ORIGINAL ARTICLE

Allantofuranone, a new antifungal antibiotic from *Allantophomopsis lycopodina* IBWF58B-05A

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In a screening for new bioactive compounds, the extract of *Allantophomopsis lycopodina* strain IBWF58B-05A, an imperfect ascomycete, was found to exhibit strong but rather selective antibiotic activity against *Paecilomyces variotii*. The bioactivity-guided isolation yielded allantofuranone, a new and uncommon γ -lactone. This compound showed antifungal activity against *P. variotii* and *Penicillium* species. This paper describes the isolation, structure elucidation and biological characteristics of allantofuranone.

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INTRODUCTION

Fungi associated with plants have to adapt to environments differing greatly in their chemical composition and varying substantially during the host's development. They have to cope both with the plants defense and competing microorganisms and have therefore developed a rich and diverse secondary metabolism as part of their survival strategy. *Allantophomopsis lycopodina* is a plant pathogen responsible for leaf lesions on lingonberry, *Vaccinium vitis-idaea*¹ and a fruit-rot pathogen (black rot) on cranberries, *V. macrocarpon*.² To our knowledge, no secondary metabolites have been reported from this species. A closer look into the compounds produced during fermentations of our strain, however, revealed the presence of an antifungal antibiotic. In the following, we describe the production, isolation, structure elucidation and biological activities of allantofuranone (**1**), a new and unusual γ -lactone.

RESULTS AND DISCUSSION

Structure elucidation

The isolation of allantofuranone (**1**) was guided by its inhibitory activity against *Paecilomyces variotii* as described in the Experimental section. Allantofuranone had a nominal molecular weight of 324 Da and an elemental composition determined by HR-MS of C₁₉H₁₆O₅, requiring 12 unsaturations. The presence of two mono-substituted phenyl residues could be deduced by their characteristic pattern in the ¹H-NMR spectrum. Their ortho protons were shifted downfield, and two of them showed a heteronuclear multiple bond correlation (HMBC) to a ketone carbonyl with a typical shift of 193.97 p.p.m. The carbonyl group itself exhibited intense correlations with two

diastereotopic methylene protons forming an AB spin system. The neighboring quaternary center resonated at δ_C 107.61 and showed an HMBC correlation with a methoxy group at δ_H 3.28. The chemical shift of the quaternary carbon gave evidence for the presence of an acetal. The methylene protons showed a ³J C,H correlation to a further quaternary carbon at 122.92 p.p.m., which is bound to the second phenyl residue. Only one of the two remaining carbon atoms showed a weak ⁴J correlation to one of the methylene protons. Their chemical shifts (167.46 and 140.19 p.p.m.) were in good agreement with those of C-2 and C-3 in a 3-hydroxyfuran-2(5H)-one ring.³ This hypothesis could later be confirmed by X-ray crystallography. Remarkably, allantofuranone crystallized in the centrosymmetric space group $P\bar{1}$, thus the natural product was racemic (Figures 1 and 2).

Biological activities

Up to 50 $\mu\text{g ml}^{-1}$, no antibiotic activity was observed in the serial dilution assay against: *Micrococcus luteus*, *Bacillus brevis*, *B. subtilis*, *Mycobacterium phlei*, *Pseudomonas aeruginosa*, *Athrobacter citreus*, *Corynebacterium insidiosum*, *Escherichia coli*, *Enterobacter dissolvens*, *Absidia glauca* (+), *A. glauca* (–), *Ascochyta pisi*, *Aspergillus ochraceus*, *Fusarium oxysporum*, *F. fujikuroi*, *Nematospora coryli*, *Zygorhynchus moelleri* and *Mucor miehei*. Compound **1** exhibited antifungal activity against *P. islandicum* and *P. notatum* at 25 $\mu\text{g ml}^{-1}$ and against *P. variotii* starting from 10 $\mu\text{g ml}^{-1}$.

In the agar diffusion assay, the antifungal activity of **1** against *P. notatum*, *P. islandicum* and *P. variotii* was more pronounced (Table 1). Activity was observed against *Magnaporthe grisea* (15 μg per disc) and *P. lilacinus* (30 μg per disc), whereas *P. farinosa* was not inhibited. Interestingly, *P. lilacinus* started forming reddish pigments beginning

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at 5.0 µg per disc of **1**. The germination of *P. infestans* and *M. grisea* spores was inhibited at an IC_{100} value of 50 µg ml⁻¹. The spore germination of the other fungal species was not inhibited (up to 100 µg ml⁻¹). No interference with respiration (*P. notatum* and *P. variotii*) and no phytotoxic, cytotoxic and nematocidal effects were observed at concentrations of up to 100 µg ml⁻¹.

