Glisoprenins C, D and E, New Inhibitors of Appressorium Formation in *Magnaporthe grisea*, from Cultures of *Gliocladium roseum*

1. Production and Biological Activities

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Glisoprenins C, D, and E, new glisoprenin derivatives, were isolated together with glisoprenin A from submerged cultures of the deuteromycete *Gliocladium roseum* HA190-95. All glisoprenins inhibited appressorium formation in *Magnaporthe grisea* on inductive (hydrophobic) surfaces. The compounds exhibited moderate cytotoxic, but no antifungal, antibacterial or phytotoxic activities.

Blast disease, caused by the ascomycete *Magnaporthe grisea* (anamorph *Pyricularia grisea*) is considered to be the principal fungal disease of rice (*Oryza sativa*)[1]. Prerequisite for a successful infection of the plants is the development of a highly melanized appressorium. Its formation occurs in response to environmental stimuli, such as surface hardness[2] and hydrophobicity[3]. Under laboratory conditions, differentiation to infective structures can be observed on the hydrophobic surface of parafilm or GelBond sheets. Cutin monomers[4], wax compounds[5] or cAMP[6] can induce appressorium formation on hydrophilic surfaces. The signal transduction leading to differentiation is still poorly understood. Disruption of cAMP dependent protein kinase A in *M. grisea* resulted in mutants unable to form appressoria[7]. MAP kinase was reported to be essential for appressorium formation and successful infection of the plants[8] and *MPG1*, a gene coding for a hydrophobin, is expressed at high levels during appressorium formation[9]. In *MPG1*- mutants a low level of appressorium formation occurred and could be suppressed by yeast extract indicating that additional factors play a role[10].

Inhibitors of signal transduction might not only be valuable tools in investigations on mechanisms and pathways leading to appressorium formation and revealing new targets, such compounds might also be of great interest as potential plant protectants. A test system following appressorium formation on an inductive surface was used to screen higher fungi for the production of inhibitors. Extracts from submerged cultures of *Gliocladium roseum*, HA190-95, were found to inhibit appressorium formation on the inductive surface of a GelBond sheet. Bioactivity-guided isolation yielded glisoprenins A, C, D and E as inhibitors. Glisoprenin A had been isolated before from cultures of a *Gliocladium* species as an inhibitor of acyl-CoA : cholesterol acyltransferase[11,12], while glisoprenins C, D and E are new fungal secondary metabolites. In this report we describe the fermentation, isolation and biological activities of glisoprenins A, C, D, and E. The structure determination will be described in a second paper[13].

Materials and Methods

General

Materials used for preparative HPLC were obtained from Jasco. Preparative HPLC was performed with a Jasco PU980 equipped with a Jasco MD910 diode array detector. Analytical HPLC was carried out on a Hewlett-Packard HP 1090 Type II equipped with a diode array detector. Malt extract was purchased from Kirner

Vitaborn Werk, Kirn, Bacto yeast extract from Difco Lab., Detroit, and glucose from Deutsche Maizenawerke, Hamburg.

Producing Organism

The strain HA190-95 was isolated from a decaying fruiting body of a basidiomycete. The culture is grown at 25°C on YMG agar containing (g/liter): glucose 4, yeast extract 4, malt extract 20, agar 20 and kept as a lyophilisate in the strain collection of the Department of Biotechnology, University of Kaiserslautern.

Fermentation

Fermentations were carried out in 20 liters of malt medium (malt extract 40 g/liter tap water) in a Braun Biostat U appartus (Braun, Melsungen) at 24°C with an aeration rate of 3 liters/minute and agitation (120 rpm). As inoculum, a well grown culture (200 ml in a 500 ml Erlenmeyer flask incubated on a rotary shaker with agitation at 120 rpm at 22°C) in the same medium was used. The production of the biologically active compounds was followed using the test system for appressoria development described below. Aliquots of the culture fluid were extracted twice with ethyl acetate. The combined extracts were dried with Na_2SO_4. After evaporation of the solvent *in vacuo* (40°C), the oily residue was dissolved in 1 ml methanol. For HPLC analysis 10 μl of the methanolic solution were used.

Biological Assays

Antimicrobial activity was determined in the plate diffusion assay as described previously[14]. Cytotoxic activities against L1210 cells (ATCC CCL 219), HL60 cells (ATCC CCL 240), COS-7 (ATCC CRL 1651) and B16-F1 cells (ATCC CRL 6323) were determined according to ZAPF *et al.*[15]. Inhibition of growth of germinated seeds of *Setaria italica* and *Lepidium sativum* was tested as described by ANKE *et al.*[14]. Nematicidal activity was measured as described by STADLER *et al.*[16].

