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Antibacterial Activity of Acylglucinol Derivatives against *Flavobacterium columnare*

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Abstract

Columnaris (caused by *Flavobacterium columnare*) is one of the most common bacterial diseases affecting the pond-raised channel catfish (*Ictalurus punctatus*) in the southeastern United States of America resulting in annual losses of millions of dollars. As part of our continuing effort to discover environmentally benign compounds for the control of columnaris disease, acyl derivatives of phloroglucinol were synthesized and tested against *F. columnare* using a rapid bioassay. Among the analogs that were tested, diacyl analogs showed very high antibacterial activity against *F. columnare* in the laboratory bioassay. Diisovaleryl and diisobutyryl analogs were found to have the strongest activity against *F. columnare* (isolate ALM-00-173) based on 24-h 50% inhibitory concentration (IC_{50}) and minimum inhibitory concentration (MIC). Diisovaleryl and diisobutyryl analogs had IC_{50} values 0.82 mg/L and 0.80 mg/L, respectively, whereas the drug control florfenicol had an IC_{50} value of 0.81 mg/L. Diisovaleryl and diisobutyryl analogs also had 24-h relative-to-drug-control IC50 values around 1.0 indicating activities similar to florfenicol, which is included in medicated feed and is one of the current management approach for columnaris.

Keywords

Acylphloroglucinol, Columnaris Disease, *Flavobacterium columnare*, Antibacterial, Aquaculture

1. Introduction

Columnaris disease caused by *Flavobacterium columnare* is one of the most common bacterial diseases of pond-raised channel catfish (*Ictalurus punctatus*) in the southeastern United States of America (USA) with mor-

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talities as high as 50% - 60% in pond populations of catfish [1]. Direct economic losses to catfish producers are likely millions of dollars annually from single or mixed infections involving *F. columnare* [2]. The Gram-negative, rod-shaped bacterium *Flavobacterium columnare* is the cause of columnaris disease, and two genomovars of *F. columnare* have been identified, with genomovar II isolates attributed as being more pathogenic towards channel catfish [3]. The U.S. Food and Drug Administration (FDA) has granted conditional approval of Aquaflor® (50% florfenicol active ingredient) for treating columnaris disease in channel catfish [4].

There are numerous reports about antibacterial activity of phloroglucinol derivatives [5]-[8]. Acylphloroglucinols isolated from *Hypericum olympicum* have shown antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug resistant (MDR) strains of *Staphylococcus aureus*, Mycobacterium species and *Salmonella enterica* [9]. Monomeric and polymeric phloroglucinols are secondary metabolites that occur in certain plant species. These derivatives occur in other organisms such as brown algae or bacteria *Ecklonia stolonifera*, *Eisenia bicyclis* [10] or species in the genus *Zonaria* [11], which produce phloroglucinol and phloroglucinol derivatives. The bacterium *Pseudomonas fluorescens* produces phloroglucinol, phloroglucinolcarboxylic acid, and diacetylphloroglucinol [12]. Acyl phloroglucinols have been isolated from coastal woodfern *Dryopteris crassirhizoma* [13]. Some phloroglucinol analogs have been isolated from *Eucalyptus* species [14]. The pharmacological activity of St. John's wort (*Hypericum perforatum*) is due to phloroglucinol derivatives hyperforin and adhyperforin [15] [16]. Humulone is a phloroglucinol derivative with two prenyl groups and one isovaleryl group found in the resin of mature hops (*Humulus lupulus)* used as flavoring and stability agents in manufacture of beer to provide a bitter, tangy flavor [17]. Diacylphloroglucinols have been shown to possess antiviral and antibacterial activity [18]. Diisobutyrylphloroglucinol has been shown to have a strong anti-bacterial activity against *Bacillus subtilis* (IFO 3734) and a moderate activity against *Escherichia coli* (IFO 3301) [18].

