The Journal of Antibiotics
https://doi.org/10.1038/s41429-019-0154-3

JARA Japan Antibiotics Research Association
The Society for Actinomycetes Japan

BRIEF COMMUNICATION

Secondary metabolites produced by the citrus phytopathogen *Phyllosticta citricarpa*

Daiani C. Savi[1,2] · Khaled A. Shaaban[2,3] · Prithiba Mitra[2] · Larissa V. Ponomareva[2,3] · Jon S. Thorson[2,3] · Chirlei Glienke[1] · Jürgen Rohr[2]

Received: 2 November 2018 / Revised: 29 December 2018 / Accepted: 25 January 2019

Abstract

The isolation and structure elucidation of one new fungal metabolite, phenguignardic acid butyl ester (**1a**), and four previously reported metabolites (**1b**, **2a**, **3**-**4**) from the citrus phytopathogen *Phyllosticta citricarpa* LGMF06 are described. The new dioxolanone phenguignardic acid butyl ester (**1a**) had low phytotoxic activity in citrus leaves and fruits (at dose of 100 µg), and its importance as virulence factor in citrus black spot disease needs to be further addressed. Beside the phytotoxic analysis, we also evaluated the antibacterial (against methicillin sensitive and resistant *Staphylococcus aureus*) and cytotoxic (A549 non-small cell lung cancer, PC3 prostate cancer and HEL 299 normal epithelial lung) activities of the isolated compounds, which revealed that compounds **1a**, **1b** and **2a** were responsible for the antibacterial activity of this strain.

Introduction

Phyllostica citricarpa is an important pathogen associated with citrus black spot (CBS) disease, causing severe economic losses to citrus producers in South Africa, Brazil and the United States [1, 2]. Besides CBS, *Phyllosticta* species are responsible for diseases in several crops including grape, horse chestnut, papaya, pomelo fruit and banana [3]. To control diseases caused by *Phyllosticta* species, it is important to understand the mechanisms involved in this pathosystem. Experiments with pathogen culture filtrates have shown that tissue response in vitro may correlate with disease reaction of the host species and the isolated compounds produced by the phytopathogen may allow to understand the virulence and pathogenicity mechanisms, as well as to select important traits to disease control [4]. The investigation of dioxolanone as a virulence factor in four species of *Phyllosticta* were performed by Buckel et al. [5]. The authors demonstrated that phenguignardic acid (**1c**) and guignardic acid are involved in the development of grape black rot, a serious disease in grapes. Guignardic acid was described as the first member of a new class of natural compounds containing a dioxolanone moiety in 2001, since then, dioxolanone derivatives have been isolated from *Phyllosticta* species and other genera, such as *Aspergillus*, [6–8] as potential virulence factors. Based on these data we isolated and characterized the secondary metabolites produced by *P. citricarpa* LGMF06, a fungus strain isolated from lesions of CBS in Brazil, in order to identify the major secondary metabolites produced by this phytopathogen.

After a large-scale fermentation of the fungus strain LGMF06 in malt extract medium (Fig. S1), the extraction of the filtrate resulted in 752 mg of brown oil crude extract. Purification of the obtained crude extract (752 mg) using various chromatographic techniques afforded one new compound, phenguignardic acid butyl ester (**1a**), along with

These authors contributed equally: Daiani C. Savi, Khaled A. Shaaban

Supplementary information The online version of this article (https://doi.org/10.1038/s41429-019-0154-3) contains supplementary material, which is available to authorized users.

✉ Chirlei Glienke
ch.glienke@gmail.com

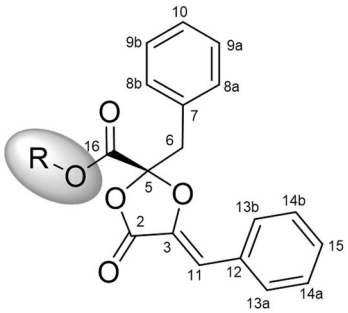
✉ Jürgen Rohr
jrohr2@uky.edu

[1] Department of Genetics, Universidade Federal do Parana, Av. Coronel Francisco Heráclito dos Santos, 210. CEP, Curitiba, Parana 81531-970, Brazil

