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Two New Cytotoxic Cytochalasins from *Xylaria obovata*

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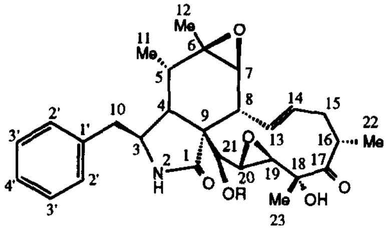
Abstract: Two new cytotoxic cytochalasins were isolated by brine shrimp bioassay-guided fractionation from a culture of the wood inhabiting fungus, *Xylaria obovata*. Their structures were determined as 19,20-epoxycytochalasin Q (**1**) and its deacetyl analog **2** by the application of spectroscopic techniques and chemical correlation with cytochalasin R. Acetylation of **2** yielded **1**. Both **1** and **2** were cytotoxic but were found to be inactive in an HIV-protease inhibitory assay and a mechanism-based DNA damaging yeast assay.

The cytochalasins constitute a small group of toxic fungal metabolites with marked cytotoxic effects on mammalian cells in tissue culture and it was only recently that one of them, 18-deoxycytochalasin H, was reported to have HIV-protease inhibitory activity.[1] In continuing our studies of biologically active natural products, we have investigated the growth medium of the Xylariaceous wood fungus, *Xylaria obovata* (Berk.) Fr. and herein we report the bioassay-guided isolation and structures of two new closely related cytotoxic cytochalasins **1** and **2**. This constitutes the second report of the occurrence of a cytochalasin in a *Xylaria* species, the first being the isolation of cytochalasin D from *X. cubensis*.[2]

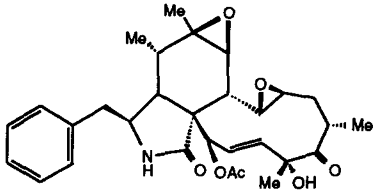
RESULTS AND DISCUSSION

The wood-inhabiting tropical fungus, *X. obovata*, was grown on sterile moist rice. The culture was soaked in aqueous MeOH, filtered, and the filtrate extracted with $CHCl_3$. Evaporation gave an extract showing a lethal effect on brine shrimp (*Artemia salina*) indicating that it may contain cytotoxic components.[3] Bioassay-guided fractionation afforded two compounds, the structures of which were established as the cytochalasins **1** and **2** as described below.

Compound **1** had the composition $C_{30}H_{37}NO_7$ as determined by HREIMS and ^{13}C NMR spectroscopy. The IR spectrum indicated amide and ester carbonyl (1680 and 1740 cm^{-1}, respectively) functions. The occurrence of two major fragment ions due to the loss of 91 and 59 Da from the M^+ in its MS suggested the presence of benzyl and acetyl groups. The ^1H NMR spectrum confirmed the presence of amide NH [δ 9.58, s (1H), exchangeable with D_2O], benzyl [5 aromatic H at δ 7.23, d (1H); 7.34, t (2H); 7.40, d (2H) and the benzylic methylene at δ 2.87 dd (1H) and 3.08 dd (1H)] and acetate [δ 2.18 s (3H)] functions, and also had two methyl doublets at δ 0.63 and 1.07, two methyl singlets at δ 1.17 and 1.64, and two olefinic proton signals (1H each) at δ 5.83 and 6.64, in addition to several other well defined signals (Table 1).



1 R = Ac
2 R = H



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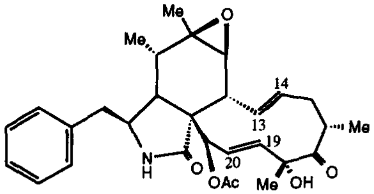
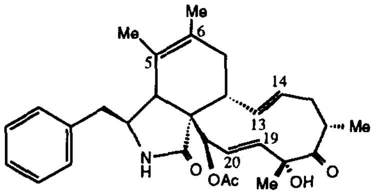


4 13(14)-β-epoxide ; 19(20)-α-epoxide
5 13(14)-β-epoxide ; 19(20)-β-epoxide
6 13(14)- and 19(20)-unsaturation



