*Chaetomium globosum*	70
Chaetoglobosin J	**45**	*Chaetomium globosum*	70
Chaetoglobosin K	**46**	*Diplodia macrospora*	37
Cytochalasin K	**47**	*Chalara microspora*	49



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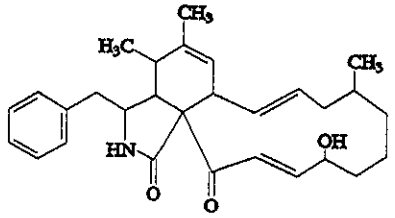

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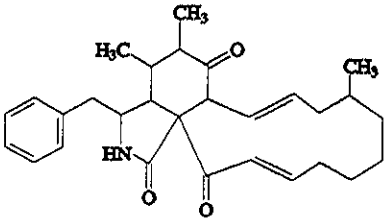

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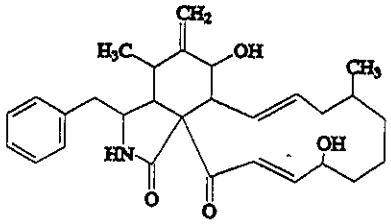

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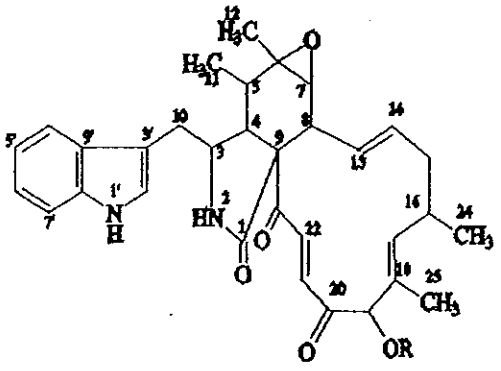
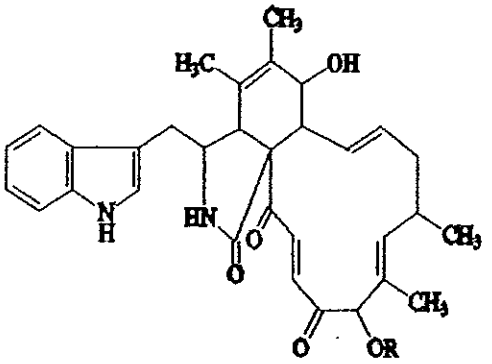
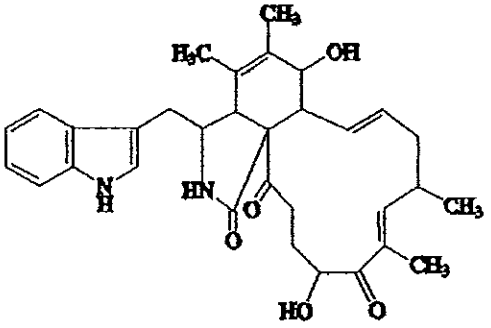


35 R = H
36 R = $COCH_3$



37 R= H
38 R= $COCH_3$



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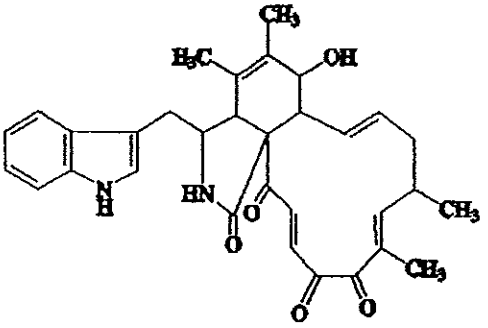

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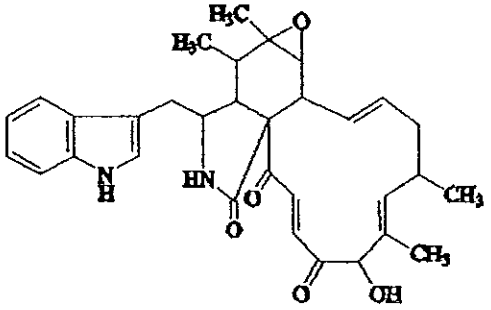

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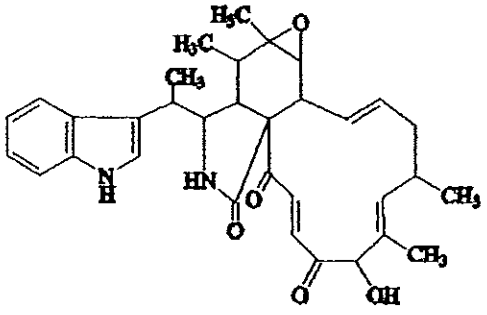

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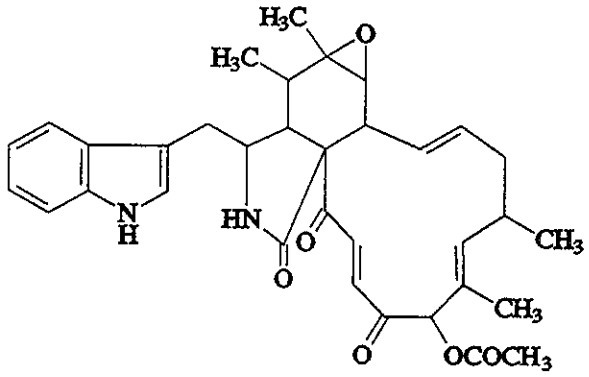


47

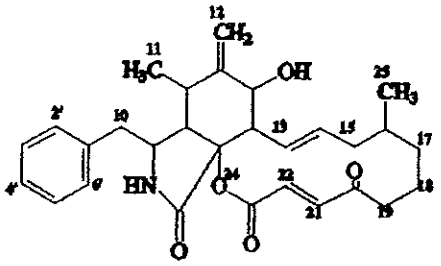
Figure 8. Structures of [13]cytochalasans.

