MYRIOCIN, A NEW ANTIFUNGAL ANTIBIOTIC FROM *MYRIOCOCCUM ALBOMYCES*

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A new crystalline antifungal compound, myriocin, has been isolated from culture filtrates and mycelium of the thermophilic ascomycete *Myriococcum albomyces*. Myriocin is strongly active *in vitro* against yeasts and dermatophytes. This paper deals with the production, isolation, and purification, as well as the physical, chemical and biological properties of myriocin and one of its derivatives, anhydromyriocin. The elucidation of its chemical structure is also reported. The compound appears to be too toxic for therapeutic purposes.

There are only few published reports on antimicrobial agents isolated from thermophilic fungi. Of a large number of thermophilic microorganisms screened for their antibiotic activity an ascomycete, identified as *Myriococcum albomyces*[1], was found to produce a principle mainly active against *Candida albicans in vitro*. This new antifungal compound was isolated from the fermentation broths of this microorganism grown in submerged culture, and called myriocin. The strain reported in this paper has been deposited in the culture collection of the Northern Utilization Research and Development Division, United States Department of Agriculture, Peoria, Ill., and assigned the number NRRL 3858.

Production of Myriocin

Myriococcum albomyces NRRL 3858 was maintained in sterile soil at room temperature or in lyophilized form. From these stock cultures suspensions were prepared with sterile water and used to inoculate slants containing a medium consisting of glucose, 2 %; Trypticase (BBL, Baltimore, Md.), 0.5 %; Bacto-Peptone (Difco Laboratories, Detroit, Mich.), 0.5 %; Bacto-Agar, 2 %; pH 5.7. The slants were incubated for seven days at 52°C (70 % relative humidity). Spore suspensions from slants served as inoculum for the vegetative medium, which had the following composition: "blackstrap" molasses, 0.8 %; Bacto-Malt extract, 1.5 %; Bacto-Yeast extract, 0.5 %; glycerol, 1 %; NaCl, 0.5 %; $MgSO_4 \cdot 7H_2O$, 0.05 %; $ZnSO_4 \cdot 7H_2O$, 0.01 %; sperm oil, 0.5 %; ingredients were dissolved in tap water. pH was adjusted to 6.5 before sterilization. Incubation was carried out for 30~40 hours at 47°C on a rotary shaker (240 rev./min., 2″-stroke). The vegetative growth was used to inoculate the production medium consisting of: "cerelose" (Corn Products Corp., New York), 1.0 %;

soluble starch, 1.0 %; Bacto-Yeast extract, 0.5 %; $NaNO_3$, 0.3 %; K_2HPO_4, 0.1 %; $MgSO_4 \cdot 7H_2O$, 0.025 %; KCl, 0.05 %; $FeSO_4 \cdot 7H_2O$, 0.002 %, lard oil, 1 %. pH was corrected to 6.0 before sterilization.

Fermentation was carried out in 250-liter fermenters (New Brunswick Co., New Jersey, Model F-250) which were seeded with 2 % of the vegetative culture. Incubation was run at 47°C for the first 24 hours to allow maximal growth of the organism, then at 40°C for 6 more days. Aeration rate for optimal antibiotic production was found between 0.3 to 0.5 vol/vol/min., while best agitation was achieved at 300 rev/min. During the last two days of fermentation it was found advantageous to control the pH between 7.0 and 7.2 by periodic additions of 30 % sulfuric acid.

Assay Procedure

Myriocin concentration was determined by the disc-plate agar diffusion method. A stock solution of 1,000 μg/ml was prepared in methanol. Working solutions were made by diluting the stock solution in distilled water. Paper discs, 12.7 mm diameter (Schleicher and Schuell) were dipped into each dilution, drained, and dried at room temperature. The discs were placed on SABOURAUD's dextrose agar inoculated with *Saccharomyces cerevisiae* AY F-190. The diameters of the zones of inhibition were measured to the nearest mm after a 24-hour incubation at 28°C, and plotted against concentrations to draw a standard curve. Unknown solution or broths were treated similarly and their concentrations were read by interpolation on the standard curve.

