A NEW IMMUNOSUPPRESSIVE CYTOCHALASIN ISOLATED FROM A *Pestalotia* sp.

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In the course of screening for immunomodulators we have discovered a new cytochalasin produced in the fermentation broth of a *Pestalotia* sp. AB 1942R-114. The cytochalasin mycotoxins inhibit glucose transport and a wide variety of fundamental prokaryotic and eukaryotic cellular processes mediated by actin microfilaments.[1] Compounds closely related to cytochalasin B bind to the glucose transporter and actin, while most other cytochalasins bind preferentially to actin sites.[2] Cytochalasins that bind monomeric actin uncouple intrinsic actin ATPase activity from polymerization and inhibit both the rate of polymerization and the interaction of actin filaments in solution, resulting in inhibition of cellular motility and changes in morphology.[1,3,4] In addition, cytochalasins interfere with immune function, with effects including inhibition of antigen-induced B-cell activation,[5] inhibition of macrophage-lymphocyte interactions during antigen recognition,[6] and suppression of the murine *in vivo* allogeneic antitumor response.[7] We report the discovery, isolation, and structural identification of a new 10-phenyl[11]cytochalasin with *in vitro* immunosuppressant activity.

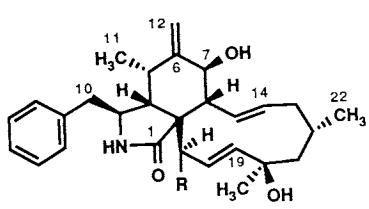
The known compounds, cytochalasin H, epoxycytochalasin H, cytochalasin J, and the new compound, cytochalasin U, were isolated from the fermentation broth of *Pestalotia* sp. AB 1942R-114. This fungus was isolated from a partridge pea plant (*Cassia fasciculata*) collected in Missouri, U.S.A. It has been previously reported that cytochalasins H and J and epoxycytochalasin H are produced by *Phomopsis paspalli* and *Phomopsis sojae*, respectively. Interestingly, both *Phomopsis* and *Pestalotia* are plant-associated imperfect fungi belonging to the class Coelomycetes.[8]

A 42-liter stirred fermenter was charged with 30 liters of a medium consisting of dextrin 2%, glucose monohydrate 1%, dried distiller's solubles (Brown Foreman) 0.5%, primary yeast (Universal Foods) 0.5% and $CaCO_3$ 0.2%. The pH was adjusted to 7.0 before sterilization at 121°C and 1.05 kg/cm^2 for 1 hour. Inoculum for the fermentation was prepared by seeding (0.5%) medium I of GOETZ *et al.*[9] with frozen (−80°C) vegetative growth from a previous seed culture. Incubation of the inoculum (600 ml medium in 2-liter Erlenmeyer flasks) was 72 hours at 28°C on a rotary shaker operated at 225 rpm. The resulting growth was used at 5% to inoculate the fermenter. During fermentation the temperature was controlled at 22°C. Agitation was 250 rpm, the air flow was 0.7 vol/vol/minute and head pressure was maintained at 0.35 kg/cm^2. Antifoam was a silicone defoamer, XFO 371 (Ivanhoe Industries), added initially at 0.01% and then available on demand. The fermentation was terminated after nine days. A 2-liter aliquot was used for the initial isolation work.

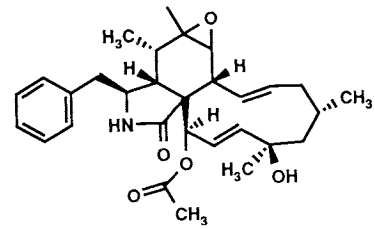
The whole broth (2 liters) was centrifuged and the supernatant adsorbed in batch onto SP-207 (obtained from Mitsubishi Kasei Corp.). The resin was washed in a fritted glass filter with water, 50% methanol-water, and methanol, successively. The active methanol eluate was concentrated to dryness under reduced pressure and the residue was triturated with ethyl acetate. The soluble material was purified chromatographically on a C-8 column (1 × 24 cm, Lobar obtained from E.M. Science) using a methanol-water gradient consisting of: 0~20 minutes, isocratic 50% methanol-water, 20~70 minutes, linear gradient 50 to 100% methanol. The flow rate was 5 ml/minute. Four active bands were collected at 31 (**1**), 39 (**2**), 44 (**4**), and 47 (**3**) minutes. Final purification of **4** was achieved by HPLC using 5% isopropanol-hexane over silica (0.39 × 30 cm, Waters μBondapak).

