SHORT REPORT

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Lactones from *Glomerella cingulata* Cultivated in Rice: Structural Studies and Antimicrobial Evaluation

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Abstract: Extracts of the endophytic fungus *Glomerella cingulata* grown in polish rice, were fractionated by chromatographic procedures including preparative scale HPLC. The isolated compounds were structurally elucidated through spectroscopic analysis, mainly ^{1}H and ^{13}C NMR and HRMS. These analyzes allowed the identification of pestalotin-1 (**1**) and the phthalides (3R*,8S*)-5,7-dihydroxy-3-(1-hydroxyethyl)-phthalide (**2**) and (3R*,8R*)-5,7-dihydroxy-3-(1-hydroxyethyl)-phthalide (**3**). The extracts, their fractions and isolated substances were tested against several bacteria. The lactone **2** showed some activity against lineages of *E. coli* and *Enterococcus faecalis* while its diastereomer **3** and pestalotin-1 were inactive.

Keywords: Endophytic fungus; *Glomerella cingulata;* antimicrobial activity.

1. Microorganism Source

The endophytic fungus *G. cingulata* was isolated from the plant *Virola surinamesis* by inoculating surface-sterilized leaves into Petri dishes containing potato-dextrose-agar (PDA) medium. The fungus was identified in comparison with our *Glomerella*/*Colletotrichum* collection (c.a. 230 isolates), and stored in the mycoteca of the Laboratory of Micromolecular Biochemistry of Microorganisms (LaBioMMi) at the Federal University of São Carlos (UFSCar) and registered under the code LaBioMMi 1394.

2. Previous Studies

Microorganisms, especially those associated with plants, usually produce substances with diverse biological activities and pharmacological functionalities [1,2]. The search for these substances is of particular interest in fungal species, as they are fertile sources of natural bioactive products [3-5]. Belonging to the class Ascomycetes, the genus *Glomerella* has diverse species, such as *G. acutata*, *G. salicis*, and *G. cingulata*. In the asexual phase, these fungi become known as *Colletotrichum sp.* They

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cause anthracnose in plants [6,7] and can act as biological control agents [8-10]. In addition, *Glomerella* sp. presents an endophytic phase characterized by a mutualistic association with plants. In this phase, the fungi inhabit the internal tissues of plants without damage to the host, benefiting both counterparts. In general, endophytic microorganisms have promising biotechnological and medicinal applications [11,12].

Endophytic fungi produce secondary metabolites of several classes [13,14], which attract attention in the scientific community because of their bioactive potential against human pathogenic bacteria [15,16]. In the literature, reports show different metabolites produced by *G. cingulata*, such as terpenoids [17]. The glomeremophilane sesquiterpenes (A-D) found in *G. cingulata* have shown potential to control neurological diseases such as Alzheimer's and Parkinson's [18]. Despite the relatively large amount of knowledge about the chemistry of *G. cingulata*, the link between its different classes of secondary metabolites with biological activities is still not fully understood. Studies performed by an Indian group showed that extracts of a strain of *G. cingulata* presented no antibacterial activity against gram positive and gram-negative bacteria at any concentration [19], although they did show antifungal activity. Here in this manuscript, we report the production, isolation, and structural identification of the δ-lactone pestalotin (**1**) and a pair of diastereoisomeric γ-lactones (**2** and **3**) from extracts of *G. cingulata* grown in rice. Extracts, fractions, and pure compounds were tested against bacterial strains. Curiously, only one of the γ-lactones diastereomer (**2**) showed antibacterial activity.

3. Present Study

3.1. General Procedures

The ^{1}H and ^{13}C NMR spectra (1D and 2D) of **1** dissolved in deuterated chloroform ($CDCl_3$), were acquired on a DRX-400 Bruker spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). The NMR spectra of compounds **2** and **3** were obtained on a MERCURY-300 Varian spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C) in deuterated methanol (CD_3OD). Chemical shifts (δ) were recorded in ppm using the TMS (Tetramethylsilane) signal as the internal reference. Compound **1** was analyzed by high-resolution mass spectrometry (HRMS) using an Agilent 6545 LC/Q-TOF spectrometer and compounds **2** and **3** in a Waters Xevo G2-S Q-TOF spectrometer. Silica gel 60-254 mesh (Merck) was used in the separations by open-column chromatography. Chromatoplates (Merck) were used for analysis by thin-layer chromatography, which was revealed after elution by exposure to ultraviolet light (360 or 254 nm) and by immersion in vanillin methanol solution followed by heating. The HPLC-DAD data for compound **1** were obtained using a Shimadzu chromatograph, model SIL-10 ADPV, in a Phenomenex Luna column (5mm phenyl hexyl 4.6 x 150 mm) eluting in an exploratory gradient mode composed of water and acetonitrile at a flow of 0.8 mL/min. For compounds **2** and **3**, a C_{18} Gemini column (4.6 x 250 mm x 5mm) was used with a mobile phase composed of water and acetonitrile in the isocratic mode at 1.0 mL/min.

