Phylum-specific fungitoxicity of new disulfide compounds

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Anderson, J., R. F. Langler & F. Bärlocher (2002). Selective fungitoxicity of new disulfide compounds. – Sydowia 54(2): 121–128.

Three disulfides, two of which are related to the natural product dysoxysulfone, were tested for antibiotic activity against 10 species of 3 phyla classified as true Fungi (Zygomycota, Ascomycota, Basidiomycota) and two species of Oomycota (Stramenopila). With a disk diffusion test, the highest concentration of 100 µg failed to inhibit the two Oomycetes. The two Zygomycetes were much less sensitive than the other representatives of the true Fungi. Supplementing the assay medium with ergosterol, an essential sterol of true Fungi, did not mitigate growth inhibition by the three compounds.

Keywords: disulfide compounds, antifungal, dysoxysulfone relatives, ergosterol supplement.

Fungi are the most important class of plant pathogenic organisms (Agrios, 1997). Fungal diseases of crops can be devastating and disease symptoms have been described since farming became the major source of our food and fiber supply. By contrast, the incidence of human diseases caused by fungi is less than that of bacterial and viral infections (Rippon, 1988). However, in the past two decades, opportunistic fungal infections in humans have increased dramatically (Pfaller & Wenzel, 1992; Graybill, 1996). This has been attributed to rising numbers of immunocompromised patients, to evolving resistance of established pathogens such as *Candida* to classical antibiotics, and to newly emerging fungal pathogens (e.g., species of *Fusarium*, *Alternaria*, *Aspergillus*). Consequently, there is "an urgent need for safer, more efficient antifungal agents" (Mims & al., 1993). New lead structures are often discovered by following up on the use of medicinal plants in folk or alternative medicine (Köller, 1992a; Stevens & Lartey, 2000). Crushed leaves of one such plant, *Dysoxylum richii* Gray (Meliaceae) have long been used by Fijian natives and are said to cure various

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ailments such as fevers and infections (Parham, 1943). A biologically active disulfide was isolated from chromatographic fractions (Jogia & al., 1989) and was shown to have antibiotic activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Candida albicans* (Block & al., 1994). Through X-ray diffraction analysis, the active disulfide was identified as 2,4,5,7,9-pentathiadecane 2,2,9,9-tetraoxide or dysoxysulfone (Jogia & al., 1989). It was first synthesized by Block & al. (1994).

Dysoxysulfone is a sulfur-containing antifungal of the general formula $C_nH_{2n+2}S_nO_x$. Investigations into structure-activity relationship of this and related compounds were initiated by Bärlocher & al. (1999); they included the synthesis of antifungals with submicrogram toxicity against *Aspergillus niger* and *A. flavus* (Langler & al., 1999; Bärlocher & al., 2000a, b; 2001; 2002a, b). The objective of the current study was to expand the phylogenetic scope by including 10 species from 3 phyla of the Kingdom Fungi and 2 representatives of the phylum Oomycota (Kingdom Stramenopila). We tested their susceptibilities against three potent antifungal compounds synthesized during our research.

Materials and methods

Cultures of *Alternaria alternata* (Fr.) Keissler (85 W 4133), *Aspergillus niger* van Tiegh. (85 W 4100), *A. flavus* Link (85 W 4090), *Candida albicans* (Robin) Berkhout (85 W 4150), *Phycomyces blakesleeanus* Burgeff (85W 1198), *Rhizopus stolonifer* (Ehrenberg ex Fries) Vuillemin (85 W 4900), *Rhodotorula rubra* (Demme) Lodder (85 W 6065), *Saccharomyces cerevisiae* Meyen ex Hansen (85 W 5005) and *Schizophyllum commune* Fr. (85 W 4950) were obtained from Ward's Supply Company (St. Catharines, ON, Canada), while *Saprolegnia* sp. (BA-15-6270), *Pythium irregulare* Buisman (BA-15-6214) and *Sordaria fimicola* (Roberge ex Desmazières) Cesati & de Notaris (BA-15-6290) were obtained from Carolina Biological Supplies (Burlington, NC, USA). All fungi were maintained on Sabouraud Dextrose Agar (Difco 210950); the three yeasts were also maintained in malt extract broth (1% malt extract, Sigma M-0383, and 0.25% peptone, Difco 0885-17-1), and *Saprolegnia* sp. and *Pythium irregulare* on corn meal agar (Difco 0386-01-3).