Compounds such as xenofuranones A and B (**2**, **3**),³ butyrolactone II (**4**)⁴ or WF-3681 (**5**)⁵ show structural similarities to **1**. It was reported that they exhibited no antibacterial and only weak cytotoxic activity. This corresponds to our findings. The xenofuranones and WF-3681

showed no activity against yeasts, which also matches our results. Unfortunately, there was no information about their activity against filamentous fungi.

EXPERIMENTAL SECTION

General

Melting points were determined with a Dr Tottoli apparatus and are uncorrected. Optical rotations were measured with a Krüss P8000 polarimeter at 589 nm. UV and IR spectra were measured with a Perkin-Elmer Lambda-16 spectrophotometer and a Bruker IFS48 FTIR spectrometer, respectively. NMR spectra were recorded with a Bruker Avance-II or with a Bruker DRX-500 spectrometer. The spectra were measured in CDCl₃ and the chemical shifts were referenced to the residual solvent signal (CDCl₃: δ_H =7.26 p.p.m. and δ_C =77.16 p.p.m.).⁶ APCIMS spectra were measured with a Hewlett Packard MSD1100. FD-MS spectra were recorded on a Finnigan MAT 95 spectrometer. ESI-HR-MS spectra were recorded on a MicroMass/Waters Q-TOF Ultra 3 spectrometer equipped with a LockSpray interface using NaI/CsI or trialkylamines as external reference.

1 Allantofuranone

2 Xenofuranone A (R = Me)
3 Xenofuranone B (R = H)

4 Butyrolactone II

5 WF 3681

Figure 1 Allantofuranone and related compounds.

Table 1 Antifungal activity of allantofuranone (**1**) in the agar plate diffusion assay

Organism	Diameter inhibition zone (mm) µg per disc ^a							
	0.5	1	2.5	5.0	10	15	20	40
<i>Penicillium notatum</i>	—	—	10i	13i	17i	20i	21i	22i
<i>P. islandicum</i>	—	—	—	12i	23i	27d	29d	35d
<i>Paecilomyces variotii</i>	—	12i	27d	29d	38d	42d	45d	47d
<i>P. lilacinus</i>	—	—	—	—	—	—	12i	14i
<i>Magnaporthe grisea</i>	—	—	—	—	—	12i	15i	20i

Abbreviations: d, inhibition zone diffuse; i, inhibition zone incomplete.

^aDiameter 6 mm.

Figure 2 Crystal structure of **1** (ORTEP, ellipsoids drawn at 50% probability).

Producing organism

The filamentous ascomycete IBWF58B-05A was isolated from a strongly rotten piece of wood from a deciduous tree collected in France. It was identified by microscopy and sequencing of its internal transcribed spacer (ITS). The ITS sequence shows homologies of 99.8% to *A. lycopodina* IFO 32908 (GenBank accession no. AB041243). The microscopic characteristics fit the description by Carris.⁷ The spores are inequilateral/light lunate shaped, 2.5–3 µm × 10–13 µm and are produced in conidiomata (diameter 100–300 µm in YMG agar culture). The agar culture is dark grey-brown to black. Strain IBWF58B-05A has been deposited in the culture collection of the Institute of Biotechnology and Drug Research (IBWF e.V.), Kaiserslautern, Germany. For maintenance, the fungus was grown on agar slants on YMG agar (yeast extract 4.0 g l⁻¹, malt extract 10 g l⁻¹, glucose 10 g l⁻¹, the pH value was adjusted to 5.5 before autoclaving). Solid media contained 2.0% of agar.

