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Phenguignardic Acid and Guignardic Acid, Phytotoxic Secondary Metabolites from *Guignardia bidwellii*

Daniel Molitor,[†,‡] Johannes C. Liermann,[§] Beate Berkelmann-Löhnertz,[‡] Iris Buckel,[⊥] Till Opatz,[§] and Eckhard Thines*[,⊥]

[†]Department Environment and Agro-Biotechnologies, Centre de Recherche Public−Gabriel Lippmann, 41 Rue du Brill, L-4422 Belvaux, Luxembourg

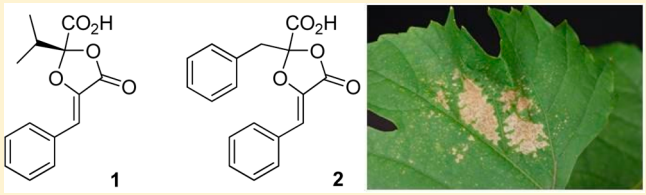
[‡]Department of Phytomedicine, Geisenheim Research Center, Von-Lade-Strasse 1, D-65366 Geisenheim, Germany

[§]Institute of Organic Chemistry, Johannes-Gutenberg-University, Duesbergweg 10-14, D-55128 Mainz, Germany

[⊥]Institute of Biotechnology and Drug Research, Erwin-Schrödinger-Strasse 56, D-67663 Kaiserslautern, Germany

Supporting Information

ABSTRACT: Bioactivity-guided isolation led to the identification of phenguignardic acid (**2**), a new phytotoxic secondary metabolite from submerged cultures of grape black rot fungus, *Guignardia bidwellii*. The compound is structurally related to guignardic acid (**1**), a dioxolanone moiety-containing metabolite isolated previously from *Guignardia* species. However, in contrast to guignardic acid, which is presumably synthesized from deamination products of valine and phenylalanine, the biochemical precursor for the biosynthesis of the new phytotoxin appears to be exclusively phenylalanine. Guignardic acid was also found in extracts of cultures from *Guignardia bidwellii*. The phytotoxic activities of both compounds were assessed in plant assays using either detached vine leaves or intact plants. Antimicrobial and cytotoxic activities of phenguignardic acid were determined.



Black rot is one of the most devastating diseases of grapes in North America.[1] The causal agent of the disease is the fungus *Guignardia bidwellii*, anamorph *Phyllosticta ampelicida*.[2] The disease occurs worldwide throughout humid viticultural production regions[3] and has been known in Europe for more than 100 years.[4] It can result in crop losses ranging from 5% to 80%.[5] In viticulture endorsed by integrated pest management programs the disease is controlled by the application of modern and selective fungicides. However, disease outbreaks are regularly observed in organic viticulture in the areas around the Moselle and Nahe Rivers and in the Middle Rhine Valley in Germany.[6] It is believed that climate change, e.g., increased spring temperatures, contributes to the enhanced frequency of occurrence observed in Germany since 2002. The abandoned vineyards at the Moselle River ("Drieschen") are significant reservoirs for the fungus.[7] All commercially important *Vitis vinifera* cultivars and most of the interspecies crosses are reported to be susceptible to the disease.[6]

The infection-related morphogenesis of the fungus including sporulation, spore germination, and appressorium formation has been studied in order to understand the basis of the plant−pathogen interaction.[2,8] Interestingly, disease symptoms are exclusively observed on younger and still expanding leaves. Under optimal temperature conditions lesions can be observed 10 to 14 days after inoculation. Since the fungus initially grows biotrophically before switching to necrotrophic growth,[2] *G. bidwellii* can be considered to be a hemibiotrophic fungus, such as *Magnaporthe grisea*, *Phytophthora infestans*, and *Colletotrichum* spp.[9]