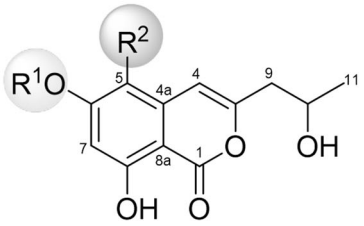
[2] Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, KY 40536, USA

[3] Center for Pharmaceutical Research and Innovation, College of Pharmacy, University of Kentucky, Lexington, KY 40536, USA

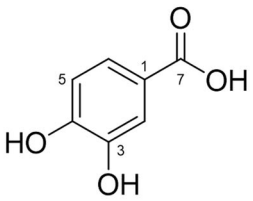
Published online: 21 February 2019



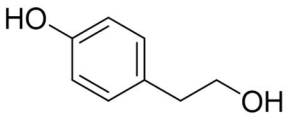
1a: R=$CH_2CH_2CH_2CH_3$; Phenguignardic acid butyl ester
1b: R=CH_3; Phenguignardic acid methyl ester
1c: R=H; Phenguignardic acid



2a: R^1=H, R^2=OH; Peniisocoumarin G
2b: R^1=R^2=H; Orthosporin
2c: R^1=CH_3, R^2=H; Diaporthin



3: Protocatechuic acid



4: Tyrosol

Fig. 1 Chemical structures of compounds **1a**-**4**

four previously reported compounds [phenguignardic acid methyl ester (**1b**), peniisocoumarin G (**2a**), protocatechuic acid (**3**) and tyrosol (**4**)], (Fig. 1 and supplementary file, Fig. S1). The chemical structure of the new metabolite (**1a**) was determined by cumulative 1D and 2D NMR spectroscopy, high resolution mass spectrometry (HRMS), chemical synthesis and by comparison with related structures [5–9].

The physicochemical properties of compounds **1a**, **1b** and **3**-**4** are summarized in the experimental section (see Supplementary Information). Compound **1b** (15.0 mg) was isolated as brown oil using standard chromatographic techniques (Supplementary Information, Fig. S1), and its spectroscopic and spectrometric data were consistent with phenguignardic acid methyl ester, a dioxolanone originally isolated and characterized from the endophytic fungus *Aspergillus flavipes* AIL8 [8]. The physicochemical properties of compound **1a** (6.3 mg) were similar to those of **1b**. The molecular formula of **1a** was deduced as $C_{22}H_{22}O_5$ on the basis of (+)-HRESI-MS [*m/z* 384.1809 $[M + NH_4]^+$ (calcd for $C_{22}H_{26}N_1O_5$, 384.1805)] and NMR, with a molecular mass of 42 amu higher than that of **1b**, which is corresponding to C_3H_6. The proton NMR data of **1a** in CD_3OD (Table 1) were almost identical to those of **1b**, except for the missing of a singlet methoxy at δ_H 3.84 (in **1b**) and the presence of a new signals at δ_H 4.27 (2H, CH_2-18), δ_H 1.63 (2 H, CH_2-19) and δ_H 1.33 (2 H, CH_2-20), along with a triplet methyl signal at δ_H 0.87 (3 H, CH_3-21). Likewise, the ^{13}C NMR and HSQC spectra of **1a** (Table 1) differed from that of **1b**: a methoxy group of **1b** (δ_C 53.9, CH_3-18) was replaced by four up-field shifted signals at δ_C 68.0 (CH_2-18), δ_C 31.6 (CH_2-19), δ_C 20.1 (CH_2-20) and δ_C 14.0 (CH_3-21), consistent with the replacement of the methoxy group of **1b** (16-OCH_3) with an butoxy group (–$OCH_2CH_2CH_2CH_3$) in **1a** (Fig. 1, Supplementary Information, Fig. S2, and Table 1). Cumulative analyses of the ^1H,^1H-COSY/HMBC/TOCSY/NOESY spectra (Supplementary Information, Fig. S2) also confirmed the presence of the butyl group in **1a**, and a key $^3J_{C\text{-}H}$ HMBC correlation from the methylene group at δ_H 4.27 (CH_2-18) to C-16 (δ_C 166.8); Fig. S2 confirmed the attachment of the butoxy group at 16-position. All of the remaining HMBC correlations (Fig. S2) and NMR data (Table 1) are in full agreement with structure **1a**. The absolute configuration of **1a** stereocenter was established by comparison of the NMR shifts, optical rotation to those of **1b** (which have been isolated/characterized from the same strain crude extract), and by its total synthesis (Table 1, Experimental Section and Supplementary Information, Fig. S4). Compounds **1a** and **1b** were chemically synthesized from phenguignardic acid (**1c**) following the procedure previously described by Stoye et al. [7]. Treatment of **1c** with methyl iodide and *n*-butyl bromide in acetone using K_2CO_3 as the base yielded the phenguignardic acid methyl ester (**1b**, 65%) and phenguignardic acid butyl ester (**1a**, 55%), respectively (Fig. S4). The ^1H NMR data of **1a** and **1b** were in full agreement to those of our isolated natural products (Figs S8, S9 and S26, S29) and the reported data of **1b** [7, 8]. In summary, cumulative analyses of 1D (^1H, ^{13}C) and 2D (HSQC, ^1H,^1H-COSY, TOCSY, HMBC and NOESY)

