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Characterization of an antifungal glycolipid secreted by the yeast *Sympodiomycopsis paphiopedili*

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Abstract

An antifungal glycolipid was purified from the culture liquid of the ustilaginomycetous yeast *Sympodiomycopsis paphiopedili* by column and thin-layer chromatography. According to nuclear magnetic resonance and mass-spectroscopy experiments it was a cellobioside containing 2,15,16-trihydroxypalmitic acid as an aglycon. The minimal effective concentrations leading to ATP leakage and growth inhibition were 45 and 160 μg ml^{-1} for *Cryptococcus terreus* and *Candida albicans*, respectively.

Keywords: *Sympodiomycopsis paphiopedili*; Fungicide; Glycolipid; ATP leakage; Yeast; NMR

1. Introduction

By now, two types of antifungal agents secreted by yeasts are known, glycoproteins (mycocins) [1] and glycolipids [2–4]. Mycocins have been investigated for at least 40 years, whereas the fungicidal activity of glycolipids has recently come to light. The glycolipids secreted by yeasts commonly have been considered as emulsifying agents associated with consumption of low-soluble nutrients [5–8]. The most-studied of them are sophorolipids [5,6] and mannosylerythritol lipids [7,8]. The antifungal activity of extracellular glycolipids has been observed for *Cryptococcus humicola* [2], *Pseudozyma fusiformata* [3], and *Pseudozyma flocculosa* [4]. The glycolipids of *Cr. humicola* [2] and *Ps. flocculosa* [4] are cellobiose lipids differing in aglycon structures.

Recently, we have revealed that *Sympodiomycopsis paphiopedili*, a yeast-like organism, displays an antifungal activity against 84% of 464 tested strains of yeasts and yeast-like fungi belonging to 86 genera and including phytopathogenic and medically important species [9].

This study was aimed at the characterization of the chemical composition and some properties of the antifungal compound produced by *S. paphiopedili*.

Abbreviations: NMR, nuclear magnetic resonance; ^{1}H,^{1}H COSY, ^{1}H,^{1}H-correlated spectroscopy; TOCSY, total-correlation spectroscopy; ROESY, rotation frame Overhauser effect spectroscopy; ^{1}H,^{13}C HSQC, heteronuclear single-quantum coherence; HMBC, heteronuclear multiple-bond correlation.

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2. Materials and methods

2.1. Strains and culture conditions

The yeast strains were obtained from the Russian Collection of Microorganisms (VKM) and the Japan Collection of Microorganisms (JCM). Strains were maintained on malt agar slants at 5 °C.

The strain *Sympodiomycopsis paphiopedili* VKM Y-2817 was grown for one month without shaking at 24 °C in a liquid medium containing glucose, 1.0%; $(NH_4)_2SO_4$, 0.1%; $MgSO_4$, 0.005%; yeast extract, 0.05%; succinic acid, 0.8%; pH 4.0, adjusted by NaOH. During the cultivation neither cell lysis nor contamination was observed by light microscopy.

Cryptococcus terreus VKM Y-2253 and *Candida albicans* JCM 1542 were grown to the middle-logarithmic growth phase as described [3].

2.2. Purification of glycolipid

After growth of *S. paphiopedili* VKM Y-2817, culture liquid was separated from biomass by centrifugation (5000*g*, 30 min) and filtered through a glass microfiber filter GF/A (Whatman, UK). The filtrate was lyophilised and suspended in methanol. Insoluble compounds were removed by filtration. After evaporation, the remaining substance was suspended in cold deionized water. The insoluble compounds were separated by centrifugation (4000*g*, 15 min), washed twice with water under the same conditions and dissolved in methanol. The methanol solution was applied to an LH–Sephadex column (25×1.5 cm) and eluted with methanol (flow rate 15 ml h^{-1}). Fractions (4 ml) were assayed for antifungal activity by measurement of the diameters of growth inhibition zones of *Cr. terreus*. Active fractions (No. 6–9) were concentrated by evaporation and purified on HP-KF Silicagel plates (Whatman, USA). Development was with a solvent system containing chloroform/methanol/water (4: 4: 0.2, v/v/v). The bands exhibiting absorption in UV light were scraped and the preparations were suspended in methanol. The antifungal activity was determined by measurement of the diameters of growth inhibition zones of *Cr. terreus*. Only one band with $R_f = 0.75$ exhibited antifungal activity. The filtered and partially evaporated extract was kept at 5 °C and used as a final preparation. After methanol evaporation, the concentration was determined by weighing.