Three compounds were used for testing: compound I, p-$CH_3(C_6H_4)SO_2CH_2SSCH_3$ (Bärlocher & al., 2000 a); compound II, p-$0_2N(C_6H_4)SSCH_3$ (Langler & al., 1999); and compound III, $CH_3(CH_2)_6SO_2CH_2SSCH_3$ (Bärlocher & al., 2002b). They were synthesized by RFL as described earlier (Langler & al., 1999; Bärlocher & al., 2000a).

Test solutions were prepared by adding 25 mg of each compound to 50 ml of acetone. Preliminary tests showed that higher concentrations (25 mg compound in 5 ml acetone) were necessary to show measurable inhibition with the two Zygomycetes, *Rhizopus stolonifer* and *Phycomyces blakesleeanus*. Test solutions were wrapped in aluminium foil to prevent exposure to light and stored at 4°C for at least 24 h to ensure homogeneity of solutions.

Antifungal activity was estimated with a disk diffusion test. Whatman #4 filter paper was cut into 7 mm disks, and 5, 10 or 20 µl of the test solution was applied (corresponding to 2.5, 5 or 10 µg per disk at the low concentration; 25, 50 or 100 µg at the high concentration). To control disks, 20 µl of acetone was applied. Before being used, disks were incubated for several hours to ensure complete evaporation of acetone.

Six agar plugs (5 mm diameter) overgrown with the fungus were cut from a 7–10 d old culture and ground to a smooth consistency in 3 ml of dist. sterile water with a manual tissue homogenizer. Aliquots (0.5 ml) of this suspension were spread evenly over 15 ml Sabouraud Dextrose Agar in 9 cm Petri plates. Each plate was supplied with 4 evenly spaced paper disks (7 mm), supplied with antifungal compounds as described above. In additional experiments, 100 mg of ergosterol (Fluka 45480) were dissolved in 100 ml of ethanol. Two ml of the solution were added to the test plate before antifungal assays. Ethanol was allowed to evaporate; control plates were treated with 2 ml of ethanol without ergosterol. This modified assay was done with the following species (all 3 compounds): *Candida albicans*, *Aspergillus flavus*, *Sordaria fimicola* and *Schizophyllum commune*.

After 48 h incubation at 20°C, the degree of inhibition was estimated by the area of no growth surrounding the disks (generally circle or ellipsis; measured by hand). For each combination of fungus and compound, 8 replicate tests (corresponding to 8 separate Petri plates) were done. Inhibition areas for the highest dosage were compared by 2-Way Factorial ANOVA (factors: Species, Compound) with SYSTAT 5.2.1 for Macintosh. Two separate analyses were run for the Zygomycetes (*Rhizopus*, *Phycomyces*) and the remaining 8 species (Ascomycota and Basidiomycota, or Dikaryomycota; Kendrick, 1992).

As there was no growth from homogenized agar plugs overgrown with *Saprolegnia*, we placed a 1 cm^2 plug of *Saprolegnia* in the centre of an agar plate, and placed the four paper disks at a distance of 0.5 cm from the fungus.

For the three yeasts (*Candida albicans*, *Saccharomyces cerevisiae*, *Rhodotorula rubra*, we used 250 µl of 3-day old cultures to inoculate the test plates.

