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A novel antimicrobial epoxide isolated from larval *Galleria mellonella* infected by the nematode symbiont, *Photorhabdus luminescens* (Enterobacteriaceae)

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Abstract—A novel antimicrobial epoxide, 2-isopropyl-5-(3-phenyl-oxiranyl)-benzene-1,3-diol (**1**), was identified from larval *Galleria mellonella* infected by a symbiotically associated bacterium–nematode complex (*Photorhabdus luminescens* C9–*Heterorhabditis megidis* 90). Its structure was determined with spectroscopic analysis and confirmed by chemical synthesis starting from a known antibiotic, 2-isopropyl-5-(2-phenylethenyl)-benzene-1,3-diol (**2**). Epoxide **1** was active against *Bacillus subtilis*, *Escherichia coli*, *Streptococcus pyogenes*, and a drug-resistant, clinical strain of *Staphylococcus aureus* (RN4220) with minimum inhibitory concentrations in the range of 6.25–12.5 μg/ml. Epoxide **1** was cytotoxic against human cancer cell lines, MCF-7 wt, H460, and Jurkat, with GI_{50} of 2.14, 0.63, and 0.42 μM, respectively, but was less toxic on normal, mouse splenic lymphocytes with a GI_{50} of 45.00 μM.

1. Introduction

Photorhabdus spp. are luminous bacteria that are symbiotically associated with soil-living, entomopathogenic nematodes, *Heterorhabditis* spp. The infective juveniles (IJs) of the nematode carry the bacterium in their gut and search for an insect host in the soil. After they enter an insect larva via natural openings or by penetrating directly through the cuticle, the IJs release the symbiotic bacteria. Working together, the nematodes and their bacterial symbiont kill the insect, usually within 48 h.

One of the fascinating phenomena about this nematode–bacterium–insect interaction is the range of bioactive metabolites, noticeably antibiotics,[1,2] that these symbiotic bacteria produce. It has been hypothesized that the antibiotics produced by the symbiotic bacteria help to minimize competition by secondary microbial contaminants within the insect cadaver, and thus provide an optimum growth environment for the nematode symbionts.[3] Paul et al.[4] reported first the production of the antimicrobial compound, 2-isopropyl-5-(2-phenylethenyl)-benzene-1,3-diol (also named 3,5-dihydroxy-4-isopropylstilbene **2**),[4] from in vitro cultures of a *Photorhabdus* sp. Stilbene derivatives are well known from plants, but not common metabolites of bacteria.[5,6] Later, 3,5-dihydroxy-4-ethylstilbene and anthraquinone derivatives also were reported from in vitro cultures.[7–9] Hu et al.[10,11] showed that compound **2** was produced also in larval *Galleria mellonella* infected by the nematode–bacterium complex at concentrations much higher than that produced in in vitro culture. By comparing the metabolites produced in vitro and in vivo, Hu et al.[12] reported that compound **2** as well as 3,5-dihydroxy-4-ethylstilbene and anthraquinone derivatives were all produced in insect cadavers infected by the *H. megidis* 90–*P. luminescens* C9 complex. In the same study, they also noticed that a possibly new antibiotic, referred to as AT, was produced.[12] Following further investigation, this paper now reports on the isolation and identification of a novel antimicrobial epoxide **1**, 2-isopropyl-5-(3-phenyl-oxiranyl)-benzene-1,3-diol from larval *G. mellonella* infected by the *H. megidis* 90–*P. luminescens* C9 complex.

2. Results and discussion

Epoxide **1** was isolated and purified from the larval *G. mellonella* infected by the nematode-bacterium

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complex, *H. megidis* 90–*P. luminescens* C9, and its structure (Fig. 1) was identified based on spectroscopic analysis and chemical synthesis (Scheme 1).

The antimicrobial activity of **1** is listed in Table 1. Epoxide **1** was active against all microorganisms tested, including the clinical, drug-resistant strain *Staphylococcus aureus* RN4220, with minimum inhibitory concentrations (MICs) around 6.25–12.5 μg/ml, except for *Pseudomonas aeruginosa*, for which the MIC was 100.0 μg/ml. The antibiotic spectrum of **1** is similar to that of the known antibiotic **2** (unpubl.).

