UNIVERSITY OF CALIFORNIA SAN DIEGO

Microbial Allies of the Reef: Chemical Profiling of Coral Symbiotic Bacteria Metabolites for Antibiotic Potential

A Thesis submitted in partial satisfaction of the requirements
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in

Marine Biology

by

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Professor Paul Jensen
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2024

The Thesis of Brianna Alexa Prieto is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

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ABSTRACT OF THE THESIS

Microbial Allies of the Reef: Chemical Profiling of Coral Symbiotic Bacteria Metabolites for Antibiotic Potential

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The prevalence of coral diseases has significantly surged in the past couple of years, particularly within the Caribbean region. Notably, certain corals exhibiting resistance harbor symbiotic bacteria, suggesting a potential role of these microorganisms in bolstering resilience. In this study, I explored four strains of symbiotic bacteria, hypothesizing their capability to produce antibiotic compounds targeting coral pathogens. Through culturing these bacteria and evaluating their extracts, we found that only *Glutamicibacter soli* displayed inhibitory effects, although without complete pathogen eradication. Further chemical analysis of *G.soli* culture extracts unveiled the

presence of two prominent molecules: cholic acid and a previously undescribed aromatic compound: *N*-[2-(4-Hydroxyphenyl)ethyl]-3-methylbutanamide. The isolation of cholic acid stands out as a unique finding, shedding light on its origin within marine bacterial extracts and expanding our understanding of symbiotic interactions in coral health.

Chapter 1 CORAL REEF ECOSYSTEMS

It is truly incredible to think that coral reefs are the result of the collective efforts of hundreds of thousands of tiny animals working together. As author Juli Berwald describes them, coral reefs can be detailed as architects, constructing intricate structures that serve as physical protection for islands and coasts. They are also manufacturers who create rock scaffolding to build their own homes. Corals also exhibit remarkable chemical abilities, producing their own protective sunscreen and complicated venoms to ward off pathogens. It is very well known that for us humans, coral reefs hold immense value. They support commercial fisheries (Grafeld *et al.* 2017), contribute to tourism and recreation (Brander *et al.* 2007), and provide essential ecosystem services, providing a home to at least 25 percent of all marine life on the planet (NOAA Coral Reef Conservation Program). It is no wonder that the protection of coral reef ecosystems is vital for the planet's well-being.

Despite their importance, coral reef ecosystems are facing significant threats and have been commonly identified as the most fragile marine ecosystem (Irudayarajan *et al.* 2023). Some of these threats include anthropogenic climate change, ocean acidification, pollution, overfishing, and most importantly disease outbreaks. Nowadays, there have been as many as 40 coral diseases reported worldwide. One of the most recent, and a deadliest disease, is stony coral tissue loss disease (SCTLD). It was first reported in 2014 in Florida, following an abnormal increase in sea surface temperature (Precht *et al.* 2016). It can progressively destroy the coral's tissue and increase the susceptibility to pathogens, capable of eradicating whole hard coral colonies. This impacts more than half the species of the Florida Keys and the Caribbean, by infecting at least 22 coral species (Ushijima *et al.* 2020). The exact cause for SCTLD is yet to be discovered, however, recent studies have demonstrated that it can be related to an infection of pathogenic

bacteria, viruses, or a combination of both. The effective use of antibiotics to stop the spread of this disease supports this hypothesis. In addition, several pathogenic bacteria have been involved with SCTLD including *Vibrio coralliilyticus* (Deutsch *et al.* 2022).

Given that antibiotics are giving positive results in field trials, there have been proposals for other methods to control pathogenic bacteria. It is well known that bacteria can become resistant to antibiotics, as has been observed in humans (Andersson, 2003). That being the case, a coral probiotic hypothesis was postulated. This states that a coral's health is directly linked to its microbial community composition and that this microbiome is capable of adapting depending on various environmental conditions (Peixoto *et al.* 2021). For corals, having an advantageous microbiota contributes to nutrient acquisition, intercellular communications, and pathogen protection. The ability to understand and use coral-associated bacteria for the development of probiotic treatments can bring a new horizon to coral protection and conservation. Successful testing of these treatments has been done in the Florida Keys, by Dr. Valerie Paul's laboratory group at the Smithsonian Marine Station in Fort Pierce, Florida (Deutsch *et al.* 2022). The mystery persists: do these specific coral-associated bacteria yield positive outcomes against SCTLD?

Chapter 2 HYPOTHESIS: A CHEMICAL FOUNDATION FOR THE PROTECTION OF CORALS

A deep dive into current research papers has given the outcome that analyses of chemical backgrounds on coral-associated bacteria have yet to be discovered. For that reason, a collaboration with Dr. Valerie Paul was established to examine a portion of coral-associated strains that were isolated from resistant corals by her team. They have been identified following the procedure described by Deutsch and collaborators (2022). These strains were suspected to be responsible for coral resistance against SCTLD. Such examination would involve the culturing, extraction of secondary metabolites, chemical analyses of each strain, and screening against known pathogens. The extracts obtained will be tested against putative coral pathogens; related to SCTLD, to isolate and define molecules with antimicrobial activities. The hypothesis of this project is that resistant corals have symbiotic relationships with bacteria that may enhance their survival by the production of antibacterial natural products.