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The ^{13}C NMR spectrum, analyzed with the aid of DEPT, showed that the compound contained 7 quaternary carbons which were attributable to 3 carbonyl groups (amide, ester and ketone), 3 oxygenated carbons and 1 aromatic carbon, 16 methines, 2 methylenes and 5 methyl carbons (Table 2). Of the 16 methine carbons, 7 were in the aromatic-olefinic region and these were assigned to 5 phenyl and 2 olefinic carbons. Assignment of the phenyl carbons (by HETCOR) leaves the signals at δ 131.6 and 132.5 for a 1,2-disubstituted double bond. The ^{13}C NMR spectrum also indicated the presence of 6 oxygenated carbons [δ 53.9 (d), 57.4 (s), 60.8 (d). 63.1 (d) 73.7 (d) and 77.4 (s)] in addition to the 3 carbonyl carbons [δ 216.3 (s) (ketone), 175.4 (s) (amide) and 170.8 (s) (ester)]. Since **1** contains only 7 oxygen atoms, two cyclic ether moieties, possibly epoxides, must be invoked to accommodate nine oxygenated carbons. The foregoing suggested a cytochalasin-type structure for **1** with a 3-benzylperhydroisoindolyl residue attached to a macrocyclic ring and containing a carbonyl group, a double bond, two oxirane rings and an acetate group. Based on biogenetic considerations and analogy with known cytochalasins, the carbonyl and acetate functions were placed at C-17 and C-21, respectively.

Compound **1** is isomeric with cytochalasin N and cytochalasin R (**3**).[4] Some similarities and differences were observed in the structural features of **1** compared with cytochalasins N and R. As noted above, **1** has only one double bond in the macrocyclic ring system as in cytochalasin R (**3**). Cytochalasin N, however, contains 2 double bonds in the macrocyclic ring. The olefinic region of the ^{1}H NMR spectrum of **1** had two signals of 1H each (δ 6.64 dd, J= 15 and 10 Hz; δ 5.83, sept, J= 15, 10 and 6 Hz) suggestive of a *trans*- 1,2-disubstituted double bond. In the ^{1}H NMR spectrum of **1**, coupling was observed between H-4 and H-5 (J= 5.8 Hz) and H-5 and H-11 (J= 7.2 Hz). These observations and comparison of the ^{1}H and ^{13}C NMR data of **1** with those of cytochalasins E,[5] F,[6] Q,[4] R,[4] K,[6] L,[7] and M[7] suggested the presence of a 6,7-epoxide moiety in **1**. The remaining epoxide functionality and the 1,2-*trans*-disubstituted double bond must be placed in the macrocyclic ring system, most probably in the biogenetically favorable 13(14) and 19(20) positions. Cytochalasin R (**3**) contains a *trans*-1,2-disubstituted double bond at the 19(20) and a *trans*-epoxide at the 13,14- positions. On TLC, **1** was found to be different from an authentic sample of cytochalasin R.[4] The comparison of the ^{1}H NMR spectra of **1** with those of cytochalasin R suggested that **1** is an isomer of cytochalasin R with the epoxide and the double bond interchanged. However, detailed ^{13}C NMR assignments for **3** were not available for comparison; only the multiplicities of the signals have been listed.[4] As such it was thought desirable to assign the ^{13}C NMR spectral data for **3** and this was done with the aid of its DEPT and HETCOR spectra. The detailed assignments are given in Table 2. Comparison of these with the ^{13}C NMR spectral data for **1** (Table 2) confirmed that **1** is an isomer of **3**.

Table 1. ^{1}H NMR Data for Cytochalasins 1, 2, 4 and 5.[a]