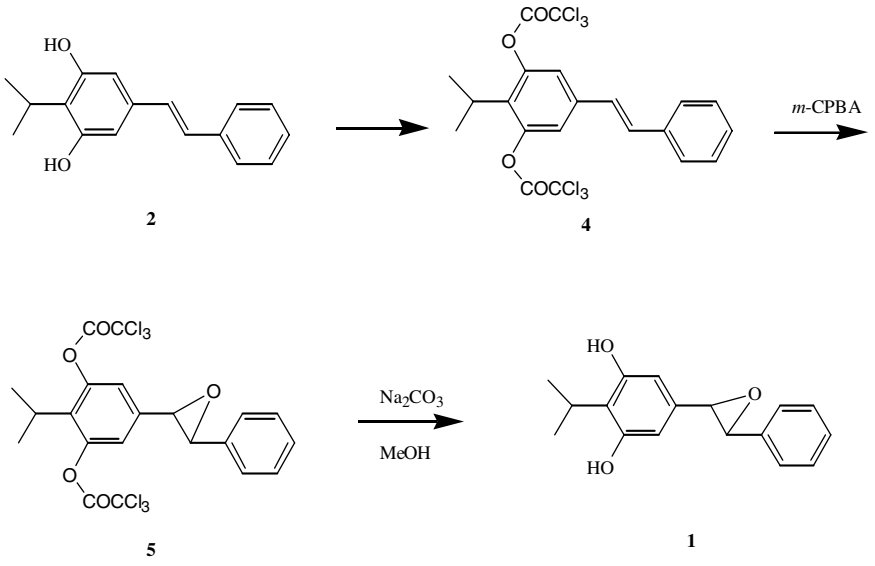
The cytotoxicity of **1** is listed in Table 2. Epoxide **1** was cytotoxic against the three human cancer cell lines, MCF-7 wt (breast cancer), H460 (lung cancer), and Jurkat (T cell leukemia), with 50% growth inhibition (GI_{50}) of 2.14, 0.63, and 0.42 μM, respectively, but was less toxic on normal, mouse splenic lymphocytes with a GI_{50} of 45.00 μM.

Although our earlier study indicated that the bacterial metabolites contained an unidentified compound that could be a new antibiotic,[12] the amount of this compound was not sufficient for study at that time. Consequently, a second study was initiated. Unfortunately, the compound was undetectable after the final purification step. Because of this, thin-layer chromatography plates were used in the last purification procedure in the third study so that the location of the compound could be closely monitored. Additional observations showed that the loss of the compound during the purification process at the second study was probably due to the unstable nature of the compound. This was expected once the final structure of the compound, epoxide **1**, had been determined (Fig. 1).

As epoxide **1** is not very stable in nature, and thus difficult to isolate in sufficient amounts and with high purity, total synthesis of **1** was pursued and used to confirm the suggested structure. The first attempt of synthesizing **1** by epoxidation of the precursor **2** using 3-chloroperoxybenzoic acid (*m*-CPBA) was not successful. Instead, the reaction yielded mainly the undesired, but expected, compound **3** in which a hydroxylation occurred on the substituted benzene ring. This result suggests that the substituted benzene ring of **2** was more reactive than the carbon–carbon double bond to the oxidant because of the activation of two hydroxyl groups. To overcome this, the two hydroxyl groups need to be protected. In addition, because the structure of **1** was not very stable, removal of the protecting groups had to be undertaken under very mild reaction conditions. So, the trichloroacetyl group was selected as the protecting group, as it can be removed later using weak bases. This new, detoured approach was successful and led to the expected compounds, 2-isopropyl-5-(2-phenylethenyl) -3-trichloroacetoxyphenyl trichloroacetate (**4**) and 2-isopropyl-5-(3-phenyloxiranyl)-3-trichloroacetoxyphenyl trichloroacetate (**5**). Deprotection of the trichloroacetyl groups in compound

1 **2**

Figure 1. The structures of epoxide **1** and its possible biosynthetic precursor **2**.