Chapter 3 RESULTS

The 1L bacterial cultures of the 4 coral-associated strains (Table 1) were extracted (Table 2) and subjected to disk diffusion growth inhibition assays for screening of antibacterial activity against coral pathogens (Table 3) that play a role in SCTLD. It is important to mention that, as shown in Figure 1 (b), the 'hazy' zone of inhibition was a behavior that was seen repeatedly in most of the samples. Further description of this result will be discussed in the following chapter. Figure 1 (b) shows the 'highest' inhibition activity seen on all the strains, this being *Glutamicibacter soli* cultured in MB medium. Strains OfavD, OfavH, and McavD did not show any relevant activity to be reported.

Table 1. Coral-Associated Bacterial Strains

Strain ID	Strain	Coral Source
OfavH	*Streptomyces laculatispora*	*Orbicella faveolata*
OfavD	*Micromonospora terminaliae*	*Orbicella faveolata*
McD-86	*Glutamicibacter soli*	*Montastraea cavernosa*
McavD	*Gordonia hongkongensis*	*Montastraea cavernosa*

Table 2. Extract amounts

Strain	Mass (mg)
OfavH	40

Table 2. Extract amounts *Continued*

Strain	Mass (mg)
OfavD	52
McD-86	44
McavD	126

Table 3. Coral Pathogens

Strain ID	Strain	Coral Source
OfT6-21	*Vibrio coralliilyticus*	Diseased *Orbicella faveolata*
OfT7-21	*Vibrio coralliilyticus*	Diseased *Orbicella faveolata*
McT4-56	*Leisingera* sp.	Diseased *Montastraea cavernosa*

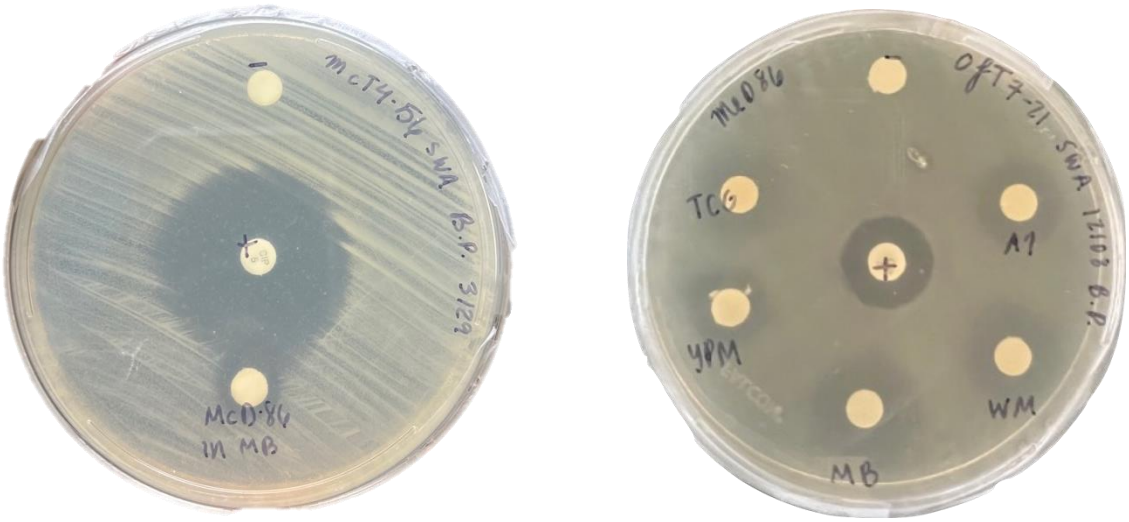

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Figure 1. (a) The agar plate showing the antibacterial activity of *Glutamicibacter soli* (McD-86) against *Leisingera* sp. (McT4-56). (b) Inhibition activity of McD-86 strain grown in 5 different media, against *Vibrio coralliilyticus* (OfT7-21). Negative control (-): MeOH. Positive control (+): ciprofloxacin.

The *Glutamicibacter soli* strain McD-86 was grown in 20L using a MB medium, and the EtOAc extract was separated by Silica Gel Chromatography. The resulting six fractions were tested, and the results are shown in Figure 2. Due to the activity exhibited by Fraction S6, further analyses and purifications were performed.

