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NOTE

GKK1032A$_2$, a secondary metabolite from *Penicillium* sp. IBWF-029-96, inhibits conidial germination in the rice blast fungus *Magnaporthe oryzae*

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The rice blast fungus *Magnaporthe oryzae* is the most important pathogen on cultivated rice because of its economic relevance and destructiveness.[1] Blast disease caused by this heterothallic ascomycete destroys about 10–30% rice yield every year.[2] In order to infect and colonize the host plant *Oryza sativa*, conidia of the fungus attach to the leaf surface and germinate by sending out a germ tube. Induced by the hard hydrophobic surface, appressoria required for penetration differentiate. Within the specialized infection structure compatible solutes, for example, glycerol, accumulate.[3] The solutes are retained within the cell by a melanin layer. As a consequence, water flows into the infection structure to generate turgor required for a mechanically penetration of the plant cuticle. As the functionality of the appressorium is an essential prerequisite for a successful infection of the host plant, the melanin layer is a pathogenicity factor in *M. oryzae*. Agrochemicals interfering with the biosynthesis of the biopolymer, for example, tricyclazole, have been successfully used in the past as protective agents.[4] After penetration, the hyphae disperse within the plant to form conidiophores and conidia. The spores are spread in the field via wind and water.[2]

Due to the resistance development against commercially available fungicides, there is a demand for lead structures for the development of novel fungicides.[4] Secondary metabolites from nature have in the past been used as lead structures for successful pesticides. The widely used class of QoI (quinone outside inhibitor)-fungicides is based on the structure of strobilurin A, a secondary metabolite found in cultures of the basidiomycete *Strobilurus tenacellus*.[5] As fungi represent an almost inexhaustible reservoir for natural compounds, screening approaches are conducted for the identification of new secondary metabolites interfering either with pathogenic differentiation or fungal vegetative growth. In this course, compound GKK1032A$_2$ (**1**) was identified from cultures of the fungus *Penicillium* sp. IBWF-029-96. The compound has been described as a metabolite from fungal cultures. In this note, we describe a biological activity of this compound, which has not been reported before.[6,7]

EXPERIMENTAL PROCEDURE

Organisms

Penicillium sp. IBWF-029-96 and the unidentified strain IBWF-012-06 are stored in the culture collection of the Institute of Biotechnology and Drug Research (IBWF e.V.), and was cultured at room temperature on yeast malt glucose medium consisting of 4 g yeast extract (Becton Dickinson GmbH, Heidelberg, Germany), 10 g malt extract (Fränkle & Eck, Fellbach, Germany), 10 g glucose and 18 g agar per 1 l tap H_2O. The pH was adjusted with 1 N HCl to 5.5 prior to sterilization.

M. oryzae strain 70-15 was obtained from the Fungal Genetics Stock Centre, Kansas City, KS, USA. The strain was cultured on complete medium (CM) medium as described previously.[8] *Botrytis cinerea*, *Fusarium graminearum* and *Phytophthora infestans* were provided by BASF SE.

Fermentation and isolation

The strain *Penicillium* sp. IBWF-029-96 was fermented in yeast malt glucose medium in a 20-l fermenter (Biostat A-20, Braun Melsungen, Melsungen, Germany) at room temperature with aeration (4.5 l min^{-1}) and agitation (120 r.p.m.). A well-grown culture (200 ml) in the same medium was used as inoculum. Daily samples were withdrawn to measure pH, glucose and maltose content as well as mycelial dry weight and in order to quantify the bioactive secondary metabolite as described below. The fermentation was stopped after 8 days, when glucose in the culture broth was depleted. The mycelium containing the bioactive compound was separated from the culture fluid by filtration and lyophilized. The compound was extracted from lyophilized mycelium (101.84 g) with 3 l of methanol/acetone (1:1). The culture fluid did not contain bioactive compounds, and was therefore discarded. The organic extract was dried with Na_2SO_4 and concentrated to yield 6.45 g of an intermediate product. Chromatography on silica gel in cyclohexane-ethyl acetate (silica gel 60, 63–200 μm, 90 g, column size 24×4.5 cm, Merck, Darmstadt, Germany) and elution with increasing concentration of ethyl acetate yielded 272 mg of a second intermediate product. The fraction containing the bioactive metabolite was eluted with 70% ethyl acetate. A third intermediate product (117 mg) was obtained by using a Chromabond (C18ec) column and elution with water-MeCN (15:85). Final purification (46.8 mg) was achieved by HPLC using a Nucleosil 100-5 C18 column (21×250 mm, 5 μm, Merck). The bioactive