Isolation and Purification of Myriocin

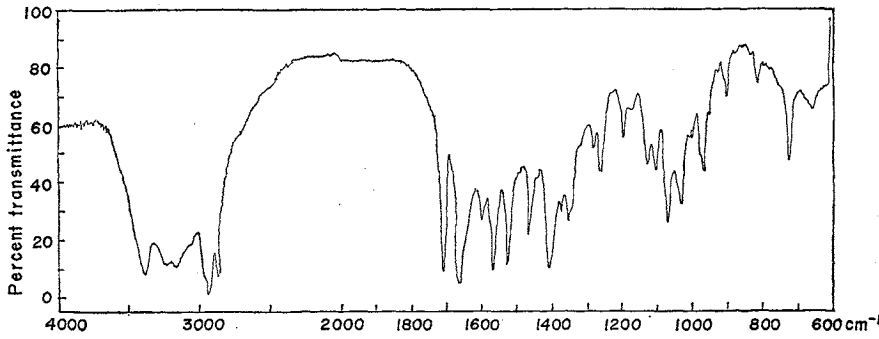
Two hundred and forty liters of fermentation broth were fed to a basket centrifuge and the mycelium recovered. The supernate was discarded, although it contained appreciable amounts of myriocin, since recovery required extractions with *n*-butanol and extensive purification by column chromatography followed by countercurrent distribution. It was more convenient to extract myriocin from the mycelium since it contained larger quantities than the supernate. The mycelium was extracted three times with five volumes of methanol per weight of wet mycelium. The methanol extracts were pooled and concentrated under vacuum to a small volume of an aqueous phase. The aqueous phase was extracted once with one volume of methylene chloride. Myriocin crystallized out at the interphase of the two solvents and was recovered by filtration. The crystals were washed once with hot distilled water (70°C), followed by two washings with acetone, and dried thoroughly under vacuum. Fifty eight grams of white crystalline material were obtained (m. p. 182~184°C), which amounts to a recovery of 85 % of the material present in the mycelium.

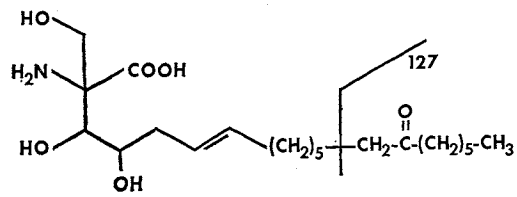
Physical and Chemical Properties of Myriocin

Myriocin (1) is slightly soluble in alcohols, but insoluble in water and all other common organic solvents. It is stable at room temperature, in solution as well as in solid state.

Myriocin analysed for $C_{21}H_{39}O_6N$ (M. W. 401): Calcd.: C 62.81, H 9.79, N 3.49; Found: C 62.84, H 9.69, N 3.37. m.p. 183~184°C; $[\alpha]_D^{24}$ +10.3° (*c* 0.386, CH_3OH).

Fig. 1. Infrared absorption spectrum of myriocin (KBr).





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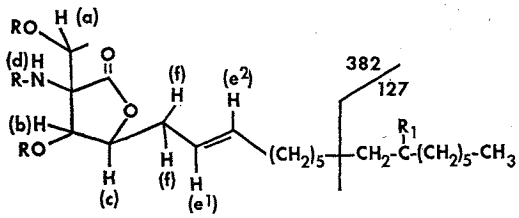
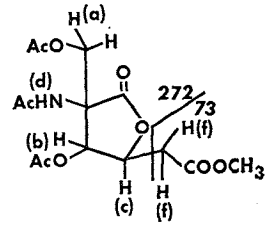


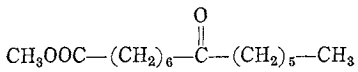


5

2 R=HR¹=O
3 R=COCH₃ R¹=OCOCH₃
4 R=COCH₃ R¹=O

Table 1. Relevant NMR signals (σ) of myriocin derivatives 2~5

Formula No.	a	b	c	d	e
2	3.61 (q)	4.15 (d)	4.66 (m)	—	5.72 (m)
3	4.50 (s)	5.74 (d)	4.7 (m)	6.2 (s)	5.35 (e^1) 5.65 (e^2)
4	4.51 (s)	5.79 (d)	4.7 (m)	6.46 (s)	5.5 (m)
5	4.48 (s)	5.70 (d)	5.24 (m)	6.3 (s)	



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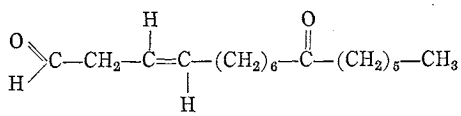

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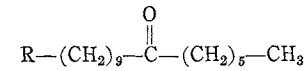


8 R= —CHO
9 R= —COOH
10 R= —COOCH₃

Fig. 2. N.M.R. spectrum of dihydroanhydromyriocin tetraacetate.