Many enzymes/proteins have been identified to play crucial roles in the infection process or within plant−pathogen interactions.[10,11] Apart from enzymes, such as cutinases and xylanases, several plant pathogenic fungi were found to produce and secrete toxic secondary metabolites contributing to the virulence of the fungus. Such toxins have been identified to be part of effective strategies to weaken and kill host plants.[12] Secondary metabolites are believed to be virulence or pathogenicity factors, as some of them cause specific disease symptoms upon application of the pure compound onto the host tissue.[13] The exploitation of dead plant tissue after secretion of phytotoxins is a strategy used by necrotrophic fungi. In contrast, biotrophic fungi exclusively colonize living plant tissue. There are different mode-of-action types of phytotoxins within the host. The compounds can interact with a range of cellular targets, modify gene expression, or destroy membrane integrity. Interactions with plant enzymes have been reported in which the phytotoxins interfere with the biosynthesis of important metabolites.[12] Several phytotoxins, e.g., phyllosinol, have been identified from *Guignardia* species.[14] Species of this genus have frequently been isolated as endophytes and have been considered to be excellent sources

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of novel lead structures. From cultures of the *Spondia mombin* endophyte *Phyllosticta telopeae* a secondary metabolite was isolated based on antibacterial activities of an extract. The compound, (−)-(*S*)-guignardic acid, was described as the first member of a new class of natural compounds containing a dioxolanone moiety.[15]

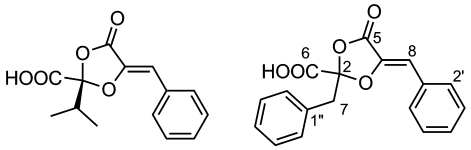
To date, no phytotoxins have been described from *G. bidwellii* strains. In the present article the existence of two phytotoxic secondary metabolites produced by the fungus in submerged cultures is reported, and the fermentation, isolation, structure elucidation, and biological activity of these compounds are described.

RESULTS AND DISCUSSION

Fermentation of *Guignardia bidwellii* and Isolation of Secondary Metabolites. Several media were used for the cultivation of *G. bidwellii* in order to optimize the synthesis of bioactive compounds (data not shown). The highest quantities of phytotoxic constituents were found upon fermentation of the fungus in malt extract medium (MEM). In this medium *G. bidwellii* was cultured for 25 days in six 2 L flasks until the free glucose within the medium was depleted and the pH increased. A typical fermentation diagram for a submerged culture of *G. bidwellii* in MEM medium is shown in the Supporting Information.

HPLC analysis revealed that from day 6 onward the secondary metabolite **1**, with a retention time of 10.83 min, was observed, and from day 14 onward a second compound, **2**, with a retention time of 13.94 min and with a UV spectrum resembling that of compound **1**, was detected. Both compounds were isolated from culture filtrate extracts by bioactivity-guided chromatographic methods. Mycelial extracts were discarded since the concentrations of the metabolites were found to be significantly lower compared to culture fluid extracts.

Structure Elucidation. The compounds **1** and **2** (Figure 1) were analyzed by mass spectrometry and NMR spectroscopy.



1 Guignardic acid **2** Phenguignardic acid

Figure 1. Structure of guignardic acid (**1**) and phenguignardic acid (**2**).

Compound **1** was identified as guignardic acid, which is already known as a metabolite of *Guignardia* species.[15] Compound **2** was found to have an elemental composition of $C_{18}H_{14}O_5$ (HRMS), and the NMR spectroscopic data suggested a related structure. The ^{1}H and ^{13}C NMR spectra showed resonances for two phenyl residues. One phenyl residue was connected to an olefinic carbon atom at δ_C 109.7, the proton of which (δ_H 6.20) gave HMBC correlations to a second olefinic carbon atom at δ_C 135.5 and a lactone carbonyl at δ_C 162.9. Additionally, a methylene group (δ_C 41.0) was found with the methylene protons forming an AB spin system. HMBC indicated that this methylene group was connected to a quaternary carbon at δ_C 105.9 and the second phenyl residue. In conclusion, the described data match a 4-benzylidene-1,3-dioxolan-5-one scaffold as found in guignardic acid (**1**), with the NMR and UV data in good correspondence with the reported data,[15] and the quaternary carbon at δ_C 105.9 should therefore be an acetalic center connected to a carboxyl group, which could be detected as a very broad resonance (δ_C 168.7) in the ^{13}C NMR spectrum. The configuration at C-2 is unknown and will be the subject of future studies.