As part of our continuing effort to discover environmentally benign compounds for the control of columnaris disease, acyl derivatives of phloroglucinol were synthesized and tested against *F. columnare* in a rapid bioassay developed by Schrader and Harries [2]. As a result of our investigation, we were able to identify several potent derivatives with activities similar to the drug control florfenicol.

2. Materials and Methods

2.1. General Experimental Procedures

All solvents were reagent grade and used without further purification. Progress of synthesis and chromatographic profile were monitored by silica gel TLC plates GF with fluorescent indicator (250 micron, Analtech, Newark, DE, USA). Iodine vapor, UV light (at 254 and 365 nm), and anisaldehyde spray reagents were used for the detection of compounds. Flash column chromatography was performed on Biotage Isolera Four (Biotage, Charlotte, NC, USA) using FLASH + silica gel cartridges with ultra violet detection at 254 and 280 nm. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury AS400 spectrometer operating at 400 MHz for ^{1}H NMR and at 100 M*Hz* for ^{13}C NMR. The HR-ESIMS was measured using Jeol ACCU TOF JMS-T1000 mass spectrometer.

2.2. Materials

Phloroglucinol, $POCl_3$, and isovaleric acid were purchased from Sigma Aldrich (St. Louis, MO, USA). $AlCl_3$ was obtained from Lancaster Chemical (Ward Hill, MA, USA). Isobutyric acid, 3-phenylpropionic acid, and 2-methylbutyric acid were supplied by Alfa Aesar (Ward Hill, MA, USA).

2.3. Synthesis

The general synthetic procedure involved reaction of the appropriate acid with $POCl_3$ in the presence of $AlCl_3$ under N_2 for 24 h (4 h at 0˚C and then allowed to come to normal room temperature gradually) (**Figure 1**). The products were purified by biotage flash column chromatography using increasing amounts of amounts of ethyl acetate in hexane (0% to 80%). The purity of these compounds was confirmed by thin layer chromatography, high resolution ESI mass spectrometry and NMR.

triisovalerylphloroglucinol (**3a**) ^{1}H NMR δ ($CDCl_3$) 0.99 (18H, d, J = 8 *Hz*), 2.25 (3H, m), 2.98 (6H, d, J = 8 *Hz*), ^{13}C NMR 22.7, 25.4, 52.7, 103.1, 175.8, 207.3 HR-ESI-MS, [M-H-]377.19357 (calcd for $C_{21}H_{29}O_6$, 377.19641)