Inhibition of Appressorium Development

The strains of *M. grisea* used in this study were obtained from Dr. B. SPEAKMAN, BASF AG, Ludwigshafen. The strains were grown on oatmeal agar at 24°C. Sporulation was induced under fluorescent light. Conidia were harvested from 10- to 14-day old cultures. After washing, the conidia were suspended in sterile water to give a final concentration of 1.25×10^5 per ml. Inhibition of appressorium formation was followed on the hydrophobic side of GelBond gel-electrophoresis sheets (Sigma, St. Louis) or in 24-well microtiter plates (hydrophilic surface) (Sarstedt, Nürnbrecht). GelBond sheets were washed in sterile water for 30 minutes. Pieces of the sheets were placed hydrophobic side up in a microtiter plate. Forty μl of the conidia suspension were added to the hydrophobic surface. After one hour, sterile water (0.96 ml) was carefully added. Extracts and chemicals (inducers) were added dissolved in methanol or ethanol to give a final solvent concentration of less than 1%. At this concentration no effects on germination, mycelial growth or appressoria development were observed. The plates were incubated at 22°C for 20 hours. Appressorium formation on a non inductive (hydrophilic) surface was induced with 8-(4-chlorophenylthio)-adenosine-3',5'-monophosphate (chlorophenylthio-cAMP, Boehringer Mannheim, Mannheim), 1,16-hexadecanediol (Aldrich, Steinbronn) or IBMX (3-isobutyl-1-methylxanthine, Sigma, St. Louis). Experiments were carried out in triplicate with 300 germinated conidia microscopically evaluated in each test.

Results and Discussion

Taxonomy of the Producing Organism

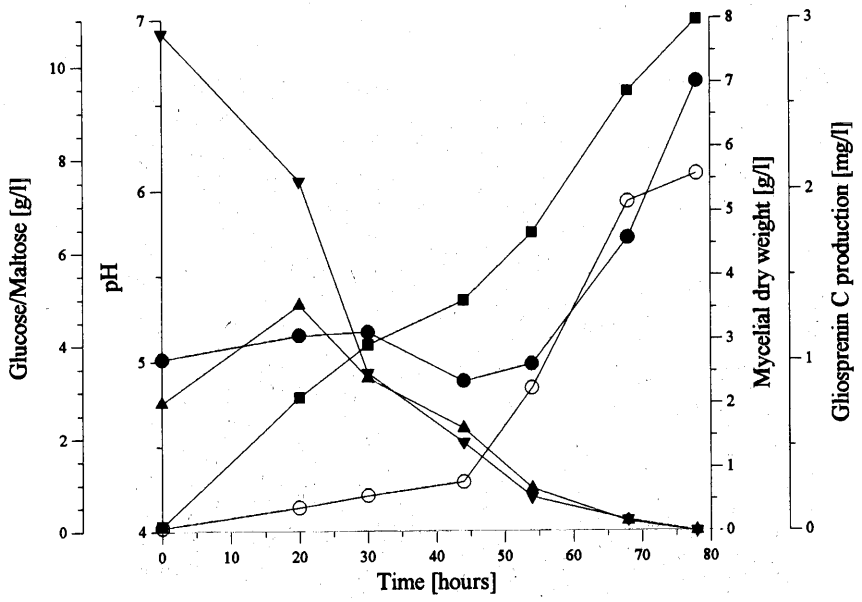
The producing organism formed white to yellowish brown colonies on the agar medium. The reverse was yellow. The colony surface was granular to felty. Two kinds of erect conidiophores were found: Primary conidiophores with divergent *Verticillium*-like branches, phialides, 25×2.5 μm, in whorls of four to five with discrete heads and densely penicillate secondary conidiophores with phialides, $12 \sim 20 \times 2.5$ μm, in whorls of three to seven. On penicillate conidiophores, conidia were produced in slimy masses and on verticillate ones in thick, short columns. Conidia were elongate, slightly asymmetrical, smooth-walled and $4.5 \sim 6 \times 2.5 \sim 3.5$ μm in size. The mycophilic habitat and the microscopical descriptions fit those for *Gliocladium roseum*[17].

Fermentation and Isolation of Glisoprenins A, C, D, and E

A fermentation diagram of *G. roseum* is shown in Fig. 1. Maltose was immediately consumed and its hydrolytic cleavage resulted in an increase of the glucose concentration; after 20 hours both carbon sources were consumed simultaneously. The production of glisoprenins started slowly and after 45 hours when the pH had reached its minimum, the production of glisoprenin C drastically increased until the carbon sources were completely used up. The amounts of glisoprenins A, D

Fig. 1. Fermentation of *Gliocladium roseum* HA190-95 in malt medium in a 20-liter fermenter.