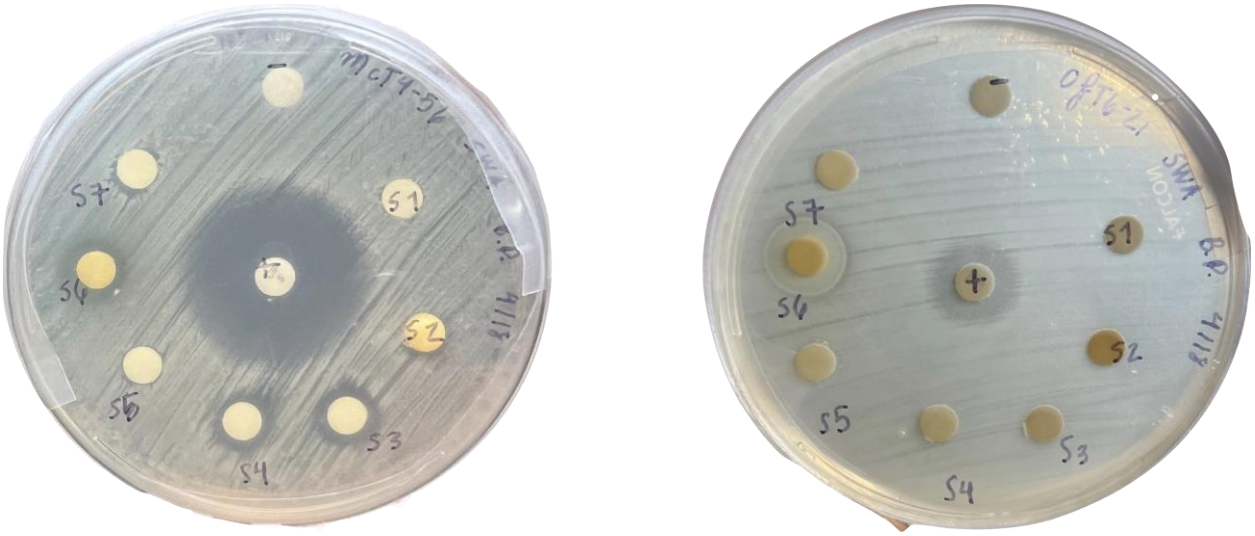

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Figure 2. The antibiotic activity plates of Fractions S1-6 from *Glutamicibacter soli* strain McD-86 against *Leisingera* sp. (McT4-56). (b) Fractions 1-6 tested against *V. coralliilyticus* (OfT6-21). Negative control (-): MeOH. Positive control (+): ciprofloxacin.

Fraction S6 was injected into HPLC for further purification, resulting in 20 fractions, as shown in Figure 3. All fractions were tested against the three putative coral pathogens and Fraction 3 exhibited antibiotic activity. To discover what molecule was responsible for such activity, further purification was performed with HPLC, NMR and LCMS.

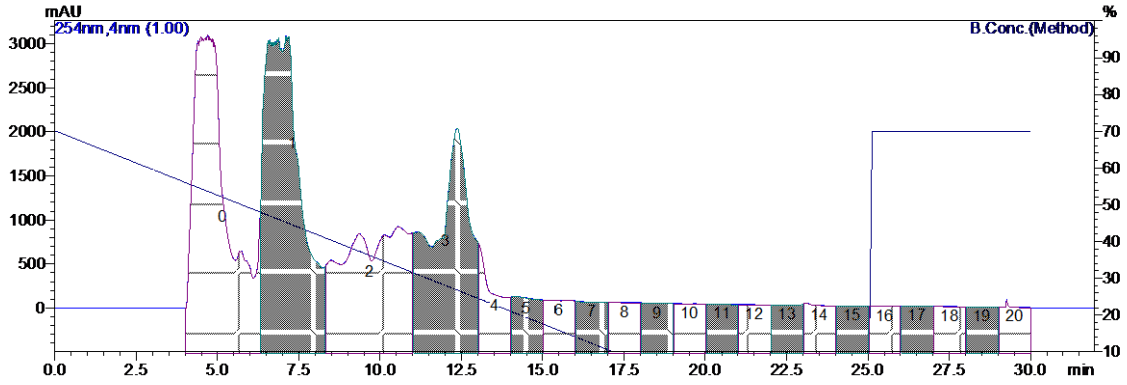

Figure 3. The HPLC chromatogram of fraction 6 from *Glutamicibacter soli* strain McD-86, absorbance 254 nm.

After the chemical analyses were executed, cholic acid was identified in S6-FR3. It is important to note that the isolation of cholic acid was guided by ion-selective LCMS primarily since this molecule is known to have very low UV absorbance (Kakiyama *et al.* 2014). LCMS data indicated that FR3 had a molecular ion of *m/z* 817.5820, reflected as $[2M+H]^+$.