H	**1**[b]		**2**[b]	**4**[b]	**5**[c]
	in d_5-pyridine	in $CDCl_3$	in d_5-pyridine	in d_5-pyridine	in d_5-pyridine
3	3.92 br t (7.0)	3.60 br t (6.8)	3.91 br t (7.0)	3.95 ddd (7,7.5,5,2)	3.95 m
4	2.52 dd (5.8,2)	2.21 m	2.10 m	2.52 dd (6,2)	2.64 dd (6,2)
5	1.89 dq (7.2,5.8)	1.60 m	1.96 m	1.82 dq (6,7.5)	1.70 m (6,7.5)
7	3.02 d (5.6)	2.71 d (5.6)	3.09 d (5.6)	3.37 d (6)	3.22 d (6)
8	2.75 m	2.32 dd	2.75 m	2.03 dd (8.5,6)	1.92 dd (8,6)
10	2.87 dd (13.2,8) 3.08 dd (13.2,6)	2.80 d (7.2)	2.85 dd (13.2,7) 3.06 dd (13.2, 5.8)	2.93 dd (13,8) 3.02 dd (13,6)	2.89 dd (13,8) 3.34 dd (13,6)
11	0.63 d (7.2)	0.80 d (7.2)	0.82 d (7.2)	0.69 d (8)	0.44 d (8)
12	1.17 s	1.18 s	1.20 s	1.20 s	1.17 s
13	6.64 dd (15.2,10)	6.08 dd (15.2,10)	6.66 dd (15.4, 9.6)	4.43 dd (8.5,2)	4.11 dd (8,2)
14	5.83 sept (15.6,10 ,6)	5.65 m	5.85 sept (15.6,10,4)	3.22 m	3.35 m
15	2.00 m, 2.75 m	2.61 dd (12,11.4)	1.97 m, 2.75 m	2.16 m, 2.22 m	2.08 m, 2.21 m
16	3.24 m	3.20 m	3.21 m	3.33 m	3.35 m
19	3.72 d (2)	3.15 br s	3.98 d (2)	4.00 d (2)	3.94 d (2)
20	4.30 dd (2,0.8)	3.52 d (1.2)	4.27 t (1.6)	4.59 dd (2,0.8)	4.17 dd (2,1)
21	6.18 br s	5.65 m	4.97 br s	6.14 br s	5.72 br s
22	1.07 d (6.8)	1.19 d (6.4)	1.06 d (6.4)	1.16 d (7.0)	1.10 d (7.0)
23	1.64 s	1.52 s	1.67 s	1.71 s	1.68 s
NH	9.58 s	5.80 br s	9.25 s	9.67 s	_[d]
OAc	2.18 s	2.10 s	--	2.08 s	2.04 s
2',6'	7.40 d (6.7)			7.00-7.40m	7.10-7.40m
3',5'	7.34 t (7.8)	7.20 m	7.20 m		
4'	7.23 d (6.7)				

[a] At 400 MHz; coupling constants (Hz) in parenthesis.
[b] Assignments based on DQCOSY (^{1}H-^{1}H COSY) and HETCOR spectra.
[c] Assignment by comparison with **1** - **4**.
[d] Signal probably hidden under solvent signal.

From the foregoing the structure of the major cytochalasin was identified as (13*E*)-21-acetoxy-6,7,19,20-diepoxy-18-hydroxy-16,18-dimethyl-10-phenyl[11]cytochalas-13-ene-1,17-dione or 19,20-epoxy-cytochalasin Q (**1**). The double bond in **1** (see above) was confirmed to be at the 13(14) position based on the results of decoupling experiments in $CDCl_3$. Thus, irradiation of the signal at δ 2.32 (H-8) reduced the doublet at δ 2.71 (H-7) to a singlet and the dd at δ 6.08 (H-13) to a doublet. Irradiation of the dd at δ 2.61 (H-15) caused the multiplet at δ 5.65 due to the olefinic H at C-14 to collapse to a br d and the complex multiplets at δ 2.61 (H-15) and 3.20 (H-16) to collapse to broad quartets.

In the ^1H NMR spectrum of **1** in d_5-pyridine, the protons of the epoxide group in the macrocyclic ring appeared as two doublets at δ 3.72 (H-19) and 4.30 (H-20) with a low coupling constant (J= 2 Hz) suggesting a *trans* stereochemical disposition of these two protons. The chemical shifts and coupling constants of H-19 and H-20 agreed reasonably well with those reported for engleromycin,[8] the only natural cytochalasin known so far with a 19,20-epoxide moiety. It was also found that irradiation of the signal at δ 4.30 (H-20) caused enhancement of the signals at δ 3.72 (H-19) and 6.18 (H-21). Interestingly, very little coupling was observed between H-21 (δ 6.18, br s) and H-20 (δ 4.30, dd, J = 2.0 and 0.8 Hz) which suggested that the dihedral angle H-C(20)-C(21)-H is close to 90°. Examination of models show that a 90° angle can be achieved without significant steric interactions only if the oxirane in **1** has the β-orientation. The structure **1** proposed for the major cytochalasin was further confirmed by analysis of its HMBC spectrum[9] (in d_5-pyridine); the connectivities observed are presented in Figure 1.