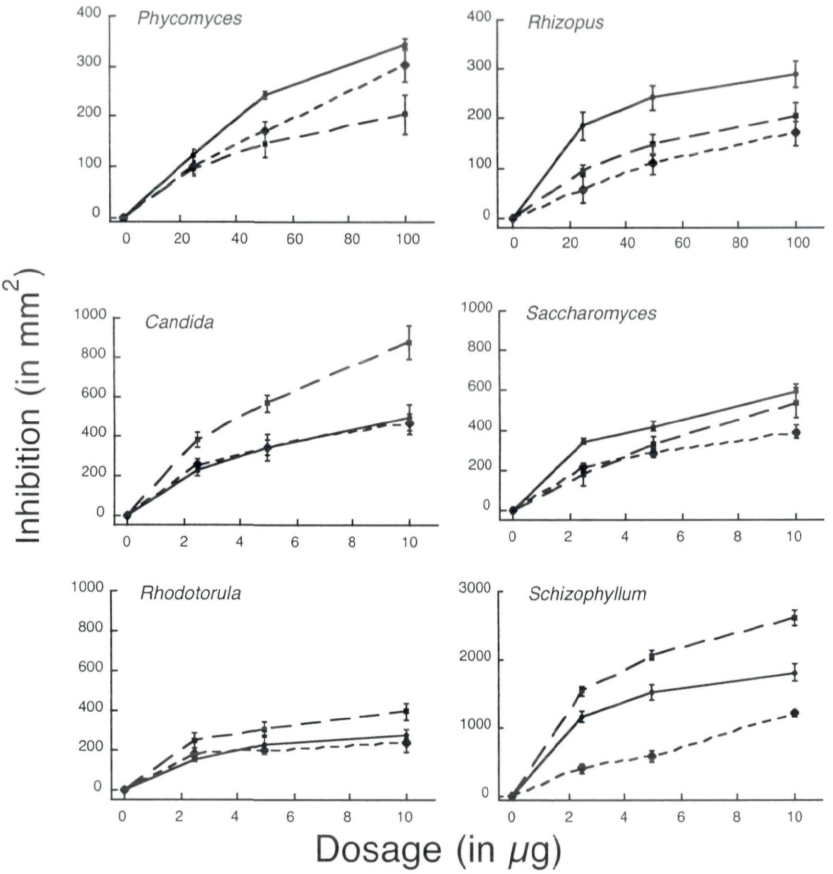

Fig. 1. – Inhibition, expressed as mycelium-free area surrounding antifungal disks, of 6 fungi when exposed to increasing amounts of 3 disulfide compounds. – N = 8, ± SEM. Scales differ among individual graphs. ● and solid lines, Compound I; ❑ and long dashes Compound II; ◆ and short dashes, Compound III.

Results

The two Oomycetes (*Saprolegnia* sp., *Pythium irregulare*) were not inhibited by any of the three compounds at the highest levels tested.

Preliminary tests had shown that the two Zygomycetes (*P. blakesleeanus*, *R. stolonifer*) were much less sensitive than the other representatives of the Fungi (Eumycota). At a concentration of up to 100 µg per disk, Compound I had the greatest inhibitory effect (Fig. 1). Species, compounds and their interaction were highly significant (2-way ANOVA, Tab. 1).

For 5 of the other 8 species, Compound I again had the greatest inhibitory effect; with *Rhodotorula*, *Candida* and *Schizophyllum*,

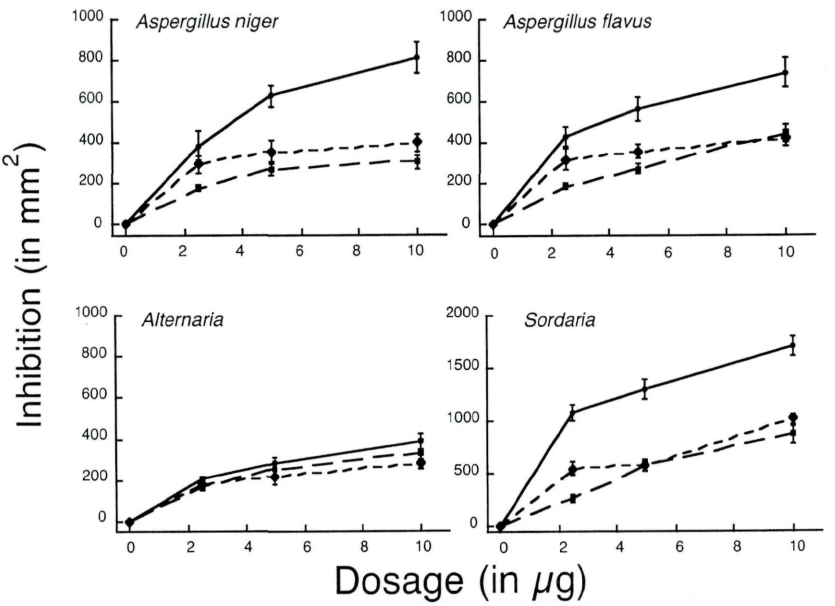

Fig. 2. – Inhibition, expressed as mycelium-free area surrounding antifungal disks, of 4 fungi when exposed to increasing amounts of 3 disulfide compounds. – N = 8, ± SEM. Scales differ among individual graphs. ● and solid lines, Compound I; ❑ and long dashes Compound II; ◆ and short dashes, Compound III.

Compound II was more potent (Figs. 1, 2). Species, compounds and their interaction were highly significant (2-way ANOVA, Table 1). Thus, *Alternaria* responded similarly to all 3 compounds; by contrast, with *Schizophyllum* and the two *Aspergillus* species inhibition by the most effective compounds was approximately twice as much as the one by the least effective compound. Within the Ascomycetes, *Sordaria* was the most sensitive species; overall, *Schizophyllum* was most strongly inhibited.