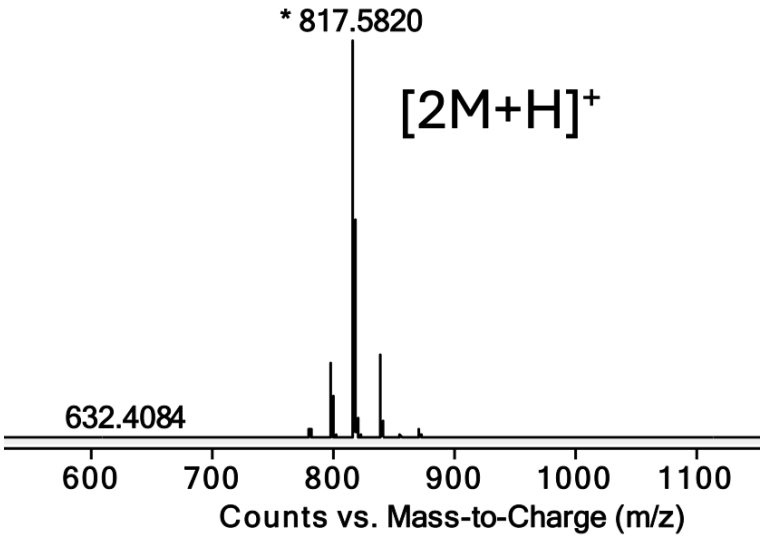

Figure 4. The positive-mode LCMS spectrum of FR3. The molecular mass of cholic acid is 408.2875 * 2 + 1.007825 (H) = 817.5828, Δ= -0.97 ppm.

As previously mentioned, cholic acid was found in FR3, however, it was not the only molecule in that sample. An antimicrobial testing of pure cholic acid (SIGMA No. C-1129) was performed and resulted in no activity in any of the coral pathogens from this study. When FR3 was tested, activity was seen, suggesting that the other molecule present had antibiotic properties. Therefore, studies were guided to find the molecule, suspected to be the aromatic metabolite observed in the NMR spectral data.

From a new 20L culture of *G. soli*, grown in MB, a much more efficient fractionation was performed by normal phase flash column chromatography using a Teledyne ISCO CombiFlash Rf+ Lumen System. This method resulted in 69 fractions of 15 mL each. To simplify the analysis, guided by the resultant chromatogram, some fractions were combined. Table 4 represents the combination of fractions and their respective masses. After an antibacterial analysis, Peak 29 was a candidate for further HPLC purification to obtain a pure compound.

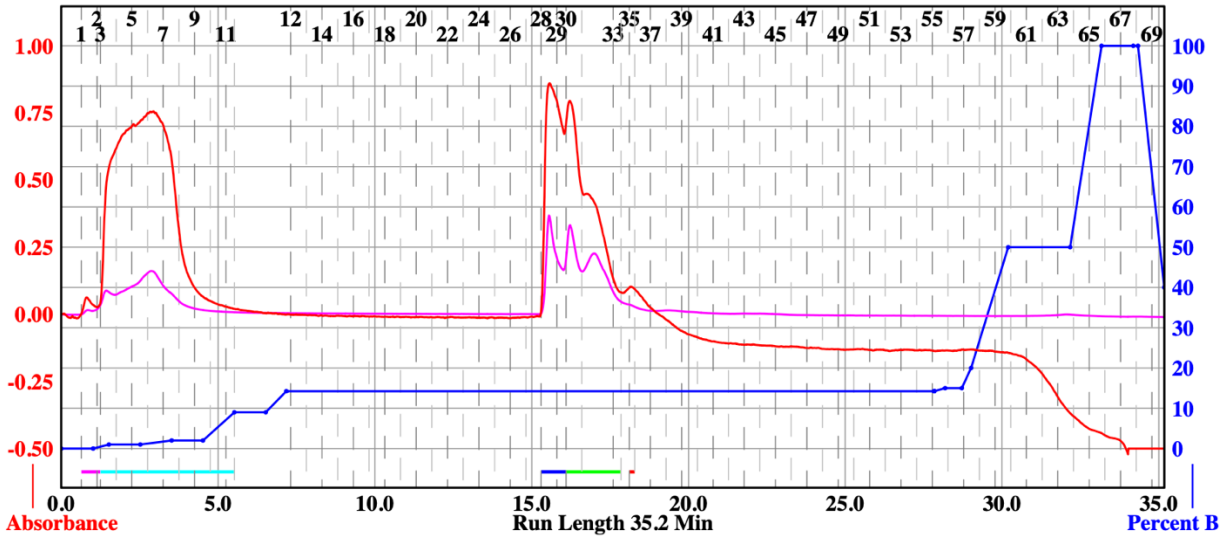

Figure 5. CombiFlash chromatogram of 20L *Glutamicibacter soli,* strain McD-86 extract. Percentage of MeOH in blue (Percent B).