● pH, ▲ Glucose, ▼ Maltose, ○ Gliosprenin C production, ■ Mycelial dry weight.



and E were too low to be followed during fermentation. The fermentation was stopped after 78 hours. The culture fluid (19 liters) was separated from the mycelia by filtration. Extraction of the culture fluid with 6 liters of ethyl acetate yielded 490 mg crude product. Chromatography on silica gel 60 (25~40 μm; column size 120 × 25 mm) in cyclohexane - methanol (1 : 1) and elution with methanol resulted in 89.5 mg of intermediate product. From this intermediate product glisoprenins A, C, D and E were obtained by HPLC (LiChrosorb, 7 μm; 250 × 25 mm; flow rate 5 ml/minute) with a water - methanol gradient (0~30 minutes: 0~50%; 30~95 minutes: 50~100%; 95~120 minutes 100%). The retention times were as follows: glisoprenin E 70 minutes, glisoprenin D 76 minutes, glisoprenin C 88 minutes and glisoprenin A 98 minutes. The isolation yielded 2.6 mg glisoprenin A, 12.7 mg glisoprenin C, 3.5 mg glisoprenin D and 2 mg glisoprenin E. Whereas glisoprenins A and B were obtained from the mycelial extracts of a *Gliocladium* species[11], mycelia of our strain contained no active compounds and therefore were discarded.

Biological Properties

Fig. 2 shows appressoria formed on an inductive surface (A) and mycelial growth on the same surface in the presence of glisoprenin C. Table 1 shows the effects of glisoprenins A and C on appressorium formation on the hydrophobic (inductive) side of a GelBond sheet in four *M. grisea* strains. Both compounds were equally effective towards all four strains. When the effects of all glisoprenins on appressorium formation under inductive and non inductive conditions were compared, data given in Table 2, it was found that glisoprenin E was ten times less active. As previously reported for glisoprenin A[18], none of the compounds affected the induction of infectious structure formation by 1,16-hexadecanediol or chlorophenyltio-cAMP. The germination of conidia and mycelial growth of *M. grisea* was not inhibited by any of the compounds at concentrations up to 100 μg/ml. The inhibition of appressorium formation on hydrophobic surfaces by the glisoprenins was not overcome by the addition of 1,16-hexadecanediol or chlorphenylthio-cAMP. Even 10 fold higher concentrations as compared to those inducing infectious structures formation on hydrophilic surface were completely ineffective.

In the agar diffusion assay with 100 μg/disc no antimicrobial activity was observed against *Paecilomyces variotii*, *Nematospora coryli*, *M. grisea*, *Mucor miehei*, *Penicillium notatum*, *Bacillus brevis*, *Bacillus subtilis* or *Enterobacter dissolvens*. None of the compounds exhibited nematicidal activity towards *Caenorhabditis elegans* or *Meloidogyne incognita*. Glisoprenins A, C, and D were moderately cytotoxic as shown in Table 3. At 25~50 μg/ml 50% of the cells were lysed. Glisoprenin E was not cytotoxic at 50 μg/ml, higher concentrations were

Fig. 2. Effect of glisoprenin C on appressorium formation in germinating conidia of *M. grisea* on the hydrophobic surface of a gelBond sheet.

A: Appressorium formation on inductive surface. B: Inhibition of appressorium formation in the presence of 5 μg/ml of glisoprenin C (Pictures were taken with an Olympus ITM-2 microscope equipped with a camera).