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compound was isolated by using an isocratic method with 78% MeCN in H_2O at a flow rate of 28 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 the multi-wavelength detector UV-1570 M. The fermentation samples were analyzed on an Agilent 1100 Series HPLC (Agilent Technologies, Waldbronn, Germany) equipped with a LiChrospher 100 C18 column (125×4 mm, 5 µm, Merck).

The appearance of the active compound GKK1032A$_2$ (**1**) in the daily samples was monitored with the help of a calibration curve created by analytical HPLC using different concentrations of purified compound.

Structural analysis was carried out on a Bruker Avance-II 400 NMR spectrometer equipped with a 5-mm BBO probehead and on a Perkin-Elmer 241 polarimeter.

Biological assays

Antimicrobial activity of the compounds was assessed by using simple agar-diffusion assays with fungi and bacteria as test organisms. The test was carried out as described previously.[9] The maximum concentration used was 100 µg per filter disc. The following organisms were tested: Bacteria: *Bacillus brevis, B. subtilis, Micrococcus luteus, Enterobacter dissolvens, Staphylococcus aureus*; Fungi: *Candida albicans, M. oryzae, Mucor miehei, Nematospora coryli* and *Paecilomyces variotii.*

Nematicidal activity against *Caenorhabditis elegans* and *Meloidogyne incognita* was assessed as described previously.[10] A maximum concentration of 50 µg of the compound per well was used.

Cytotoxic activity against Jurkat (DSMZ ACC 282) and Colo-320 (DSMZ ACC 144) cells was assayed as described previously.[11,12] Both cell lines were grown in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany).

The inhibition of conidial germination was assayed according to Rieger *et al.*[13] and carried out in triplicates. Spores of *M. oryzae* were harvested with the help of a glass spatula and distilled H_2O. The harvested material was filtered and the supernatant was centrifugated (1000 g min^{-1}). The pellet was dissolved in distilled H_2O. The activity was tested in 96-well plates (Greiner Bio-One, Kremsmünster, Austria) with an end concentration of 1×10^5 spores per ml and a total volume of 200 µl. After 24 h of incubation at room temperature, germinated and not germinated spores were counted.

Phytotoxic effects were tested as described before.[13] Seeds of *Setaria italica*, *Lepidium sativum*, *O. sativa* CO-39 and *Triticum aestivum* RIBAND were used. The assay was performed thrice. Seeds were grown in sterile 24-well plates on filter papers with 10 and 25 µg of purified compound in 250 µl H_2O. Additionally, leaves of *O. sativa* were treated with the compound.

In order to assess the protective effect of GKK1032A$_2$ (**1**) on rice plants, harvested spores of *M. oryzae* were dissolved in 0.2% gelatine to a concentration of 1×10^5 spores per ml. This suspension was mixed with the compound to get concentrations up to 10 µg ml^{-1}. Five-hundred µl of the respective suspensions were sprayed on leaf segments of 21-day-old rice plants placed on a water-agar plate. The leaf segments were incubated at 27 °C. After 5 days, appearing lesions were counted in an area of 1 cm^2. The test was carried out in triplicates.

The mitochondrial respiration of *M. oryzae* in presence of **1** was monitored as described previously.[13]

The fungal strain IBWF-029-96 was identified as *Penicillium* sp. Its conidiophores showed all morphological characteristics of the genus as described.[14] Previous fermentation experiments showed that **1** was produced upon cultivation in malt extract medium. It was furthermore shown that higher aeration shortened the time needed for the culture to reach the stationary phase. However, under these conditions the concentration of bioactive compound was found to significantly lower (data not shown). Figure 1 shows a fermentation diagram for the submerged cultivation inclusive the recorded fermentations parameters. After 8 days of cultivation, the fermentation was stopped. The production of **1** started at day 5 and the concentration increased constantly until the end of fermentation. Bioactivity-guided purification led to the isolation of 46.8 mg GKK1032A$_2$ (**1**). The structure of **1**, shown in Figure 2, was analyzed by NMR and MS techniques. The obtained spectroscopic and physical data were in accordance to those given in earlier descriptions of the compound.[6,7] The NMR data are shown in Table 1.

