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ORIGINAL ARTICLE

Caripyrin, a new inhibitor of infection-related morphogenesis in the rice blast fungus *Magnaporthe oryzae*

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Caripyrin (*trans*-5-(3-methyloxiranyl)pyridincarboxylic acid methyl ester, 1), a new pyridyloxirane, was isolated from submerged cultures of the basidiomycete *Caripia montagnei*. The compound was found to inhibit conidial germination and appressorium formation in the rice blast fungus *Magnaporthe oryzae*, whereas the infection-related morphogenesis in several other phytopathogenic fungi was not affected. In plant assays on rice, 1 was found to protect plants more efficiently against fungal infection than the structurally related fungal secondary metabolite, fusaric acid. Contrary to the latter, 1 was neither cytotoxic, antibacterial, nor nematicidal.

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INTRODUCTION

Crop losses world wide due to plant diseases are estimated to account for almost 20% of the major food and cash crops produced.[1] Among the plant diseases caused by fungal pathogens, rice blast, caused by *Magnaporthe oryzae*, is the most serious disease that infects cultivated rice and therefore a threat to the world's most important food security crop.[2] To enter and colonize the host plant, *M. oryzae* displays a remarkable morphological and physiological specialization. During the prepenetration phase, the germ tube differentiates into a melanized dome-shaped appressorium after attachment to the leaf surface and germination of the spore. This infection structure is an essential prerequisite for a successful infection, allowing the fungus to penetrate the plant cuticle in a direct manner by mechanical force.[3] Therefore, an intact melanin layer is essential for appressorial turgor generation. Melanin-deficient mutants, for example, *ALB1*, *BUF1* and *RSY1*, fail to generate appressorial turgor and are nonpathogenic.[4]

In *M. oryzae*, tetrahydroxynaphthalene or trihydroxynaphthalene reductases are targets for the successful and widely used plant protectants, such as tricyclazole and carpropamid.[5] Inhibitors of melanin biosynthesis are excellent examples for plant protectants interfering with the infection-related morphogenesis but not with vegetative growth.

To control rice blast, quinone outside inhibiting fungicides are increasingly applied in Japan. Such quinone outside inhibiting fungicides are threatened by resistance development in target pathogens.[6] As *M. oryzae* is considered as a 'high-risk' pathogen in terms of resistance to quinone outside inhibiting fungicides, inhibition of targets, essential for differentiation processes and pathogenicity, provides interesting alternatives for plant protection strategies.

Fungi are known to be a rich source for inhibitors of the infection-related morphogenesis in phytopathogenic fungi. In this paper, we describe the producing organism, its cultivation and isolation of caripyrin (*trans*-5-(3-methyloxiranyl)pyridincarboxylic acid methyl ester **1**), a new pyridyloxirane and its biological activities.

MATERIALS AND METHODS

Microorganisms

Producing organism. *Caripia montagnei* IBWF-A24-2006 was isolated from spore prints of fruiting bodies growing on decaying wood collected in French Guiana. Methods and reagents for DNA extraction and PCR amplification of the internal transcribed spacer region 1 (primers ITS1F and ITS4B) and the 5′ end of the 18S ribosomal RNA gene were as described previously.[7]

The strain was maintained at 22 °C on yeast malt glucose (YMG) medium composed of yeast extract 4 g (Hartge Incredients, Hamburg, Germany), malt extract 10 g (Fränkle & Eck, Fellbach, Germany), glucose 4 g and agar 20 g per 1 l tap H_2O. The pH was adjusted with 1 N HCl to 5.5. Mycelial cultures of *C. montagnei* are deposited in the culture collection of the Institute of Biotechnology and Drug Research (IBWF e.V.).

Test organisms. *M. oryzae* strain 70-15 was obtained from the Fungal Genetics Stock Centre, Kansas City, KS, USA. The strain was maintained on CM medium as described.[2] *Fusarium graminearum*, *Botrytis cinerea* and *Phytophthora infestans* were provided by BASF SE.