Table 1 ^{13}C (100 MHz) and ^{1}H (400 MHz) NMR spectroscopic data for compounds **1a** and **1b** (δ in ppm)

Position	Compound 1a (CD_3OD)		Compound 1a ($CDCl_3$)		Compound 1b ($CDCl_3$)		Compound 1b ($CDCl_3$) [8][a]	
	δ_C, type	δ_H (mult, *J* in [Hz])	δ_C, type	δ_H (mult, *J* in [Hz])	δ_C, type	δ_H (mult, *J* in [Hz])	δ_C, type	δ_H (mult, *J* in [Hz])
2	163.8, C		162.7, C		162.6, C		162.3, C	
3	136.9, C		135.6, C		135.4, C		135.2, C	
5	106.9, C		105.5, C		105.4, C		105.2, C	
6	41.3, CH_2	3.61 (d, 14.8) 3.55 (d, 14.7)	40.8, CH_2	3.55 (d, 14.7) 3.50 (d, 14.6)	41.0, CH_2	3.56 (d, 14.7) 3.50 (d, 14.8)	109.6, CH	6.33 (s)
7	132.6, C		130.9, C		130.7, C		132.2, C	
8a/8b	132.4, CH	7.30 (m)	131.2, CH	7.29-7.20 (m, overlapped)	131.2, CH	7.26 (m)	129.8, CH	7.67 (d, 7.5)
9a/9b	129.5, CH	7.24 (m)	128.7, CH	7.29-7.20 (m, overlapped)	128.7, CH	7.24 (m)	128.7, CH	7.44 (d, 7.5)
10	128.9, CH	7.24 (m)	128.1, CH	7.29-7.20 (m, overlapped)	128.1, CH	7.24 (m)	129.0, CH	7.38 (t, 7.5)
11	110.1, CH	6.30 (s)	109.7, CH	6.30 (s)	109.9, CH	6.29 (s)	40.8, CH_2	3.61 (dd, 15.0)
12	133.7, C		132.4, C		132.3, C		130.6, C	
13a/13b	131.0, CH	7.67 (d, 8.0)	130.0, CH	7.61 (dd, 7.0, 1.2)	130.1, CH	7.62 (d, 7.2)	131.0, CH	7.32 (m)
14a/14b	129.9, CH	7.40 (t, 7.4)	128.9, CH	7.42-7.32 (m, overlapped)	129.0, CH	7.39 (t, 7.2)	128.4, CH	7.28 (m)
15	130.3, CH	7.37 (t, 7.2)	129.2, CH	7.42-7.32 (m, overlapped)	129.3, CH	7.35 (m)	127.8, CH	7.28 (m)
16	166.8, C		165.6, C		166.0, C		165.8, C	
18	68.0, CH_2	4.27 (tt, 6.4, 2.8)	67.2, CH_2	4.22 (t, 6.6)	53.9, CH_3	3.84 (s)	53.6, CH_3	3.87 (s)
19	31.6, CH_2	1.63 (dt, 14.3, 6.6)	30.5, CH_2	1.61 (m)				
20	20.1, CH_2	1.33 (m)	19.1, CH_2	1.29 (m)				
21	14.0, CH_3	0.87 (t, 7.4)	13.7, CH_3	0.85 (t, 7.4)				

See Supplementary Information for NMR spectra. Assignments supported by 2D HSQC and HMBC experiments.