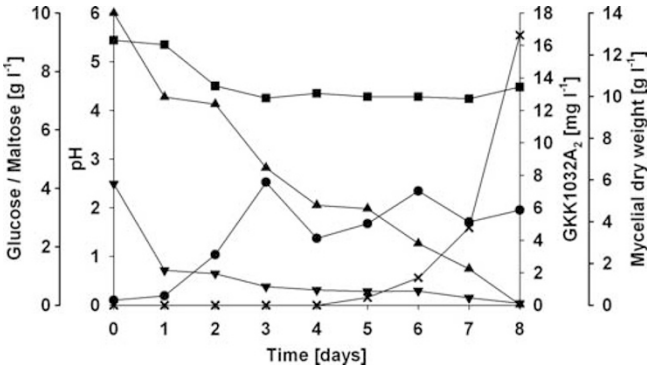

Figure 1 Fermentation diagram *Penicillium* sp. IBWF-029-96 in yeast malt glucose medium (20-l scale). ●, mycelial dry weight; ■, pH; ▲, glucose; ▼, maltose; ×, GKK1032A$_2$.

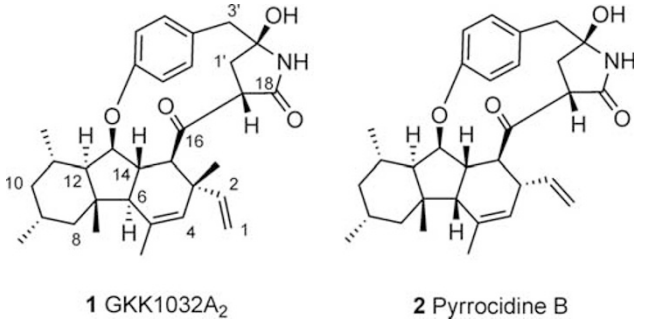

Figure 2 Structure of GKK1032A$_2$ (**1**) and Pyrrocidine B (**2**).

Resistance development and favorable ecotoxicological profiles of agrochemicals are the driving force for the development of novel plant protectants. Even though efficient fungicides for the treatment of rice blast are available, the disease caused by the fungus *M. oryzae* remains to be a serious problem. Therefore, the development of new fungicides with a new mode of action is required. Because of their enormous structural diversity, natural products are regarded to be excellent lead structures for the development of new agrochemicals. In addition, the fungal secondary metabolism represents a large source for bioactive structures.[15] In this study, natural products from fungi were screened concerning their plant-protective activity towards *M. oryzae.* Compounds interfering with the infection-related morphogenesis in phytopathogenic fungi prevent the pathogen from entering and colonizing the host plant. In this context, pathogenic differentiation prior to plant penetration appears to hold targets for fungicides. The infection structures of *M. oryzae* are located on the host surface, and are therefore easily accessible. In addition, through the inhibition of the initial step in the infection process such as the conidial germination, the arising of resistances is minimized and it allows the application of specific and environmentally safe fungicides.[4]

In the screening for inhibitors of the infection-relevant structures in *M. oryzae* culture extracts of the fungus *Penicillium* sp. IBWF-029-96 caused an inhibition of conidial germination. The secondary metabo-

Table 1 ^{1}H (400 MHz) and ^{13}C (101 MHz) NMR data of GKK1032A$_2$ in $CDCl_3$