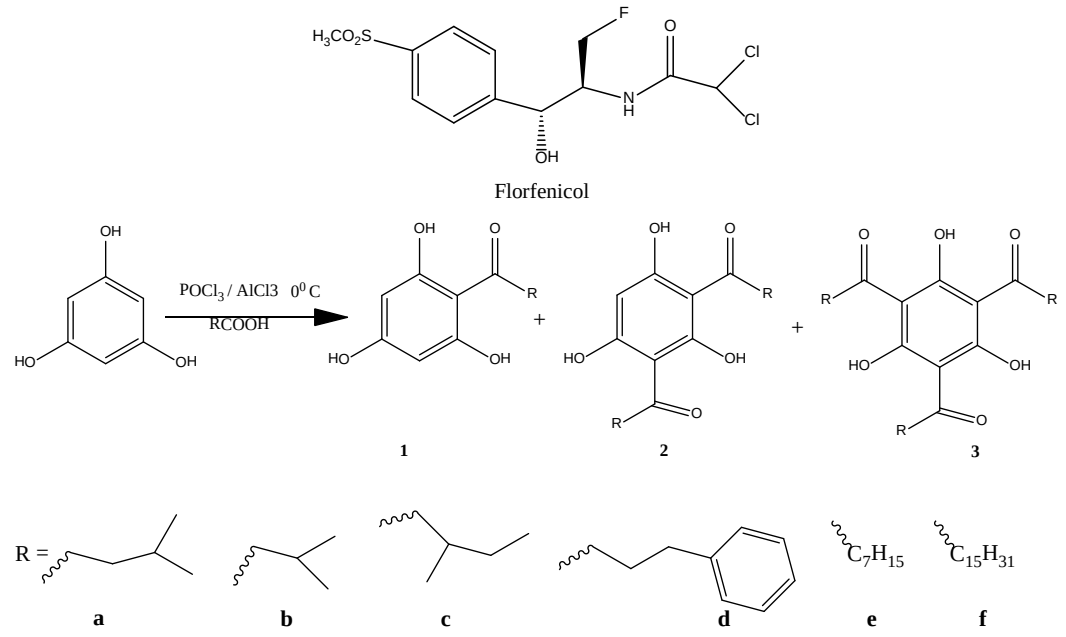

Figure 1. General synthetic procedure of phloroglucinol analogs.

diisovalerylphloroglucinol (**2a**) ^{1}H NMR δ ($CDCl_3$) 0.99 (12H, d, J = 8 *Hz*), 2.25 (2H, m), 2.98 (4H, d, J = 8*Hz*), 5.90 (1H, s), ^{13}C NMR δ 22.7, 25.4, 52.7, 103.1 125.9, 128.7, 141.7, 168.6, 205.3 HR-ESI-MS, [M-H-]293.13564 (calcd for $C_{16}H_{21}O_5$, 293.13890)

monoisovaleryl phloroglucinol (**1a**) ^{1}H NMR δ ($CDCl_3$) 0.91 (6H, d, J = 8 *Hz*), 2.29 (1H, m), 2.88 (2H, d, J = 8*Hz*), 5.83 (2H, s), ^{13}C NMR δ 22.7, 24.8, 53.3, 96.1, 104.3, 164.5, 165.7, 205.8 HR-ESI-MS, [M-H-] 209.07698 (calcd for $C_{11}H_{13}O_4$, 209.08138)

triisobutyryl phloroglucinol (**3b**) ^{1}H NMR δ ($CDCl_3$) 1.2 (18H, d, J = 8 *Hz*), 3.99 (3H, septet, J = 8 *Hz*), ^{13}C NMR HR-ESI-MS, [M-H-]347.14841 (calcd for $C_{19}H_{23}O_6$, 347.14946)

diisobutyrylphloroglucinol (**2b**) ^{1}H NMR δ ($CDCl_3$) 1.1 (12H, d, J = 8 *Hz*), 3.96 (2H, septet, J = 8 *Hz*), 5.8 (1H, s), ^{13}C NMR δ 18.2, 38.6, 94.4, 164.4, 210.2 HR-ESI-MS, [M-H-]265.11040 (calcd for $C_{14}H_{17}O_5$, 265.10760)

monoisobutyrylphloroglucinol (**1b**) ^{1}H NMRδ($CDCl_3$) 1.2 (6H, d, J = 8 *Hz*), 3.96 (1H, septet, J = 8 *Hz*), 5.9 (3H, s), ^{13}C NMR δ 19.1, 39.2, 95.6, 172.2, 172.9, 210.8 HR-ESI-MS, [M-H-]195.06472 (calcd for $C_{10}H_{11}O_4$, 195.06573)

tri-2-methylbutyrylphloroglucinol (**3c**) ^{1}H NMR δ ($CDCl_3$) 0.9 (9H, t, J = 8 *Hz*), 1.2 (9H, d, J = 4 *Hz*), 1.4 (6H, m) 3.8 (3H, m), ^{13}C NMR HR-ESI-MS, [M-H-]377.19540 (calcd for $C_{21}H_{29}O_6$, 377.19641)

di-2-methylbutyrylphloroglucinol (**2c**) ^{1}H NMR δ ($CDCl_3$) 0.9 (6H, t, J = 8 *Hz*), 1.2 (6,H, d, J = 6 *Hz*), 1.4 (4H, m) 3.8 (2H, m), 5.9 (1H, s) ^{13}C NMR HR-ESI-MS, [M-H-]293.13671 (calcd for $C_{16}H_{21}O_5$, 293.13890)