Scheme 1. Synthesis of epoxide **1** starting from antibiotic **2**.

Table 1. MICs of epoxide **1** against test bacteria

Microorganisms	MICs (μg/ml)
Bacillus subtilis	12.5
Escherichia coli	6.25
Enterococcus faecalis (ATCC29212)	12.5
Pseudomonas aeruginosa	100.0
Staphylococcus aureus (RN4220)	6.25
Staphylococcus epidermis	12.5
Streptococcus pyogenes (ATCC 19615)	12.5

Table 2. Cytotoxicity of epoxide **1** to three human cancer cell lines (MCF-7, H460, and Jurkat) and to normal, mouse spleen T lymphocytes (splenocytes) in two independent tests (means ± SD)

Cells	Cell density (cells/well)	GI_{50} (μM)	LC_{50} (μM)
MCF-7 wt	1×10^4	2.14 ± 0.73	>95.89
H460	6×10^3	0.63 ± 0.01	56.38 ± 6.75
Jurkat	1×10^5	0.42 ± 0.05	5.82 ± 1.24
Splenocytes	2×10^5	45.00 ± 11.22	>96.03

5 by treatment with Na_2CO_3 successfully yielded the target epoxide **1** (Scheme 1). As expected, all spectra of the synthesized epoxide **1** matched with the original data obtained from the natural epoxide **1**, thus fully confirming the structure of the natural epoxide **1**.

This is the first time that an epoxide has been identified from a tripartite insect–nematode–bacterium system. Epoxide **1** is likely to be derived from **2**. Previous studies,[10–12] including this one, showed that compound **2** was present in the same biological system where epoxide **1** was isolated but occurred in a much larger amount. As a chemical oxidation would only lead to hydroxylation on the substituted benzene ring of **2**, the conversion of **2** to **1** in a living system is likely to be completed by enzymatic epoxidation. Epoxide **1** is detectable in the natural environment involving nematodes, bacteria, and the insects, but not in the in vitro culture of the corresponding, isolated bacterial species.[12] The biological role of epoxide **1** inside the insect–nematode–bacterium system is not clear. Although it has antimicrobial properties as antibiotic **2**, it occurs in only small quantities and is unstable. Perhaps, the conversion from **2** to **1** in the living system represents one of the pathways in which compound **2** decomposes in its natural environment.

3. Conclusion

The study led to the discovery of a novel, antimicrobial epoxide **1**, which was then isolated, purified, identified, and chemically synthesized. The study provided additional evidence of the diverse nature of the bioactive metabolites generated by this unique, tripartite insect–nematode–bacterial complex. Like its possible biosynthetic precursor **2**, epoxide **1** had antimicrobial activity against a range of different microorganisms. It was also cytotoxic to three human cancer cell lines, and less so to normal, mouse splenic lymphocytes.

4. Experimental

4.1. Reagents and instruments

Solvents and chemicals (Sigma® and BDH®) of analytic degree were used throughout the study. Monitoring and separation of the compounds were performed by using thin-layer chromatography (TLC; Kieselgel 60 F254, EM Science) plates and flash silica gel column chromatography (silica gel 60, EM Science). The UV spectrum of the isolated epoxide was obtained on a Milton 3000 UV spectrometer. Mass spectra (Hewlett-Packard 5989 GC-MS system) and NMR (Bruker AMX500 spectrometer) data were obtained at Department of Chemistry, Simon Fraser University, Canada. Bioassays of antibacterial activity and cytotoxicity were performed on 96-well culture plates (Costar®, Corning).

4.2. Nematode and insect

Entomopathogenic nematode, *H. megidis* 90, carrying the symbiotic bacterium, *P. luminescens* C9, was originally collected from soil in British Columbia, Canada.[8] The nematode has been maintained in the laboratory by passaging through larval *G. mellonella*,[8] which were supplied by the Insectary of the Department of Biological Sciences, Simon Fraser University.