Tab. 1. – ANOVA of inhibition areas at highest dosage. Separate analyses were run for the Zygomycetes and the 8 members of the Dikaryomycota.

	Source	DF	F	p
Zygomycetes	Species	1	130	<0.0001
	Compound	2	48	<0.0001
	Species*Compound	2	15	<0.0001
Dikaryomycota	Species	7	149	<0.0001
	Compound	2	68	<0.0001
	Species*Compound	14	26	<0.0001
	Error	168		

Ergosterol was added to plates with the following species (all 3 compounds): *Candida albicans*, *Aspergillus flavus*, *Sordaria fimicola*

and *Schizophyllum commune*. Per species and compound, inhibition areas of 4 control plates and 4 plates with ergosterol were compared with a t-test. The p values (adjusted with Bonferroni's procedure) were $\geqslant 0.27$.

Discussion

The initial step in biological screening is often a simple growth inhibition test. It provides "the most integral and almost perfect biological parameter of a newly synthesized compound" (Köller, 1992a). It measures not only inhibitory potency, but also chemical stability of the compound and its penetration into the fungal cell, both essential characteristics for potential use in biocontrol. Unfortunately, there is no agreement on a standard protocol for susceptibility testing applicable to all groups of fungi (Köller, 1992a; Reyes & Ghannoum, 2000). Many of the currently used methods determine minimum inhibitory concentration (MIC) or minimum fungicidal concentrations (MFC), which, in theory, allow useful comparisons of many different compounds. Unfortunately, complex interactions between growth medium, fungal strain and compound make direct comparisons of MIC and MFC values difficult (Nicoletti & al., 1999).

We used a traditional disk diffusion assay. This test is simple to use and the results are reasonably easy to reproduce (Köller, 1992a; Vigers & al., 1991). The overall picture is unequivocal: with the methodology used, there was no measurable growth inhibition in the two Oomycetes (no longer considered to be true fungi; Alexopoulos & al., 1996). By contrast, all compounds significantly decreased growth in the 10 representatives of the true Fungi. However, their concentrations had to be increased by an order of magnitude to reveal any activity toward the two Zygomycetes.

Within the remaining 8 taxa, there were clear differences in susceptibilities. However, we did not find any simple, clear-cut connections with taxonomic status or growth-form. For example, *Schizophyllum* (Basidiomycetes) was clearly the most susceptible species. Its phylogenetically closest relative, *Rhodotorula* (basidiomycetous yeast) was among the least affected, along with the hyphomycete *Alternaria alternata*. The two other hyphomycetes (*Aspergillus flavus* and *A. niger*) were clearly less resistant. The two ascomycetous yeasts showed similar susceptibilities toward compounds I and III; but *Candida* was much more inhibited by compound II than *Saccharomyces*.

An obvious and well-established difference between Fungi and Oomycota is the absence of ergosterol in the latter (Newell 1992, 1993; Köller 1992b). It is therefore not surprising that Oomycetes are essentially unaffected by commercial sterol biosynthesis inhibitors.

In the current study, however, the addition of ergosterol to the growth medium of the true Fungi did not counteract inhibition by the 3 compounds. This makes it less likely that the primary mechanism involved interference with ergosterol synthesis or function.

Other differences between Oomycota and Fungi include details of lysine synthesis, preferred storage compounds, the structure of mitochondria and of cell walls. In Oomycota, walls consist primarily of β-glucans, as well as the amino acid hydroxyproline and small amounts of cellulose, while in Fungi, chitin or chitosan are generally present in addition to β-glucans and a variety of other polysaccharides (Alexopoulos & al., 1996). Each of these factors, or their combination, may account for the drastic differences in susceptibilies between the Oomycetes and the Fungi.

Of the Fungi, the two Zygomycetes were clearly more resistant than the other 8 species (Figs. 1, 2). This was rather surprising, since they have relatively thin, easily collapsible walls and are coenocytic, suggesting that they should be more vulnerable to entry of toxins (Kendrick, 1992; Alexopoulos & al., 1996).

The consistent differences among the remaining 8 species are difficult to interpret. It is unclear whether they are due to the ability of some fungi to selectively prevent intracellular entry of some of the compounds because of differences in wall and membrane structures. Alternatively, the degree of resistance may be based on the differential responses of cellular compartments to the presence of antifungal compounds (Köller, 1992b, Davidse & al., 1983). We are currently investigating some of these possibilities.

Acknowledgments

Financial support from the Natural Science and Engineering Research Council of Canada to FB is gratefully acknowledged.

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(Manuscript accepted 7th May 2002)