[a]The NMR assignments of **1b** have been wrongly reported in literature [they have switched the NMR data at the two aromatic rings (positions C-6 to C-15)] and this been corrected herein.

NMR and chemical synthesis established the structure of **1a** (Fig. 1) as a new dioxolanone analogue and was named as phenguignardic butyl ester. It is also important to note that, the NMR assignments of 1b was incorrectly reported in literature [the NMR data at the two aromatic rings were switched (positions C-6 to C-15)] and this has been corrected herein for the first time [8].

Compound **2a** was isolated as pale yellow solid using various chromatographic techniques (Fig. S1). The physicochemical properties and UV/VIS spectrum of **2a** (λ_{max} 230, 265, 350 nm) suggested an isocoumarin chromophore for **2a**, the molecular formula of **2a** was deduced as $C_{12}H_{12}O_6$ on the basis of (–)-HRESI-MS [*m/z* 251.0553 $[M - H]^-$ (calcd for $C_{12}H_{11}O_6$, 251.0561), 503.1187 $[2\,M - H]^-$ (calcd for $C_{24}H_{23}O_{12}$, 503.1195)]. Based on the analyses of 1D and 2D NMR spectra (Fig. S2 and Table S1), the planar structure of **2a** appears to be same as penisocoumarin G (5-hydroxy-orthosporin), an isocoumarin recently reported from the endophytic fungus *Penicillium commune* QQF-3 [10]. Finally, based on the NMR and MS data analyses, compounds **3** and **4** were identified as protocatechuic acid and tyrosol, respectively (Fig. 1), which are common metabolites previously reported from plants and fungi [11, 12].

Compound **1b** was isolated previously from *Aspergillus* sp. in 2014, and until now, there was not much information reported regarding the biological activity of this compound, neither antibacterial nor cytotoxicity data were available. Based on this information, and the isolation of a new dioxolanone analogue (**1a**), we decided to evaluate the phytotoxic activities of these dioxolanones (**1a**, **1b**) and peniisocoumarin G (**2a**) in the host plant tissues. To avail larger quantities for the biological analysis compounds **1a** and **1b** were chemically synthetized from phenguignardic acid (**1c**) (see experimental section), and **1c** was synthesized following the procedure previously described by Stoye et al. [7]. The plant assays were conducted using leaf disks of *Citrus sinensis*, *Citrus reticulate* and *Citrus limon* and the treatment with compound **1a**, at a dose of 100 µg, resulted in lesion development after 12 h of inoculation in *C. sinensis* (Fig. 2a, b), *C. reticulate*, and after 24 h in *C. limon* (Fig. S52). However, even after 48 h compound **1b** did not cause any lesion, even at the higher dose (100 µg), in any of the plants species evaluated. We also assayed the cytotoxicity of phenguignardic acid butyl ester (**1a**) in *C. sinensis* fruits, and a small necrotic zone, similar to CBS lesion, was observed in the area where compound **1a** was deposited (Fig. 2d). The epidemiological and infection processes of CBS were explored in various studies [2, 13]. It was suggested that the production of pycnidia in fruits is related to the disease symptoms, however the impact of phytotoxins in the disease development, to date, has not been addressed. Therefore, it is possible that phenguignardic acid butyl ester (**1a**), a new dioxolanone, is involved in the development of CBS by *P. citricarpa*, in view of its toxicity in citrus leaves and fruits. Phenguignardic acid

