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Identification, library synthesis and anti-vibriosis activity of 2-benzyl-4-chlorophenol from cultures of the marine bacterium *Shewanella halifaxensis*

Sarah L. Moore [a,†], Lucile Berthomier [a,†], Chriselle D. Braganza [a], Joanna K. MacKichan [b], Jason L. Ryan [c], Gabriel Visnovsky [d], Robert A. Keyzers [a,*]

[a] Center for Biodiscovery, and School of Chemical and Physical Sciences, Victoria University of Wellington, PO BOX 600, Kelburn, Wellington 6140, New Zealand
[b] Center for Biodiscovery, and School of Biological Sciences, Victoria University of Wellington, PO BOX 600, Kelburn, Wellington 6140, New Zealand
[c] Integrated Bioactive Technologies Group, Callaghan Innovation, Gracefield, PO Box 31310, Lower Hutt 5040, New Zealand
[d] Chemical and Process Engineering, University of Canterbury, Private Bag 4800, Christchurch 8140, New Zealand

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ABSTRACT

Summer Gut Syndrome (SGS) is caused by various *Vibrio* bacterial species and can have negative effects on aquaculture farms worldwide. In New Zealand, SGS is caused by *Vibrio harveyii* infecting King Salmon (*Oncorhynchus tshawytscha*). To find leads for the prevention of SGS, we screened the inhibitory effects of 16 strains of *Shewanella* upon *V. harveyii* growth in competitive solid phase cultures. The detailed investigation of *Shewanella halifaxensis* IRL548 revealed 2-benzyl-4-chlorophenol (**1**), a known, commercially available antibacterial agent, as the major bioactive component. Synthesis of a small library of congeners to confirm the natural product identity and to provide a structure–activity relationship for the observed activity was also completed. Compound **1** exhibits moderate activity against two pathogenic microorganisms.

Significant recent advances in aquaculture, especially in controlling dietary sources,[1,2] have made the production of large volumes of fish possible, although the high density of individuals maintained within a cage make the population susceptible to widespread and potentially catastrophic infection by pathogenic microrganisms.[2] In New Zealand, King (also known as Chinook) Salmon (*Oncorhynchus tshawytscha*) are a valuable aquaculture commodity farmed at various locations around the country. During the warmer summer months, Salmon with suppressed immune systems caused by diets enriched in non-animal based proteins, along with overcrowding, can result in intestinal imbalance of microbiota.[3] This in turn can lead to infection by *Vibrio harveyii*, the pathogen responsible for Summer Gut Syndrome (SGS), also known as vibriosis.[4] SGS presents symptoms such as vomiting, diarrhea, loss of appetite and if left untreated, could be fatal. Consequently, SGS is a large potential risk factor for the local King Salmon aquaculture industry.

Treating large numbers of farmed fish with antibiotics is not easily controlled in a marine aquaculture setting, nor is the incorporation of preventative antibiotics into fish-feed economically or environmentally viable. One alternative route to prevent SGS would be the inclusion of a suitable probiotic microorganism into the Salmon's fish-feed. Bacteria of the genus *Shewanella* are Gram-negative facultative anaerobes found from many marine environments. Species of *Shewanella* have already been investigated as potential probiotics for both sole[5,6] and abalone[7] aquaculture, with improved resistance and a reduction of mortality noted for treated abalone challenged with *V. harveyii*.[7] Recent work by Linington and coworkers has indicated that the gut microflora of commercial fish species could be useful reservoirs of new microbial strains,[8] therefore we investigated multiple strains of *Shewanella* from the guts of several fish species caught in New Zealand waters. Solid-phase co-culture of 16 *Shewanella* species with *V. harveyi* indicated eight inhibited *Vibrio* growth. Investigation of four of these strains resulted in the isolation of fifteen diketopiperazines, indole and 3-carboxyindole,[9] although none of these appeared to be responsible for the observed *Vibrio* inhibition. Diketopiperazines have been suggested as quorum-sensing metabolites in *Shewanella baltica* previously.[10]

Detailed investigation of the liquid culture of *Shewanella halifaxensis* strain IRL548,[11] sourced from the guts of a Bluenose fish, sequentially purified by various chromatographic processes

* Corresponding author. Tel.: +64 4 463 5117; fax: +64 4 463 5241.
E-mail address: rob.keyzers@vuw.ac.nz (R.A. Keyzers).
† Contributed equally to this project.

