Antimicrobial and Antitumor Activities of Mycosporulone

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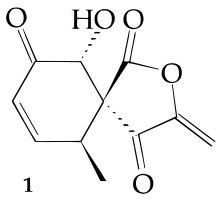
The conditions for optimal production of mycosporulone (**1**) are given. Its cytotoxic, antimicrobial, and antitumor activities are described. The biological activities of **1** were compared with those of known antibacterial, antifungal, and antitumor agents. The compound was particularly active against *Pseudomonas aeruginosa* and *Staphylococcus aureus* (resistant to penicillin). Compound **1** was not toxic to normal human cells (MRC_5), although it exhibited cytotoxic activity against the human tumor cell lines MDA-MB 231 and PC_3 and the murine L-1210 leukemia cell line.

Research on antimicrobial or antitumor compounds produced by microorganisms and plants has been widely conducted for more than 40 years, and new compounds are still being found. Interest in these compounds increases, owing to the development of resistance toward many of the biocides commonly used against pathogens in agriculture and medicine. In chemotherapy of cancer, drugs often exhibit severe side effects; acquired resistance of tumor cells is often a problem. The emergence of new diseases, such as AIDS, also requires new active compounds. As part of a program directed toward the isolation of biologically active metabolites from fungi, we previously selected several fungal strains from our collection that produce antifungal compounds devoid of cytotoxicity and were not accompanied by dangerous mycotoxins such as patulin.[1−4]

We investigated the genus *Coniothyrium* and particularly *C. sporulosum* (W. Gams & Domsch) Van der Aa (class Hyphomycetes, order Sphaeropsidales), which was selected according to the strong antifungal activity of crude culture medium extracts. Fractionation of the ethyl acetate-soluble portion of *C. sporulosum* broth, by centrifugal TLC on Si gel led to the isolation of two major compounds, coniothyriol and mycosporulone (**1**).[5,6] Mycosporulone (**1**)[6] was identified as (2-hydroxy-6-methylcyclohex-4-en-3-one)-spiro (4′-methylen-5′-oxobutanolide) [$C_{11}H_{10}O_5$]. This paper deals with the determination of optimal culture conditions for the production of **1** and the study of antimicrobial and cytotoxic properties of this compound.



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Results and Discussion

Crude extracts obtained from culture media of different strains of *Coniothyrium* grown in static malt extract cultures incubated for 8 days at 22 °C (previously standardized screening conditions) exhibited great differences in toxicity toward the target pathogenic microorganisms used: *Candida albicans*, *Cryptococcus neoformans*, *Candida tropicalis* R2 amphotericin resistant. *Coniothyrium* spp. strains 1, 2, 3, and 5 were devoid of activity, *Coniothyrium* strain 6 was active only against *C. tropicalis* R2, while *Coniothyrium* 4 and *C. sporulosum* produced extracellular compounds toxic toward the three yeasts tested. The high level of activity obtained with the extract of *C. sporulosum* was an exception when compared to results obtained with extracts of other *Coniothyrium* strains, and more generally, when compared to results obtained for extracts of the *Sphaeropsidales* species from our collection (unpublished results).

C. sporulosum was submitted to a more detailed study concerning the antimicrobial compound produced in its growing medium. As production of an antifungal compound or an antibiotic may vary according to the composition of the growing medium and the culture conditions, *C. sporulosum* was grown in nine different media under shaking and static conditions. Crude extracts of each were tested against *C. tropicalis* R2. The most active extracts were obtained from cultures using malt extract (ME) and potato dextrose media. However, results obtained with the ME medium were more reproducible. Initial pH (range 4−6) and shaking or static conditions did not influence the activity (data not shown).

A time-course study, performed in aerated ME medium, was conducted to determine the best time of culture needed for the highest activity. The evolution of both biomass and pH of the medium during the culture was monitored. Yeast bacterial, and dermatophyte human pathogens were tested as target organisms. To establish the time-course production of **1**, crude extracts from 1- to 12-day old cultures were tested. Antimicrobial activity was detected after 3 days. The highest activity was obtained during idiophase, at low pH value, between 4 and 8 days, while the highest growth (dried biomass) was at 6 days (data not shown).