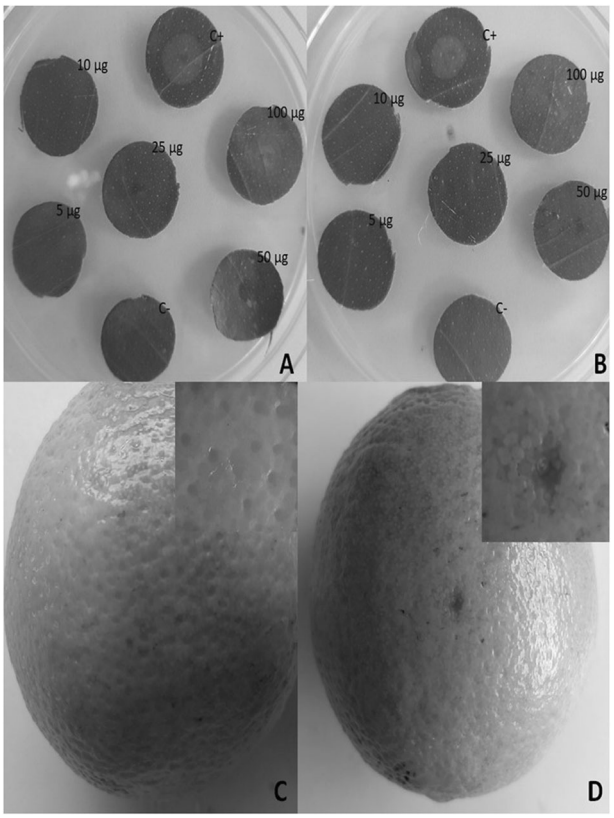


Fig. 2 Phytotoxicity assay in *Citrus sinensis* leaf disks and fruits. **a**, **b** cytotoxicity of phenguignardic acid butyl ester (**1a**) and phenguignardic acid methyl ester (**1b**) in leaves, respectively; **c** control with methanol; **d** cytotoxicity of phenguignardic acid butyl ester (**1a**) in fruits. C+ positive control, 10% of lactic acid, **c** methanol

butyl ester (**1a**) is closely related to compound phenguignardic acid (**1c**), with a butoxy group attached at 16-position (instead of an OH group, Fig. 1), and phenguignardic acid (**1c**) was described as an important virulence factor in the grape black rot disease development, caused by *Guignardia bidwellii* (*P. ampelicida*) [5]. Interestingly, the mechanism of action of this class of compounds remains unknown, and some authors suggested that these compounds could be used as an herbicide, because of their non-host specific toxicity [14]. In addition, orthosporin and closely related compounds, such as diaporthin, can be associated with necrosis in some plants [15], however, at 100 µg, 5-hydroxy-orthosporin (**2a**) did not show any toxicity in citrus leaves or fruits, suggesting that this compound is not related with CBS development.

Compounds **1a, 1b, 2a, 3** and **4** were also evaluated for their antibacterial and cytotoxic activities, i.e. against *Staphylococcus aureus* and methicillin resistant *S. aureus* and against three human cancer cell lines (A549, non-small cell lung cancer; PC3, prostate cancer; and HEL 299, normal epithelial lung). None of the compounds demonstrated cytotoxicity to human cancer cells at concentrations below 80 µM (Fig. S3). However, phenguignardic acid butyl ester (**1a**) displayed low activity against both methicillin sensitive and resistant *S. aureus*, an MIC value of 111 µg ml^{-1} (Table S2). Thus, the methoxy group attached at 16-position in **1b** reduced its antibacterial activity (MIC of 333 µg ml^{-1}), in comparison with the butoxy group in the same position in **1a** (Table S2), which suggest that modifications at 16-position of these molecules influence the antibacterial activity of this class of molecules. Peniisocoumarin G (**2a**) also displayed antibacterial activity against *S. aureus* (MIC 333 µg ml^{-1} - Table S2); however, the closely related compound orthosporin showed even lower activity in the disc diffusion analysis performed by Medeiros et al. [16]. Isocoumarins are common plant metabolites, and were reported having a broad range of biological activities, including antibacterial, antifungal and cytotoxic. For example, the isocoumarin NM-3 was in phase I of clinical tests due its antineoplastic effect [17]. Consistent with previous reports [11, 12] protocatechuic acid (**3**) and tyrosol (**4**) did not show any biological activity in our assays.