Position	d_H	d_C
1	4.88 (dd, *J*=17.6, 1.0 Hz)	112.2 (t)
	4.82 (dd, *J*=10.6, 1.0 Hz)	
2	5.42 (dd, *J*=17.6, 10.6 Hz)	146.3 (d)
3	—	41.5 (s)
4	4.90 (br s)	130.7 (d)
5	—	138.6 (s)
6	1.87 (m)	53.1 (d)
7	—	41.5 (s)
8	1.95 (m)	49.0 (t)
	0.79 (t, *J*=12.1 Hz)	
9	1.83 (m)	28.1 (d)
10	1.79 (m)	45.6 (t)
	0.61 (q, *J*=12.2 Hz)	
11	1.90 (m)	27.4 (d)
12	1.06 (dd, *J*=11.2, 7.7 Hz)	61.0 (d)
13	4.24 (dd, *J*=7.7, 4.5 Hz)	90.8 (d)
14	2.61 (ddd, *J*=13.4, 10.1, 4.5 Hz)	50.7 (d)
15	3.51 (d, *J*=10.1 Hz)	56.7 (d)
16	—	200.4 (s)
17	3.12 (dd, *J*=12.0, 4.7 Hz)	56.8 (d)
18	—	171.9 (s)
1′	2.89 (m)	33.4 (t)
	1.80 (m)	
2′	—	88.9 (s)
3′	2.92 (s)	47.0 (t)
4′	—	128.0 (s)
5′a	6.92 (dd, *J*=8.7, 1.6 Hz)	133.5 (d)
5′b	7.14 (dd, *J*=8.1, 1.6 Hz)	131.6 (d)
6′a	6.96 (dd, *J*=8.7, 2.2 Hz)	118.9 (d)
6′b	6.81 (dd, *J*=8.1, 2.2 Hz)	124.5 (d)
7′	—	159.9 (s)
3–Me	1.19 (s)	25.8 (q)
5–Me	1.86 (br s)	21.0 (q)
7–Me	1.17 (s)	16.1 (q)
9–Me	0.90 (d, *J*=6.3 Hz)	22.9 (q)
11–Me	1.10 (d, *J*=6.3 Hz)	19.9 (q)
18–NH	5.99 (br s)	—

Carbon multiplicities were detected indirectly (HSQC). The numbering scheme from Oikawa[7] was used.

Table 2 Cytotoxicity, inhibition of conidial germination and phytotoxicity of GKK1032A$_2$ and pyrrocidine B

	GKK1032A$_2$	*Pyrrocidine B*
Organism	*IC$_{50}$ [μg ml^{-1}]*	
Cell lines		
Colo-320	NE	nt
HeLa S3	nt	25
Jurkat	10	nt
Fungi		
Botrytis cinerea	NE	NE
Fusarium graminearum	NE	nt
Magnaporthe oryzae	3	2.5
Phytophthora infestans	NE	NE
Plants		
Lepidium sativum	50–100	NE
Oryza sativa	NE	nt
Setaria italica	100	NE
Triticum aestivum	>100	nt

Abbreviations: NE, no effect; nt, not tested.

Table 3 Antimicrobial activity of GKK1032A$_2$ and pyrrocidine B in agar-diffusion assays

	GKK1032A$_2$	*Pyrrocidine B*
Organism	*MIC [μg ml^{-1}]*	
Bacteria		
Bacillus brevis	NE	NE
B. subtilis	NE	nt
Micrococcus luteus	NE	nt
Enterobacter dissolvens	NE	nt
Staphylococcus aureus	NE	<10
Fungi		
Candida albicans	NE	NE
Magnaporthe oryzae	NE	<10
Mucor miehei	NE	NE
Nematospora coryli	NE	nt
Paecilomyces variotii	NE	NE

Abbreviations: NE, no effect; nt, not tested.

lite GKK1032A$_2$ (**1**) was found responsible for this activity and was isolated from the mycelium. The polyketide is a known fungal secondary metabolite, which has been identified from cultures of the genus *Penicillium* before.[6,7] The metabolite was isolated in this study, as it showed antimicrobial and antitumor activities against HeLa S3 cells. An antimicrobial activity against *Staphylococcus* and *Enterococcus* strains was also detected for pyrrocidine A and pyrrocidine B (**2**), 3,6-bisepi-3-desmethyl analogs of **1**, whereas pyrrocidine A shows higher activities.[16] An antifungal activity of these compounds was found as well, including *Aspergillus* and *Fusarium* strains.[17] In our study, GKK1032A$_2$ (**1**) failed to inhibit vegetative growth of bacteria and fungi, whereas Jurkat cells were sensitive in presence of the compound. The second tested cell line, Colo-320, was not affected (Table 2). The reason for this low susceptibility might probably be due to missing receptors.[18] Furthermore, no nematicidal activity was found.