Fermentation and isolation

Fermentations were carried out in YMG medium in a 20 l fermentor (Biostat A-20, Braun Melsungen, Melsungen, Germany) at 28 °C with aeration (5.0 l min^{-1}) and agitation (150 r.p.m.). A well-grown culture (1 l) in the same medium was used as inoculum. During fermentation, samples were taken daily

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to monitor the appearance of **1** in the broth by analytical HPLC as described below. After 16 days of fermentation, when the concentration of **1** had reached maximum and the glucose in the medium was consumed, the culture fluid (8 l) was separated from the mycelia and extracted with an equal volume of ethyl acetate. The mycelium containing no active compounds was discarded. The organic extract was dried with Na_2SO_4 and concentrated to yield 1.2 g of crude product, which was further purified by silica gel chromatography (silica gel 60, 63–200 µm, 40 g, column size 24×4.5 cm; Merck, Darmstadt, Germany). Elution with cyclohexane–ethyl acetate (7:3) yielded 190 mg of an intermediate product. Final purification was achieved by HPLC using a Nucleosil 100–5 C18 column (21×250 mm, 5 µm; Merck). Elution was carried out with 32% MeCN in H_2O at a flow rate of 15 ml min^{-1}. HPLC was performed in a preparative Jasco modular HPLC system (Jasco, Gross-Umstadt, Germany) consisting of two binary pumps (PU-1586) and a multi-wavelength detector UV-1570M. HPLC for daily samples was performed on an Agilent 1100 Series (Agilent Technologies, Waldbronn, Germany) with a LiChrospher 100 C18 column (125×4 mm, 5 µm, Merck).

Biological assays

Inhibition of conidial germination and appressorium formation. Conidia from *M. oryzae* were harvested by centrifugation (1000 *g* min^{-1}) and suspended in distilled H_2O to a concentration of 5×10^5 conidia per ml. Assays were carried out in 24-well microtiter plates (Sarstedt, Nuremberg, Germany) with 2.5×10^4 conidia per ml of distilled H_2O. After 16 h incubation at 28 °C, germinated conidia were counted using an inverted microscope (Leica DM IRB, Leica, Wetzlar, Germany). The tests were carried out in triplicates and 300 conidia were counted.

Nematicidal activity against *Caenorhabditis elegans* and cytotoxic activity against Hep G2-cells was assessed as described previously.[7,8] Antibacterial activity against *Bacillus brevis*, *B. subtilis*, *Micrococcus luteus* and *Enterobacter dissolvens* was determined in a serial dilution assay as described previously.[9]

To test the protective effect of **1** and fusaric acid (**2**), 10 ml of the solution with 5×10^4 spores per ml, 0,2% gelatine and the respective compound were sprayed on 21-day-old plants of *Oryza sativa* CO-39. For this assay, concentrations between 10 and 100 µg ml^{-1} were used for **1** and **2**. The treated plants were incubated for 24 h at 27 °C and 80% humidity in plastic bags (Rotilabo-Kordelzugbeutel, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) in an environmental test chamber (Versatile Environmental Test Chamber MLR-350H, Sanyo Electric, Munich, Germany). Then, the plants were taken, sprayed with H_2O and incubated for another 72 h under the same conditions. The assay was evaluated by counting the lesions per plant and compared with the control after a total of 96 h. In addition, a leaf segment assay was conducted as described previously.[8]

Phytotoxic activity

The phytotoxicity was monitored using a droplet assay with leaves of 28-day-old *O. sativa* CO-39. Leaves were fixed on agar plates and inoculated with droplets of 9 µl of sterile gelatine (0.2%) solution and 1 µl of MeOH and **1** or **2** in different concentrations. Toxicity was evaluated after 7 days under the microscope and compared with the control containing MeOH.

Toxicity on seeds was measured using a germination assay with seeds of *O. sativa* CO-39, *Lepidium sativum* and *Triticum aestivum* RIBAND. Seeds were grown in sterile 24-well plates on filter papers with 5 and 50 µg of **1** or **2** in 500 µl H_2O. Toxic effects on *T. aestivum* and *O. sativa* were evaluated after 8 days at 27 °C by measuring the length of sprouts and roots compared with the control grown in H_2O. Inhibitory activity against germinating seed of *L. sativum* and *Setaria italica* was assessed after 3-day growth in darkness and 1 day in light at 27 °C.

