Dibefurin, a Novel Fungal Metabolite Inhibiting Calcineurin Phosphatase Activity

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(Received for publication September 4, 1995)

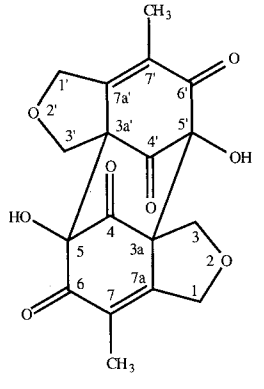
The novel calcineurin inhibitor, dibefurin, has been isolated from the fungal culture AB 1650I-759. The isolation was bioactivity-directed fractionation using an assay which measures the phosphatase activity of calcineurin. The compound was purified by countercurrent, reverse phase and gel filtration chromatographies. Several studies, including crystallographic, NMR and MS, revealed that dibefurin is a novel dimeric compound of a unique structural type.

In the course of screening microorganisms for the production of bioactive metabolites, a fungal culture (AB 1650I-759) was discovered which was found to produce calcineurin inhibition activity. Calcineurin phosphatase is a calcium/calmodulin-regulated serine/threonine phosphatase, which plays a key role in T cell activation. It has been shown that calcineurin dephosphorylates the cytosolic component of the NFAT complex, and thereby regulates nuclear translocation and dimerization.[1,2] This process is necessary for the production of interleukin-2 and other T-lymphocyte derived cytokines. The common target of the immunophilin complexes of both cyclosporin A and tacrolimus is thought to be calcineurin.[3] Compounds found in a screen for direct calcineurin inhibitors would be expected to have immunosuppressant activity independent of the requirement for cyclophilin or FKBP. Direct calcineurin inhibitors may be useful in distinguishing the immunosuppressive activity of such agents from any complications which may arise from inhibition of the peptidyl prolyl isomerase activity of the immunophilins.

Using an assay for the phosphatase activity of calcineurin, bioactivity-directed fractionation of an extract of a stationary fermentation of AB 1650I-759 provided a novel microbial metabolite of a unique dimeric structural type (**1**), dibefurin. Dibefurin appears to be a dimerization derivative of 4,5,6-trihydroxy-7-methyl-(1*H*,3*H*)-dihydroisobenzofuran. Both the monomer and the dimer are novel. The monomer from which dibefurin is derived, could itself be a derivative of flavipin (**2**), a fungistatic agent produced by *Aspergillus flavipes*.[4] Phenolic substances are commonly encountered as mold metabolites.[5] The structure of dibefurin was elucidated mainly by NMR, MS and crystallographic studies. The dibefurin producing strain characterization, fermentation, isolation, structural determination and some biological data are outlined in this paper.

Characterization of the Producing Strain

The appearance of colonies of strain AB 1650I-759 was quite different when the culture was grown under continuous fluorescent light than when grown in the dark. Although the colonies were usually quite irregular in the dark, the culture formed regular, round colonies when incubated in the light. Colonies grew faster in the light. Also, the mycelium had a sectored appearance in the dark, and different pigments were produced under each incubation condition. Strain AB 1650I-759 formed a soluble brown pigment on potato dextrose agar (PDA, Difco) in the dark but not in the light. No reproductive structures were ever observed under any growth con-



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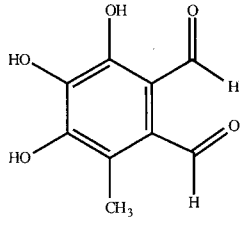


(2)

dition. It appears that strain AB 1650I-759 is the sterile mycelium of an unidentified mushroom.

The radial growth of strain AB 1650I-759 when grown under continuous fluorescent light on BLAKESLEE's malt extract agar (Special Yeast and Mold Medium, Difco) was 42~45 mm. Colonies on this medium were pale pink (7) and had a downy to woolly texture. The reverse of the colonies was deep yellowish brown (75) in the center and deep red orange (38) away from the center. On PDA, the colonies measured 54~58 mm in diameter. The center was raised, and the center and outer margin had a felty texture. The rest of the colony was floccose with clear strong orange yellow (68) exudate. The colonies were zonate. The center and edge were yellowish white (92). The rest of the colony was colored deep orange yellow (69). The reverse was dark reddish orange (38) to strong reddish brown (40) with a light orange yellow (70) edge. No soluble pigment was produced on either of these media.

In the dark, growth was irregular on BLAKESLEE's malt extract agar, and the colonies measured 24~34 mm in diameter at different points. The colonies had a cottony texture. They were colored purplish white (232) with a strong orange yellow (68) center. The reverse was dark grayish brown (62) in the center, and the rest of the colony was mottled moderate brown (58) and grayish yellow (90). On PDA in the dark, the colonies were also irregular with radial growth measuring 23~33 mm in diameter. The mycelium was very dense with a woolly texture and cottony overgrowth. The colonies had deep radial furrows. The colonies were colored grayish yellow (90) to pinkish white and often appeared sectored. The reverse was dark grayish brown (62), and the culture produced a light brown soluble pigment (57).