GKK1032A$_2$ (**1**) seems to inhibit *M. oryzae* in a specific manner, as shown in Table 2. The spore germination in the phytopathogenic fungi *F. graminearum*, *B. cinerea* and *P. infestans* was not affected. In contrast, the vegetative growth of *M. oryzae* on CM plates was not inhibited (Table 3). However, the compound seems not to influence the respiration of the fungus. Pyrrocidine B (**2**), isolated from the unidentified fungal strain IBWF-012-06, inhibits *M. oryzae* in the same manner as **1**, as shown in Table 2. Interestingly, **2** shows activity against the vegetative growth of *M. oryzae* as well (Table 3). Furthermore, we could confirm the activity of **2** against *S. aureus*, an effect not found for GKK1032A$_2$ (**1**). For pyrrocidine A, we cannot provide any further data.

Phytotoxic effects of **1** towards the host plant *O. sativa* were not observed. Sprout or root growth was not affected by the treatment. Furthermore, neither lesion formation nor necrosis was detected on leaves of *O. sativa* when exposed to the metabolite. In *L. sativum* and *S. italic* sprout growth was inhibited when exposed to 25 μg of **1**. In

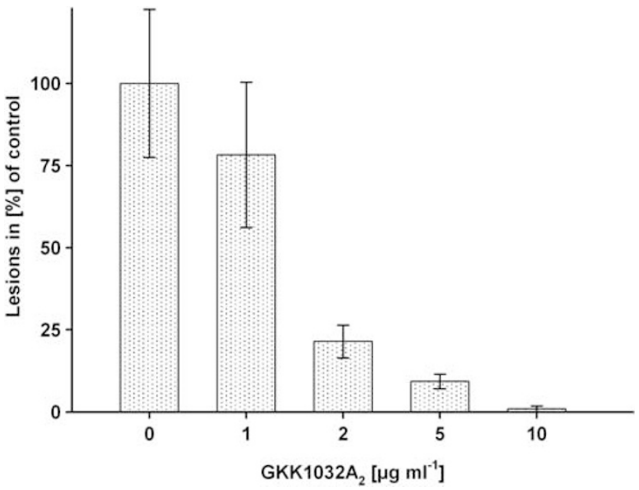

Figure 3 Protective effect of GKK1032A$_2$ on rice plants inoculated with a *M. oryzae* spore suspension.

contrast, decreased root growth was observed in *T. aestivum* upon exposure to 25 µg of the compound. As *T. aestivum* and *O. sativa* are of economic importance, they appear to be the major application area for fungicides. Therefore, a low toxicity as found for **1** is desirable. The compound was found not to affect intact rice plants. Therefore, a protective treatment of rice plants appears to be possible. In our experiments, GKK1032A$_2$ (**1**) was found to show good plant-protective activity. At a concentration of 2 µg ml^{-1}, lesion formation was reduced by 75%. A complete protection was observed at a concentration of 10 µg ml^{-1} (Figure 3). Showing an IC$_{50}$ of 3 µg ml^{-1}, the germination assay emphasizes this result.

Refering to the chemical structures, the pyrrolidinone function of GKK1032A$_2$ (**1**) and the pyrrocidines has already been found in other antifungal compounds such as talaroconvolutin A.[19] But the macrocycle containing ether, phenyl, pyrrolidinone and ketone functions seems to be very rare in natural compounds. However, there exist some studies about this tricarbocyclic systems.[20,21] GKK1032A$_2$ (**1**) and the 3,6-bisepi-3-desmethyl analogs pyrrocidines A and B (**2**) show similar bioactivity. In the case of **1**, there is a lack of antimicrobial activity. Furthermore, a different affect on the vegetative growth of *M. oryzae* could be shown for **1** and **2**. Although the chemical structures are almost identical, there seems to be major differences in the biological activities.

Considering the costs of agrochemicals, the complex structure of the GKK1032 members does not appear to be a suitable lead for the development of a plant protectant. However, it could serve as a chemical lead for the identification of the molecular target and for toxophore studies. In this context, further experiments concerning the mode of action of the compound are required.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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