Large scale production and purification of **1** for detailed studies of its antimicrobial and cytotoxic properties were conducted as previously described.[6] Good reproducibility was obtained, from one preparation to another, concerning the yield of **1** in the crude extract (0.213 ± 0.003 g/g dry mycelium, $n = 10$) and its antimicrobial activity.

The minimum inhibitory concentrations (MIC) of **1** toward several pathogenic bacteria are shown in Table 1. Compound **1** was active against *Escherichia coli*, *Pseudomonas aeruginosa*, and a penicillin-resistant strain of *Sta-*

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Table 1. Minimal Inhibitory Concentrations (MIC) of **1**, Kanamycin, Streptomycin, and Penicillin G toward Pathogenic Bacteria

pathogen	**1**[a]	kanamycin[a]	streptomycin[a]	penicillin G[a]
Escherichia coli	14	28	28	560
Pseudomonas aeruginosa	14	140	140	560
Staphylococcus aureus (r)[b]	14	14	14	560
S. aureus	420	140	420	14
Streptococcus faecalis	280	560	420	14

[a] Results are means of three determinations and are expressed as μg/mL. [b] Resistant to penicillin.

Table 2. Minimal Inhibitory Concentrations (MIC) of **1**, Ketoconazole, Amphotericin B, and Nystatin toward Pathogenic Yeasts

pathogen	**1**[a]	ketoconazole[a]	amphotericin B[a]	nystatin[a]
Candida albicans	14	280	420	14
C. glabrata	420	14	140	28
C. parapsilosis	28	560	560	560
C. tropicalis	14	560	560	14
C. tropicalis R2[b]	14	140	>560	14
Cryptococcus neoformans 1	280	56	140	14
Cr. neoformans 2	14	280	140	14

[a] Results are means of three determinations and are expressed as μg/mL. [b] Resistant to amphotericin.

Table 3. Cytotoxicity (MRC_5) and Antitumor Activity (MDA-MB 231, PC3, L-1210) of **1** as Compared with Taxol and Doxorubicin

compound	MRC_5	MDA-MB 231	PC_3	L-1210
taxol[a]	nd	0.0030 ± 0.0002	0.0027 ± 0.0001	0.0140 ± 0.0001
doxorubicin[a]	nd	0.0900 ± 0.0050	0.1900 ± 0.0010	0.0200 ± 0.0002
1[a]	>100	7.70 ± 0.20	2.60 ± 0.17	0.30 ± 0.01

[a] Results are means of three determinations and are expressed as lethal doses giving 50% of mortality (μg/mL) ± standard deviation.

phylococcus aureus. Compound **1** was compared to a few microbially produced antibacterial platforms: kanamycin, streptomycin, and penicillin G. The bacteria chosen for these tests, especially *P. aeruginosa* and *S. aureus*, belonged to the family of potentially opportunistic germs often responsible for severe human nosocomial infections in various pathological situations. Numerous examples may be cited, including urinary tract infections,[7] pulmonary complications occurring in cystic fibrosis,[8,9] and in HIV infection.[10] The microorganisms involved often become resistant to usual chemotherapy.[9,11,12] Thus, mycosporulone (**1**) is of interest.

Mycosporulone (**1**) was also active against pathogenic yeasts as shown in Table 2. The most sensitive were *Candida albicans*, *C. tropicalis* (sensitive and resistant to amphotericin), one strain of *Cryptococcus neoformans*, and *C. parapsilosis*. On the whole, **1** was more active than ketoconazole and amphotericin B and gave results comparable to those obtained with nystatin, except for *C. glabrata* (less active) and *C. parapsilosis* (most active). On the other hand, **1** and reference standards (ketoconazole, clotrimazole, and nystatin) gave similar results when tested against six common dermatophytes: *Epidermophyton floccosum*, *Microsporum canis*, *Trichophyton mentagrophytes* var. *interdigitale*, *T. mentagrophytes* (2 strains), *T. rubrum*, *T. tonsurans* (data not shown).