3.2. Fermentation, Extraction and Purification

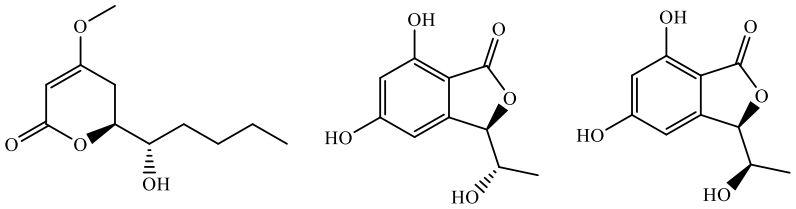
The fungus was cultivated in solid medium using 3 kg of parboiled rice, divided into thirty erlenmeyers (500 mL) containing 85 mL of distilled water. This medium was autoclaved twice for 15 minutes at 121°C and 1 atm at an interval of 24 hours. Inoculation was performed by adding three 5 mm diameter circular slices of PDA containing mycelium. The biomass was extracted after thirty days of cultivation with 150 mL of 99.5% ethyl alcohol and taken to the ultrasound bath for 1 hour. The solution was filtered and then evaporated under reduced pressure, yielding the ethanolic extract (70 g). This extract was chromatographed on silica gel under vacuum, using mobile phases composed of hexane (Hex) and ethyl acetate (EtOAc) in polarity gradient mode, to obtain fractions of FHA-A (Hex:EtOAc - 90:10, 10, 3 mg), FHA-B (Hex:EtOAc - 70:30, 2.01 g) and FA-C (EtOAc - 100%, 751.3 mg); and ethyl acetate with methanol (MeOH) to obtain FAM-D (EtOAc: MeOH - 90:10, 412.5 mg), FAM-E (EtOAc: MeOH - 70:30, 3.6 g) and FM- F (100% MeOH, 9.18 g). The FA-C fraction was chromatographed and eluted with an eluent system composed of Hex:EtOAc in gradient mode, from which it was possible to obtain the FA-C-3 subfraction (Hex:EtOAc - 1:1, 140.2 mg). This sample was purified by preparative HPLC equipped with a reversed-phase column (Phenylhexyl, 10µm, 250 x 21.20mm) and a diode array

detector (DAD). The mobile phase composed of deionized water (A) and acetonitrile (B) was used in gradient elution, ranging from 20 to 80% B in 45 min, remaining constant at 100% B for 25 min. Compounds were detected at 210 and 271 nm. From the FA-C-3 fraction, it was possible to isolate 35 mg of 1. Other fractions from the 100% ethyl acetate elution were also subjected to preparative HPLC separation in a condition similar to that described above for 1, leading to the isolation of the substances 2 (12.0 mg) and 3 (70.0 mg).

3.3. Structural Studies

The molecular structures of **1**, **2** and **3** were drawn in ChemDraw (Level Pro, V. 14.0.0.117) and assigned with a proper 3D orientation by ChemBio3D (Pro, V. 14.0.0.117) and also by Chemcraft (Chemcraft comprises a set of graphical tools for facilitating working with quantum chemistry computations). All the structures were analyzed for connection error in bond order. The energy of the molecules was minimized using Avogadro [20] with MMFF94 force field.

Although natural products **1**, **2**, and **3** (Figure 1) have been described for other fungi, they were not yet found in *Glomerella* species. These three compounds belong to the same general biosynthetic group and are classified as pentaketides (C_{10}), but they show slightly different skeletons. It isn't a big challenge to identify the planar structures of these polyketides by inspection of their spectroscopic data, mainly the NMR with the help of MS data. However, as they all contain two vicinal stereocenters, the identification of their correct stereochemistry is still at least a nontrivial task.



