CORNEXISTIN: A NEW FUNGAL METABOLITE WITH HERBICIDAL ACTIVITY

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(Received for publication March 8, 1991)

Cornexistin†, a new compound demonstrating promising herbicidal activity, was purified from the culture filtrate of a newly-isolated fungus identified as *Paecilomyces variotii* SANK 21086. The compound was extracted with organic solvents from the culture filtrate, purified using column chromatography on Sephadex LH-20 and finally crystallized from methylene chloride.

Following analysis of its physico-chemical properties it was identified to be a new compound belonging to the nonadride group. Chemical structure elucidation was conducted by analyses of various spectral data and the structure was finally confirmed by means of X-ray crystallographic analysis. Based on its herbicidal characteristics cornexistin may be classified as a postemergence herbicide active against certain young annual and perennial monocotyledonous and dicotyledonous plants with selective protection for corn.

In the course of new compounds screening of microbial products for herbicidal efficacy a new compound named cornexistin demonstrated promising activity as a postemergence herbicide with selectivity to corn. Cornexistin was purified from the culture filtrate of a fungus (*Paecilomyces variotii* SANK 21086) previously isolated from a sample of deer feces collected in Canada. This paper describes the identification of the producing organism, fermentation, purification, physico-chemical and biological properties of this unique compound.

Materials and Methods

Identification of Producing Organism

Strain SANK 21086 was freshly isolated from a deer dung sample collected a Nojak, Alberta, Canada in 1982.

From the characters described below, the fungus was identified as *P. variotii* SANK 21086[1,2]. This strain was deposited as FERM BP-1351.

† Jpn. Kokai 38085 ('89), Feb. 8, 1989.

Paecilomyces variotii Bainier

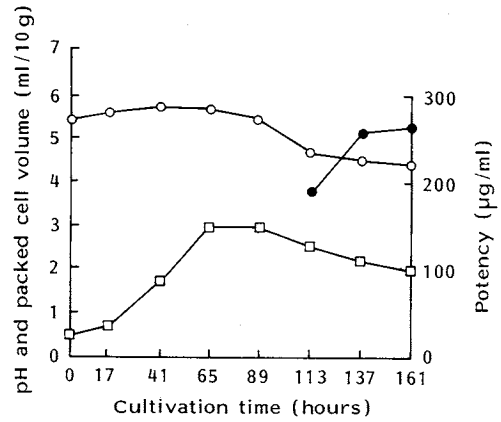
Colonies on malt extract agar medium grow rapidly, up to 35~43 mm i.d. for 7 days at 24°C; consisting of a dense felt of conidiophores, giving a powdery appearance. Straw yellow (3C4)[3] in color. Reverse of colonies are grayish orange (5B3).

Colonies on CZAPEK agar medium grow well, up to 18~22 mm i.d. for 7 days at 24°C and are similar in appearance to those on malt extract agar medium. At 37°C, growth is very poor, but conidiogenesis is observed. Vegetative mycelia are nearly hyaline and smooth walled, 2.0~5.0 μm i.d.

Conidiophores are formed both from aerial and vegetative mycelia, 20~120 × 2.5~4.0 μm in size, consisting of dense whorls of verticillately or irregularly arranged branches, smooth walled or slightly roughened. Phialides in whorls or solitary, variable in size, nearly smoothed 10~40 × 2.5~4.0 μm, tapering below into a long cylindrical neck. Conidia are hyaline to light brown and yellow-brown in mass, smooth-walled, variable in shape but mostly subglobose to ovoid, 3.0~5.5 × 2.0~4.0 μm in size.

Fig. 1. Fermentation of cornexistin.

○ pH, ● potency, □ packed cell volume.



Fermentation

One loopful of growth of *P. variotii* SANK 21086 from an agar slant was inoculated into a baffled 500-ml Erlenmeyer flask containing 100 ml of a medium comprised of glycerol 5%, potato 5%, yeast extract 0.5%, malt extract 0.5% (pH 6.0, before sterilization) and incubated as the first seed culture on a rotary shaker at 200 rpm for 5 days at 26°C. Twenty-five ml of the seed culture were transferred to a baffled 2-liter Erlenmeyer flask containing 500 ml of the medium described above and incubated for an additional 3 days at 26°C. Four 600-liter fermenters each containing 300 liters of the medium were inoculated from the 1.5 liters of seed culture and were agitated at 108 rpm, and at an air-flow rate of 300 liters/minute for 161 hours. The maximum potency in the culture broth reached about 260 μg/ml at the end of fermentation (at 161 hours) as shown in Fig. 1.

