A NEW NAPHTHAQUINONE ANTIBIOTIC FROM A NEW SPECIES OF YEAST

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A fusiform yeast producing limited pseudomycelium and limited true mycelium on malt extract agar has been isolated. This non-fermentative yeast has hyaline cell walls but produces a thick, black oily exudate which is water insoluble and gives the colony a smooth black lacquered appearance. On the basis of morphology and physiology, this organism is distinctive enough to warrant the designation of a new genus. During stationary phase of cultures on chemically defined medium, a deep red substance is produced which has strong antibiotic activity against *Staphylococcus aureus in vitro*. The substance has been identified as a new natural naphthaquinone of the empirical formula $C_{16}H_{16}O_7$.

Yeasts with black colonies are usually considered representatives of the genera *Aureobasidium, Rhinocladiella* or *Exophiala*[1,2]. These fungi have black colonies due to the accumulation of highly polymerized melanin-like pigments in their cell walls. These pigments are insoluble in water and organic solvents. Often, the young colonies of *Aureobasidium* are white to cream colored and then pass through a series of color changes which lead to dark brown or black colonies.

During the process of isolating fungi from senescent Royal Palm inflorescences, a black yeast colony arose on one of the malt extract agar plates. Subsequently, washings of senescent inflorescences from the same location followed by dilution plating on malt extract agar, failed to yield any similar colonies. Consequently, it is questionable whether the original isolate came from a palm inflorescence or was a rare aerial contaminant. In any case, the morphological and biochemical characteristics of this isolate (BY1) are given below. It is believed that they are sufficiently distinctive to warrant the designation of a new genus of yeasts.

Materials and Methods

Colonies were isolated on malt extract agar (Blakeslee formula)[3] and they were maintained and stored at 4°C on the same agar. Growth was also tested on yeast extract - Soytone - glucose agar (yeast extract 1 g, Soytone 1.5 g, glucose 15 g, agar 20 g, distilled water 1,000 ml). Dalmau cultures were carried out on Difco Cornmeal Agar. Fermentation and assimilation tests were carried out using both auxanograms and liquid tube cultures with Difco Yeast Nitrogen Base and Yeast Carbon Base. Carbon and nitrogen compounds used with the basal media were from either Sigma Chemical Co. or Difco Laboratories. Concentrations used followed LODDER[1].

For antibiotic production, synthetic medium of the following formulation was used: glutamine 2 g, glucose 30 g, magnesium sulfate heptahydrate 0.5 g, disodium phosphate dodecahydrate 1 g, monopotassium phosphate 0.6 g, distilled water 1,000 ml, and thiamine 200 μg delivered in 1 ml 70% ethanol after autoclaving. Cells were transferred by loop from fresh agar cultures to 300 ml of this synthetic medium in 1,000 ml flasks and cultivated for 14 days at approximately 30°C on a gyrotary

shaker at 200 rpm. After this time, pigments and antibiotic material were sequentially extracted from whole cultures with equal volumes of chloroform until little color appeared in the chloroform fraction. The chloroform extracts were filtered through phase separating paper (Whatman), pooled and evaporated to a gummy residue which was then sequentially extracted with hexane (J. T. Baker) until no more red color appeared in the extract. The hexane extracts were pooled and evaporated to dryness. The residue was chromatographed on a cellulose powder (Whatman CF11) column with carbon tetrachloride (J.T. Baker) as the eluting solvent. The fraction constituting the main red band from this column was evaporated to dryness and the residue was taken up in chloroform from which crystals for X-ray crystallographic analysis were obtained.

Thin-layer chromatography was done with commercially prepared silica gel 60 plates from E. Merck, Darmstadt using chloroform - methanol (3: 1) as the running solvent.

Bioautography using *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Serratia marcescens* (ATCC 27117) and *Candida albicans* (CBS 5736) was performed using standard methods[4].

The UV spectrum was recorded with a Shimadzu (UV 240) UV-Visible Spectrophotometer; the IR spectrum was recorded with a Beckman IR 20A Spectrophotometer; the MS was recorded with a DuPont 21-490B GC-MS instrument; and the low resolution NMR spectrum was obtained using a Varian EM 360L spectrometer.

Details of the X-ray crystallographic analysis are given in the Results and Discussion.

Results and Discussion

Like *Aureobasidium,* initial growth of the new isolate BY1 produced white colonies. Within a few days, these colonies turned black and assumed a shiny, smooth lacquered appearance. In contrast to *Aureobasidium,* the color did not arise from the presence of pigments in the cell walls of the fungus. Rather, the cell walls were hyaline, and the black color arose from an extracellular water-insoluble but ethanol-soluble oily material that accumulated surrounding the cells. The colonies further differed from those of *Aureobasidium* in that mycelial development was very limited in extent and the yeast cells were fusiform. Photomicrographs of cells and exudate from malt extract cultures are shown in Fig. 1.