Inhibition of respiration

Inhibition of mitochondrial respiration, that is, oxygen-uptake from oxygen-saturated cell suspensions, was measured polarographically with a Clark electrode. The assay was carried out as described by Strathkelvin Instruments with an Oxigen MeterModel 782 (Fa. Strathkelvin Instruments, North Lanarkshire, Scotland). Strobilurin A was used as a positive control. The compounds were tested up to a concentration of 100 µg ml^{-1} with conidia of *M. oryzae*.

General methods

The melting point was determined using a Dr Tottoli apparatus (Büchi AG, Flawil, Switzerland) and is uncorrected. The optical rotation was measured with a Krüss P8000 polarimeter (KRÜSS Optronic GmbH, Hamburg, Germany) at 589 nm. UV and IR spectra were measured with a Perkin-Elmer Lambda-16 spectrophotometer (Perkin-Elmer, Waltham, MA, USA) and a Bruker IFS48 FTIR spectrometer (Bruker, Ettlingen, Germany), respectively. NMR spectra were recorded with a Bruker AMX 400 spectrometer. The spectra were measured in $CDCl_3$ and the chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H=7.26 p.p.m., δ_C=77.16 p.p.m.[10]). Electrospray ionization (ESI)-MS spectra were recorded on a Finnigan Mat95 spectrometer (Finnigan MAT GmbH, Bremen, Germany). ESI-high-resolution mass spectra were recorded on a MicroMass/Waters ESI Q-TOF mass spectrometer (MicroMass/Waters, Milford, MA, USA) equipped with a LockSpray interface using NaI/CsI or trialkylamines as external reference.

Caripyrin A

Cream-colored solid, m.p. 43–45 °C. $[\alpha]_D^{25}$ +25.6 (*c* 0.75, $CDCl_3$). UV λ_{max}^{MeOH} (log ε) 223 (sh, 3.83) nm, 236 (3.98) nm, 271 (3.70) nm and 278 (sh, 3.42) nm. IR ν_{max} (KBr) (cm^{-1}) 3442, 2956, 1725, 1438, 1312, 1024 and 709. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.67 (d, *J*=2.2 Hz, 6-H), 8.10 (d, *J*=8.1 Hz, 3-H), 7.67 (dd, *J*=8.1, 2.2 Hz, 4-H), 4.00 (s, OCH_3), 3.68 (d, *J*=1.9 Hz, 2′-H), 3.06 (qd, *J*=5.1, 1.9 Hz, 3′-H) and 1.50 (d, *J*=5.1 Hz, 3′-CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.6 (C=O), 147.9 (C-6), 147.7 (C-2), 137.6 (C-5), 133.9 (C-4), 125.1 (C-3), 59.8 (C-3′), 57.0 (C-2′), 53.1 (OCH_3) and 18.0 (3′-CH3). ESI-MS *m/z* 193.9 (10) $[M+H]^+$, 215.9 (100) $[M+Na]^+$, 231.9 (42) $[M+K]^+$ and 409.0 (23) $[2M+Na]^+$. ESI-HRMS *m/z* 216.0631 (calcd for $C_{10}H_{11}NO_3+Na^+$ 216.0637).

RESULTS

Producing organism

The fruiting bodies of *C. montagnei* IBWF-A24-2006 showed all morphological characteristics of the genus and species as described.[11] The internal transcribed spacer region 1 sequence of the 5.8S ribosomal RNA gene was identical to the sequence published for *C. montagnei* in the GenBank.[12]

Fermentation of *C. montagnei* IBWF-A24-2006 and isolation of 1

To optimize fermentation conditions for the biosynthesis of bioactive compounds, strain IBWF-A24-2006 was cultured in several media. The highest production rate for the bioactive constituent was found in YMG medium at high agitation (150 r.p.m.). At lower agitation rates, the fungus failed to produce **1** in submerged culture. A fermentation diagram for a submerged culture of *C. montagnei* is shown in Figure 1. After ~4–5 days of fermentation the production of **1** started while glucose and to a lower extent maltose were consumed. Fermentations were stopped when the glucose in the medium was consumed and the production rate had reached its maximum. Bioactivity-guided isolation led to the isolation of 10.1 mg of a novel compound, which we named caripyrin (**1**).