Appendix 3. Natural 24-oxa-[14]cytochalasans.

Trivial name	Structural formula	Microorganism	Ref.
Cytochalasin A = 20-dehydrophomin	**48**	*Phoma exigua* *Helminthosporium dematioideum*	71, 6 7

Appendix 3 contd.

Cytochalasin B = Phomin	**2**	*Phoma exigua* *Helminthosporium dematioideum* *Hormiscium* sp.	71 7 72, 73
7-O-acetylcytochalasin B	**49**	*Phoma exigua var. heteromorpha*	39
Cytochalasin F	**50**	*Helminthosporium dematioideum* *Phoma exigua var. heteromrpha*	26
Cytochalasin L	**51**	*Chalara microspora*	49
Cytochalasin M	**52**	*Chalara microspora*	49 39
Cytochalasin T	**53**	*Phoma exigua var. heteromorpha*	39



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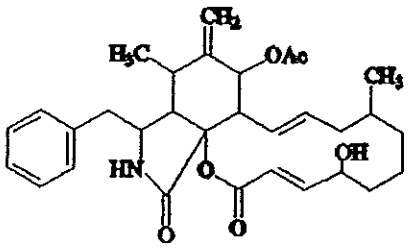

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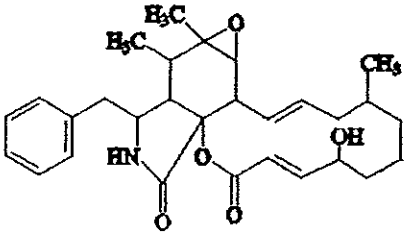

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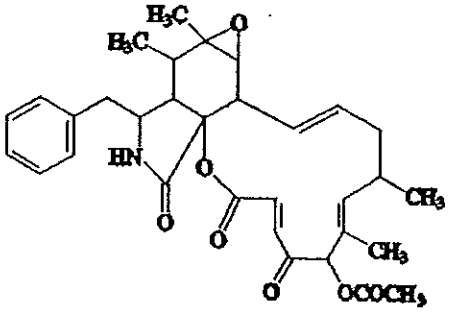

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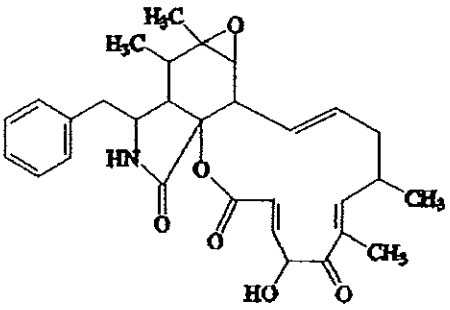

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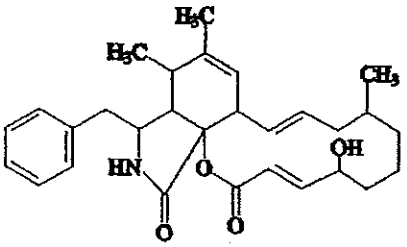


53

Figure 9. Structures of 24-Oxa-[14]cytochalasans.

Appendix 4. Natural 21,23-dioxa-[13]cytochalasan.

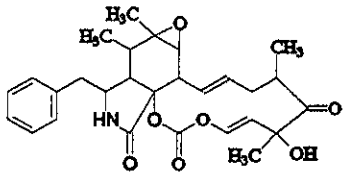
Trivial name	Structural formula	Microorganism	Ref.
Cytochalasin E	**54**	*Rosselina necatrix* *Aspergillus clavatus*	26, 47

Appendix 5. Natural 25-oxa[15]cytochalasan.

Trivial name	Structural formula	Microorganism	Ref.
Cytochalasin V	**55**	*Phoma exigua var. heteromorpha*	14

Appendix 6. Natural 25,26-dioxa[16]cytochalasan.

Trivial name	Structural formula	Microorganism	Ref.
Cytochalasin U	**56**	*Phoma exigua var. heteromorpha*	14



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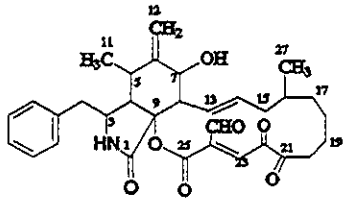

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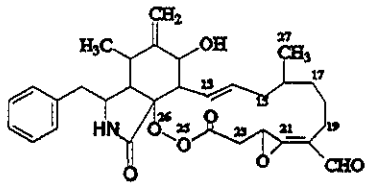

56

Figure 10. Structures of 21,23-Dioxa-[13]cytochalasan, 25- Oxa-[15]cytochalasan and 25,26-Dioxa-[16]cytochalasan.

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