Biological Activity. Initially, plant assays using leaf disks of *Vitis vinifera* were conducted in order to identify fractions containing or compounds showing phytotoxic activity. Treatment of the leaf disks with the pure compounds resulted in rapid lesion development (Figure 2). Within 4 h after application of the phytotoxic metabolites necrotic lesions were visible. In this assay guignardic acid seemed to be as active as phenguignardic acid. Lesions were recorded at concentrations up to 10 μg per droplet/leaf disk.

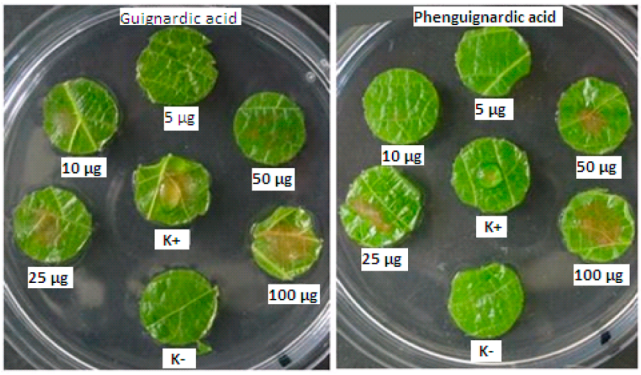


Figure 2. Phytotoxicity assay with both compounds on vine (*Vitis vinifera*) leaf disks. Amounts ranging from 5 to 100 μg were applied. K +, positive control (30 μL of 5% H_3PO_4); K−, negative control (30 μL of 100% acetonitrile).

In greenhouse experiments using intact plants both compounds were found to induce lesion formation (Figure 3), whereas phenguignardic acid was found to be more active. After 24 h, lesions were detected at a tested concentration of 1 μg mL^{-1} per plant.

Figure 3. Phytotoxic assay on intact *Vitis vinifera* plants. A 1 mL amount of phenguignardic acid solution (1 μg mL^{-1}) was applied per plant.

In order to investigate whether the phytotoxic activity of **1** and **2** is specific to vines, *Oryza sativa*, *Setaria italica*, and *Lepidium sativum* were used as additional test plants. Since lesion development was observed in assays using *O. sativa* leaves, it was indicated that both metabolites are non-host-specific toxins (Figure 4). Furthermore, seedling germination experiments using *S. italica* and *L. sativum* revealed that both compounds are phytotoxic in a non-host-specific manner. At

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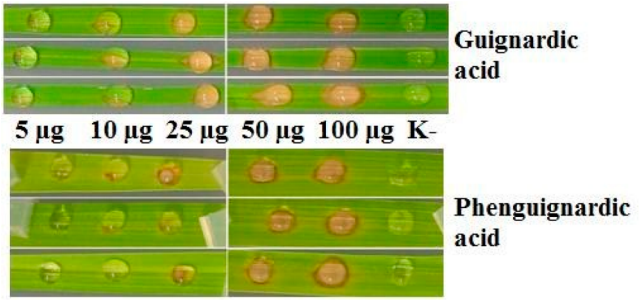

Figure 4. Phytotoxicity assay on *O. sativa* leaf segments. Concentrations of 5, 10, 25, 50, and 100 μg dissolved in acetonitrile were applied. K−: 1 μL of acetonitrile in 9 μL of 0.2% gelatin solution.

concentrations higher than 50 μg per filter disk for **1** and 100 μg for **2** germination was inhibited in both non-host plants.

No antimicrobial activity was observed for either compound **1** or **2**. Up to 100 μg mL^{-1} neither inhibition of vegetative growth, colony growth, nor effects on conidial germination in fungi were found. Furthermore, neither compound was cytotoxic against HeLa S3 or Jurkat cells at a concentration of 50 μg mL^{-1}.

General Discussion. The emergence of new diseases is a topic of importance for plant pathology and crop protection.[13] Even though black rot disease on vines is well known, occurring in viticultural production areas worldwide, it has attracted more attention within the past decade due to its frequent occurrence in some vine-growing areas in Germany. Although the infection-related morphogenesis of the causal agent *G. bidwellii* has been intensively studied in the past, the impact of phytotoxins for the pathogenic development of the fungus has to date not been addressed. (*S*)-Guignardic acid and phenguignardic acid were found to produce disease symptoms on the host plant *Vitis vinifera* (cv. Riesling) in greenhouse experiments. Whereas both compounds were equally active in the leaf disk assays, in the greenhouse experiment on potted vines compound **2** was about 5 to 10 times more active.