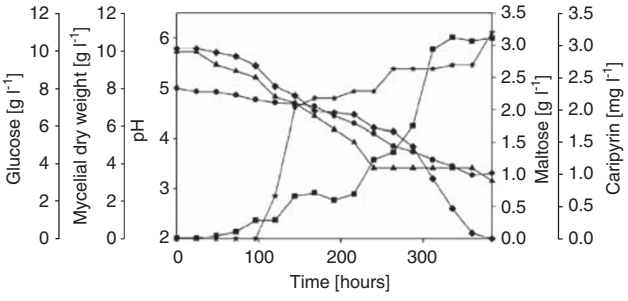

Figure 1 Fermentation of caripyrin-producing strain *Caripia montagnei* IBWF-A24-2006 in yeast malt glucose medium. —●— pH; —■— mycelial dry weight; —◆— glucose; —▲— maltose; —★— caripyrin.

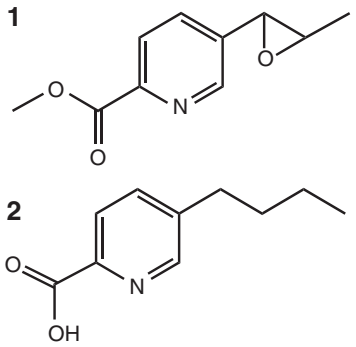

Figure 2 Structure of caripyrin (**1**) and fusaric acid (**2**).

Structure elucidation

Mass spectrometric data gave an elemental composition of $C_{10}H_{11}NO_3$, requiring five double bond equivalents. The ^{13}C NMR spectrum indicated three methine carbons at 147.9, 133.9 and 125.1 p.p.m. along with two quaternary carbons at 147.7 and 137.6 p.p.m. The HETCOR showed that the protons corresponding to these methine carbons resonated at downfield chemical shifts. These data strongly suggested a 2,5-disubstituted pyridine core, with an acceptor group bound to C-2. This substituent proved to be a methyl ester, apparent from a carbonyl group at 165.6 p.p.m. and a methoxy group at 53.1 p.p.m. (similar to methyl fusarate).[13]

The substituent at C-5 was a propyl chain with two downfield-shifted carbons, and the characteristic coupling constant between the two methine protons of 1.9 Hz indicated the presence of a *trans*-configured epoxide. The diol formally resulting from hydrolysis of **1** has been described as CJ-14877 from *Marasmiellus* sp.,[14] whereas to the best of our knowledge, the only example of an oxirane-substituted pyridine of natural origin is epoxy-6,7-racemigerine, which was isolated from *Scaevola racemigera*.[15] Figure 2 gives the structure of **1** and **2**.

Biological activity of 1

In a screening for inhibitors of infection-related differentiation processes in *M. oryzae*, extracts of the basidiomycete *C. montagnei* IBWF-A24-2006 were found to interfere with conidial germination. Compound **1** isolated from these extracts was found to inhibit conidial germination in the rice blast fungus, whereas differentiation processes in *B. cinerea*, *P. infestans* and *F. graminearum* were not affected up to 100 μg ml^{-1}. In contrast, the structurally closely related fungal metabolite **2** interfered with spore germination in *P. infestans* and *F. graminearum*. The results of the spore germination assays are summarized in Table 1.

Caripyrin (**1**) prevented germination of *M. oryzae* conidia more efficiently than **2**. In total, 90% of the *M. oryzae* conidia failed to germinate at 5 μg ml^{-1} of **1**, whereas 50 μg ml^{-1} of **2** was needed for a comparable level of inhibition. In contrast, **2** was found to inhibit spore germination in *F. graminearum* and, more efficiently, in *P. infestans*.