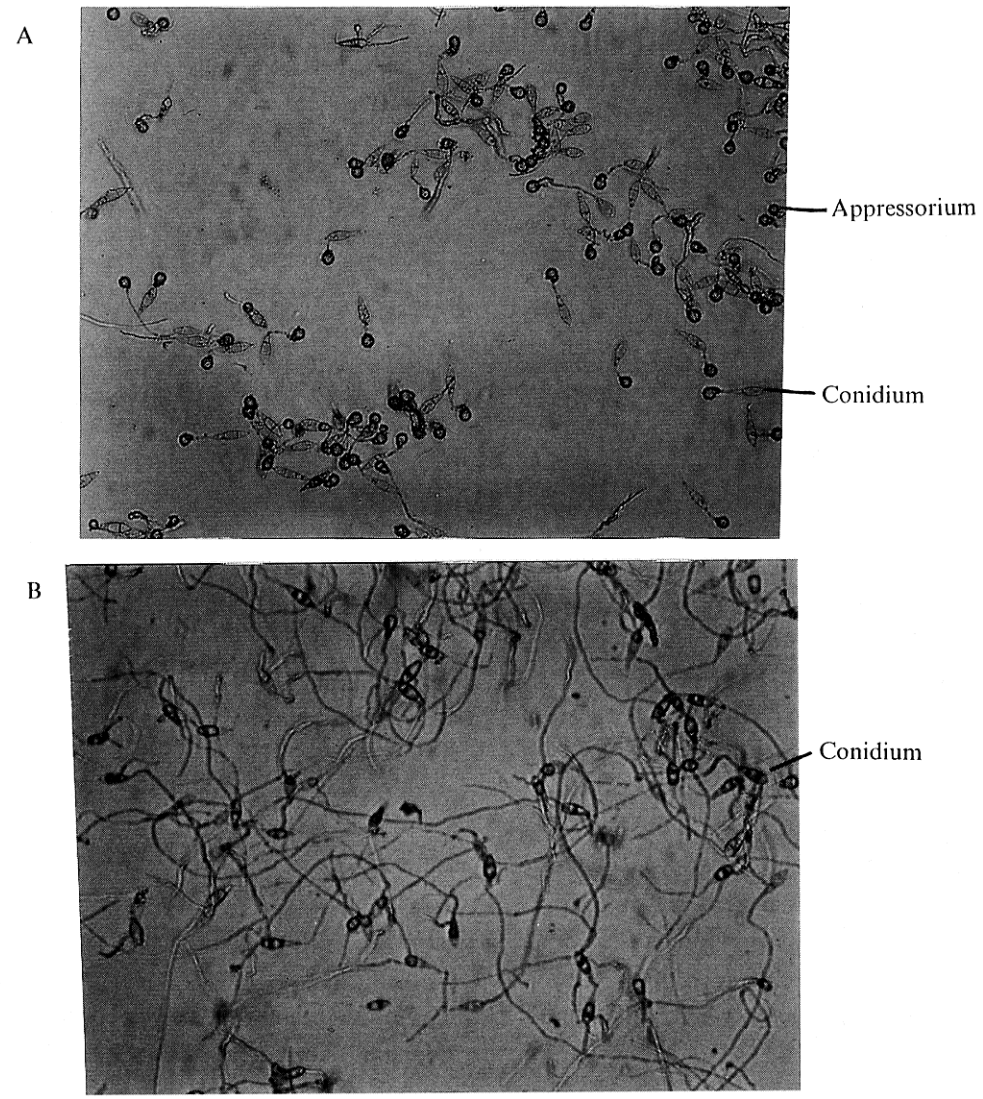


Table 1. Effects of glisoprenins A and C on appressorium formation in *Magnaporthe grisea* test strains on the hydrophobic surface of a GelBond sheet.

Strain	Appressorium formation (%)						
		Glisoprenin A			Glisoprenin C		
	0	2	5	10	2	5	10 (μg/ml)
M. grisea BASF Taiwan	98±1.6	50±4.2	17±4.1	0	51±3.8	9±3.9	1
M. grisea BASF Hoechst	97±2.4	49±1.9	9±5.2	1±0.5	53±1.9	11±5.5	1±0.7
M. grisea BASF Spain V6	98±1.7	47±5.9	6±2.9	2±1.0	50±4.4	9±4.9	2±1.0
M. grisea BASF Spain V4	97±3.2	50±3.5	7±2.7	1	53±2.2	8±3.7	1

Table 2. Effects of gliosprenins on appressorium formation in *M. grisea* (strain Taiwan) on different surfaces and with different inducers.

Surface	Glisoprenin A AIC_{50}	Glisoprenin C AIC_{50}	Glisoprenin D AIC_{50}	Glisoprenin E AIC_{50} (μg/ml)
GelBond[a]	2	2	2	25
Parafilm M[a]	2	2	2	25
Hydrophilic surface[b]	>100	>100	>100	>100
Hydrophilic surface[c]	>100	>100	>100	>100
Hydrophilic surface[d]	>100	>100	>100	>100

The concentration at which 50 ($\pm$5)% of the conidia formed an appressorium was determined as AIC_{50}. In the controls more than 95% of the germinated conidia formed appressoria.

[a] Induction on the hydrophobic surface of a GelBond sheet or on Parafilm (either side).

[b] Induction with 100 ng/ml 1,16-hexadecanediol in a microtiter plate.

[c] Induction with 20 μg/ml chlorophenylthio-cAMP in a microtiter plate.

[d] Induction with 100 μg/ml IBMX in a microtiter plate.

Table 3. Cytotoxicities of glisoprenins A, C and E against different mammalian cell lines.

Cell line	Glisoprenin A IC_{50}	Glisoprenin C IC_{50}	Glisoprenin D IC_{50} (μg/ml)
L1210	10~25	10~25	25~50
HL-60	25~50	25~50	25~50
COS-7	10~25	25~50	10~25
B16-F1	25~50	25~50	25~50

not tested due to the small amounts available.

The effects of glisoprenins on appressorium formation in other pathogenic fungi and the exact mode of action during signal transduction leading to appressorium formation remain to be elucidated. Work on the antagonistic effects between second messengers of eukaryotic signal transduction and glisoprenins is currently carried out in our lab[19].

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