Table 4. Fractionation of 20L *Glutamicibacter soli* (McD-86) extract

Fractions	ID	Mass (mg)	Fractions	ID	Mass (mg)
1-11	Pool 1	558	34	Peak 34	7
12-27	Pool 2	7.4	35	Peak 35	4.4
28	Peak 28	115	36	Peak 36	9
29	Peak 29	37	37	Peak 37	7
30	Peak 30	78	38	Peak 38	6.5
31	Peak 31	30	39-44	Pool 3	16
32	Peak 32	23	45-60	Pool 4	11
33	Peak 33	6	61-69	Pool 5	10

Peak 29 (37 mg) was further purified by HPLC in a C18 column. The chromatogram is shown in Figure 6, leading to three new fractions; Fr0 (1.2 mg, t_R 17.5 min), Fr1 (2 mg, t_R 18.7 min), Fr2 (2 mg, t_R 19.5 min), Fr3 (3 mg, t_R 21.8 min).

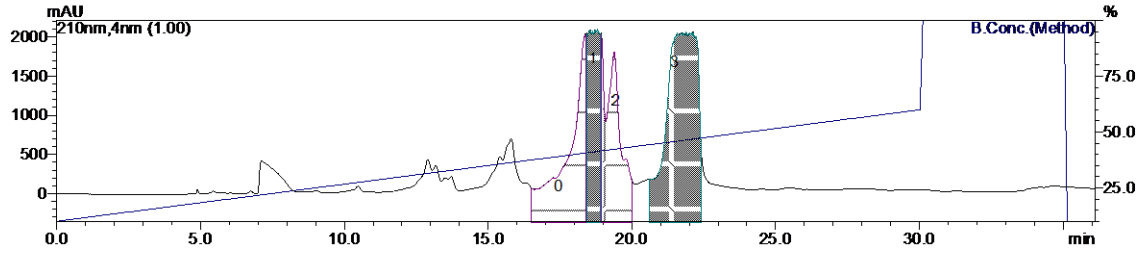

Figure 6. The HPLC chromatogram of Fraction 29 from *Glutamicibacter soli* McD-86 extract, absorbance 210 nm.

Further NMR spectral analysis gave the conclusion that Fr3 contained an aromatic metabolite identified as *N*-[2-(4-Hydroxyphenyl)ethyl]-3-methylbutanamide. Literature analysis showed that this compound has only been observed from a marine bacterial extract, *Vibrio* sp. (Hel11), by GCMS, described by Böröczky and collaborators in 2006. This was not an appropriate structure assignment. MS analysis was done in an EI-MS instrument, and a mass of 221 (2, M^+) was reported. It should be noted that the authors only detected this compound by GCMS with an increment system to deduce the position of the Me group that was described by them before. Similar molecules have been isolated, from *Cystobacter ferrugineus* by Zander and collaborators (2011). This study is the first to report the isolation, NMR spectral data, and antibiotic testing of the aromatic metabolite. The mass obtained from Peak29-FR3 by LCMS is shown in Figure 9. It was tested against pathogens (Figure 8), at a concentration of 1 mg/mL.

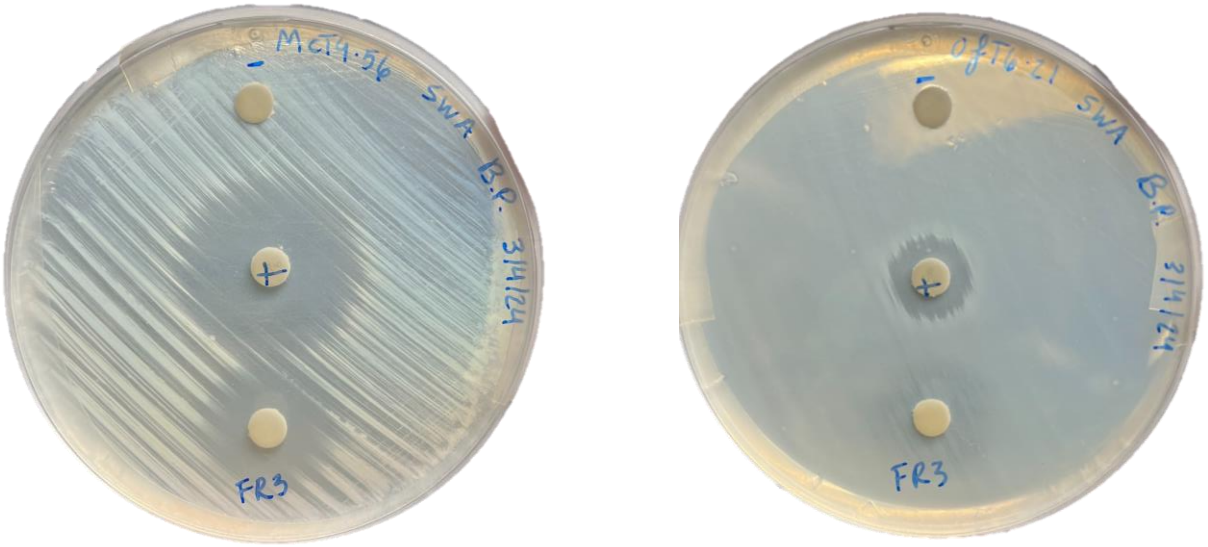

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Figure 7. a) The agar plate shows the antibacterial activity of N-[2-(4-Hydroxyphenyl)ethyl]-3-methylbutanamide against *Leisingera* sp. (McT4-56). (b) Inhibition activity of *N*-[2-(4-Hydroxyphenyl)ethyl]-3-methylbutanamide against *Vibrio coralliilyticus* (OfT7-21). Negative control (-): MeOH. Positive control (+): ciprofloxacin.