Fermentation process was monitored by mycelial growth measurement, expressed by packed cell volume specified by a solid volume in 10 g of the culture broth after centrifugation at 3,000 rpm, for 15 minutes or by HPLC analysis of cornexistin itself.

Quantitative Analysis of Cornexistin

The amount of cornexistin in the culture broth in the course of fermentation was determined by HPLC analysis under the following conditions. In preparing the specimen for HPLC analysis from culture broth the active principle was extracted from the culture filtrate with the same volume of ethyl acetate at pH 2.5 and an appropriate volume of the organic layer was concentrated to dryness. After dissolving in a constant volume of acetonitrile it was subjected to HPLC analysis. Its average Rt is 6.7 minutes. Column: Senshu Pak ODS H-2151B, solvent: 41% aqueous acetonitrile, flow rate: 1.5 ml/minute and UV: 220 nm.

Isolation Procedures

Combined culture broth from three 600-liter fermenters was filtered with a total of 25 kg of Celite 545. The culture filtrate (920 liters) obtained was extracted with the same volume of ethyl acetate three times at pH 8.0 to remove neutral or basic lipophylic impurities. The aqueous layer was then extracted once, with the same volume of ethyl acetate and an additional two times with a half the volume of the organic solvent at pH 2.5. After washing the organic layer with 600 liters of NaCl saturated aqueous solution it was concentrated *in vacuo* to dryness to produce an oily substance. This substance was then dissolved in 20 liters of methylene chloride and the active principle re-extracted three times using 50 liters of 0.1 M Na_2HPO_4 solution in each extraction. The combined aqueous extract, approximately 150 liters

of aqueous solution, was then re-extracted with 50 liters of CH_2Cl_2 two times after adjustment of pH to 2.1. One-hundred liters of the methylene chloride extract were diluted with 50 liters of methylene chloride and washed twice with 50 liters of Na_2HPO_4 solution followed by washing with 100 liters of NaCl saturated water. The organic layer was concentrated to 40 liters *in vacuo* and then concentrated to dryness after dehydration over anhydrous Na_2SO_4.

The resulting oily substance was dissolved in a minimum amount of a 1 : 1 mixture of CH_2Cl_2 - EtOAc and applied on 2.5 liters of a Sephadex LH-20 column packed and equilibrated with the same solvent mixture, and then developed and eluted using the identical solvent.

The active fractions were pooled and evaporated to dryness. The resulting solid was then dissolved in a small volume of CH_2Cl_2 and was crystallized to from 115 g of needles of cornexistin.

Results and Discussion

Physico-chemical Properties

In its purified form cornexistin exists as a colorless, neutral lipophylic, crystalline substance, soluble in alkaline aqueous solution, methanol, acetone, ethyl acetate, methylene chloride and chloroform, but insoluble in *n*-hexane. In an aqueous solution (culture broth) cornexistin behaves as an acidic substance as with two p*K*a values of 4.10 and 5.95 and demonstrates positive color reactions on a silica gel TLC plate (Kieselgel 60 F_{254}) with surfuric acid and potassium permanganate.

On the basis of various spectral analyses such as HR- of FAB-MS as well as ^{13}C NMR spectroscopy the MW and molecular formula for cornexistin were derermined to be 308 (FAB-MS $(M+H)^+$ 309) and $C_{16}H_{20}O_6$, respectively. Characteristic UV absorption maxima are shown at 238 and 280 (sh) nm. Physico-chemical characteristics of cornexistin are summarized in Table 1.

The IR and ^1H and ^{13}C NMR spectra are also shown in Figs. 2, 3, and 4.

The IR absorption band appeared at 1820 to 1760 cm^{-1} suggesting the existence of an acid anhydride moiety in its structure. Based on the analysis of ^1H NMR spectrum, the presence of a propyl group due to a triplet methyl at 0.95 ppm in conjunction with two multiplet methylenes at 1.3 and 2.0 ppm and also, the existence of an ethylidene group due to a doublet methyl at 1.7 ppm, and quartet olefinic methine at 5.3 ppm were easily deduced. The ^{13}C NMR spectrum revealed the presence of all 16 carbon atoms including two methyl carbons at 13 ppm, 4 methylene carbons at around 22~45 ppm, a methine carbon at 42 ppm, two methine carbons adjacent to an oxygen baering carbon at around 70~80 ppm, 4 olefinic carbons at around 130~150 ppm, 2 carbonyl carbons due to two acid anhydride structures at 166 ppm and a ketone carbon at 212 ppm.