Thin layer chromatography of the preparation gave one major band on Kieselgel 60 F_{254} plates (Merck, Germany) in the solvent systems containing chloroform/methanol/water 4:4:0.2, 5:3:0.2, or 3:5:0.2 (v/v/v) (not shown). The chromatograms were stained by 5% H_2SO_4 in 95% ethanol at 100 °C.

The glycolipid complex of *Cryptococcus humicola* strain 9-6 was obtained as described in [2].

2.3. Assay for antifungal activity

Sterile 5-mm diameter glass microfiber filter discs GF/C (Whatman, UK) were placed onto the surface of a solid medium (0.5% glucose, 0.2% yeast extract, 0.25% peptone, 2% agar, 0.04 M citrate–phosphate buffer, pH 4.0) in Petri dishes inoculated with *Cr. terreus* VKM Y-2253. Aliquots of glycolipid solution were pipetted onto discs. The plates were incubated at 24 °C until growth of the lawn strain appeared, and the diameters of growth inhibition zones were measured.

The measurement of ATP leakage from yeast cells treated with the glycolipid preparation was performed by the luciferine–luciferase method [3].

2.4. Sugar analysis

For sugar analysis, glycolipid samples were hydrolyzed with aqueous 3M trifluoro-acetic acid (100 °C, 6 h) and assayed using a carbohydrate analyzer LC-2000 (Biotronic, Germany).

2.5. NMR spectroscopy

One-dimensional ^1H NMR, ^{13}C NMR, two-dimensional ^1H,^1H COSY, TOCSY, ROESY and ^1H, ^{13}C HSQC and HMBC spectra were recorded with a Bruker DRX-500 spectrometer (Germany) at 30 °C using standard Bruker software (XWINNMR 1.2). The sample was examined as 5% solution in methanol–D_4 with the abundance of deuterium 99.5%. Chemical shifts were referenced to the signals CD_2H and CD_3 of the solvent (δ_H 3.25 and δ_C 49.0, respectively). A mixing time of 100 ms was used in the two-dimensional TOCSY experiment. The two-dimensional HMBC experiment was optimized for the coupling constant $J_{H,C}$ 8 Hz.

2.6. ESI-MS analysis

The glycolipid was mass-analyzed by electrospray mass spectrometry in the negative-ion mode using a Finnigan LCQ (USA) instrument. The sample was dissolved in methanol and sprayed at a flow rate of 3 μl min^{-1}.

3. Results and discussion

3.1. NMR spectroscopy and ESI-MS analysis

The purified antifungal preparation of *S. paphiopedili* was investigated by NMR spectroscopy. The ^1H NMR spectrum contained intensive signals for two protons at anomeric carbon atoms (doublets at δ 4.35 and

4.25; $J_{1,2}$ 8 Hz), signals for protons at carbon atoms bearing one oxygen in the resonance region of 3.2–3.7 ppm, and high-field signals for protons at carbon atoms without electronegative substituents (1.2–1.7 ppm). It should be noted that the spectrum contained also minor signals of impurities, for example a broadened singlet at 4.51 ppm.

The ^{13}C NMR spectrum displayed 19 intensive signals, some of them of multiple integral intensity. The presence of a low-field signal at δ 176.4 ppm attributable to a carbonyl group indicated that an acid residue was present in the glycolipid. The signal of double intensity at δ 104.6 ppm obviously belonged to anomeric carbon atoms of two sugar residues. The APT spectrum [10] made it possible to separate the signals that belong to carbons bearing an odd (1 or 3) or even (0 or 2) number of protons. The spectrum revealed ten signals of methylene groups in the resonance region of the carbon atoms, bearing one oxygen signal (71.4–80.7 ppm) and three signals of O-methylene groups at δ 61.8, 62.3 and 75.6 ppm. The high-field region of the spectrum contained signals of –C–*C*H$_2$–C– groups only; four of them (at δ 36.2, 34.3, 26.5 and 26.4 ppm) were of single intensity, and the integral intensity of the fifth signal at δ 30.7 ppm was estimated as belonging to eight –C–*C*H$_2$–C– groups.