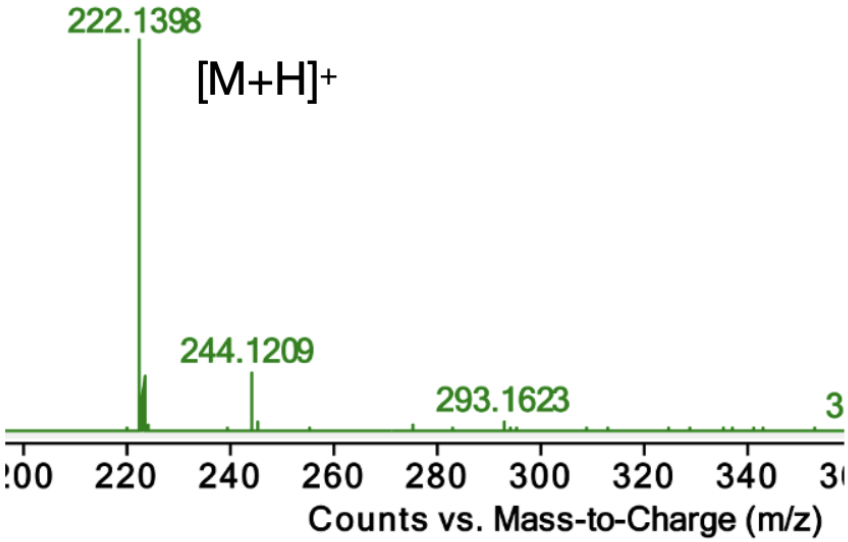

Figure 8. The positive-mode LCMS spectrum of Fraction29-FR3. The theoretical mass of the compound is 221.1415 + 1.007825 (H) = 222.1493, Δ= -0.43 ppm. Note the characteristic $[M+Na]^+$ peak at 244.1209.

5. 2 1D AND 2D NMR SPECTRAL DATA

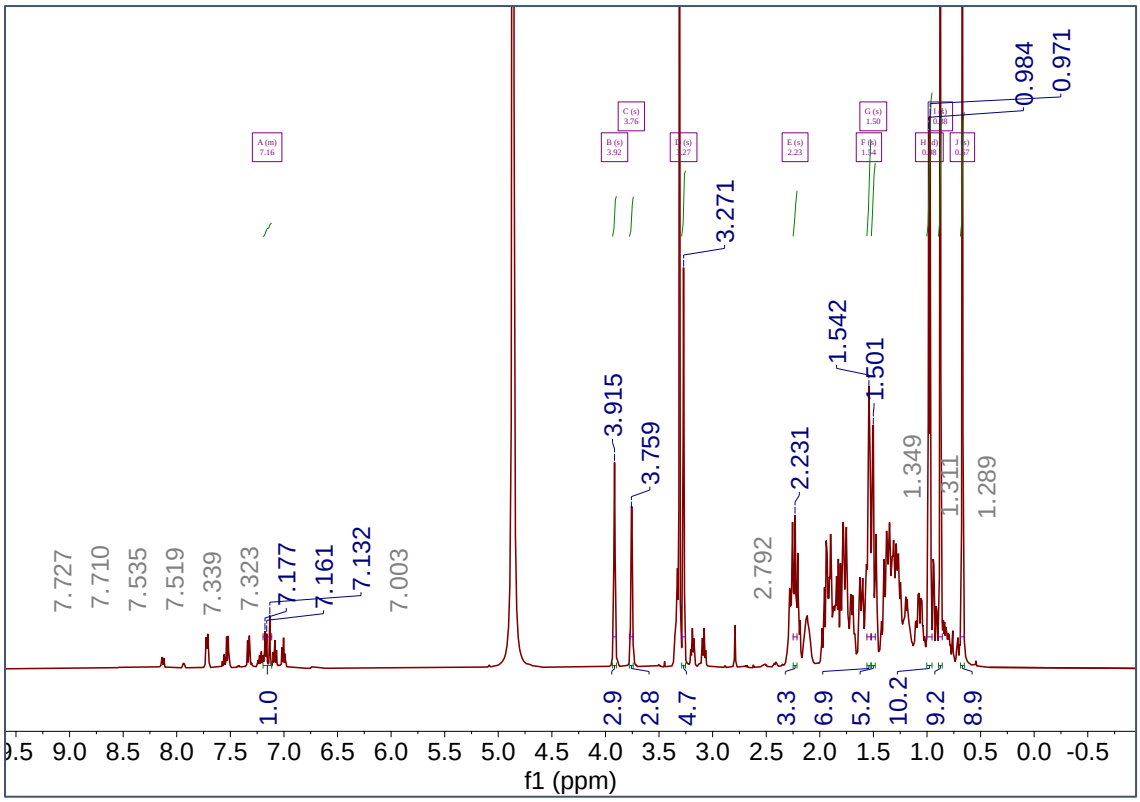

Figure 9. The ^{1}H NMR spectrum of FR3 from *Glutamicibacter soli,* strain McD-86 Fraction 6.