Interestingly, conidial germination in *M. oryzae* in CM medium was not affected by **1**. Under these conditions, **1** was ineffective even at concentrations exceeding the inhibitory concentration in H_2O. However, conidial germination in *M. oryzae* in H_2O on treatment with **1** was not restored by addition of the constituents of CM medium such as peptone, glucose, yeast extract, nitrate salts or trace element solution. Addition of single amino acids such as L-cysteine did not reduce the biological activity of **1** in equimolar concentrations (data not shown).

Table 1 Inhibitory effect of caripyrin and fusaric acid (ED, μg ml^{-1}) on conidial germination and vegetative growth of sprout and root of plant seed

	Caripyrin		*Fusaric acid*	
Test organism	ED_{50}	ED_{100}	ED_{50}	ED_{100}
Plants				
Lepidium sativum	5	>50	<5	<5
Oryza sativa	5	>50	5	>50
Triticum aestivum	>50	>50	<50	>50
Fungi				
Fusarium graminearum	>100	>100	>50	100
Magnaporthe grisea	<5	10	20	40
Phytophthora infestans	>100	>100	>10	20

Abbreviation: ED, effective dose.

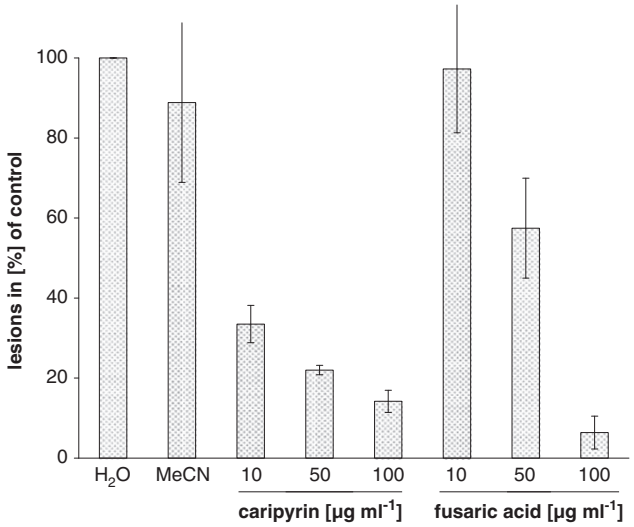

Figure 3 Protective effect of caripyrin and fusaric acid on *O. sativa* against the phytopathogenic fungus *M. oryzae*.

Plant protective activity in spray tests

To investigate the plant protective potential of **1** in comparison with **2**, rice plants were sprayed with conidia of *M. oryzae* and the compounds. As shown in Figure 3, **1** was more effective than **2**. Treatment with a 10 μg ml^{-1} solution of **1** (10 ml) reduced the number of lesions to 50%, whereas application of 10 ml of a 50 μg ml^{-1} solution reduced the number of disease symptoms by 80%. However, total protection was not observed. The same amount of **2** was hardly effective.

Phytotoxic activities toward *O. sativa*, *L. sativum* and *T. aestivum* were recorded for both compounds. As shown in Figure 4, at 50 μg ml^{-1} root length in *O. sativa* was reduced by 60% when exposed to caripyin (**1**) and by 90% when treated with **2**. The sprout length was less affected at the same concentrations. A reduction in length of 30% was observed when treated with **1** and 50% when incubated with **2**. Effects on growth of *L. sativum* and *T. aestivum* were similar to those observed for rice (data not shown).

Further biological activities

In a cytotoxicity assay with *HepG2* cells, **1** did not show toxic activity at a concentration of 100 μg ml^{-1}.[16] At the same concentration, no antibacterial activity against *B. brevis*, *B. subtilis*, *M. luteus* and *E. dissolvens* was observed in a serial dilution assay for both

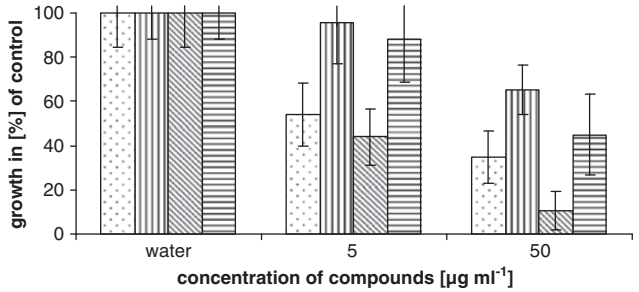

Figure 4 Growth of root ⬚▨ and sprout ▥▤ of *O. sativa* when treated with caripyrin (**1**) ⬚▥ and fusaric acid (**2**) ▨▤.

compounds. In contrast to **2**,[17] **1** was not found to inhibit respiration in *M. oryzae*, in conidia or in mycelia. Furthermore, no nematicidal activity toward *C. elegans* was found.

