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Production and toxicity of 2,3-dihydro-5-hydroxy-2-methyl-4H-1-benzopyran-4-one by *Phialophora gregata*

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Abstract

2,3-Dihydro-5-hydroxy-2-methyl-4H-1-benzopyran-4-one, was isolated from 3 week old rice cultures of *Phialophora gregata*, a soybean pathogen, grown at 25°C. This material in repeated bioassays prevented callus formation on soybean hypocotyl sections at 100 and 200 μg/ml callus culture medium. This is the first report of the production of this metabolite by a member of the *Phialphora* genus and of its toxicity to soybeans. Published by Elsevier Science Ltd.

Keywords: *Phialophora gregata*; *Glycine max*; *Leguminosea*; Soybean; Mycotoxin

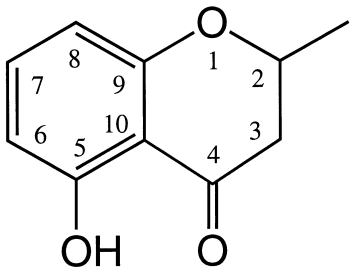
1. Introduction

Phialophora gregata (Allington and Chamberlain) W. Gams is a vascular pathogen of soybean plants (*Glycine max* (L.) Merr.) in the midwest (Allington & Chamberlain, 1948). In previous work it was shown that isolates of this fungus produce a toxic metabolite, gregatin A (Kobayashi & Ui, 1975; Kobayashi & Ui, 1977; Taylor, Peterson, & Gray, 1985). This metabolite shows anti-fungal and anti-bacterial activity as well as causing browning of internal stem vascular tissue when plants are exposed to the material (Kobayashi & Ui, 1977). During routine isolations of gregatin A from rice cultures, another unknown metabolite was consistently produced by *Phialophora gregata*. Herein we report on its identification and show that it has biological activity against soybean cells.

2. Results and discussion

2.1. Identification of 2,3-dihydro-5-hydroxy-2-methyl-4H-1-benzopyran-4-one (*1*)

Hexane extraction of a rice culture of the soybean pathogen, *Phialophora gregata*, furnished two principal compounds, the known gregatin A (Kobayashi & Ui, 1975; Kobayashi & Ui, 1977; Taylor et al., 1985) and a new metabolite **1** (also known as 5-hydroxy-2-methyl-chroman-4-one (Anderson, Edwards, & Whalley, 1983)) to the *Phialophora* genus. The yield from a typical 250 ml rice culture flask (60 g, wet weight) was 6 mg **1** and 9 mg gregatin A following 3 weeks incubation at 25°C of an inoculated flask. After TLC isolation, **1** was analysed by GC–MS showing it to be a single component with an apparent molecular ion of m/z 178. Characterization by ^{1}H NMR spectroscopy (Table 1) revealed essentially the same data reported for **1** previously (Anderson et al., 1983; Arnoldi, 1984). The present data, collected at higher magnetic field strength, permitted more detailed



2,3-dihydro-5-hydroxy-2-methyl-4H-1-benzopyran-4-one (**1**)

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Table 1
^{1}H NMR and ^{13}C NMR spectral data and coupling constants for **1**

H/C position	^{1}H NMR	^{13}C NMR
2	4.56m, 1H	73.8
2-Me	1.50d, 3H, $J_{2,2Me}$6.3	20.8
3a	2.73dd, 1H, $J_{3a,3b}$17.1, $J_{2,3a}$12.0	43.8
3b	2.66dd, 1H, $J_{3a,3b}$17.1, $J_{2,3b}$3.7	43.8
4	–	198.5
5	–	162.1[a]
5-OH[d]	11.70s, 1H	–
6	6.48[c]dd, 1H, $J_{6,7}$8.3, $J_{6,8}$1	109.1[b]
7	7.33dd, 1H, $J_{6,7}$8.3, $J_{7,8}$8.3	138.2
8	6.41[c]dd, 1H, $J_{7,8}$8.3, $J_{6,8}$1	107.3[b]
9	–	161.7[a]
10	–	108.0

[a,b,c] Shifts with the same superscript could be interchangeable for assignment.
[d] Proton exchanges with $2H_2O$.

analyses of coupling. Previously unreported ^{13}C NMR assignments are found in Table 1.