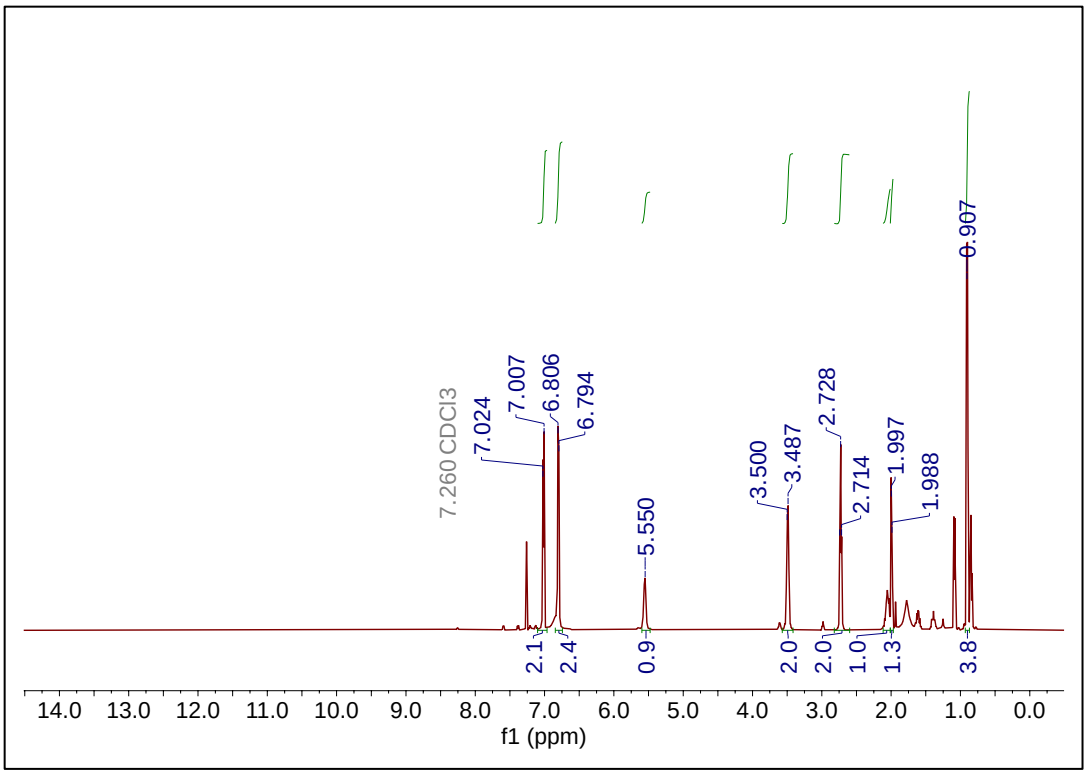

Figure 10. The ^{1}H NMR spectrum of *N*-[2-(4-Hydroxyphenyl)ethyl]-3-methylbutanamide from Fraction29-Fr3.