Fermentation and isolation

The fungus was grown in YMG medium in a 20-l fermenter (Biolaftite C6) at 22–24 °C with agitation (130 r.p.m.) and aeration (3 l min⁻¹). For inoculation, a well-grown shake culture (500 ml) in the same medium was used. When the carbon source was depleted after 7 days, the fermentation was stopped. The culture fluid (16.5 l) was separated from the mycelia by filtration and extracted twice with an equal volume of EtOAc. After evaporation of the solvent, the oily crude extract (4.5 g) was applied onto a column with silica gel (Merck 60, 0.063–0.2 mm, 150 g). Elution with cyclohexane/EtOAc (3:1) yielded 693 mg of an intermediate product. Pure **1** was obtained by preparative HPLC (Merck, Lichrosorb RP 18, 7 µm, column 250 mm × 25 mm) with a MeCN/H₂O gradient (50–62% MeCN in 12 min, flow: 20 ml min⁻¹; yield: 191.3 mg (Rt 10.5–12.0 min)).

Allantofuranone (**1**)

Yellow crystals, m.p. 147–149 °C; no significant optical rotation was detected; UV λ_{max}^{MeOH} (nm) (log ε) 227 (4.08), 246 (4.21), 289 (4.26); λ_{min}^{MeOH} (nm) (log ε) = 219 (4.04), 231 (4.06), 262 (4.03); IR ν_{max} (KBr) (cm⁻¹) 3304, 1755, 1695, 1394, 1304, 1216, 1177, 1152, 1122, 989, 766, 689; FD-MS *m/z* 324.1 (M)⁺, 648.6 (2M)⁺; ESI-HRMS *m/z* 347.0891 (calcd for C₁₉H₁₆O₅ + Na⁺ 347.0890). ¹H- and ¹³C-NMR data are presented in Table 2.

X-ray crystallographic data of **1**

Crystals of **1** were grown by slow evaporation of a methanolic solution of **1**. Data were obtained at 100 K on a Bruker APEX diffractometer with graphite-monochromated Mo-K_α radiation. Formula C₁₉H₁₆O₅, crystal size 0.02 × 0.10 × 0.38 mm³, triclinic, space group Pī, *a* = 8.779(4) Å, *b* = 9.672(4) Å, *c* = 10.765(5) Å, α = 75.886(8)°, β = 76.808(7)°, γ = 79.356(7)°, *V* = 816.4(6) Å³, *Z* = 2, *D* = 1.319 g cm⁻³, *R* = 0.0638, *R*_w = 0.1417. CCDC-717550 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; Fax: +44-1223/336-033; E-mail: deposit@ccdc.cam.ac.uk).

Biological assays

Antimicrobial activities against bacteria and fungi were determined in the agar plate diffusion or serial dilution assay as described previously.⁸ Cytotoxicity was assayed as described previously.⁹ The cell lines Jurkat (ATCC TIB 152), Colo-320 (DSMZ ACC 144) and L-1210 (DSMZ ACC 123) were grown in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany). Neuro-2A (DSMZ ACC 148) and SH-SY5Y (DSMZ ACC209) were grown in DMEM medium (Invitrogen). All media were supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 65 µg ml⁻¹ of penicillin G and 100 µg ml⁻¹ of streptomycin sulfate. The viability was measured photometrically with XTT (suspension cell lines) or with Giemsa staining (monolayer cells). The influence of **1** on the oxygen uptake by *P. variotii* and *Penicillium notatum* was tested as described previously.¹⁰ Phytotoxicity was tested with *Setaria italica* and *Lepidium sativum*.¹¹ The spore germination was tested with *M. grisea* as described

Table 2 ¹H- (400 MHz) and ¹³C-NMR (101 MHz) of **1** in CDCl₃

Position	δ _H	δ _C
2	—	167.46 (s)
3	—	140.19 (s)
4	—	122.92 (s)
5	—	107.61 (s)
6	3.90, (d, <i>J</i> = 16.5 Hz) 3.67, (d, <i>J</i> = 16.5 Hz)	43.97 (t)
7	—	193.97 (s)
8	—	136.99 (s)
9/13	7.83 (m)	128.33 (d)
10/12	7.40 (m)	128.71 (d)
11	7.52 (m)	133.54 (d)
1'	—	129.17 (s)
2'/6'	7.89 (m)	127.72 (d)
3'/5'	7.45 (m)	129.20 (d)
4'	7.37 (m)	129.45 (d)
5-OCH ₃	3.28 (s)	50.16 (q)

previously.¹² This method was adapted for the spore germination assay with *Phytophthora infestans*, *Botrytis cinerea* and *Fusarium graminearum*. The nematocidal activity was tested with *Meloidogyne incognita* and *Caenorhabditis elegans* as described earlier.¹³

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