In contrast to compound **1**, which has been isolated from *Guignardia* species before and also been prepared by chemical synthesis, **2** is a novel secondary metabolite.[15] Both compounds could result from the condensation of two amino acid derivatives, the amino acids being phenylalanine and valine for **1** and two molecules of phenylalanine for **2**. While furanones originating from the dimerization of two amino acid derivatives are well known as fungal metabolites,[16−19] to our knowledge, guignardic acid (**1**) and phenguignardic acid (**2**) remain the only examples of dimers of the dioxolanone type. Feeding experiments with phenylalanine (final concentration: 5 mM) led to an enhanced biosynthesis rate of **2**, supporting the hypothesis that phenylalanine is the sole precursor for this secondary metabolite.

In our studies we found that **1** and **2** are non-host-specific (NHS) toxins since they were toxic not only on vines but also on rice. NHS toxins could serve as lead structures for potential broad-spectrum herbicides.[20] There is a need for the development of new herbicides, since in some cases synthetic herbicides are considered to have a negative environmental impact and may be less biodegradable than compounds from natural sources and because of resistance development.[20,21] The phytotoxic compounds **1** and **2** belong to a new class of natural compounds containing a dioxolanone moiety, for which the site of action is unknown and may be a new molecular target.

EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotation was measured on a Krüss P8000 polarimeter at 589 nm. UV spectra between 210 and 450 nm were recorded using an UV/vis spectrophotometer (Lambda 16, Perkin-Elmer, Langen, Germany) by dissolving 13.16 μg of **1** and 27.6 μg of **2** in 1 mL of methanol. IR spectra were recorded using a Bruker IFS-48 spectrometer (Karlsruhe, Germany). For this purpose 35 mg of KBr was used as a matrix to embed 100 μg of the individual pure compound. Spectra were recorded between 400 and 4000 cm^{-1}. CD spectra were recorded on a Jasco J-815 spectrometer. NMR spectra were recorded on a Bruker Avance-II 400 MHz spectrometer equipped with a 5 mm BBO probe head using standard pulse sequences. For referencing, the respective solvent signals of $CDCl_3$ (δ_H 7.26, δ_C 77.16) were used. The molecular weight of the pure substances was determined using a HPLC-MS (Series 1100, Hewlett-Packard, Waldbronn, Germany). The mass spectra were recorded using atmospheric pressure chemical ionization with positive and negative polarization. A Superspher RP_{18} (125 × 2 mm; 4 μm, Merck KGaA, Darmstadt, Germany) column was used at 40 °C and a flow rate of 0.45 mL min^{-1}. For every run 1 μL of a sample at a concentration of 1 mg mL^{-1} was injected. The elution was performed with a gradient of H_2O + 0.1% v/v formic acid and acetonitrile. For preparative HPLC a Zorbax Eclipse XDB Phenyl column (250 × 9.4 mm, Agilent, Waldbronn, Germany) with a flow rate of 7 mL min^{-1} was used. The compounds were fractionated isocratically with H_2O + 0.1% v/v formic acid and acetonitrile (60:40). In order to quantify the phytotoxins, fermentation samples were taken at daily intervals and analyzed by HPLC (Agilent 1100 Series) equipped with an Eclipse XDB Phenyl column (3 × 150 mm; 3.5 μm, Agilent, Waldbronn, Germany) and an elution gradient composed of H_2O + 0.1% v/v phosphoric acid and acetonitrile. Compounds were detected via UV at 300 nm.