The cytotoxicity and antitumor activity of **1** were also evaluated (Table 3). Compound **1** was not toxic to the nontumor line of human lung fibroblasts MRC_5, up to a concentration of 100 μg/mL. To assess potential antitumor activity, **1** was compared to Taxol and Adriamycin, two classical compounds used in chemotherapy of cancer, and was considerably less active. However, for each of the cell lines PC_3 and L-1210, the LD_{50} obtained was below 4 mg/mL, which is the threshold value required by the National Cancer Institute to consider a purified product efficient and interesting.[13] Thus, mycosporulone (**1**), a recently described structure[6] seemingly devoid of excessive toxicity toward normal cells, exhibits interesting potential as an antimicrobial and antitumor compound and should be studied further for therapeutic purposes.

Experimental Section

General Experimental Procedures. TLC was carried out on precoated Si gel $60F_{254}$ plastic sheets (Merck). Separation by centrifugal TLC on Si gel used a Chromatotron apparatus (Harrisson Research). Reversed-phase HPLC was conducted on a Shimadzu apparatus (injector, pump, UV−vis detector and recorder) using a C_{18} column. NMR spectra were recorded with an AC200 Bruker spectrometer. These analyses were conducted as previously described.[6] Cellulose disks used for antimicrobial tests were obtained from Sobioda. The microassay plates were recorded on a multiscanner spectrophotometer (JBio model, LP400). Kanamycin, penicillin G, streptomycin, amphotericin B, ketoconazole, nystatin, clotrimazole, Taxol, and doxorubicin (Adriamycin) were purchased from Sigma Chemical Co.

Fungal Material. *Coniothyrium sporulosum* (W. Gams & Domsch) Van der Aa was isolated from a crab shell from Malmö (Sweden), deposited in our laboratory collection as CMPG (Collection Mycologie Pharmacie Grenoble) 741, and maintained on ME (1.5%) and agar (1.5%) (MEA) medium at 4 °C. The CMPG contains also six additional strains of *Coniothyrium* spp., which were assayed in a preliminary test. Strains 1−3 were isolated from Cormoran grotto (Ain, France); strain 1 was from water, strains 2 and 3 from soil, strain 4 was isolated from an olive tree (Sicily), strain 5 was isolated from a crab shell (Malmö, Sweden; same origin as *C. sporulosum*), strain 6 was isolated from soil from the Dead Sea shore (Israel). To obtain inoculum for studies in liquid media, the fungi were previously grown on MEA in Petri dishes (90 mm in diameter) at 24 °C.

Pathogenic Microorganisms. A panel of microorganisms, potentially pathogenic for humans, was used to assess the antimicrobial activity. The five bacteria used were purchased from the American Type Culture Collection (ATCC): *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 14502 (Gram-negative), *Staphylococcus aureus* penicillin-resistant ATCC 9144, *S. aureus* ATCC 6548P, and *Streptococcus faecalis* ATCC 10541 (Gram-positive). The fungi belonged to the CMPG, they were yeasts: *Candida albicans* CMPG 684, *C. glabrata* CMPG 681, *C. parapsilosis* CMPG 680, *C. tropicalis* CMPG 678, *C. tropicalis* R2 (polyene-resistant strain from Pasteur Institute, France) CMPG 849, *Cryptococcus neoformans* 1 CMPG 682, *C. neoformans* 2 CMPG 683 and filamentous dermatophytes: *Epidermophyton floccosum* CMPG 601,

Microsporum canis CMPG 600, *Trichophyton mentagrophytes* var. *interdigitale* CMPG 599, *T. mentagrophytes* 1 CMPG 598, *T. mentagrophytes* 2 CMPG 705, *T. rubrum* CMPG 597, and *T. tonsurans* CMPG 694. Bacteria were grown on bactocasitone 15 g; soya phytone 5 g, NaCl 5 g, distilled H_2O 1 L, pH 7.2; yeasts, on yeast extract 10 g, peptone 20 g, glucose 15 g, distilled H_2O 1 L, pH 6.0; other fungi were grown on ME medium. For solid media, agar (1.5%) was added. The media were sterilized by autoclaving (120 °C, 20 min).