The ^{1}H NMR spectrum of the specimen was completely assigned using 2D COSY and TOCSY experiments (Table 1). Spin-systems for two pyranoses having the β-*gluco* configuration were identified on the basis of characteristic coupling-constant values $J_{1,2}$, $J_{2,3}$, $J_{3,4}$ and $J_{4,5}$ [11]. The analysis of cross-peaks in the 2D spectra revealed also that three oxygen-bearing carbon atoms belong to a fatty acid residue in the glycolipid under investigation. Moreover, the analysis showed that the fatty acid contains vicinal –CH$_2$ O– and –CH–O– groups (the corresponding peaks at δ 3.70/3.83 and 3.70/3.33 in the COSY spectrum). The proton of another –CH–O– group (at δ 3.81) showed the correlation peaks with protons of the –C–CH$_2$–C– group only. As a consequence, the fatty acid should contain the fragments –OCH$_2$–CHO– and –CH$_2$–CHO–COOH. Taking into account the number of carbon atoms in the residue, this acid may be considered as 2,15,16-trihydroxypalmitic acid.

The sequence of the residues was established by the 2D ROESY spectrum, where the inter-residue correlation peaks H-1″/H-4′ and H-1′/H-16 were observed.

The ^{13}C NMR spectrum was obtained by a 2D ^{1}H, ^{13}C HSQC experiment (Table 2). The spectrum revealed the presence of a terminal -Glc*p* residue, a 4-substituted β-Glc*p* residue (C-4′ at δ 80.7 ppm), and confirmed the substitution of a lipid residue at the -O-CH$_2$- group (C-16 at δ 75.6 ppm). The sequence of the residues was finally confirmed by 2D ^{1}H, ^{13}C HMBC spectrum, due to the presence of correlation peaks δ_H/δ_C 4.35/80.7 (H-1″/C-4′) and 4.25/75.6 (H-1′/C-16).

The analysis of impurity signals in the 1D and 2D spectra revealed that the minor component of the preparation was represented by a glycoside with the following structure: β-Man*p*-(1 → 1)-Gro (Gro = Glycerol). The content of this glycoside in the preparation

Table 1
^{1}H NMR data of the glycolipid of *S. paphiopedili* (δ, ppm)

Residue	Chemical shift for							
β-Glcp-(1 →	H-1″	H-2″	H-3″	H-4″	H-5″	H-6″		
	4.35	3.17	3.31	3.25	3.28	3.82		
→ 4)-β-Glcp-(1 →	H-1′	H-2′	H-3′	H-4′	H-5′	H-6′		
	4.25	3.23	3.47	3.52	3.35	3.60		
						3.81		
→ 16)-2,15,16-trihydroxypalmitic acid	H-2	H-3,3′	H-4,4′	H-5-12	H-13,13′	H-14	H-15	H-16,16′
	3.81	1.67, 1.50	1.37, 1.33	1.2-1.3	1.40, 1.29	1.38	3.70	3.83, 3.33

Table 2
^{13}C NMR data of the glycolipid of *S. paphiopedifli* (δ, ppm)

Residue	Chemical shift for								
β-Glcp-(1→	C-1″	C-2″	C-3″	C-4″	C-5″	C-6″			
	104.6	75.0	77.9	71.4	78.1	62.4			
→4)-β-Glcp-(1→	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′			
	104.6	75.0	76.2	80.7	76.5	61.8			
→16)-2,15,16-trihydroxypalmitic acid	C-1	C-2	C-3	C-4	C-5-12	C-13	C-14	C-15	C-16
	176.4	73.5	36.2	26.4	30.7	26.5	34.3	71.7	75.6

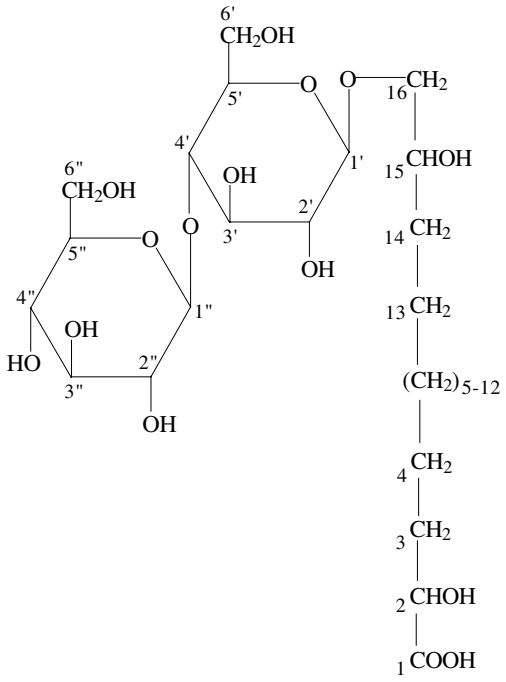

Fig. 1. The putative structure of the glycolipid produced by *Sympodiomycopsis paphiopedili* VKM-2817.