Microscopically, the hyphae grown in the light at 25°C for seven days were 1~3 μm in diameter when mounted in lactophenol. They were branched and septate. No clamp connections were observed. No reproductive structures were ever formed on a variety of media either in the light or dark.

Fermentation

Dibefurin was produced by solid state fermentation in a modification of a procedure described previously.[6] Fungal strain AB 1650I-759 was maintained as frozen mycelium at −70°C and used at 1% to inoculate 500-ml Erlenmeyer seed flasks containing 100 ml of a tomato paste-oat flour medium described by GOETZ *et al.*[7] The seed flasks were incubated on a rotary shaker at 225 rpm at 28°C for seventy-two hours. Four 20-liter glass carboys were used as the fermentation vessels. Spoon Size Shredded Wheat, which had been defatted with ethyl acetate, was dispensed at 600 g per carboy, and the carboys were sterilized for forty-five minutes at 121°C. The fermentation medium consisting of maltose 1.6%, ammonium tartrate 0.27%, KH_2PO_4 0.08%, $MgSO_4 \cdot 7H_2O$ 0.04%, NaCl 0.008%, $CaCl_2$ 0.008%, $FeSO_4 \cdot 7H_2O$ 8 ppm, $ZnSO_4 \cdot 7H_2O$ 7 ppm, $CuSO_4 \cdot 5H_2O$ 0.3 ppm, $MnSO_4$ 0.05 ppm, H_3BO_3 0.05 ppm and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.03 ppm was prepared in distilled water. The medium was dispensed at 720 ml in 2-liter Erlenmeyer flasks and sterilized for forty-five minutes at 121°C. For inoculation of each carboy, 120 ml of the 72-hour seed growth was mixed with 720 ml of fermentation medium. The inoculated medium was transferred to the Shredded Wheat in the carboy. Following mixing to distribute the inoculum, the carboys were incubated at 20°C for twenty-one days. At harvest, 600 ml acetone was poured onto the fungal growth in each carboy. After six hours, 750 ml of an ethyl acetate-toluene mixture (1:1) was added to each vessel. The carboys were held at 4°C overnight.

Isolation and Bioactivity

After the culture growth and media substrate in each carboy had been soaked in 750 ml of an ethyl acetate-toluene mixture at 4°C overnight, the carboy contents were soaked and extracted with an additional 16 liters (total) of acetone and then 16 liters (total) of methanol-acetone (1:1). The extracts were combined and concentrated on a circulating flash evaporator until 1.5 liters of an aqueous residue remained. The residue was then extracted successively with three equal volumes of methylene chloride and three equal volumes of ethyl acetate. The active combined ethyl acetate extracts were concentrated under vacuum and the oily residue was subjected to preparative droplet countercurrent chromatography in the solvent system H_2O-MeOH-$CHCl_3$ (2:4:5), with the lower phase stationary. The active fractions, which eluted early in the run, were combined (2.84 g) and subjected to C_{18} flash chromatography (Bondesil 40 μm, Analytichem International, 100 g of packing). The active component was eluted in the 20% methanol/water fraction, which was concentrated under vacuum to yield 850 mg. This material was chromatographed on a 2.5×85 cm Toyopearl HW-40S column (Tosohaas) in methanol. From the active fractions, 57~62 (10 ml fractions), 45.9 mg of a crystalline material was formed. The compound, which was identified as the novel metabolite dibefurin, was found to have an IC_{50}

concentration of 16 μg/ml in the calcineurin assay (Fig. 1). Dibefurin inhibited the mixed lymphocyte reaction assay with an IC_{50} concentration of 2.5 μg/ml and lymphocyte toxicity assay with an IC_{50} concentration of 33.0 μg/ml.

Characterization and Structure Determination

Purified dibefurin is moderately soluble in DMSO, but only sparingly soluble in any other organic solvent or water. It has UV absorption maxima at 223 nm ($\varepsilon=7600$) and 270 nm ($\varepsilon=3900$) in methanol. Significant IR bands are seen at $\nu_{max}=1755$, 1670 and 1648 cm^{-1} (Fig. 2). ESI-MS yielded an $(M+H)^+$ m/z parent peak of 361, an $(M+NH_4)^+$ signal at 378 and an $(M+Na)^+$ signal at 383. High resolution CI-MS gave an exact measured mass of 361.0924 (calc. 361.0923, $C_{18}H_{17}O_8$), indicating a molecular formula of $C_{18}H_{16}O_8$.