From these spectral analyses, the structure of cornexistin was determined to be related to the so-called nonadride group such as rubratoxin B; however, it was clearly differentiated from rubratoxin B as well as other members of this group and finally confirmed by X-ray crystallographic analysis.

The crystals are orthorhombic, space group $P2_12_12_1$, with $a=13.444(1)$, $b=15.287(1)$ $c=7.718(1)$ Å, $U=1586.2$ Å^3, $Z=4$, $D_{calc}=1.29$ g cm^{-3}. Intensity data were obtained on a Rigaku AFC-5R diffractometer with graphite monochromatized CuKα radiation using the $\theta-2\theta$ scan technique

Table 1. Physico-chemical properties of cornexistin.

MP	100~103°C
$[\alpha]_D^{25}$ (*c* 1.0, $CHCl_3$)	+168.3°
Molecular formula	$C_{16}H_{20}O_6$
MW	308
Solubility	Soluble in MeOH, acetone, $CHCl_3$ Insoluble in *n*-hexane
p*K*a	4.10, 5.95
TLC (Merck Art. No. 5715)	Rf 0.35 (*n*-hexane - acetone, 1 : 1)
Color reaction	Positive: H_2SO_4
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	238 (131), 280 (sh)
IR (KBr) cm^{-1}	1820, 1761, 1719, 1269, 1002, 922

Fig. 2. IR spectrum of cornexistin (KBr).

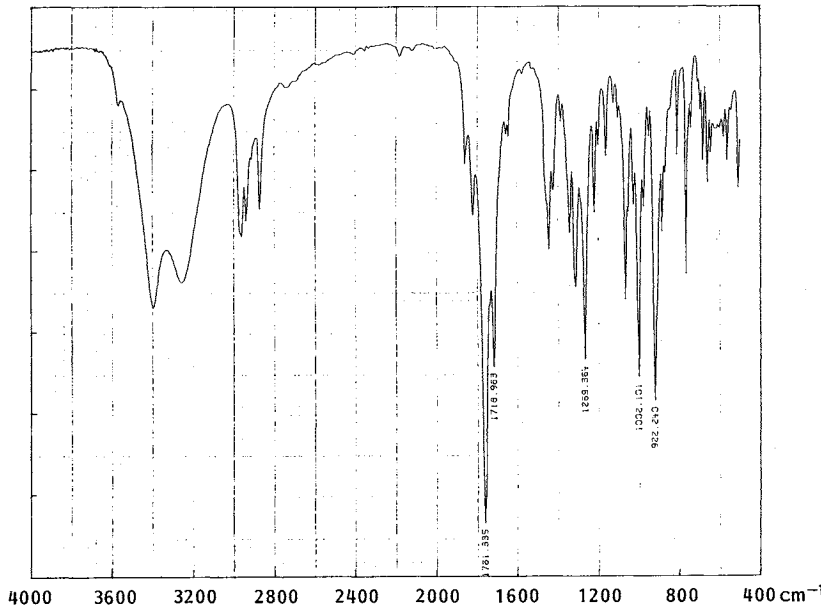
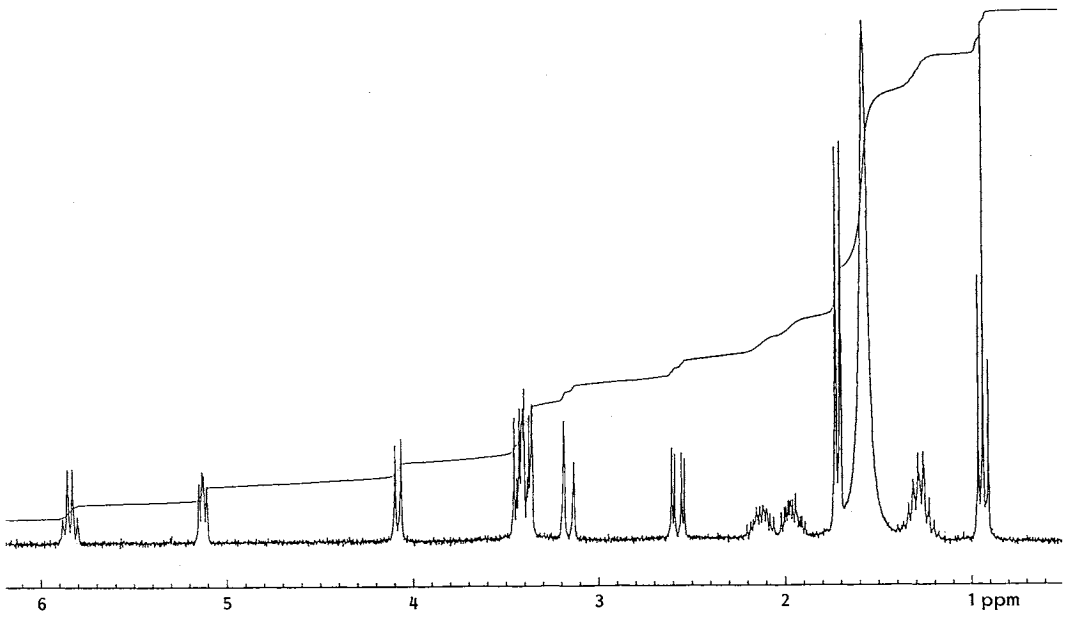