Derivatives of **1** were prepared to ascertain the structure. Although the product of reduction of **1** by $NaBH_4$ was not recovered, possibly due to the increased water solubility of the reduced compound, preparation of its methoxime derivative confirmed the presence of one ketone group. Upon examination of the GC–MS, the methoxime afforded a molecular ion at m/z 207 with fragmentation ions indicative of the methoxime (loss of MeOH, NOMe+Me and MeO+Me). GC–MS of the trimethlsilyloxy ether derivative afforded a molecular ion of m/z 250 and a base ion of 235 $[M–Me]^+$ indicating the presence of one hydroxyl group. The absence of either a carboxylic acid or lactone functionality was shown by the complete stability of **1** to 10% H_2SO_4 in methanol for 5 h at 60°C. The latter result rules out the presence of the isomerically related mellein, 3,4-dihydro-8-hydroxy-3-methyl-4H-2-benzopyran-1-one, previously isolated from *Aspergillus melleus* (Boit, 1975).

The UV spectrum of **1** was consistent with data published previously (Anderson et al., 1983), except that there was additional end absorption detected at λ_{max} 206 nm (MeOH).

2.2. Plant toxicity assay

Soybean callus formation was reduced when hypocotyl sections of Century soybean plants were exposed to **1** in a tissue culture assay for 7 days at 25°C. Two separate experiments with soybean tissue culture assay were completed and in both cases callus formation was reduced (Table 2). In preliminary studies, TLC plates containing separated mixtures of gregatin A and metabolite **1** were overlaid in separate tests with agar containing either *Bacillus subtilis* or *Saccharomyces cerevisiae* (data not shown). Gregatin A inhibited growth of both *B. subtilis* and *S. cerevisiae* whereas metabolite **1** only inhibited the growth of *S. cerevisiae*. These results agree with previously published work that showed gregatin A inhibited the growth of bacteria and yeast (Kobayashi & Ui, 1977) whereas metabolite **1** inhibited the growth of yeast (Anke, Stadler, Mayer, & Sterner, 1995). From the results of the present soybean callus formation assay, metabolite **1** does not appear to be highly toxic to soybean cells. In comparison, 6-methoxymellein and 6-hydroxymellein (Marinelli, Zanelli, & Ronchi, 1996) have been shown to reduce carrot (*Daucus carota* L.) suspension cell viability at concentrations from 0.06 to 0.3 mM and in other work (Kurosaki, Matsui, & Nishi, 1984) 6-methyoxymellein at concentrations of 0.5 to 1 mM caused an 80% decrease in carrot suspension cell viability. Compound **1** in our present work was used at higher concentrations with intact soybean hypocoty tissue pieces rather than soybean cell suspensions so factors such as metabolite tissue penetration differences between intact tissue and individual plant cells could be a source of higher metabolite concentration of compound **1** for comparable inhibition. Metabolite **1** was found to have low water solubility in our present work (unpublished data), so the effective concentration in the soybean tissue culture assays are unknown. Metabolite **1** has been isolated from *Daldinia concentrica* (Allport & Bu'Lock, 1960) and also from one strain of *Hypoxylon howeianum* (Anderson et al., 1983). Since both **1** and gregatin A have been shown to be produced by *Phialophora gregata* and these materials are toxic to soybean cells, they may play an important role in development of symptoms in diseased soybean plants. Future work needs to be done to determine their exact role in disease development in infected soybean plants.

Table 2
Mean soybean hypocotyl section fresh weight in mg after 1 week exposure to **1**

Concentration (μg/ml)	Experiment 1[a]	Experiment 2[b]
0	111.5	121.4
100	73.4	43.4
200	30.6	20.6
300	11.5	16.4
LSD ($P \leqslant 0.05$)	40.3	13.8

[a] Each treatment was performed in duplicate and data is presented as the mean.
[b] Each treatment was performed in triplicate and data is presented as the mean.

3. Experimental

3.1. Instrumentation

NMR spectra were obtained with a Bruker model ARX-400 spectrometer (^{1}H NMR, 400 MHz; ^{13}C NMR,

100 MHz). NMR spectra were recorded with $CDCl_3$ as internal standard and solvent. GC–MS was completed with a Hewlett-Packard model 5890 gas chromatograph interfaced with a model 5971 mass-selective detector operating at 70 eV. The capillary WCOT GC column was a Hewlett-Packard HP-5MS cross-linked 5% phenyl methyl silicone (0.25 mm × 30 m, film thickness 0.25 μm). The temperature was programmed from 65–260°C at 10°C/min; He flow was 0.675 ml/min (retention time of **1** = 11.2 min).