Table 2. ^{13}C **NMR Spectral Data of 1 - 4.**[a]

C	**1**[b,c]	**2**[b]	**3**[b]	**4**[d]	C	**1**[b,c]	**2**[b]	**3**[b]	**4**[d]
1	175.4 s	177.2 s	175.5 s	175.4 s	16	42.2 d	42.2 d	38.2 d	40.4 d
3	54.9 d	54.6 d	54.8 d	55.3 d	17	216.3 s	216.8 s	213.2 s	215.9 s
4	51.2 d	52.8 d	50.8 d	54.0 d	18	77.4 s	77.7 s	78.8 s	74.2 s
5	37.3 d	37.5 d	37.5 d	37.3 d	19	60.8 d	60.8 d	130.5 d	59.5 d
6	57.4 s	57.6 s	56.1 s	56.2 s	20	53.9 d	56.1 d	132.2 d	58.1 d
7	63.1 d	63.6 d	62.2 d	61.5 d	21	73.7 d	73.3 d	76.2 d	77.8 d
8	45.7 d	44.7 d	44.8 d	43.9 d	22	19.2 q	19.2 q	20.4 q	20.3 q
9	55.6 s	56.7 s	55.2 s	54.3 s	23	22.7 q	22.8 q	24.8 q	23.4 q
10	46.1 t	46.2 t	46.2 t	46.0 t	1'	138.2 s	138.3 s	138.2 s	138.0 s
11	12.4 q	12.7 q	12.5 q	12.8 q	2',6'	130.2 d	130.1 d	130.1 d	130.2 d
12	19.6 q	19.8 q	19.5 q	19.7 q	3',5'	128.9 d	128.9 d	129.0 d	129.0 d
13	132.5 d	132.9 d	59.1 d	59.3 d	4'	127.1 d	126.1 d	127.1 d	127.1 d
14	131.6 d	131.1 d	61.8 d	61.5 d	$\mathit{C}H_3CO$	20.4 q	--	20.5 q	19.3 q
15	37.9 t	38.1 t	38.8 t	38.9 t	$CH_3\mathit{C}O$	170.8 s	--	170.6 s	170.5 s

[a] In d_5-pyridine at 100.57 MHz; multiplicity from DEPT experiment.

[b] Assignment based on ^1H-^{13}COSY (HETCOR) data.

[c] Assignment supported by HMBC data.

[d] Assignment by comparison with **1** - **3**.

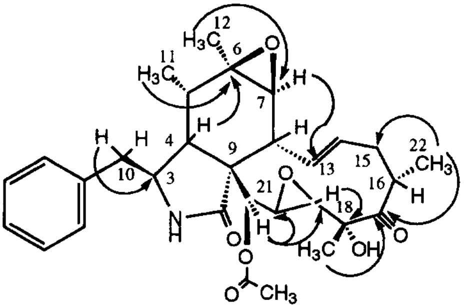

Fig. 1. Some Selected HMBC Correlations of **1**.

Epoxidation of **1** with excess m-CPBA afforded two products in the ratio of 10:1, and the major product was purified by Si gel TLC. Spectral data [MS, ^1H NMR (Table 1)] indicated it to be the triepoxide **4**. Examination of models suggests that the attack of a bulky reagent such as m-CPBA is possible mainly from the β-face of **1** yielding the 13,14-β-epoxide as the major product. Epoxidation of cytochalasin R (**3**) under identical conditions was found to be slower than that observed for **1** and afforded two triepoxides in the ratio of 1.5:1. The minor product was shown to be identical (TLC, ^1H NMR) with the major triepoxide **4** derived from **1** (see above). The major triepoxide derived from **3** must therefore be diastereomer **5**. Examination of models indicates that attack on the 19(20) double bond of **3** by the bulky m-CPBA is more likely to occur from the α-face suggesting that the minor product is the 19,20-β-epoxide (**4**), which is in conformity with the β-epoxide structure **1** proposed for the major natural cytochalasin.