1 **2** (3*R**, 8*S**)- **3** (3*R**, 8*R**)-

Figure 1. Lactones isolated from *G. cingulata* grown in rice

The ^{13}C NMR spectrum of **1** contains three signals for sp^2 hybridized carbons whose chemical shifts (δ_C 90.0, C-3'; 166.7, C-5'; 173.1, C-4') will prompt associate with an α,β-unsaturated carboxyl derivative, containing a very polarized double-bound. These signals, along with the methylene at δ_C 32.4 and the oxygen-bearing carbon at δ_C 72.4 can rapidly identify a 4-methoxy-2H-pyran-2-one partial structure (Figure S1-S6). Similarly, the identification of the 1-penthanol group attached at position C-6' of the pyran-2-one comes from the signals observed in the ^1H NMR. Further, the HRMS (*m/z* 215.1277 $[M+H]^+$; 215.1283 calcd. for $C_{11}H_{19}O_4$) corroborates the identification of the planar structure of **1** (Figure S7).

The identification of the exact stereocenters present in pestalotin-1 structure (**1**) is still not that easy by NMR spectra inspection. It seems that only the isomer (1'S*,6R*)- has been reported as a natural fungal metabolite, although researchers have already synthetized the other three stereoisomers, named (-)-epipestalotin and (+)-pestalotin. In the present work, we optimized the three-dimensional structure of pestalotin-1 in order to find out any important correlation with its ^1H NMR spectrum so that at least the relative stereochemistry could be assigned. The 3D structure of **1** (Figure 2, **1a**) reveals that the 2H-pyran-2-one is a semi-planar ring, and that both H-5 (a and b) are equidistant regarding H-6, i.e. they are *gauche*-oriented in a Newman projection (**1b**). This configuration results in the same coupling constant among H-6 (δ_H 4.31, dt, J = 4.0 and 12.8 Hz) and both H-5a (δ_H 2.25, dd, J = 3.9 and 17.1 Hz) and H-5b (δ_H 2.81, dd, J = 3.9 and 17.1 Hz). Therefore, it is relatively safe to use this information for C-6 relative stereochemistry identification. These data are in agreement with those found in the literature [22,23] for pestalotin-1 (**1**). In the 3D-structural calculations, it was considered the possibility of internal hydrogen bonding. These interactions are more effective when a sample is dissolved in non-polar solvents such as $CDCl_3$. The best structural conformation found is represented by **1c**, where H-6 and H-7 appear *gauche*-oriented, which would result in a medium-coupling constant. The alternative stereochemistry, keeping the *n*-buthyl group in the same equatorial position due to steric hindrance, let H-7 and H-6 disposed in anti-, resulting in a bigger coupling.

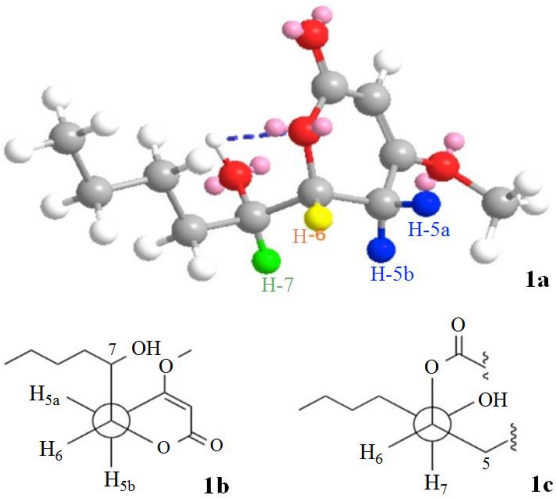


Figure 2. Optimized 3D-structure for pestalotin-1 (**1**)

The fungal metabolites **2** and **3** are part of a great polyketide group known as resorcylic acid lactones. The resorcylic acid (2,4-dihydroxybenzoic acid) portion in these molecules is easily recognized through the two meta-coupled aromatic hydrogens (δ_H 6.29, d, J = 1.8 Hz; δ_H 6.45, dd, J = 0.9 and 1.8 Hz, for **2** and δ_H 6.28, d, J = 1.5 Hz; δ_H 6.44, dd, J = 0.6 and 1.5 Hz, for **3**), seven sp^2 carbons [δ_C 172,1 (171.2), 166.8 (166.7), 159.7 (159.5), 152.8 (153.0), 105.0 (105.0), 103.7 (103.6) and 102.7 (102,4) for **2** and **3**] in its ^{1}H and ^{13}C NMR (Figure S8-S20) respectively (Table S1 and S2). Curiously, these resorcylic acid lactones fungal secondary metabolites are found to contain different ring sizes, being those of 5, 6, 12, and 14 atoms the most frequently found in the literature. The HRMS (Figure S21) analysis of these two lactones showed a prominent ion at *m/z* 211.0606 ($[M+H]^+$), corroborating the molecular formula $C_{10}H_{10}O_5$. There are two groups of isomeric resorcylic lactones with this molecular formula, the γ- and δ-lactones (called phtalides and isocoumarins respectively). The occurrence of a deshielded ^{13}C signal at c.a. 85 ppm, which is characteristic of carbon-bearing oxygen in a 5-membered ring, is a decisive data enough for the differentiation of the two structural possibilities. Therefore, according to our data and those found in the literature [24], lactones **2** and **3** are phthalides. As mentioned above, what is still a challenge in these lactones structures is the assignment of the vicinal stereocenters. In the present work, we made some efforts to identify the relative stereochemistry in these molecules using NMR data interpretation, since they showed different responses in the antibacterial bioassays. For this purpose, the three-dimensional structures of phthalides **2** and **3** were optimized, also considering the occurrence of internal hydrogen bonding. Thus, the hydrogens H-3 and H-8 appear on the opposite side (*anti*-) in the optimized phthalide **2** structure (Figure 3, **2a**), and in *gauche*- for phthalide **3** (Figure 3, **3a**). As a consequence of these spatial arrangements, the coupling constants of H-3 with H-8 are higher (c.a. 5.5 Hz) for the (3*S**,8*R**)- isomer (**3**), and half of this value (c.a. 2.5 Hz) for its diastereoisomer **2** (3*S**,8*S**)-. This is in agreement with the work carried out by other researchers after the synthesis of the four isomers.