Microorganisms. The producing organism, *Guignardia bidwellii* CBS 111.645, was obtained from the Centraalbureau voor Schimmelcultures, CBS Fungal Biodiversity Centre. The strain was maintained at 27 °C on oatmeal agar composed of 74.5 g of Difco oatmeal agar (Becton, Dickinson and Company, Sparks, USA) per 1 L of deionized H_2O. The test organisms *Penicillium notatum*, *Paecilomyces variotii*, *Mucor miehei*, *Nematospora coryli*, *Magnaporthe oryzae*, *Fusarium graminicola*, *Phytophthora infestans*, *Botrytis cinerea*, *Bacillus brevis*, *Bacillus subtilis*, *Enterobacter dissolvens*, and *Sarcina lutea* were maintained as described previously.[22,23]

Plants. Two-eye cuttings of *Vitis vinifera* cv. Riesling were collected from mature shoots after the first frost, i.e., after being stratified. They were disinfected by soaking in 0.5% Chinoplant solution (active substance: 8-hydroxyquinoline) for 12 h. Thereafter, they were stored at 4 °C and 95% humidity until use. Shortly before use, the cuttings were soaked in lukewarm water for half an hour in order to gain sufficient moisture. Cuttings were then reduced at both ends by cutting their edges (about one inch) in order to remove dried parts. The lower eye was removed, and the cuttings were put into boxes filled with a mixture of 50% perlite and 50% standard soil mixture. At 10 to 12 weeks, plants were potted in MCI-17 pots filled with standard soil ED 73. Plantlets were fertilized during irrigation once a week with 1 g L^{-1} Flory 3 Mega (18 g of N, 12 g of P, 18 g of K, 2 g of Mg).

Fermentation and Isolation. Agar plugs of well-grown *G. bidwellii* cultures were aseptically transferred as inoculums to 2 L Erlenmeyer flasks containing 1 L of liquid malt extract medium composed of malt extract 20 g (Lindenmeyer GmbH & Co. KG, Heilbronn, Germany), peptone 1 g (Becton, Dickinson and Company, Sparks, USA), and glucose 20 g per 1 L of tap H_2O. Fermentation was carried out at 22 °C on an orbital shaker at 120 rpm. During the fermentation, samples were taken daily to monitor the occurrence of the natural compounds. Also glucose content and pH value were measured daily. For the semiquantitative measurement of glucose, test strips were used (Diabur-Test 5000, Roche Diagnostics, Mannheim, Germany). The pH value was measured by a pH meter (MP220, Mettler Toledo, Schwerzenbach, Switzerland). After 25 days of fermentation, glucose was depleted in the medium and the

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fermentation was stopped. The mycelium was separated from the culture fluid by filtration via a Büchner funnel. After extraction of the culture filtrate with an equal volume of EtOAc the organic solvent was dried with Na_2SO_4 and evaporated *in vacuo* to dryness. The mycelium was lyophilized and extracted with methanol. The solution was filtered and evaporated *in vacuo* to dryness. Constituents of the crude products were separated using silica gel-60 chromatography with a cyclohexane−EtOAc−methanol gradient, followed by a solid-phase extraction on a Chromabond column (C_{18} ec-column, Macherey-Nagel, Düren, Germany) with a H_2O + 0.1 volume % formic acid−acetonitrile gradient. Final purification of the bioactive secondary metabolites was achieved by preparative HPLC.

Phytotoxic Activity. Leaf disks were collected from intact young leaves of *Vitis vinifera* (cv. Riesling) and placed on H_2O−kinetin agar containing 0.3% agar and 0.2 ppm kinetin solution per 1 L of deionized H_2O. The pure compounds were dissolved in acetonitrile at 1 mg mL^{-1} and applied onto the leaf disks to give amounts ranging from 5 to 100 μg. A 30 μL amount of 5% phosphoric acid was used as the positive control, and 30 μL of acetonitrile was used as the negative control. Incubation was carried out at 22 °C for approximately 24 h (16 h light, 8 h dark). Phytotoxicity assays using intact, greenhouse-grown potted vines (cv. Riesling) were carried out with 1 μg mL^{-1} pure compound applied in a volume of 1 mL of acetonitrile. Seed germination assays using *Setaria italica* and *Lepidium sativum* as test plants were also conducted. Compounds dissolved in acetonitrile were applied onto 12 mm filter disks (Macherey-Nagel, Düren, Germany). After evaporation of the solvent the filters were transferred into an incubation chamber (Ø 14 mm, 4 cm height). Six seeds of each plant and 150 μL of tap H_2O were added. The chambers were initially incubated for 3 d at 27 °C in the dark with a wet atmosphere and afterward for 24 h under artificial light. As controls, filters inoculated with only solvent were used. Phytotoxic effects were assessed after 96 h by measuring the length of the shoot. Phytotoxic activity was concluded when differences of >6 mm compared to the control were observed.