The minor cytochalasin, $C_{28}H_{35}NO_6$, had a ^1H NMR spectrum (Table 1) almost superimposable with that of **1** except for the absence of the resonance due to the acetyl group in the former. The IR spectrum of the minor cytochalasin lacked the absorption due to ester carbonyl as compared with **1**. The foregoing data suggested that the minor cytochalasin is the deacetyl analog **2** of the major cytochalasin **1**. This was confirmed by its ^{13}C NMR spectral data (Table 2) and by acetylation (Ac_2O/pyridine), which afforded the monoacetyl derivative identical (TLC, ^1H NMR, IR and MS) with **1**.

Biosynthetically **1** may arise from cytochalasin Q (**6**) as a result of biological epoxidation, a process commonly encountered in the biogenesis of secondary metabolites. In a study to establish the ease and site of chemical epoxidation, Edwards *et al.* treated cytochalasin C (**7**) with m-CPBA and found the following order of reactivity; 5(6) > 19(20) > 13(14).[4] However, the natural occurrence of both 19,20-epoxides [e.g. **1** in *Xylaria obovata* (present work)] and 13,14-epoxides [e.g. **3** in *Hypoxylon terricola*][4] suggests that enzymatically controlled biological epoxidation can occur at either of the two available sites in the macrocyclic ring of cytochalasin C.

Cytochalasins **1** and **2** were lethal to brine shrimp (LC_{50} 2.5 μg/ml) and cytotoxic to HL-60 cells at 1μg/ml. They also inhibited mammalian cell growth with high potency as demonstrated in the Vero monkey cell growth inhibition (XTT) assay for cytotoxicity[10] (IC_{50} for **1**, 0.46 μg/ml; for **2**, 1.9 μg/ml). However, both cytochalasins were inactive in an HIV-protease inhibitory assay[11] and in our mechanism-based yeast anticancer bioassay.[12]

EXPERIMENTAL

General Experimental Procedures. These are identical to those reported in ref. 12; IR spectra on a Perkin-Elmer 727B spectrophotometer; visualization on TLC was by spraying with 50% ethanolic H_2SO_4, heating at 100-110° and viewing under UV light at 366 nm.

Collection and Production of Xylaria obovata. Fruit bodies of the fungus *Xylaria obovata* were collected from the Munsea forest in Arsi Province, Ethiopia. A voucher specimen of the fruit body is deposited under the cipher ADA 228 at the National Herbarium, Addis Ababa University. To each of three 5 l Erlenmeyer flasks was added 500 g of rice and 330 ml of distilled water. The rice was allowed to soak for 3 hr and then sterilized at 121° for 30 min. 150 ml of *X. obovata* inoculum, grown for 5 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. The flasks were shaken intermittently by hand for the first 7 days to prevent clumping. When all the seeds were invaded and the mycelial mat had turned black, 18 days after inoculation, the mouldy rice was ground with a mortar and pestle.

Extraction and Purification of Cytochalasins 1 and 2 from X. obovata. The ground moldy rice was soaked overnight in a total volume of 2 litres of MeOH-water (55:45). It was then filtered and the filtrate extracted with $CHCl_3$. Removal of the solvent yielded 4.8 g of crude extract. The crude extract (4 g) was partitioned between 80% aq. MeOH and hexane and the aqueous portion extracted with $CHCl_3$. Removal of $CHCl_3$ yielded 2.8 g of extract, which was applied to a Si gel column and eluted with a hexane-acetone gradient system. Ten fractions, each 300 ml, were collected, and fractions 4-5 (250 mg) after further purification by preparative TLC, yielded cytochalasins **1** (500 mg) and **2** (70 mg).