Mycosporulone Preparation Process. In preliminary tests, a culture plate was used for each strain of *Coniothyrium*. Colonies (mycelium and spores) were inoculated in 70 mL of liquid ME (1.5%) medium in 250-mL Erlenmeyer flasks. Cultures were incubated for 8 days under static conditions at 24 °C. Each assay was performed in triplicate. *C. sporulosum* was then grown in nine different liquid media. The fungus (mycelium and spores) was inoculated in 250-mL Erlenmeyer flasks, containing 70 mL of the following media: ME or potato dextrose (Difco), used either at their normal pH (= 6) or adjusted to pH 4; saccharose (4%)−yeast extract (2%) pH 6.5 medium; synthetic medium of Galzy and Slonimski[14]-glucose (1%) added with or without corn steep liquor (1%); Sabouraud dextrose (Difco); Tryptone soya (Difco). Cultures were run under static and shake conditions at 24 °C, respectively, for 8 and 5 days. Media were separated from the mycelia by filtration and extracted by 2 × 35 mL EtOAc. Organic phases were pooled and dried at 40 °C under reduced pressure. Crude extracts were dissolved in 3 mL EtOAc. A time-course study of the appearance of the antimicrobial activity in crude extract was run in aerated ME medium (3-L fermentors) at 24 °C for 1 to 12 days. Large-scale production, isolation, and purification procedures were as previously described.[6] The fermentors used were 10 L. Cultures were run for 4 days at 24 °C, and purified mycosporulone (**1**) was dissolved in EtOAc. The product was monitored by TLC and HPLC and was identified by MS and ^{1}H and ^{13}C NMR spectra.[6]

Antimicrobial Activity. Assessment of antimicrobial activity of crude extracts in EtOAc was performed on solid media using the disk (diameter, 6 mm; 50 mL of crude extract) diffusion method previously described.[15−17] Inhibition diameters were recorded after 24−72 h of incubation, depending on the target microorganism assayed. For purified mycosporulone (**1**), the determination of minimal lethal concentration (MLC) and minimal inhibitory concentration (MIC) for bacteria, yeasts, and filamentous dermatophytes was conducted using microtitration plates[16] and antimicrobial reference compounds as controls (kanamycin, penicillin G, and streptomycin for bacteria; amphotericin B, ketoconazole, and nystatin for yeasts; clotrimazole, ketoconazole, and nystatin for dermatophytes); 96-well microtest plates were prepared by dispensing 100 μL of liquid medium into each well. Calibrated suspensions (10^6 CFU/mL) of target strains were uniformly added (20 μL). MeOH solution of mycosporulone (10 μL) was added in increasing concentrations by a progressive ratio of 2 (final concentration: 14 to 560 μg/mL). Medium blanks, positive controls, and MeOH controls were run simultaneously. Each assay was carried out in triplicate. Microplates were incubated at 24 °C under shaking (200 rpm) for 72 h. Growth was followed by recording the microassay plates every 12 h at 620 nm. MLC was the first concentration for which microorganisms did not grow when they were transferred into a medium devoid of the tested compound (MLC 100%).

Cytotoxicity Test. Cytotoxicity was researched on MRC_5 cell line (ATCC CCL 171, Flow Laboratories, Uxbridge, Great Britain), using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) modified colorimetric micromethod of Mossmann.[18] The test was run in 96-well microtest plates as previously described,[19] with a maximal concentration of 100 μg/mL. For antitumor activity, cell culture conditions and evaluation of the inhibition of L-1210 murine leukemia cells, tumor clonogenic human cells (MDA-MB 231), and PC_3 cells from human prostatic adenocarcinoma[20] were as previously described,[4,21] using the colorimetric method derived by Mossmann.[18] In these tests, Taxol and doxorubicin were used as references. Cells were incubated 5 days with the products. Results were expressed as lethal doses giving 50% of mortality (LD_{50}).

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