Since the ^{13}C NMR indicates only nine carbons and the ^1H NMR only eight protons, in view of the mass spectroscopic data, one can assume that dibefurin is a symmetric dimer. The proton NMR in DMSO-d_6 consists of two pairs of methylene doublets and two singlets (Table 1). In a long range COSY and a ROESY experiment the C-1 methylene protons show cross-correlation peaks to the C-7 methyl and the C-3 methylene protons. HMQC/HMBC experiments indicate the fusion position of this dihydrofuran moiety on the six-membered ring with important three bond couplings seen between the C-4 carbonyl and the C-3 methylene protons, and between the C-7 olefinic carbon and the C-1

Fig. 1. Inhibition of phosphatase activity of calcineurin by dibefurin.

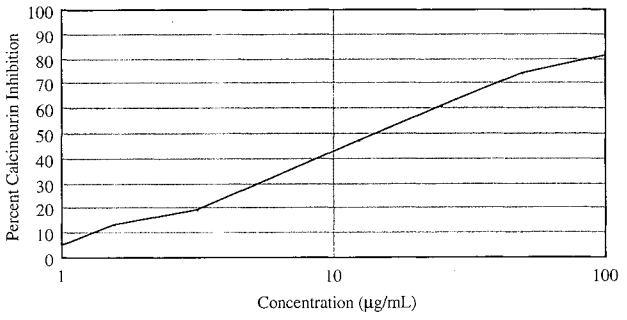


Table 1. ^{13}C and ^1H NMR data for dibefurin in DMSO-d_6.

Number	^{13}C NMR Data[a]	^1H NMR Data[b]
1	69.8, CH_2	4.58 d, 4.63 d, $J=15$ Hz
3	64.3, CH_2	4.24 d, 4.36 d, $J=9.3$ Hz
3a	65.9 Q	—
4	195.8 Q	—
5	88.6 Q	—
5-OH	—	7.16 s
6	192.2 Q	—
7	128.0 Q	—
7a	157.9 Q	—
7-CH_3	12.3, CH_3	1.70 s

[a] 75 MHz, including multiplicity assignment on the basis of distortionless enhancement by polarization transfer (DEPT) summary.
[b] 500 MHz.

Fig. 2. The FTIR spectrum of dibefurin in a KBr pellet.

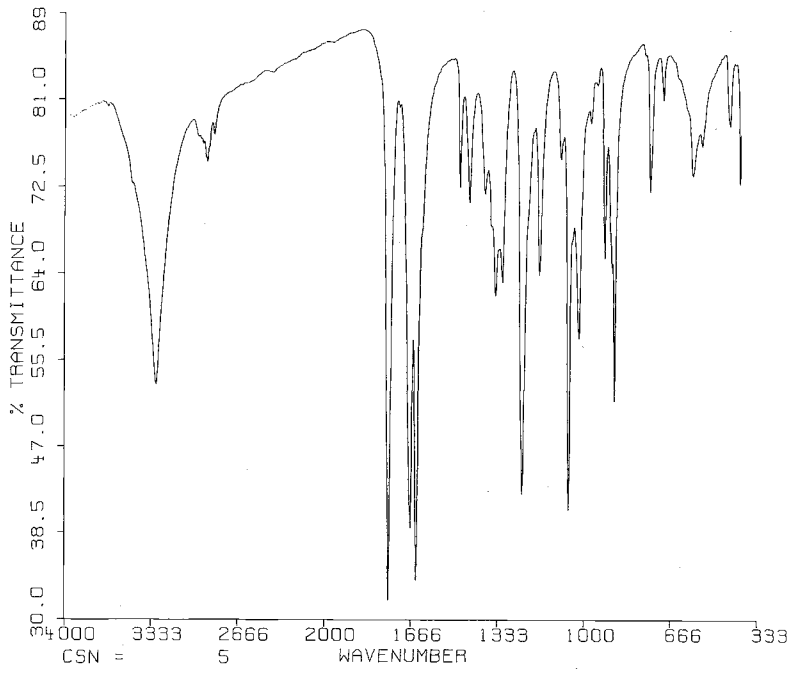


Fig. 3. Summary of HMBC and ROESY correlations for dibefurin.