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(Supplementary materials), led to the isolation of 2-benzyl-4-chlorophenol (**1**) as the main bioactive component. To the best of our knowledge, although **1** is a known antibacterial agent used in hospital disinfectants,[12] this is the first Letter of it as a natural product. To confirm the structure of the compound and to probe a possible structure–activity relationship (SAR), the synthesis of **1** and a small series of analogues was performed, and the bioactivity of both the natural product and analogues assessed against three pathogenic bacterial isolates. The results of the isolation, synthesis and bioactivity profiling of **1** and analogues are presented herein.

A 20 L culture of *S. halifaxensis* IRL548 was grown using marine medium under aerobic conditions. This culture was clarified by centrifugation after which the supernatant was passed through a column of HP-20 poly-(styrene-divinylbenzene) copolymer that was subsequently eluted using 30%, 75% and 100% mixtures of acetone in water. The 75% acetone elution was subsequently re-suspended in methanol and filtered, with the methanol-soluble portion being chromatographed using a stepped gradient of 50% methanol in water to methanol using HP-20ss. The methanol fraction was finally separated using C_{18} HPLC to yield compound **1** as a colorless solid.

The chemical formula of **1** ($C_{13}H_{11}OCl$) was determined from the deprotonated molecule detected using high mass accuracy ESI-MS (det. *m*/*z* 217.0434; calcd *m*/*z* 217.0426), and in particular the presence of chlorine identified from the distinctive 3:1 M:M +2 ratio of chlorine isotopomers. The structure of **1** was rapidly determined from interpretation of comprehensive 1D and 2D NMR data. In particular, only 11 ^{13}C resonances were detected, 10 of which were indicative of aromatic carbon signals, suggesting the presence of either a mono- or *para*-disubstituted benzene ring due to internal symmetry arguments. A monosubstituted benzene ring was inferred from the characteristic doublet/triplet/triplet ^{1}H NMR multiplicities in a 2:2:1 integration ratio. This left both a *meta*- and an *ortho*-coupled doublet, and an *ortho*,*meta*-coupled doublet of doublet, and a highly deshielded methylene to be assigned. Through the use of chemical shift arguments in conjunction with key HMBC correlations (Fig. 1), the structure of **1** could be assigned as 2-benzyl-4-chlorophenol (Table 1). A thorough literature survey using several commercial databases (MarinLit,[13] AntiBase,[14] SciFinder Scholar[15]) could find no evidence that **1** has been reported as a natural product previously although it is a well-known synthetic material, having been synthesized as early as 1932.[16,17]

To confirm the structure of **1** and to establish any potential SAR, Friedel–Crafts alkylation of *ortho*, *meta* and *para*-chlorophenol with benzyl bromide was used to provide material for biological evaluation. The method of Klarmann et al.[16] was used to prepare an authentic standard of **1** for spectroscopic analysis from *para*-chlorophenol. The other two possible regioisomers were also used to provide simple analogues for comparison of bioactivity. All three regioisomers also provided bis-benzyl adducts in addition to the expected mono-addition products (Scheme 1). The spectroscopic data of both natural and synthetic **1** were identical, confirming the structure of the isolated metabolite (Figs. S1 and S2).

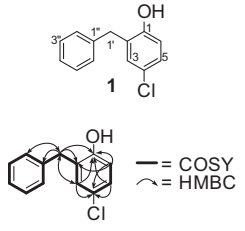
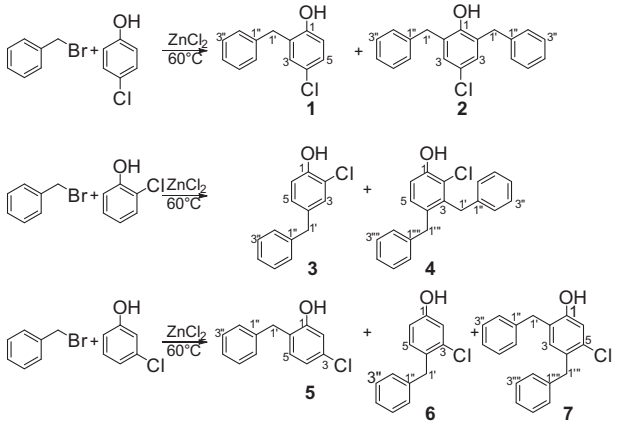


Figure 1. Key COSY and HMBC correlations used to establish the structure of **1**.

Table 1
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of 2-benzyl-4-chlorophenol (**1**), CD_3OD

Position	δ_C	δ_H	Multiplicity, *J* (Hz), rel. int.
1	155.1	—	—
2	131.3	—	—
3	130.9	6.91	d, (2.6), 1H
4	124.8	—	—
5	127.8	6.98	dd, (8.5, 2.6), 1H
6	117.1	6.73	d, (8.6), 1H
1′	36.4	3.89	s, 2H
1″	141.9	—	—
2″/5″	129.9	7.20	d, (7.4), 2H
3″/6″	129.3	7.25	t, (7.4), 2H
4″	127.0	7.16	t, (7.1), 1H



Scheme 1. Library synthesis of benzylchlorophenol analogues from benzyl bromide and various chlorophenols.