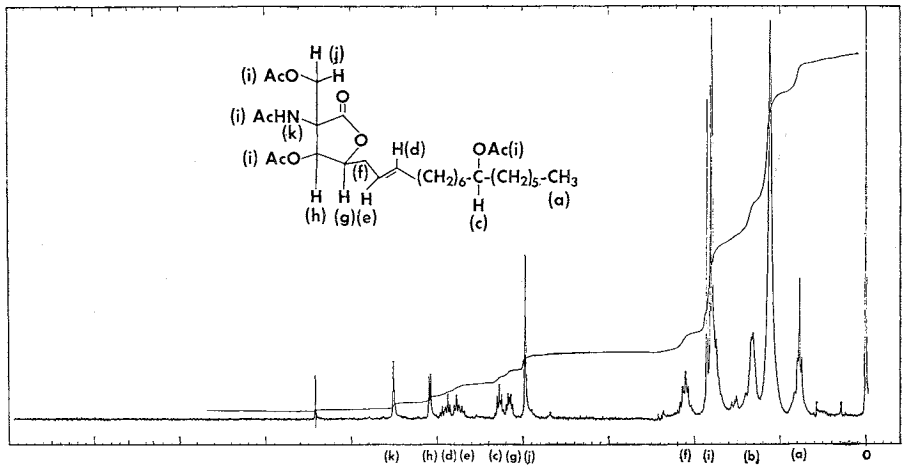
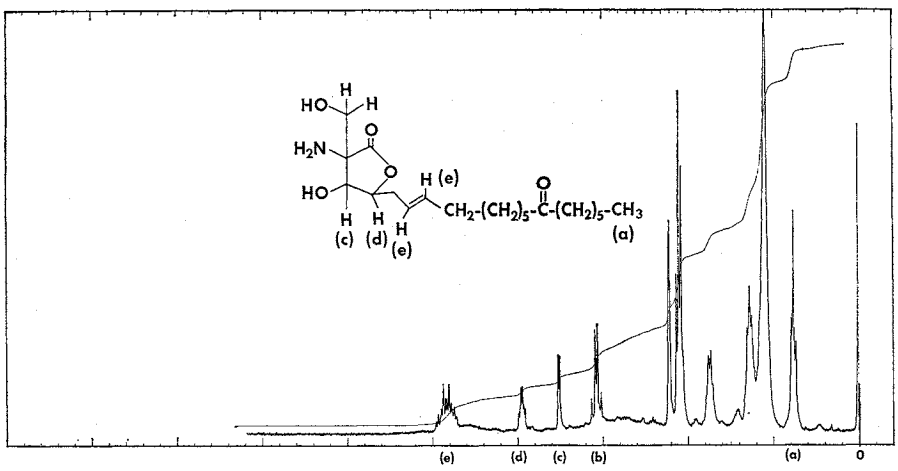


Fig. 3. N.M.R. spectrum of anhydromyriocin.



The infrared spectrum (KBr) has a broad hydroxylic absorption and characteristic bands at 1702 and 1665 cm^{-1} (Fig. 1). The mass spectrum exhibits no molecular ion peak, the highest ion peak being located at M^+-(*m/e* 383), with a base peak at M^+-(127+18), (*m/e* 256). Myriocin has no characteristic UV absorption, and no satisfactory NMR spectrum could be obtained because of its low solubility in common solvents. Myriocin exhibits a positive ninhydrin reaction.

When myriocin was refluxed in *tert*-amyl alcohol overnight, it was transformed into anhydromyriocin (**2**). This is characterized by the appearance of a new band at 1773 cm^{-1} in the infrared spectrum, which indicates a γ-lactone carbonyl. The NMR spectrum (in $CDCl_3$, 22 MHz, Table 1) shows two olefinic and four carbinolic protons. The 5.72 σ signal is attributable to protons on a disubstituted double bond. The *trans* geometry follows from: (i) J=16 Hz in the NMR spectrum of **3** (Fig. 2),

and (ii) by the presence of 950 cm^{-1} band in the infrared spectrum of myriocin and its derivatives.

Anhydromyriocin is slightly more soluble than myriocin in alcohols. A NMR of anhydromyriocin is shown in Fig. 3. The mass spectrum shows a molecular ion peak M^+ (*m/e* 383). Elemental analysis for M.W. 383: Calcd for $C_{21}H_{37}O_5N$: C 65.76, H 9.72 N 3.65. Found: C 65.79, H 9.79, N 3.40; m.p. 78~79°C, and $[\alpha]_D^{24}$ +33.4° (*c* 0.718, CH_3OH).

Acetylation of myriocin (**1**) and anhydromyriocin (**2**) yields the same acetate (**4**) which corresponds to $C_{27}H_{39}O_8N$ (509): Calcd.: C 63.63, H 8.51, N 2.75. Found: C 63.63, H 8.80, N 2.91. Dehydration of **1** during acetylation may be rationalized as follows:

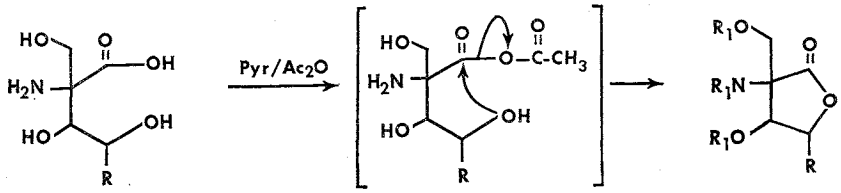
Table 2. Antifungal activity of myriocin and anhydromyriocin