Cultivation of BY1 on yeast extract - Soytone - glucose medium (liquid or solid) led to rapid loss of viability such that no viable cells could be recovered after two weeks. This could have resulted because of a rise in pH of the culture medium to approximately 7.2 during growth on this medium.

Dalmau cultures on cornmeal agar[1] revealed limited pseudomycelium and limited true mycelium with predominant yeast growth. In some areas under the coverglass, multiseptate cells arose with slightly pigmented thick walls after one week. In some respects, these were reminiscent of *Aureobasidium* or *Rhinocladium*[5] (see Fig. 1).

Fermentation tests with glucose were negative and assimilation tests gave the results shown in Table 1. Nitrate assimilation was positive and there was no apparent absolute vitamin requirement for growth, although thiamine appeared to improve it. Growth at 37°C was negative.

Based on the preceding information, this new anamorphic isolate BY1 should be designated the representative of a new form genus of yeasts. Fungi in this new genus would differ from members of the genus *Candida* by the formation of black or darkly pigmented colonies, and from members of other black yeast genera by the formation of black pigments which are extracellular and organic solvent soluble rather than cell wall confined and organic solvent insoluble.

Growth on liquid malt medium yielded black cultures in 7 to 10 days. Aliquots of this culture fluid had strong antibiotic activity against *S. aureus* and weak activity against *C. albicans.* Cells could be sedimented by low speed centrifugation (3,000 rpm, 10 minutes) leaving a black supernatant liquid.

Fig. 1. Photomicrographs of BY1 from malt extract agar cultures and from cornmeal agar cultures.

a) A loop of cells from a colony on malt extract agar. Note that the cell walls are hyaline while the cells are surrounded by dark amorphous material which forms into spherical viscous droplets when dispersed in water. b) Cells from a liquid malt extract culture. c) A microcolony on agar. d) Photomicrograph of pseudomycelium from a Dalmau culture on cornmeal agar. e) Multiseptate cells from a Dalmau culture on cornmeal agar. f) Photomicrograph of crystals from a culture of BY1 in synthetic liquid medium at 10 days.

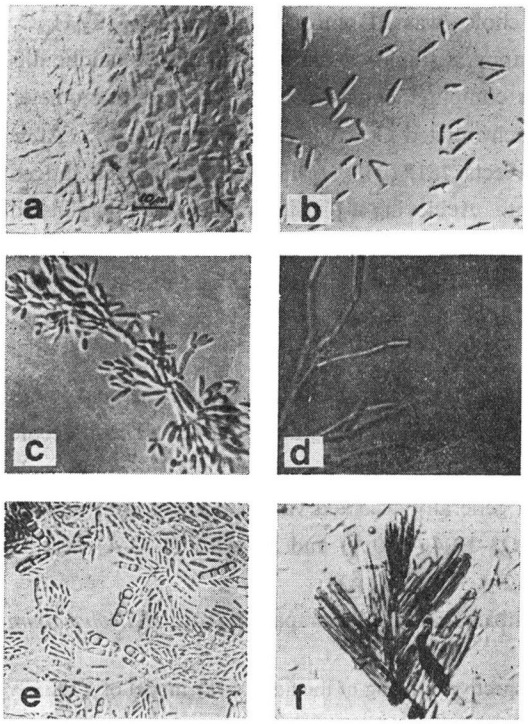


Table 1. Assimilation of various carbon compounds by isolate BY1.

Arabinose	+	Maltose	+
Cellobiose	+	Mannitol	+
Dulcitol	−	Rhamnose	+
Fructose	+	Sorbitol	−
Galactose	−	Starch	−
Glucose	+	Sucrose	+
Inositol	+	Trehalose	+
Lactose	−	Xylose	+

Further high speed centrifugation (15,000 rpm, 20 minutes) of this black supernate brought down a black oily layer and left a clear, greenish supernate. The strongest antibiotic activity resided in the black layer.

Cultures in liquid synthetic medium were similar to those in liquid malt medium. The culture fluid was initially cloudy white to cream but turned brown and then black within 7 days. After 10 days cultivation the pH of the medium dropped from 6.5 to 3.5 and abundant brownish birefringent crystals could be seen in the medium (Fig. 1). Chloroform extraction of the whole culture fluid removed these crystals and the black pigment. By adjusting the pH of the medium to 8 with sodium hydroxide, the crystals turned blue and dissolved. At the same time, pigments could no longer be extracted from the medium with chloroform. Reacidification to pH 3.5 with hydrochloric acid restored extractability.

Sequential chloroform extraction of cultures in liquid synthetic medium depleted the antibiotic activity of the medium. Chromatography of the chloroform concentrate on silica gel plates yielded several colored compounds (red, orange, purple, green and brown to black). By bioautography, only one of these spots, the purple one, had antibiotic activity. This compound and its antibiotic activity could be extracted from the chloroform concentrate residue with hexane, yielding a deep red solution upon concentration. This concentrate still showed several spots on thin-layer chromatography.

The dried hexane extract was chromatographed on a cellulose powder column with carbon tetrachloride as the eluting solvent. Several colored bands were separated on this column. The major one was magenta in color and fractions from it showed antibiotic activity. Concentrates from two other bands also had antibiotic activity.