Chlorophenols are known to exert their antiseptic bioactivity by interfering with the cell membrane, and by uncoupling of oxidative phosphorylation.[18] All seven synthesized compounds were assessed for their bioactivity against *Escherichia coli*, *Staphylococcus aureus*, and most importantly for the purposes of this project, *Vibrio harveyii*. Although **1** is a known antibiotic compound, we could find only one previous Letter of the bioactivity of **1** versus a species of *Vibrio* (*V. fischeri*)[19] hence it was of interest to assess the activity of the compound against the more relevant pathogenic isolate.

All of the synthesized compounds were moderately active against both *E. coli* and *V. harveyii*, although somewhat surprisingly, only the dibenzylated analogues **2**, **4**, **6** and **7** were active against the Gram positive pathogen *S. aureus* (Table 2). This result was unexpected given the use of **1** as a clinical disinfectant within hospitals and within antibacterial cosmetics and other personal grooming products. Intriguingly, the natural product was also the least active compound tested. No other clear SAR could be determined.

Table 2
Bioactivity of the synthetic benzylchlorophenol library

Compound	IC_{50} (μM)		
	S. aureus	*E. coli*	*V. harveyii*
1	NS	54.9	41.2
2	19.4	35.6	24.3
3	NS	50.3	38.9
4	13.9	35.6	29.1
5	NS	50.3	38.9
6	NS	50.3	36.6
7	6.5	35.6	24.3

NS = not sensitive.

Also commonly known as chlorophene, **1** is used clinically as a medical disinfectant and within personal grooming products.[12,20] To probe the biosynthesis of **1**, attempts were made to culture *Shewanella halifaxensis* IRL548 with NaBr instead of NaCl, to try and promote production of a brominated analogue however the bacteria would not grow with this salt replacement. To validate that the isolated compound is in fact a true natural product and was not the product of the highly unlikely and inadvertent contamination of the culture, a repeat culture was prepared and the same compound was detected by both NMR and MS analyses in the culture supernatant (Fig. S18).

Chlorophene has a wide range of observed bioactivities, including antimicrobial properties, hence its use as a disinfectant. It has been suggested, however, to be a carcinogen and possible fertility disruptor[20] and hence its widespread use where the potential for human exposure, including within a food product, should be limited. The production of **1** as a microbial metabolite is unexpected yet given its broad bioactivities, the ecological advantages to *S. halifaxensis* in controlling its local microenvironment are potentially high. Although the inhibition of *V. harveyii* by **1**, determined by our study, is only modest in potency, this may be enough to control local populations of the pathogen in vivo.

As several strains of *Shewanella* have already been trialed as aquaculture probiotics,[5–7] the use of *S. halifaxensis* IRL548 in a similar role has precedence. Improved rates of survival by abalone treated with *S. colwelliana* WA64 and *S. olleyana* WA65 challenged with *V. harveyii*[7] gives a promising outlook for the use of *S. halifaxensis* in New Zealand with farmed King Salmon. Such a use, however, must first be evaluated in light of the wide variety of bioactivities associated with the compound although indications are that **1** should not bioaccumulate in the fatty tissue of fish and hence may pose minimal risk following human consumption, especially as it exhibits only a low oral toxicity.[20] The long-term effect of exposing King Salmon to *Shewanella halifaxensis* IRL548 would need to be the first step in the evaluation of the probiotic potential of this common bacteria.

In conclusion, a known, commercially available disinfectant compound, 2-benzyl-4-chlorophenol (**1**) was isolated from a culture of *Shewanella halifaxensis* IRL548 as a natural product for the first time. This bacterial strain had been selected for investigation due to its inhibition of *Vibrio harveyii*, the pathogen responsible for Summer Gut Syndrome in farmed New Zealand King Salmon, and hence it may be a useful lead strain for inclusion as a probiotic. Synthesis of **1** and analogues allowed for the bioassay testing of the library, with all compounds showing activity against *V. harveyii* and *E. coli*. This study reveals that species of *Shewanella* have biosynthetic capacity to produce unusual bioactive compounds and may be a valuable resource for both probiotics themselves, and also for discovering new natural product leads for other biotechnological uses.

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Supplementary data

Supplementary data (full experimental details of the culture of *S. halifaxensis* IRL548, isolation of **1**, synthesis of **1**–**7** and NMR spectra of all compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.05.002.

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