4.3. Infection of the insects

Infective juveniles of the nematode emerging from already infected larval *G. mellonella* were collected in water in Petri dishes and allowed to pass through two layers of wet strength paper tissue to collect only active nematodes.[10] They were then concentrated in distilled water. About 1000 IJs in 1 ml water were pipetted onto the surface of the filter paper in a polystyrene Petri dish (100 × 15 mm), and about 30 last-instar, larval *G. mellonella* were added. Hundreds of larvae were prepared this way. The Petri dishes with nematodes and insects were incubated at 25 °C in the dark for 7 days. By this time all larval *G. mellonella* had died and turned the typical, red-brown color of insects infected by the *Heterorhabditis–Photorhabdus* complex. The cadavers were then separated from filter paper and weighed (103 g).

4.4. Extraction of the bacterial metabolites

The larval insect cadavers were homogenized in a mortar with acetone and the extracted solution was collected. The process was repeated until the extract was colorless.[10] The combined extract was then dried under vacuum using a rotary evaporator, to generate a dark brown, oily mixture (35.6 g), which was then re-extracted with ethanol to obtain a brown mixture (7.7 g) for further processing.

4.5. Separation and purification of the epoxide 1

The following procedures including drying the sample were all performed under relatively dim light. The brown mixture was dissolved in dichloromethane

and loaded into a silica gel column (silica gel 60, 30 × 6.4 cm), which was stabilized with the solvents, ether and hexane (6:4, v/v). The same solvent was used also as an elute solvent for the monitoring. Eluted samples were monitored closely using TLC plates. Sample collection using test tubes (18 × 150 mm) started at the appearance of the first metabolite, which was a yellow pigment, and continued until the 61st tube, thereafter, pure methanol was used as elute solvent and the collected samples were combined. Samples from the 5th to 37th tubes, containing the epoxide, were combined based on TLC analysis. The sample was dried under vacuum using a rotary evaporator to generate a brown solid (2.3 g) that was further purified.

The above brown solid was dissolved in dichloromethane, and loaded into a silica gel column (silica gel 60, 31.5 × 4.8 cm), and a mixture of dichloromethane and ether (95:5, v/v) was the elute solvent. Sample collection using test tubes (18 × 150 mm) started at the appearance of a yellow pigment. Samples from the 18th to 22nd tubes, containing epoxide, were combined based on TLC analysis and dried under vacuum using a rotary evaporator to generate a dark brown solid (0.15 g). This dark brown solid was further purified using TLC plates. The partially purified solid was dissolved in ethyl acetate and applied as a narrow, straight line 1 cm from the edge of the plates. The TLC plates were developed in a sealed glass container (29.5 × 10 × 27.5 cm) containing the developing solvent (methanol and chloroform, 3:97, v/v) until the front line of the solvent was about 1 cm from the top of the plate. The plates were monitored with a UV detector and the band corresponding to the epoxide was located by gently drawing lines along the edge of the band. The band was carefully cut from the TLC plates and then cut into small, 1 cm pieces, which were re-extracted with methanol. The extract was filtered to remove the silica gel and dried with a rotary evaporator to yield a light brown solid (21 mg), the epoxide **1**. It has a R_f of 0.48 (ethyl acetate:hexane = 2:8), and a single, maximum peak of UV absorbance at 208 nm (Fig. 2). It is soluble in ethanol and methanol. Molecular formula: $C_{17}H_{18}O_3$. ^{1}H NMR (500 MHz; $CDCl_3$) δ (ppm): 1.35 (d, 6H, J = 7.5 Hz), 3.45 (m, 1H, J = 7.5 Hz), 3.7 (d, 1H, J = 2 Hz), 3.8 (d, 1H, J = 2 Hz), 4.9 (s, 2H), 6.33 (s, 2H), 7.34 (m, 5H). MS (CI) 271 (M+1). ^{13}C NMR (500 MHz) ($CDCl_3$) δ (ppm): 20.76 (C-15, 16), 24.43 (C-17), 50.71 (C-8), 51.03 (C-7), 107.22 (C-10, 12), 126.64 (C-11), 127.09 (C-9, 13), 128.55 (C-4, 6), 128.65 (C-14), 141.65 (C-5), 142.51 (C-2), 154.74 (C-1, 3).