Fig. 3. ^{1}H NMR spectrum of cornexistin in CD_3OD (400 MHz).



($2\theta < 128°$). Structure was solved by direct methods using the MULTAN84[4] series of programs, with RANTAN being used to obtain the phases. Positions of the hydrogen atoms were estimated from standard geometry except for those of the two hydroxy groups which could not be located. The final refinements using blockdiagonal least-squares methods with anisotropic temperature factors for the non-hydrogen atoms and isotropic temperature factors for the hydrogen atoms lowered R value to 0.076, 1,387 observed

Fig. 4. ^{13}C NMR spectrum of cornexistin in CD_3OD (100 MHz).

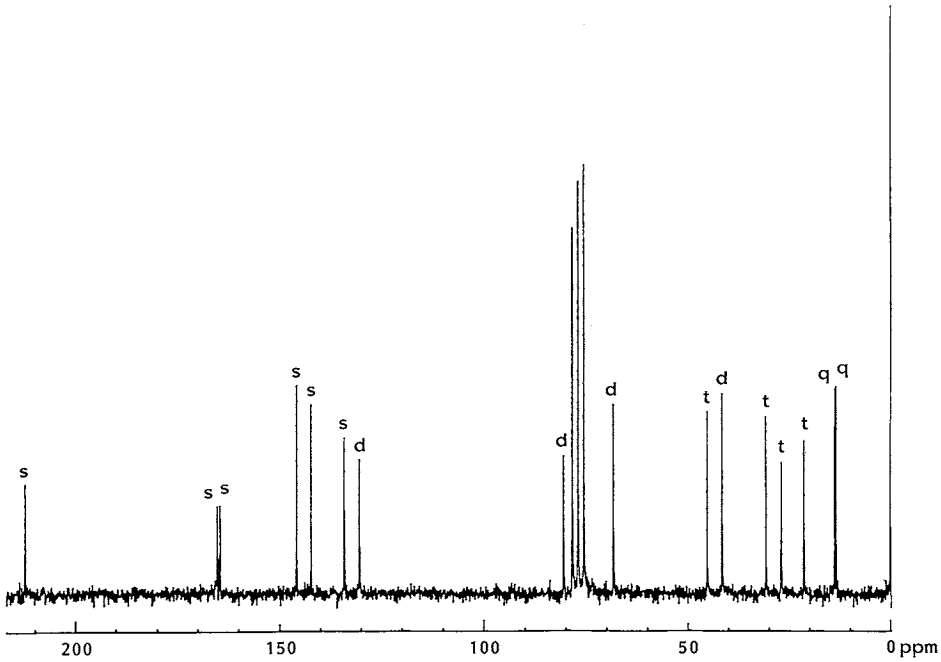
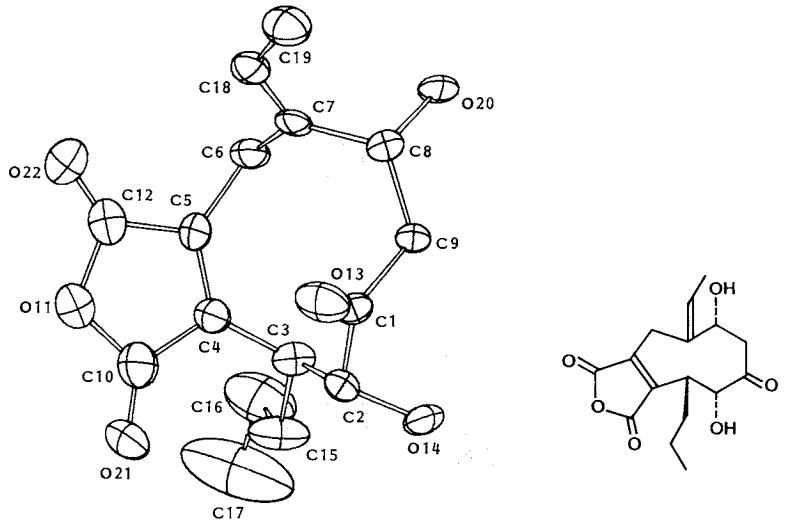


Fig. 5. X-Ray structure of cornexistin.



reflections with $Fo > 2\sigma$ (Fo). Final atomic parameters, tables of bond lengths and angles have been deposited with the Cambridge Crystallographic Data Centre.