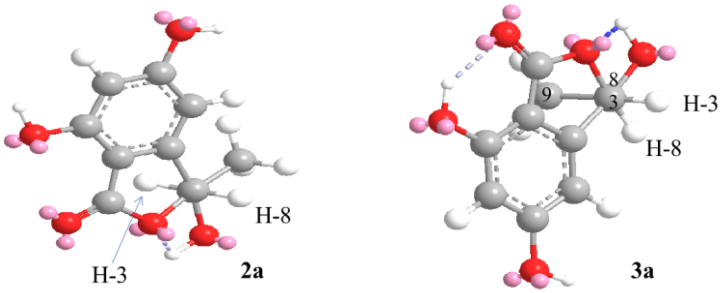


Figure 3. Optimized 3D-structure for phthalides **2** and **3**

3.4. Evaluation of Antimicrobial Activity

The antibacterial activity was evaluated by determining the minimum inhibitory concentrations (MIC) using the microdilution assay against strains of *Listeria monocytogenes*, *Salmonella enterica*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Escherichia coli*. The fractions and compopunds **1**, **2**, and **3** were dissolved in dimethyl sulfoxide (DMSO) to reach a concentration of 10,000 μg/mL. Each solution was serially diluted in a 96-well plate containing Mueller-Hinton (MH) medium to obtain a gradient of concentrations ranging from 2500 μg/mL to 4.98 μg/mL. Ciprofloxacin suspension (50 μg/mL) was used as a positive control. The bacterial inoculum was prepared in autoclaved glass tubes with a screw cap containing 3 mL of sterile phosphate-buffered saline (PBS), at 1.5 × 108 CFU/mL, with turbidity equivalent to 0.5 on the McFarland. Posteriorly, the wells received 10 μL of a microbial suspension. After 24h of incubation at 37ºC, the bacterial growth was checked using 30 μL of 0.03% resazurin sodium solution. The MIC was defined as the lowest concentration able to inhibit bacterial growth, seen by a change in color from blue to pink [21].

The results showed that the fractions FHA-A, FHA-B, and FA-C presented the lowest inhibitory concentration, which corresponds to the value 625.0 μg/mL. The solutions of FHA-A and FHA-B presented inhibitory activity against the *E. coli*, while FA-C was active against the *S. aureus*. Furthermore, the other tested fractions had a high MIC (1250 μg/mL and 2500 μg/mL) for all the tested bacteria. Despite the high MIC value, the non-negligible antibacterial activity of this extract was further investigated. The ethyl acetate extract, in particular the solution with 500.0 μg/mL, presented bacteriostatic activity against the *E. faecalis* strain. The compound **2** showed bactericidal activity against *E. coli* (250.0 μg/mL) and *S. aureus* (500.0 μg/mL) as well as bacteriostatic activity against *E. coli* (62.5 μg/mL) and *E. faecalis* (31.2 μg/mL). Curiously, the compound **3**, a diastereoisomer of **2**, didn't show any inhibitory activity on the pathogens tested (Table S3, see supporting information). In contrast to the results described by K. Harakishore group [20], where no antibacterial activity was shown by any concentrations of *G. cingulata* crude extract, we observed a non-negligible antibacterial activity presented by the fungal biomass extracts of the *G. cingulata* when cultivated in rice, and also determined the differential activity of a pair of diasteromeric lactones.

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Supporting Information

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