mono-2-methylbutyrylphloroglucinol (**1c**) ^{1}H NMR δ ($CDCl_3$) 0.9 (3H, t, J = 8 *Hz*), 1.1 (3,H, d, J = 8 *Hz*), 1.7 (2H, m) 3.8 (1H, m), 5.8 (2H, s) ^{13}C NMR HR-ESI-MS, [M-H-]219.15953 (calcd for $C_{11}H_{23}O_4$, 219.159635)

tri-3 phenylpropionylphloroglucinol (**3d**) ^{1}H NMR δ (CDCl3) 2.8 (6H, t, J = 8 *Hz*), 3.0 (6H, t, J = 8 *Hz*), 6.69 (3OH, s), 7.2-7.34 (9H, m), 7.28 - 7.32 (6H, m), ^{13}C NMR δ 30.8, 35.9, 112.7, 126.5, 128.4, 128.6, 139.9, 151.0, 170.5 HR-ESI-MS, [M-H-]521.19659 (calcd for $C_{33}H_{29}O_6$, 521.19641)

di-3-phenylpropionylphloroglucinol (**2d**) ^{1}H NMR δ (CD3OD) 2.9 (4H, t, J = 8 *Hz*), 3.34 (4H, t, J = 8 *Hz*), 5.91 (1H, s), 7.18 - 7.3 (10H, m),^{13}C NMR δ 30.3, 45.6, 95.26, 103.9, 125.9, 128.7, 141.7, 168.6, 205.3 HR-ESI-MS, [M-H-]389.13176 (calcd for $C_{24}H_{21}O_5$, 389.13890)

mono-3-phenylpropionylphloroglucinol (**1d**) ^{1}H NMR δ (CD3OD) 2.92 (2H, t, J = 8 *Hz*), 3.31 (2H, t, J = 8 *Hz*), 5.83 (2H, s), 7.10 - 7.24 (5H, m),^{13}C NMR δ 30.3, 45.6, 95.26, 103.9, 125.9, 128.7, 141.7, 168.6, 205.3 HR-ESI-MS, [M-H-]257.08098 (calcd for $C_{15}H_{13}O_4$, 257.08138)

dioctanoylphloroglucinol (**2e**) ^{1}H NMR δ (CD3OD) 0.9 (6H, t, J = 8 *Hz*), 1.2 (12H, br S), 1.3-1.4 (8H, m), 2.3

(4H, t, J = 8 *Hz*), 5.9 (1H, s),^{13}C NMR δ 14.1, 22.7, 29.2, 29.4, 31.8, 44.2, 95.15, 172.1, 219.2 HR-ESI-MS, [M-H-]377.2391 (calcd for $C_{22}H_{33}O_5$, 377.23280)

dipalmitylphloroglucinol (**2f**) ^{1}H NMR δ (CD3OD) 0.9 (6H, t, J = 8 *Hz*), 1.2-1.3 (24H, br S), 1.4 (4H, m), 2.9 (4H, t, J = 8 *Hz*), 5.9 (1H, s),^{13}C NMR δ 14.1, 22.7, 29.2, 29.4, 31.8, 44.2, 95.15, 172.1, 219.2 HR-ESI-MS, [M-H-]601.48343 (calcd for $C_{38}H_{65}O_5$, 601.48320)

3. Antibacterial Bioassay

An isolate of *F. columnare* [isolate ALM-00-173 (genomovar II)] was obtained from Dr. Covadonga Arias (Department of Fisheries and Allied Aquacultures, Auburn University, Alabama, USA). Cultures of the *F. columnare* isolate were maintained separately on modified Shieh (MS) agar plates (pH 7.2 - 7.4) in order to assure purity. Prior to bioassay, single colony of *F. columnare* was used to prepare the assay culture material by culturing in 75 mL of MS broth (24 h) at 29˚C ± 1˚C at 150 rpm on a rotary shaker (model C24KC; New Brunswick Scientific, Edison, New Jersey, USA). After overnight incubation, a 0.5 McFarland standard of *F. columnare* assay material was prepared by transferring cells from the broth culture to fresh MS broth [2].