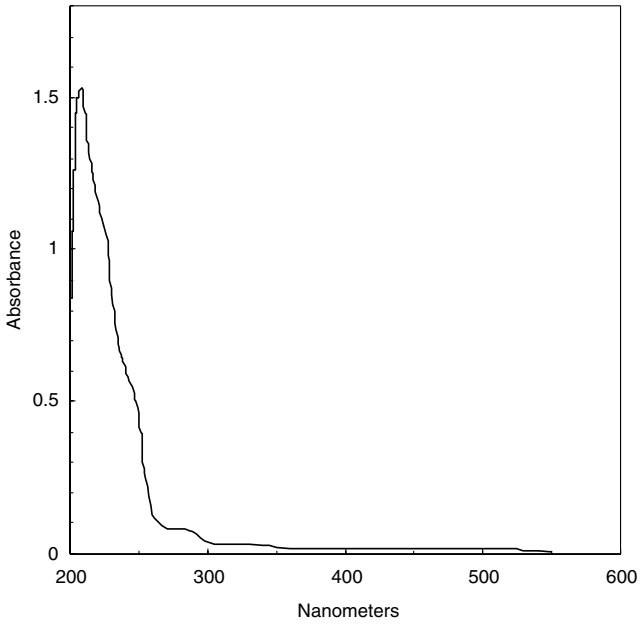


Figure 2. UV spectrum of epoxide **1**.

4.6. Synthesis of epoxide 1

4.6.1. 2-Isopropyl-5-(2-phenylethenyl)-3-trichloroacetoxyphenyl trichloroacetate (4). To a solution of 300 mg of **2** and 0.5 ml of trichloroacetyl chloride in 50 ml of dichloromethane, 0.5 ml of triethylamine in 5 ml of dichloromethane was added dropwise at room temperature in 10 min. The resulting reaction mixture was stirred overnight at room temperature. Reaction was checked by TLC with 5% of ether in hexanes as the eluent. R_f value is 0.4. When TLC showed the completion of the reaction, solvent was evaporated under reduced pressure. The product was purified by flash silica gel column chromatography with 5% ether in hexanes as the eluent. A total of 610 mg of **4** (white solid) was obtained (yield 95%). Molecular formula: $C_{21}H_{16}F_6O_4$. ^{1}H NMR (500 MHz; $CDCl_3$) δ (ppm): 1.35 (d, 6H, J = 7.5 Hz), 3.45 (m, 1H, J = 7.5 Hz), 6.55 (s, 1H), 6.63 (s, 1H), 7.02 (s, 2H), 7.42 (m, 5H). MS (CI) 543 (M+1).

4.6.2. 2-Isopropyl-5-(3-phenyl-oxiranyl)-3-trichloroacetoxyphenyl trichloroacetate (5). The mixture of **4** (400 mg) and *m*-chloro perbenzoic acid (300 mg) in 20 ml of dichloromethane was stirred at room temperature for 24 h. TLC showed that the reaction was not completed. Another 300 mg of *m*-chloro perbenzoic acid was added. The mixture was stirred for another 24 h by which time the start material had disappeared. Product (R_f was 0.3 by TLC with 5% ether in hexanes as the eluent) was purified by flash silica gel column chromatography with 5% ether in hexane as the eluent. A total of 240 mg of pure compound **5** (white solid) was obtained at a yield of 53%. Molecular formula: $C_{21}H_{16}F_6O_5$. ^{1}H NMR (500 MHz; $CDCl_3$) δ (ppm): 1.37 (d, 6H, J = 7.5 Hz), 3.46 (m, 1H, J = 7.5 Hz), 3.8 (d, 1H, J = 2 Hz), 3.9 (d, 1H, J = 2 Hz), 6.51 (s, 2H), 7.42 (m, 5H). MS (CI) 559 (M+1).