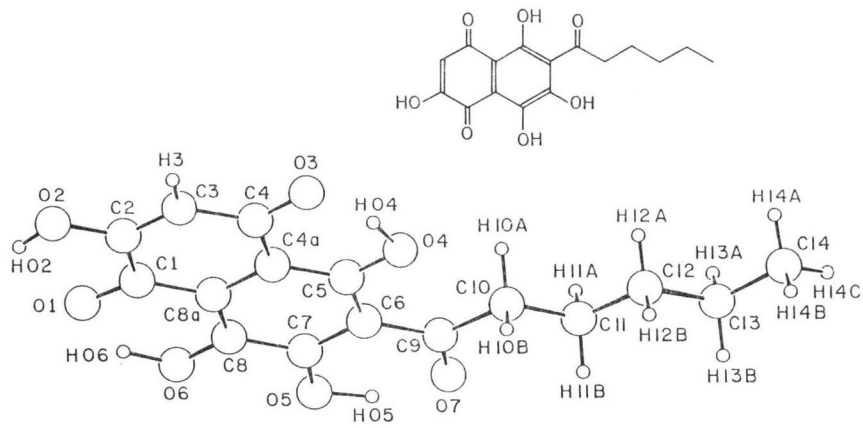
Crystallization of the red compound from chloroform yielded deep red needles (mp 171 ~ 174°C) (approximately 200 mg from 4.5 liters of culture broth), and the compound was designated Y1005. Due to the poor solubility in organic solvents, the NMR spectrum of Y1005 was obtained in pyridine-d_5

and it showed two triplets at δ 3.18 (2H) and 0.77 (3H), two sets of multiplets centered at δ 1.83 (2H) and 1.32 (4H) and one aromatic proton singlet at δ 6.90. The presence of OH groups in Y1005 was evident in its IR spectrum where absorption maxima were observed at 3380, 3320 cm^{-1}. UV absorptions were recorded at λ 225 nm (ε 5,024), 267 (4,704), 313 (4,408) and 542 (1,920). Due to poor volatility, the mass ion of Y1005 could not be detected by mass spectrometry measurement. Preliminary X-ray photographs from single crystal diffraction analysis of Y1005 showed only triclinic symmetry, and accurate lattice constants, determined by a least squares fit of diffractometer-measured angles for fifteen reflections, were a=4.763(1), b=17.238(2), c=8.774(1) Å, α=93.55(2), β=96.15(2) and γ=93.94(2)°. There was some uncertainty about the molecular formula at this stage of the analysis, but density considerations suggested that for most of the plausible formulae, z=2 in the space group $P\bar{1}$ or P1. Ultimately it was clear that the correct choice was $P\bar{1}$ with one molecule of $C_{16}H_{16}O_7$ forming the asymmetric unit. For X-ray crystallography, a single crystal was cut and all unique diffraction maxima with $2\theta \leq 114°$ were collected on a computer controlled four circle diffractometer using a variable speed, 1° w-scan and graphite monochromated CuK$\bar{\alpha}$ radiation (1.54178 Å). After correction for Lorentz, polarization and background effects, 1815 (94%) of 1923 reflections were judged observed ($I \geq 36(I)$) and used in subsequent refinement. Achieving a phasing model was complicated by the hypercentric nature of the data but it was ultimately successful using direct methods techniques[6~10]. Block diagonal least squares refinement with anisotropic nonhydrogen atoms and isotropic hydrogens converged to a conventional crystallographic residual of 0.053 for the observed reflections*.

Fig. 2 shows a structural formula and a computer generated perspective drawing of the final X-ray model of Y1005. As can be seen, it is 6-hexanoyl-2,5,7,8-tetrahydroxynaphthaquinone. The molecule has a planar naphthaquinone coupled to a fully extended hexanoyl chain so that the entire molecule is planar. The molecular geometry agrees well with generally accepted values. There are intramolecular hydrogen bonds between O1–H06 (1.62 Å), O3–H04 (1.50 Å) and O7–H05 (1.52 Å) and an intermolecular hydrogen bond between O1–H02 (1-x, 2-y, 1-z) (1.89 Å).

The new natural naphthaquinone somewhat resembles one called mompain[11] from *Helicobasidium*

Fig. 2. Structural formula and computer generated perspective drawing of the final X-ray model of Y1005.



* Tables of fractional coordinates, thermal parameters, bond distances and bond angles have been deposited with the Cambridge Crystallographic Data Centre and are also available from J.C.

mompa, a violet root rot in the order Auriculariales. Mompain differs from Y1005 by lacking the six-carbon keto side chain.

This new compound has fairly strong antibiotic activity against *S. aureus* (approximately 100 μg/ml gave an inhibition zone equivalent to 1 μg/ml streptomycin) and mild activity against *C. albicans.* The crude preparation was also active against *B. subtilis, Escherichia coli* and *S. marcescens.*

Acknowledgments

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