Compound **2** afforded crystals and by X-ray crystallography was identified as cytochalasin H.[10] Two others were identified using spectroscopic methods as cytochalasins J (**1**) and epoxycytochalasin H (**3**).[11]

The mass spectrum (DCI-NH_3) of **4** showed an $(M+H)^+$ at *m/z* 494 and by high resolution peak match was determined to be 494.2924. This corresponded to the formula $C_{30}H_{40}NO_5$ (calcd 494.2906) and indicated that **4** is isomeric with cytochalasin H. Comparison of their ^1H and ^{13}C NMR data (DEPT, COSY, HMBC and HMQC) confirms their structural similarity except at positions 6, 7 and 12. In **4**, the ^{13}C vinyl signal at



1 R=OH Cytochalasin J
2 R=OAc Cytochalasin H

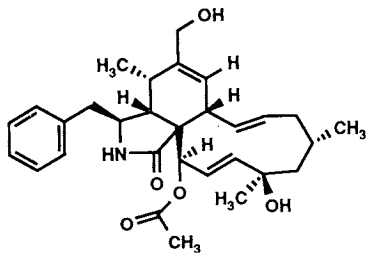


3 Epoxycytochalasin H

Table 1. Comparative ^{13}C NMR assignments (ppm).

Position	DEPT	Cytochalasin H[a]	Cytochalasin U[b]
6	C	152.06	143[c]
7	CH	73.55	130.14
12	CH_2	114.36	63.16

[a] In deuterated methanol.
[b] In deuterated methanol-deuterated acetone (1:1).
[c] Chemical shift assigned from 2D HMBC map.



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130.14 ppm was that of a methine; its proton coupling partners (8 and 12) are indicative of its position at 7. The coupling patterns, in the heteronuclear and homonuclear correlation experiments, of the methylene signal at 63.16 ppm in the ^{13}C and 4.03 ppm in the ^{1}H spectra were indicative of its position at 12. Chemical shifts are well suited for a methylene disubstituted with an olefin and hydroxyl. Though the ^{13}C chemical shift of 6 was ~9 ppm upfield relative to cytochalasin H, due to changing from an exocyclic (152.06 ppm) to an endocyclic olefin (143 ppm), its coupling pattern in the HMQC experiment identified it as such. The structure of **4**, which we named cytochalasin U, is shown above.

The *in vitro* immunosuppressive activity of cytochalasin U was compared to four known cytochalasins. The ability of compounds to inhibit [^{3}H]thymidine uptake in mixed BALB/c and C57BL/6 spleen cell suspensions was determined as previously described.[12] As shown in Table 2, cytochalasins B (Sigma, St. Louis, U.S.A.), J. H, epoxy-H, and U inhibited the murine mixed lymphocyte reaction (MLR) with IC_{50} concentrations of less than 10 μg/ml. The rank-order potency for inhibition of the murine MLR by cytochalasins H, J, and B correlates with that previously reported for inhibition of actin filament elongation and decreases in actin filament viscosity, implicating actin involvement in immunosuppressive activity.[13] In addition, cytochalasin H which differs from cytochalasin U and the other compounds by the presence of a hydroxyl group at C-7 was approximately 25-fold more potent in inhibiting the MLR. The presence of an unsubstituted 7-hydroxyl has been previously shown to be important for binding of cytochalasins to actin, for inhibition of actin gelation, and inhibition of the generation of cytolytic T lymphocytes.[2,14]

Table 2. *In vitro* immunosuppressive activity of cytochalasins.

Compound	MLR IC_{50} (μg/ml)
Cytochalasin U	5.2±2.4[a]
Cytochalasin B	8.3±2.8
Cytochalasin H	0.2±0.1
Epoxycytochalasin H	4.0±1.0
Cytochalasin J	3.7±0.2

[a] IC_{50} for inhibition of [^{3}H]thymidine uptake in mixed murine splenocyte cultures (mean±S.D., n=3 determinations).

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