Pure compounds (>98% pure) were evaluated for antibacterial activity using a rapid 96-well microplate bioassay and following the procedures of Schrader and Harries [2]. Florfenicol was included as a positive drug control. In addition, control wells (no test compound) were included in each assay. All pure compounds were dissolved separately in technical grade 100% methanol. Drug controls were dissolved in technical grade ethanol. Final concentrations of test compounds and drug controls were 0.01×10^4 µM. Three replications were used for each dilution of each test compound and controls. In order to determine the 24-h 50% inhibitory concentration (IC_{50}) and minimum inhibitory concentration (MIC), sterile 96-well polystyrene microplates (Corning Costar Corp., Acton, Massachusetts, USA) with flat-bottom wells were used. Initially, reconstituted test compounds or drug control were aspirated separately into individual microplate wells (10 µL/well), and solvent was allowed to completely evaporate at room temperature before 0.5 MacFarl and bacterial culture (prepared as described by Schrader and Harries [2]) was added to the microplate wells (200 µL/well). Microplates were incubated at 29˚C ± 1˚C (VWR model 2005 incubator; Sheldon Manufacturing, Inc., Cornelius, Oregon, USA). A Packard model Spectra Count microplate photometer (Packard Instrument Company, Meriden, Connecticut, USA) was used to measure the absorbance (630 nm) of the microplate wells at time 0 and 24 h.

The means and standard errors of absorbance measurements were calculated, graphed, and compared to controls to determine the 24-h IC_{50} and MIC of each test compound [2]. The 24-h IC_{50} and MIC results were divided by the respective 24-h IC_{50} and MIC results (in ppm) obtained for the positive control, to determine the relative-to-drug-control florfenicol (RDCF) values.

4. Results and Discussion

According to results of the antibacterial bioassay of the phloroglucinol analogs diisovalerylphloroglucinol (**2a**), dioctanoylphloroglucinol (**2e)**, and dipalmityl phloroglucinol (**2f)**, **2a** had similar activity against *F. columnare* ALM-00-173 as the drug control florfenicol, with a 24-h IC_{50} of 0.82 µg/mL (**Table 1**). Florfenicol had a 24-h IC_{50} of 0.81 µg/mL. While the minimum inhibitory concentrations (MIC) of diisovalerylphloroglucinol and florfenicol were 2.94 µg/mL and 0.36 µg/mL, respectively, and would indicate an approximate order of magnitude less activity compared to florfenicol, the MIC test concentrations were in a ten-fold dilution pattern (1000, 100, 10, 1, 0.1, 0.01 µM), and, therefore, it is possible that the actual MIC may be between two dilution values 0.29 µg/mL and 2.94 µg/mL. Therefore, in bioassay, the 24-h IC_{50} values provide a more comprehensive evaluation of the test compounds to the drug control, while the MIC values provide a more general guide for the evaluation of antibacterial activities.

The acyl analogs with long chains **2e** and **2f** (with C7 and C15, respectively) showed weak or no activities at the tested concentrations. Based on this preliminary data, several acyl analogs of phloroglucinol were synthesized to determine if a more active analog of **2a** could be produced and also determine the potential structure-activity relationships of the test compounds. According to the 24-h IC_{50} values (**Table 1**), diisobutyrylphloroglucinol derivative **2b** had the highest activity among the compounds tested with a 24-h IC_{50} of 0.80 µg/mL. The relative-to-drug-control florfenicol (RDCF) 24-h IC_{50} and MIC values for **2b** 1.0 and 7.39, respectively, which were the lowest among all the analogs were tested. Analogs **2a**, and **2c** also showed strong activities, with

Table 1. Results of the bioassay evaluation of phloroglucinol and derivatives for toxicity against *Flavobacterium columnare* ALM-00-173. Numbers in parentheses are the standard error of the mean.