Test organism	Myriocin MIC* (μg/ml)	Anhydromyriocin MIC* (μg/ml)
Candida albicans AY F-598**	0.32	0.8
Candida albicans ATCC 11651	2.5	1.6
Candida albicans AY F-610	12.5	6.25
Candida albicans AY F-611	12.5	6.25
Candida albicans AY F-612	12.5	12.5
Candida albicans AY F-613	6.25	12.5
Candida albicans AY F-614	3.2	3.2
Candida albicans AY F-615	25.0	25.0
Candida albicans AY F-616	1.6	3.2
Candida albicans AY F-617	1.6	3.2
Trichophyton granulosum AY F-604	1,000	4.0
Microsporum gypseum AY F-605	1,000	8.0

* MIC: Minimum inhibitory concetration
** Ayerst culture collection numbers

Table 3. Acute toxicity (LD_{50}) of myriocin and anhydromyriocin

Test species	Route of administration	Myriocin (mg/kg)	Anhydromyriocin (mg/kg)
Mice	i. p.	5~10	75~100
Mice	p. o.	300~400	100~200
Rats	i. p.	2~5	37



$R = -CH_2-CH_2-CH=CH-(CH_2)_6-\overset{O}{\overset{\|}{C}}-(CH_2)_5-CH_3$
$R_1 = -COCH_3$

The mass spectrum shows a molecular ion peak M^+ (*m/e* 509) and fragments $M^+ -60$ (*m/e* 449), $M^+ -127$ (*m/e* 382), $M^+ -(60+127)$ (*m/e* 322). The NMR signals of interest are listed in Table 1. The presence of the isolated ketone in the parent antibiotic was confirmed by its disappearance upon borohydride reduction. The resulting alcohol was characterized as its acetate (**3**).

Ozonolysis of the triacetate **4** followed by oxidative work up with hydrogen peroxide and esterification yielded two compounds: (i) A crystalline product of the formula $C_{14}H_{19}O_9N$, m.p. 174~175°C having infrared bands (in $CHCl_3$) at 3400, 3350, 1780~1725 and 1675 cm^{-1}. The mass spectrum shows a molecular ion M^+ (*m/e* 345) and fragments $M^+ -73$ (*m/e* 272) and $M^+ -43$ (*m/e* 302). The NMR spectrum ($CDCl_3$,

100 Hz) exhibits in addition to the bands shown in Table 1 a doublet at 2.9δ (J=7 Hz) attributable to the α-methylene group of the newly generated ester. The compound was assigned structure **5** based on the above evidence. (ii) Product **6** which was identified as 8-ketotetradecanoic acid methyl ester by comparison with an authentic sample of this compound[4]*.

Treatment of myriocin (**1**) with periodic acid (3 mol) in a mixture of ether and water yielded a major product (84.5 %) which was assigned structure **7** based on spectral data. The ultraviolet spectrum of **7** in neutral medium showed a weak end absorption. However, on alkalization a maximum developed at 233 mμ (ε 5100). Furthermore, spin decoupling of the NMR (100 MHz, $CDCl_3$) demonstrated that 1H triplet (9.65δ, aldehydic) collapsed to a singlet when observed during the irradiation with the resonance frequency of the α-methylenic protons (3.09δ, multiplet). The following transformations confirm the structural assignment for aldehyde **7**. Catalytic reduction of aldehyde **7**, followed by the silver oxide oxidation of the resulting saturated product **8** yielded the acid **9**. This acid and its methyl ester were identical in all respects with an authentic sample[3]* of 11-ketoheptadecanoic acid and its ester.

The above physical and chemical data are best fitted in the expression **1** for myriocin.

Antimicrobial Activity *In Vitro*

Myriocin and anhydromyriocin do not exhibit any significant antibacterial activity. Their antifungal activity was determined by two-fold dilution and points in SABOURAUD's dextrose agar using pathogenic fungi and *Candida albicans* as test organisms. The inoculated broth was incubated for 7 days at 37°C for *C. albicans* and for 14 days at 28°C for the fungi, and examined for growth of the miccro-organisms. The results are given in Table 2.

Myriocin, but not anhydromyriocin, also exhibited activity *in vitro* against some plant pathogenic fungi such as *Endothia parasitica* at concentrations of 100 μg/ml and *Botrytis cinerea* at 0.5 μg/ml.

Toxicity of Myriocin and Anhydromyriocin

The acute toxicity of myriocin and anhydromyriocin was studied in mice and rats; the results are give in Table 3.

Oral toxicity of myriocin in mice was much lower than intraperitoneal toxicity, which may be explained by poor absorption. No such difference appeared for anhydromyriocin. Tolerance studies of myriocin in dogs resulted in death of the animals 48~72 hours after subcutaneous injection of 0.25 mg/kg.

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