DISCUSSION

Conidial germination is an initial step in the infection process of many phytopathogenic fungi and subsequently essential for a successful colonization of host plants. Therefore, inhibition of this early differentiation process offers potential targets for plant protection strategies with specific and environmentally safe fungicides.[18] In screening extracts of the basidiomycetes, *C. montagnei* IBWF-A24-2006 were found to inhibit conidial germination in *M. oryzae*, whereas vegetative growth was not affected. The producing organism, *C. montagnei*, is a fungus occasionally seen in forests forming club-shaped, flat-topped white fruiting bodies on dead branches. The fungus was identified on the basis of its morphological features and by a genetic analysis. The internal transcribed spacer sequence of our *C. montagnei* shows high sequence homology to other basidiomycetes, such as *Micromphale brassicolens* and *Gymnopus iocephalus*, and to a shorter sequence of another strain of the species (data not shown). To date no secondary metabolites have been recorded from this species.[19]

Caripyrin (**1**), a structurally new pyridyloxirane, was identified in this study as specific inhibitor of conidial germination in the rice blast fungus *M. oryzae*. It was produced in submerged cultures with high agitation in YMG medium. The compound is structurally closely related to **2**, a fungal secondary metabolite identified from other fungal species belonging to the genus *Fusarium*, but not from basidiomycetes.[20]

Fusaric acid (**2**) has been described to be produced in *Fusarium verticillioides* under stress conditions, notably, nutrient limitations.[21] The metabolite has been described as an inhibitor of metal-containing oxidative enzymes, such as dopamine-β-hydroxylases, phenylethanolamine methyltransferase and catechol oxidase.[22] Nonpathogenic *Fusarium oxysporum* species have been described as an potential biocontrol agents, producing only **2** and dehydrofusaric acid, but no other toxins.[23] An antifungal activity toward *P. infestans* has recently been reported.[24] Whether **2** is required for the antagonistic interaction remains to be elucidated. In this study, we found that **2** prevents spore germination in our *F. graminearum* strain, but fails to inhibit vegetative growth of all fungal test organisms.

In contrast to **2**, **1** is produced in complex media before nutrient limitation. Caripyrin (**1**) was found to inhibit conidial germination in *M. oryzae* under conditions resembling those found on the plant surface. Neither vegetative growth nor differentiation processes in all other fungi tested were affected. Such selectivity matches the requirements for a modern plant protectant because of the low toxicological impact on other fungi. When *C. montagnei* was tested for antagonistic activity toward phytopathogenic fungi, neither *M. oryzae* nor *F. graminearum*, nor *B. cinerea* were inhibited.

The protection of host plants from fungal colonization was more efficient for **1** compared with **2**. Compound **1** was found to reduce the number of lesions caused by the rice blast fungus on rice leaves, but a complete protection of the plants has not been observed. Therefore, the compound does not seem to be a promising candidate for the development of an agro fungicide.

Phytotoxic effects have been observed in seeds of rice, wheat and *L. sativum* when exposed to **1** or **2**. However, all seed/plants used were less susceptible to **1** than to **2**. Phytotoxic activity has been reported for **2** toward *Lemna minor*, *Oryza* sp. and other plants.[25,26] It was found that the metabolite could elicit various plant defense responses at a concentration of 100 nM without toxic effects in *Arabidopsis thaliana* cell cultures.[27] Addition of L-cysteine in equimolar concentrations did not reduce the biological activity of **1**. Thus, the epoxide moiety does not seem to be responsible for the activity against *Magnaporthe grisea*.

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