19,20-Epoxycytochalasin Q (1). Mp 266-268°; $[\alpha]_D$ -73° (c = 1.1, $CHCl_3$); IR (KBr): 1220, 1370, 1450, 1690, 1740, 2970, 3440 cm^{-1}; HRMS, *m/z* (rel. int. %): 523.2648 $[M]^+$(8) (calc. for $C_{30}H_{37}NO_7$: 523.2569), 495 $[M - CO]^+$ (17), 480 $[M - CH_3CO]^+$ (62), 464 $[M - OAc]^+$ (90), 432 $[M - C_7H_7]^+$ (37), 338 $[M - HOAc - C_7H_7]^+$ (77), 91 $[C_7H_7]^+$ (100); R_f (TLC): 0.68 (hexane-*i*-PrOH; 8:2); ^1H NMR (400 MHz, $CDCl_3$); δ 0.8 (3H, d, *J* = 7.2 Hz, Me-11), 1.18 (3H, s, Me-12), 1.19 (3H, d, *J* = 6.4 Hz, Me-22), 1.52 (3H, s, Me-23), 1.60 (1H, m, H-5), 2.10 (3H, s, CH_3CO), 2.10 (1H, m, H-15), 2.21 (1H, m, H-4), 2.32 (1H, dd, *J* = 10 and 5.6 Hz, H-8), 2.61 (1H, dd, *J* = 12 and 11.4 Hz, H-15), 2.71 (1H, d, *J* = 5.6 Hz, H-7), 2.84 (2H, d, *J* = 7.2 Hz, H-10), 3.15 (1H, br s, H-19), 3.22 (1H, m, H-16), 3.52 (1H, br d, *J* = 1.2 Hz, H-20), 3.60 (1H, br t, *J* = 6.8 Hz, H-3), 4.06 (1H, br s, OH, exchangeable with D_2O), 5.63 (2H, m, H-14 and H-21), 5.80 (1H, br s, NH), 6.08 (1H, dd, *J* = 15.2 and 10 Hz, H-13), 7.20 (5H, m, Ar-H); ^1H NMR (see Table 1); ^{13}C NMR (see Table 2).

Deacetyl-19,20-Epoxycytochalasin Q (2). Mp 119-121°; IR (KBr): 1370, 1450, 1685, 2970, 3400 cm^{-1}; HRMS, *m/z* (rel. int. %): 481.2464 $[M]^+(5)$ (calc. for $C_{28}H_{35}NO_6$: 481.2467), 390 $[M - 91]^+$ (100), 338 $[M - H_2O\text{-}C_7H_7]^+$ (96); R_f: (TLC) 0.74 (hexane-*i*-PrOH; 8:2); ^1H NMR (see Table 1); ^{13}C NMR (see Table 2).

Acetylation of 2. **2** (1.5 mg) in dry pyridine (0.2 ml) was treated with Ac_2O (0.15 ml) for 24 h at room temperature. Usual work-up afforded the acetyl derivative (1.2 mg) which was found to be identical (TLC, $[\alpha]_D$, ^1H NMR and MS) with the major cytochalasin **1**.

Epoxidation of 1. m-CPBA (50-60%; 100 mg) was added to a solution of **1** (20.0 mg) in CH_2Cl_2 (3 ml). After 24 h (TLC control) excess CH_2Cl_2 was added and the solution was washed with satd. aq. $NaHCO_3$, dried, and evaporated to yield the crude product (22.3 mg) which was purified by prep. TLC (Si gel; 20% i-PrOH in hexanes; triple elution) to obtain the triepoxide **4** (10.7 mg) and a minor product (1.0 mg). Data for **4**: ^1H NMR (see Table 1); ^{13}C NMR (see Table 2); HRFABMS, *m/z* 540.2605 $[M+H]^+$ (calc. for $C_{30}H_{38}NO_8$: 540.2597).

Epoxidation of Cytochalasin R (3). m-CPBA (50-60%; 20.0 mg) was added to a solution of **3** (4.0 mg)[4] in dichloromethane (1 ml). After 48 h (TLC control) it was worked up as above affording a crude product mixture (3.8 mg). Separation of this by prep TLC (reversed-phase; 20% H_2O in MeOH) gave the triepoxides **4** (1.1 mg) and **5** (1.7 mg). Data for **5**: ^1H-NMR (see Table 1); HRFABMS, *m/z* 540.2593 $[M+H]^+$ (calc. for $C_{30}H_{38}NO_8$: 540.2597).

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