Biological Properties

The herbicidal activity of cornexistin against some species of common annual weeds is shown in Table 2.

Table 2. Herbicidal activity of cornexistin against annual weeds.

Plant	Annual weed	1,000	500	100
		ppm		
Monocotyledonous	Barnyardgrass	5	5	2
	Crabgrass	5	5	4
	Foxtail green	5	5	5
	Johnson grass	5	5	5
Dicotyledonous	Night shade black	5	5	5
	Cocklebur	5	5	5
	Ragweed	5	5	5
	Morningglory, tall	5	5	4
	Velvetleaf	5	5	5

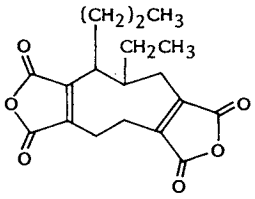
Rating scale of herbicidal activity: 5=95~100% (plant growth inhibition) 4=80~94%, 3=50~79%, 2=20~49%, 1=5~19%, 0=0~4%.

Photo. 1. Selective herbicidal activity of cornexistin.

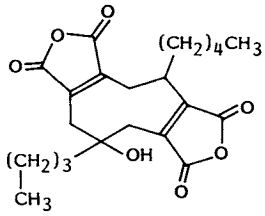
Seed of various annual mono- and di-cotyledonous plants were sown on the surface of soil in plastic pots (150 cm^2 in surface area, 7.5-cm in height). The resulting seedlings were grown in a greenhouse for 14 days and then sprayed with cornexistin at various concentrations to determine postemergence herbicidal efficacy. Fourteen days after treatment cornexistin showed a high herbicidal activity on many of the plants shown in Table 2; however, corn seedlings exhibited tolerance to the chemical. This herbicidal spectrum suggests that the chemical may be useful for postemergence weed control with selective protection of corn as shown in Photo. 1.

Photo. 1 also clearly shows the excellent control of annual weeds growing in association with corn

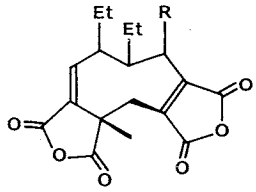
Fig. 6. Structure of nonadride group compounds.



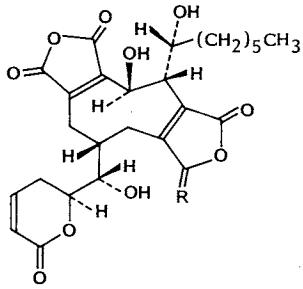
Heveadride



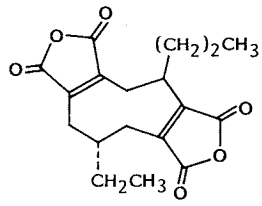
Scytalidin



Glauconic acid R=OH
Glaucanic acid R=H



Rubratoxin A R=H, OH
Rubratoxin B R=O



Byssochlamic acid

when cornexistin was sprayed over the seedlings at 0.5 kg/ha 9 days after planting.

Antimicrobial Activity

Cornexistin did not show any antimicrobial activity against about 30 species of various microorganisms at concentrations up to 1,000 μg/ml using the paper-disc agar diffusion assay method.

Acute Toxicity

Acute toxicity studies with cornexistin were conducted in mice and its LD_{50} value given orally was greater than 1 g/kg; when given intraperitoneally it was greater than 100 mg/kg.

The compounds related to cornexistin in the nonadride group such as rubratoxins A and B[5], heveadride[6], scytalidin[7], byssochlamic acid[8], glauconic acid[9] and glaucanic acid[9] are reported in the literatures. However, the difference in chemical structure between cornexistin and rubratoxins, which are clearly demonstrated to have either one or two molar of acid anhydride moiety, is shown in Fig. 6.

Of these fungal metabolites rubratoxin B is the only compound reported to demonstrate herbicidal activity; however, its activity is very weak compared to that of cornexistin. In comparative acute toxicity studies cornexistin showed lower toxicity to mice than rubratoxin B.

Based on its promising weed control efficacy and selectivity to corn, cornexistin is expected to be quite useful as a herbicide for corn fields and development studies are currently ongoing.

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