Acknowledgements This work was supported by NIH grants CA 91091, GM 105977 and an Endowed University Professorship in Pharmacy to J.R. This work was also supported by National Institutes of Health grants R24 OD21479 (JST), the University of Kentucky College of Pharmacy, the University of Kentucky Markey Cancer Center and the National Center for Advancing Translational Sciences (UL1TR001998). It was also supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico–Brazil grant 424738/2016-3 and CNPq309971/2016-0 to C.G., and CAPES-Brazil – grant to D.C.S. We thank the College of Pharmacy NMR Center (University of Kentucky) for NMR support.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Glienke C, et al. Endophytic and pathogenic *Phyllosticta* species, with reference to those associated with citrus black spot. Persoonia. 2011;26:47–56.
2. Guarnaccia V, et al. First report of *Phyllosticta citricarpa* and description of two new species, *P. paracapitalensis* and *P. paracitricarpa*, from citrus in Europe. Stud Mycol. 2017; 87:161–85.
3. Wikee S, et al. *Phyllosticta capitalensis*, a widespread endophyte of plants. Fungal Div. 2013;60:91–105.
4. Amusa NA. Microbially produced phytotoxins and plant disease management. Afr J Biotechnol. 2006;5:405–14.
5. Buckel I, et al. Phytotoxic dioxolanones are potential virulence factors in the infection process of *Guignardia bidwellii*. Sci Rep. 2017;7:8926.

6. Buckel I, et al. Phytotoxic dioxolanone-type secondary metabolites from *Guignardia bidwelli*. Phytochemistry. 2013;89: 96–103.
7. Stoye A, Kowalczyk D, Opatz T. Total Synthesis of (+)-Phenguignardic acid, a phytotoxic metabolite of *Guignardia bidwellii*. Eur J Org Chem. 2013; 5952–60.
8. Bai Z-Q, et al. New phenyl derivates from endophytic fungus *Aspergillus flavipes* AIL8 derived of mangrove plant *Acanthus ilicifolius*. Fitoterapia. 2014;95:194–202.
9. Molitor D, et al. Phenguignardic acid and guignardic acid, phytotoxic secondary metabolites from *Guignardia bidwelli*. J Nat Prod. 2012;75:1265–9.
10. Cai R, Wu Y, Chen S, Cui H, Liu Z, Li C, She Z. Peniisocoumarins A-J: isocoumarins from *Penicillium commune* QQF-3, an endophytic fungus of the mangrove plant *Kandelia candel*. J Nat Prod. 2018;81:1376–83.
11. Meepagala KM, et al. Isolation of a phytotoxic isocoumarin from *Diaporthe eres*-infected *Hedera helix* (English Ivy) and synthesis of its phytotoxic analogs. Pest Manag Sci. 2018;74:37–45.
12. Teichmann K, et al. In vitro inhibitory effects of plant-derived by-products against *Cryptosporidium parvum*. Parasite. 2016;23:41.
13. Goulin EH, et al. Identification of genes associated with asexual reproduction in *Phyllosticta citricarpa* mutants obtained through *Agrobacterium tumefaciens* transformation. Microbiol Res. 2016; 192:142–7.
14. Molitor D, Beyer M. Epidemiology, identification and disease management of grape black rot and potentially useful metabolites of black rot pathogens for industrial applications-a review. Appl Biol. 2014;165:305–17.
15. Lee IK, Seok SJ, Kim WG, Yun BS. Diaporthin and orthosporin from the fruiting body of *Daldinia concentrica*. Mycobiology. 2006;34:38–40.
16. de Medeiros AG, et al. Bioprospecting of *Diaporthe terebinthifolii* LGMF907 for antimicrobial compounds. Folia Microbiol (Praha). 2018;63:499–505.
17. Oikawa T, et al. Effects of cytogenin, a novel microbial product, on embryonic and tumor cell-induced angiogenic responses in vivo. Anticancer Res. 1997;17:1881–6.