4.6.3. 2-Isopropyl-5-(3-phenyl-oxiranyl)-benzene-1,3-diol (1). To a solution of 240 mg of **5** in 40 ml of methanol, 100 mg of K_2CO_3 was added while stirring. One hour later, the reaction mixture was plunged into 200 ml of water and the mixture was extracted twice, each time with 100 ml of ethyl acetate. Extracts were combined, washed with 200 ml of water, and evaporated to dryness with a rotary evaporator. A total of 110 mg of epoxide **1** (off white solid) was obtained that was pure by TLC analysis (yield 90%). It is soluble in methanol, ethanol, and dichloromethane, and has the same UV spectrum

as the isolated epoxide (Fig. 2). Molecular formula: $C_{17}H_{18}O_3$. ^{1}H NMR (500 MHz; $CDCl_3$) δ (ppm): 1.35 (d, 6H, $J = 7.5$ Hz), 3.45 (m, 1H, $J = 7.5$ Hz), 3.7 (d, 1H, $J = 2$ Hz), 3.8 (d, 1H, $J = 2$ Hz), 4.9 (s, 2H), 6.33 (s, 2H), 7.34 (m, 5H). MS (CI) 271 (M+1). ^{13}C NMR (500 MHz) ($CDCl_3$) δ (ppm): 20.76 (C-15, 16), 24.43 (C-17), 50.71 (C-8), 51.03 (C-7), 107.22 (C-10, 12), 126.64 (C-11), 127.09 (C-9, 13), 128.55 (C-4, 6), 128.65 (C-14), 141.65 (C-5), 142.51 (C-2), 154.74 (C-1, 3).

4.6.4. Antimicrobial activity of epoxide 1. Microorganisms used in the study are listed in Table 1. In brief, MICs of **1** against the test bacteria were determined by twofold broth dilution series in tryptic soy broth (Difco). One hundred microliters of test solution of the isolated epoxide **1** in medium and 100 μl of bacterial suspension were mixed in each well of 96-well plates. The final density of the test microorganisms in the test solutions was 2×10^5 CFU/ml. There were three replicates for each concentration. The plates were incubated at 37 °C in the dark, and MICs were visually determined after 24 h incubation. The experiment was repeated twice.

4.6.5. Cytotoxicity of epoxide 1. Human cancer cell lines, MCF-7 wt (breast cancer), H460 (lung cancer), and Jurkat (leukemia), as well as normal, mouse spleen cells (Table 2) were used for cytotoxicity assays by the MTT method.[13,14] In brief, cells were grown and tested in RPMI1640 medium, excluding MCF-7 which is in DMEM, with 10% heat-inactivated fetal bovine serum and antibiotics (penicillin, 100 U/ml, and streptomycin, 100 μg/ml) at 37 °C in 5% CO_2. For H460 or MCF-7 cells, 100 μl of cell suspension was transferred in triplicate to each well of a 96-well flat-bottomed microtiter plate and incubated for 24 h. One hundred microliters of test solution (solvent dimethylsulfoxide $\leqslant 1\%$) of varying concentrations of the synthetic epoxide **1** in the medium was then added to each well of the plate and the plate was incubated for 48 h followed by the MTT assay. Jurkat cells were assayed as above except that round-bottomed plates were used. Splenic lymphocytes from female Balb/c mice were assayed in the same way as Jurkat cells except that there was not a 24 h pre-incubation period; cell suspension and test solution were added simultaneously, and the cells were stimulated with concanavalin A (5 μg/ml). In all the assays, cells at time zero served as the baseline controls and cells without test compound served as negative controls.[14,15] GI_{50} and LC_{50} values were calculated according to the methods established by the National Cancer Institute, USA.[14,15] All the assays were done twice.

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