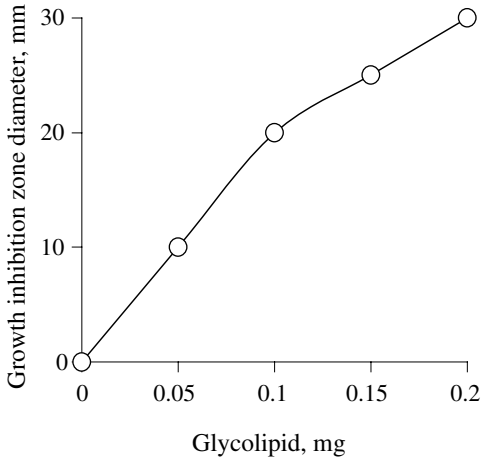

Fig. 2. Effect of glycolipid amount on the diameter of the growth inhibition zone for *Cryptococcus terreus* VKM Y-2253. The mean values of three determinations are shown. The standard deviations did not exceed 15% of the mean values.

was low. Sugar analysis revealed glucose (~95%) and mannose as a minor component (~5%) in the hydrolysate.

The negative-ion mass spectrum comprised one major band (*m*/*z* 627.3) indicating the molecular mass of the main compound of the preparation to be 628.3 Da.

In agreement with the NMR spectroscopy data, the main component of the glycolipid preparation of *S. paphiopedili* was a cellobiose lipid with a putative chemical structure depicted in Fig. 1.

The glycolipid of *S. paphilopedili* has the simplest structure among the known yeast glycolipids with antifungal properties. Its cellobiose backbone has no additional substituents by –OH groups. The cellobiose lipids of *Cr. humicola* possess highly acetylated cellobiose (up to five acetyl groups) linked to the 2,16-dihydroxy palmitate or 2,18-dihydroxy (2,17,18-trihydroxy)-stearate [2]. The glycolipid of *Ps. flocculosa* is 2-(2′,4′-diacetoxy-5′-carboxy-pentanoyl) octadecyl cellobioside [4]. *Ustilago maydis* secreted glycolipids in which cellobiose (acylated with acetic, hexanoic, 3-hydroxyhexanoic or 3-hydroxyhexanoic acid) is esterified with 15,16-dihydroxyhexadecanoic or 2,15,16-trihydroxydecanoic acid [12–14].

3.2. Antifungal action of the glycolipid secreted by S. paphiopedili

The purified cellobiose lipid inhibited the growth of *Cr. terreus* VKM Y-2253 (Fig. 2), which was used as a model sensitive organism in experiments with glycolipids of *Cr. humicola* [2] and *Ps. fusiformata* [3]. Similar to the cellobiose lipids of *Cr. humicola* [2] and *Ps. fusiformata* [3], the glycolipid of *S. paphiopedili* leads to ATP leakage from the cells of *Cr. terreus* VKM Y-2253 (Figs. 3–5). ATP leakage and the loss of cell viability of *Cr. terreus* occurred at similar concentrations (not shown). ATP leakage was maximal at 20–30 °C (Fig. 3).

The glycolipids of *S. paphiopedili* and *Cr. humicola* showed similar pH-dependence with the pH optima 4.0–4.5 (Fig. 4). The minimal effective concentrations of both glycolipid preparations were ~45 and 120–160 μg ml^{-1} for *Cr. terreus* and *C. albicans*, respectively