Rice (*Oryza sativa*) leaves at 21 days old were inoculated on water agar (20 g of agar per 1 L of deionized H_2O). Droplets (9 μL) of sterile 0.2% gelatin solution were applied on the leaves. The pure compound was dissolved in acetonitrile at different concentrations, and 1 μL of each concentration was added into the gelatin solution droplet. A 1 μL amount of acetonitrile in 9 μL of 0.2% gelatin solution was used as a negative control. Incubation was carried out at room temperature for approximately 24 h.

Antimicrobial Activity. Antimicrobial activity of the isolated compounds against *Bacillus brevis*, *Bacillus subtilis*, *Enterobacter dissolvens*, *Sarcina lutea*, *Penicillium notatum*, *Paecilomyces variotii*, *Mucor miehei*, and *Nematospora coryli* was determined by agar diffusion assays carried out as described previously.[22] The compounds were tested at concentrations of 5, 10, 25, 50, and 100 μg per filter disk.

Inhibition of conidial germination in fungi was monitored using the test organisms *Magnaporthe oryzae*, *Fusarium graminicola*, *Phytophthora infestans*, and *Botrytis cinerea* as described before.[23] Both compounds were applied in these assays at concentrations up to 100 μg mL^{-1}.

Cytotoxic Activity. Cytotoxic effects of both compounds were assessed using Jurkat and HeLa S3 cells as described previously.[24] The metabolites were tested at concentrations ranging from 5 to 50 μg mL^{-1}.

Phenguignardic Acid (2): brown oil, $[\alpha]^{23}_D$ +116 (*c* 0.18, $CDCl_3$); UV (MeOH) λ_{max} (log ε) 223 (3.74), 231 (3.65), 296 (4.06), 311 (3.99) nm; CD (NH_4^+ salt in MeOH, *c* 4.47 × 10^{-4} M) λ ($\Delta\varepsilon$) 209 (+0.50), 228 (−0.93), 233 (−1.27), 300 (+1.10), 312 (+0.84) nm; IR (KBr) ν 3437, 2930, 1796, 1631, 1361, 1263, 1182, 761, 692 cm^{-1}; ESIMS *m*/*z* (%) 355 (100) $[M - H + 2Na]^+$, 333 (48) $[M + Na]^+$; HRESIMS *m*/*z* 333.0748 (calcd for $C_{18}H_{14}O_5$+Na^+ 333.0739).

Table 1. ^1H (400 MHz) and ^{13}C (101 MHz) NMR Data of Phenguignardic Acid (2) in $CDCl_3$

position	δ_C, type	δ_H (*J* in Hz)	HMBC[a]
2	105.9, qC		7
4	135.5, qC		8
5	162.9, qC		8
6	168.7, qC		
7	41.0, CH_2	3.51, d (14.7)	
		3.44, d (14.7)	
8	109.7, CH	6.20, s	2′/6′
1′	132.3, qC		3′/5′
2′/6′	130.0, CH	7.59, m	8, 2′/6′, 4′
3′/5′	128.9, CH	7.34, m	2′/6′, 3′/5′
4′	129.2, CH	7.31, m	2′/6′, 3′/5′
1″	130.7, qC		7, 3″/5″
2″/6″	131.2, CH	7.22, m	7, 4″, 3″/5″
3″/5″	128.6, CH	7.16, m	3″/5″
4″	127.9, CH	7.16, m	2″/6″

[a]HMBC correlations are from the indicated proton(s) to the carbon stated.

■ ASSOCIATED CONTENT

Supporting Information

Fermentation diagram of *G. bidwellii* and 1D and 2D NMR spectra of phenguignardic acid (**2**). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +49 631/31672-20. Fax: +49 631/31672-15. E-mail: thines@ibwf.de.

Notes

The authors declare no competing financial interest.

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