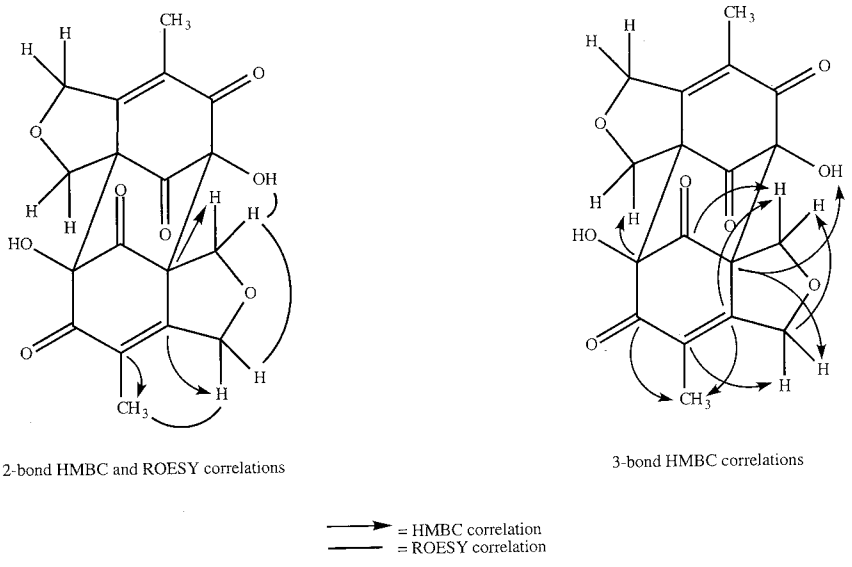
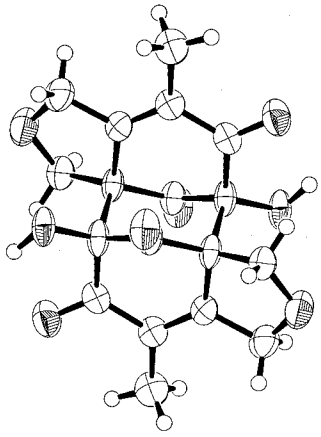


Fig. 4. The crystallographic ORTEP drawing of dibefurin.



methylene protons (Fig. 3). Chemical shift considerations and the HMBC experiment establish the six membered ring portion of the molecule, noting the coupling between the C-6 carbonyl and the C-7 methyl protons. Three bond couplings seen between the C-5 carbon and the C-3′ methylene protons, as well as between the C-3a carbon and the 5′-OH hydroxyl proton, illustrate the dimeric nature of this compound.

The structure of dibefurin was confirmed and the relative stereochemistry determined by a single crystal X-ray experiment. Dibefurin was crystallized from methanol. The crystallographic data are summarized as follows: Triclinic space group P-1, a=7.460(2) Å, b=7.504(2) Å, c=7.081(2) Å, β=109.99(2)°, V=372.3(2) Å^3, Z=1, Dcal=1.303 g/cm^3. The structure was solved with SHELX86. The structure was refined by the full matrix least-squares method. The final full-matrix least-squares, with anisotropic temperature factors for all non-hydrogen atoms, converged with an r factor of 0.093 (Rw-0.104, S=9.13). Fig. 4 is an ORTEP drawing of dibefurin with thermal ellipsoids scaled at the 50% probability level for non-hydrogen atoms.

Materials and Methods

The Producing Strain

The producing fungus, strain AB 1650I-759, was obtained by aseptically excising and culturing a small portion of the internal mycelium of an unidentified mushroom which was collected in Lindenhurst, Illinois, U.S.A. Strain AB 1650I-759 was grown for characterization at 25°C both in the dark and under continuous fluorescent light for seven days. The culture was deposited at the National Center for Agricultural Utilization Research, United States Department of Agriculture, 1815 North University Street, Peoria, Illinois, 61604 U.S.A. The accession code at this depository is NRRL 25063. The colors and numbers in parenthesis in the culture description follow the ISCC-NBS Centroid Color Charts.

Assays

The assay for phosphatase activity of calcineurin has been previously described.[8] Bovine brain calcineurin and calmodulin (Sigma) were used.

The two-way mixed lymphocyte reactions were performed using splenocytes from Balb/c and C57BL/6 mice as previously described.[9] The lymphocyte toxicity is measured using formazan dye as previously described.[9]

Instrumentation

The droplet countercurrent device used in the isolation of dibefurin consists of 100 spiral Teflon loops with an approximate total volume of 800 ml. Approximately 90% of this volume is retained as stationary phase during use, when a mobile phase flow rate of 1~1.5 ml/minute is maintained.

NMR spectra were acquired employing a Varian Unity 300 or 500 spectrometer. Mass spectra were recorded on a Finnigan-MAT-TSQ700 or VG70-SEQ spectrometer. UV spectra were recorded on a Hitachi U-2000 spectrophotometer, and IR spectra on a Nicolet 5SXC FT-IR instrument using a KBr pellet. Crystallographic data collection was performed on a Rigaku AFC5R difractometer.

Acknowledgments

The authors express appreciation to DAVID N. WHITTERN for NMR studies and RICHARD F. ANDERSON for IR analysis.

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