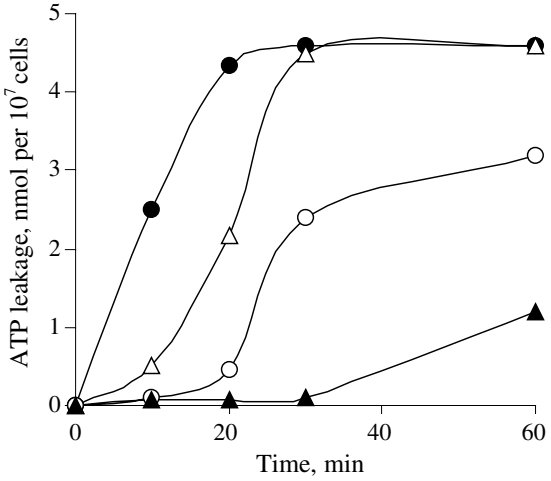

Fig. 3. Changes in the extracellular level of ATP during incubation of *Cryptococcus terreus* VKM Y-2253 cells with glycolipid of *Sympodiomycopsis paphiopedili* (60 μg ml^{-1}) at 0 °C (▲), 10 °C (○), 20 °C (△), 30 °C (●) and pH 4.0. No ATP leakage was observed in the control samples with the appropriate methanol concentration. The mean values are shown. The standard deviations did not exceed 5–10%.

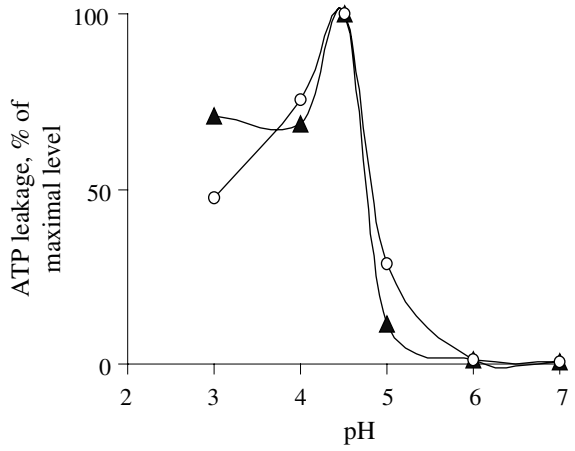

Fig. 4. Effects of pH on ATP leakage (for 30 min at 20 °C) from cells of *Cryptococcus terreus* VKM Y-2253 treated with glycolipids of *S. paphiopedili* (▲) and *Cr. humicola* (○). The glycolipid concentration was 60 μgml^{-1} at. No ATP leakage was observed in the control samples with the appropriate methanol concentration. The mean values are shown. The standard deviations did not exceed 5–10%.

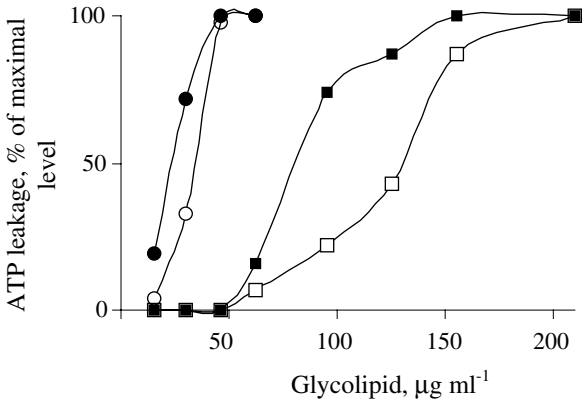

Fig. 5. The effect of glycolipid concentration on ATP leakage from yeast cells at pH 4.0 and 20 °C. The cells of *Cryptococcus terreus* VKM Y-2253 were treated by glycolipid of *S. paphiopedili* (○) and *Cr. humicola* (●). The cells of *Candida albicans* JCM 1542 were treated by glycolipids of *S. paphiopedili* (□) and *Cr. humicola* (■). The mean values are shown. The standard deviations did not exceed 5–10%.

(Fig. 5). After 30 min treatment of the cells (3×10^7 cells ml^{-1}) of *Cr. terreus* and *C. albicans* with the above concentrations of *S. paphiopedili* glycolipid, no growth on glucose-peptone agar was observed. It should be noted that the concentrations of glycolipid in the culture medium of *S. paphiopedili* in our experiments varied from 50 to 70 μgml^{-1}.

Earlier, the membrane-damaging activity of the cellobiose glycolipid of *Cr. humicola* has been investigated by a number of physicochemical methods [2]. It has been shown that the cellobiose glycolipids can intercalate themselves in the lipid bilayer, resulting in its permeabilization or physical disruption [2,4]. The similarity in the structure and ability to make target cells leaky for ATP suggests that the mode of action of *Cr. humicola* and *S. paphilopedili* glycolipids may be the same. The significance of peculiarities of their structures for antifungal activity, in particular acetylation of the cellobiose backbone and the number of hydroxyl groups in the fatty acid, needs further investigation.

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