Test compound	24-h IC_{50}[a]	MIC[b]	24-h IC_{50} RDCF[c]	MIC RDCF[c]
Florfenicol	0.81 (0.06)	0.36 (0)		
2a	0.82 (0.06)	2.94 (0)	1.03 (0.01)	7.67 (0.5)
2b	0.8 (0)	2.66 (0)	1.0 (0.07)	7.39 (0)
2c	0.87 (0.04)	2.94 (0)	1.08 (0.1)	8.17 (0)
2d	1.37 (0.2)	21.45 (17.55)	1.72 (0.36)	59.58 (48.75)
3a	13.65 (1.05)	21.0 (0)	17.13 (2.48)	58.33 (0)
3b	53.9 (4.9)	107.8 (88.2)	66.85 (1.52)	299.44 (245.0)
3c	56.7 (2.1)	21.0 (0)	70.59 (2.21)	58.33 (0)
3d	41.28 (0)	25.8 (0)	51.52 (3.52)	71.67 (0)
1a	>378.0	>378.0	nd	nd
1b	0.96 (0.02)	3.36 (0)	1.19 (0.1)	9.33 (0)
1c	1.87 (0.02)	3.78 (0)	2.34 (0.19)	10.5 (0)
1d	>552.0	>552.0	nd	nd
2e	28.2 (0.19)	20.82 (17.03)	57.48 (7.67)	57.84 (47.31)
2f	>602.9	>602.9	nd	nd
phloroglucinol	>126.0	>126.0	nd	nd

[a]24-h IC_{50} = 50% inhibitory concentration in mg/L. [b]MIC = Minimum inhibitory concentration in mg/L. [c]RDCF = Relative-to-drug-control florfenicol; numbers below "1" indicate greater activity as compared to the drug control. nd = not determined.

24-h IC_{50} of 0.82 and 0.87 µg/mL, respectively, and 24-h IC_{50} RDCF values of 1.03 and 1.08, respectively, and MIC of 2.94 µg/mL and MIC RDCF values of 7.67 and 8.17, respectively.

Several additional analogs also had moderate strong activities based on 24-h IC_{50} results. These include mono-3-phenylpropionylphloroglucinol (**2d**), monoisobutyryl phloroglucinol (**1b**) and mono-2-methylbutyryl phloroglucinol (**1c**) which had 24-h IC_{50} of 1.37, 0.96, and 1.87 µg/mL, respectively. Monoacyl and triacyl analogs showed very weak activity with IC_{50} values in the range from 13.65 to >378, except for **1b** and **1c,** which possess isobutyryl and isovareryl moieties. The parent molecule phloroglucinol was inactive.

According to our bioassay results, diacylphloroglucinol analogs showed strong activity. Triacylphloroglucinol analogs showed the weakest activity and the monoacylanalogs showed a moderate activity. Diacyl analog **2b** with isobutyryl moieties showed the highest activity. Diisovaleryl (**2a**) and di-2-methylbutyryl (**2c**) analogs also showed comparable activities to **2b**. Introduction of a phenyl moiety (**2d**) significantly reduced the activity so as introduction of longer carbon chains containing acyl moieties. Thus there is an optimum size for the acyl group and lipophilicity for antibacterial activity. The current antibiotic florfenicol that is being used to treat columnaris disease is amide. The diacylphloroglucinols are a different class of molecules that have the potential to replace florfenicol.

This study is the first one to evaluate phloroglucinol derivatives for antibacterial acitivities against *F. columnare*. In summary, **2a**, **2b**, and **2c** were highly active against *F. columnare* and as active or nearly as active as the current drug florfenicol used in medicated feed for managing columnaris disease. Similar analogs occur in plants and other organisms as monomers and dimers [5]-[11]. Synthesis of these analogs in bulk is feasible as the reaction involved one-pot synthesis with two stages and inexpensive as the starting materials are inexpensive and available in bulk quantities and they could potentially be incorporated into fish feed as an alternative to florfenicol. More testing is needed regarding toxicity to fish and other non-target organisms in the pond envi-

ronment before assessing the incorporation of acylphloroglucinols in fish feed as a management approach for columnaris disease. In addition, palatability studies would also need to be conducted before any efficacy studies (challenge studies) are initiated.

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