3.2. Cultures

All isolates of *P. gregata* used in this study originated from single conidium cultures. Cultures were maintained on slants of soybean stem extract agar at 20°C. For all experiments, new cultures were prepared from stock cultures and grown at 20°C on soybean stem agar plates. Production of certain metabolites by different isolates were examined in sterile autoclaved white rice (*Oryza sativa L.*). In each 250 ml flask, 25 g of white rice was added and enough distilled water to cover the rice. Each flask was then microwaved 3 times on a high setting to pre-wet the rice. The excess water was drained from each flask and the flasks were autoclaved for 25 min. A 1.5 × 0.5 cm piece of fungal agar culture was placed in a sterile plastic test tube and macerated with 4 ml of sterile water. The macerated fungal mycelium plus spores was then distributed over the surface of the sterile rice culture. Each flask was incubated at 20°C for 3 weeks in the dark.

3.3. Extraction and purification of metabolites

Each rice culture was placed in a glass beaker and 200 ml hexane was added. The rice was stirred with a glass rod and left for 30 min with occasional stirring. The hexane solubles were filtered and the solution subsequently evaporated to dryness with a flash evaporator. The oily residue results were redissolved in a minimum volume of EtOAc, transferred to a pre-weighed glass vial and the contents evaporated under a stream of N_2 until constant weight was obtained. All samples were then maintained in the dark at −20°C until work-up. The sample of crude extract was applied to silica gel 60 TLC coated with fluorescent indicator (Aldrich) and the plate was developed in a solvent mixture of hexane–EtOAc–*iso*-PrOH (15:4:1). The plates were examined under UV light (254 nm) separating **1** (R_f 0.80) from gregatin A (R_f 0.70) and each band was extracted with EtOAc.

Metabolite **1** was re-isolated by silica gel 60 TLC and plates were developed with hexane–EtOAc (4:1). **1** was isolated as a band at R_f 0.68 separating it from residual gregatin A at R_f 0.39.

3.4. 2,3-Dihydro-5-hydroxy-2-methyl-4H-1-benzopyran-4-one (*1*)

^1H and ^{13}C NMR data are given in Table 1. EIMS *m/z* (rel. int.): 178 $[M]^+$ (92), 163 $[M–Me]^+$ (44), 136 $[M–CH_2CO]^+$ (100), 108 $[M–MeCHCHCO]^+$ (58), 80 (10), 69 (4), 52 (10), 39 (9).

3.5. Methoxime of *1*

1 mg of **1** was treated with 2 mg methoxylamine·HCl (Aldrich) in 0.2 ml dry pyridine for 3 h affording the methoxime of **1**. EIMS *m/z* (rel. int.): 207 $[M]^+$ (100), 192 $[M–Me]^+$ (2), 175 $[M–MeOH]^+$ (9), 161 $[M–MeO–Me]^+$ (4), 147 $[M–MeON–Me]^+$ (14), 135 (66), 117 (23), 107 (14), 89 (9), 78 (7), 63 (6), 51 (7), 41 (23).

3.6. Trimethylsilyloxy (TMSiO) ether of *1*

1 mg of **1** was treated with 30 μl trimethylchlorosilane–hexamethyldisilazane–dry pyridine (3:2:2) for 30 min and then the reagent was evaporated under a stream of N_2 for dissolution of the TMSiO derivative in hexane. EIMS *m/z* (rel. int.): 250 $[M]^+$ (1), 235 $[M–Me]^+$ (100), 217 (4), 189 (3), 161 $[M–TMSiO]^+$ (5), 117 (3), 110 (3), 75 (21), 73 $[TMSi]^+$ (13).

3.7. Plant toxicity assay

Toxicity of the metabolite in question was examined in a soybean tissue culture assay. The inhibition of callus initiation was determined at concentrations of 100, 200 and 300 μg per ml of tissue culture medium (Murashige & Skoog, 1962). Sterile hypocotyl sections from soybean cultivar Century (4 per well) were placed in 1.5 ml of soybean tissue culture medium in 24 well culture plates and incubated at 25°C under continuous light on a platform shaker for 7 days. Metabolite **1** purified by the same procedures used for the chemical structure determinations was placed on sterile 1.25 cm antibiotic assay discs, the EtOAc solvent evaporated and then the discs were placed into individual wells of a 24 well tissue culture dish. Samples of soybean hypocotyl sections (4 per sample) were weighed initially and an average of duplicate samples was used to determine the average initial sample weight. At 7 days, samples of each assay concentration were removed and weighed. The soybean toxicity was assayed by two separate experiments (once in duplicate, once in triplicate) and data for each concentration is reported in Table 2.

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