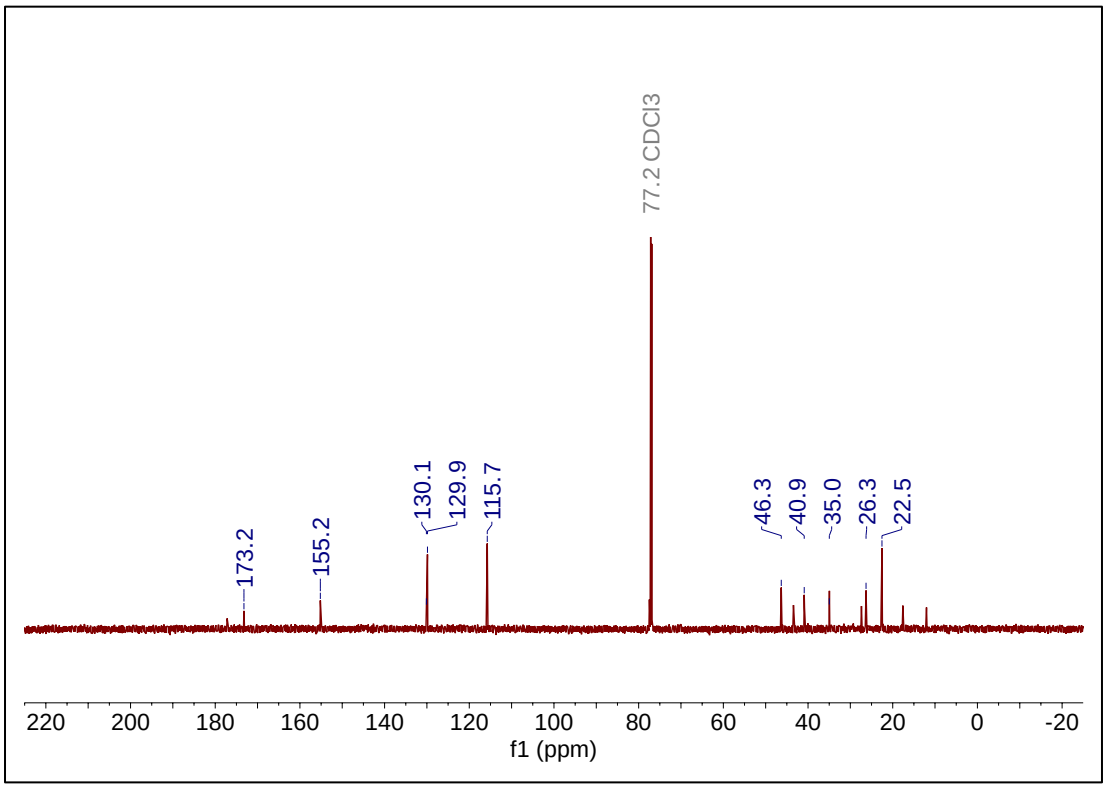

Figure 11. The ^{13}C NMR spectrum of *N*-[2-(4-Hydroxyphenyl)ethyl]-3-methylbutanamide from Fraction29-Fr3.

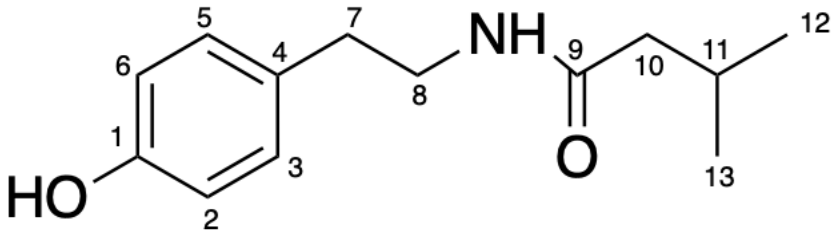

Figure 12.Chemical structure of *N*-[2-(4-Hydroxyphenyl)ethyl]-3-methylbutanamide.

Table 5. NMR data of *N*-[2-(4-Hydroxyphenyl)ethyl]-3-methylbutanamide

Position	**δ_H mult (*J* in Hz)**	**δ_C**
1		155.2, C
OH	0.84, t (7.5)	
2,6	7.00, d (6.2)	129.9, CH
3,5	6.79, d (6.0)	115.8, CH
4		130.2, C
7	2.73,t (7.0)	34.9, CH2
8	3.48, qd (7.0, 3.1)	41,CH2
NH	5.54, s	
9		173.2, C
10	2.01, d (7.3)	46.3, CH2
11	2.05, d (13.1, 6.6)	26.3, CH
12,13	0.9, d (6.4)	22.5, CH3

Chapter 4 GENOME SEQUENCING

SeqCenter was responsible for the DNA extraction and genomic sequencing of *Glutamicibacter soli* (McD-86). DNA extraction followed the ZymoBIOMICS™ DNA Miniprep Kit instructions. Sample McD-86 was submitted as cell pellets; they were resuspended in 750 µL of lysis solution. Following all the steps of the DNA Miniprep Kit, concentrations were determined via Qubit. For Illumina sequencing, libraries were prepared using the tagmentation-based and PCR-based Illumina DNA Prep kit and custom IDT 10bp unique dual indices (UDI). Sequencing was performed on an Illumina NovaSeq X Plus sequencer, producing 2x151 bp paired-end reads. Illumina sequence had a total read of 5927390 pairs. As for Nanopore Sequencing methods, libraries were prepared using the PCR-free Oxford Nanopore Technologies (ONT) Ligation Sequencing Kit (SQK-NBD114.24) with the NEBNext R Companion Module (e7180L). Nanopore sequencing was performed on an Oxford Nanopore a MinION Mk1B sequencer. Nanopore trimmed reads resulted in 2560109. Combo assembly and annotation were performed via Porechop, to trim residual adapter sequence from the ONT reads. De novo genome assemblies were generated from the ONT with Flye under the nano-hq model. Subsequent polishing used the Illumina read data with Pilon under default parameters. Assembled contigs were evaluated via circulator using the ONT long reads. Assembly annotation was then performed with Bakta.

The genome annotation was done using Prokka and Interpro, the DNA map is shown in Figure 13. The genome has a size of 3,616,107 base pairs in total, with a G+C content of 74. *G.soli* genome hosts at least 7105 features.

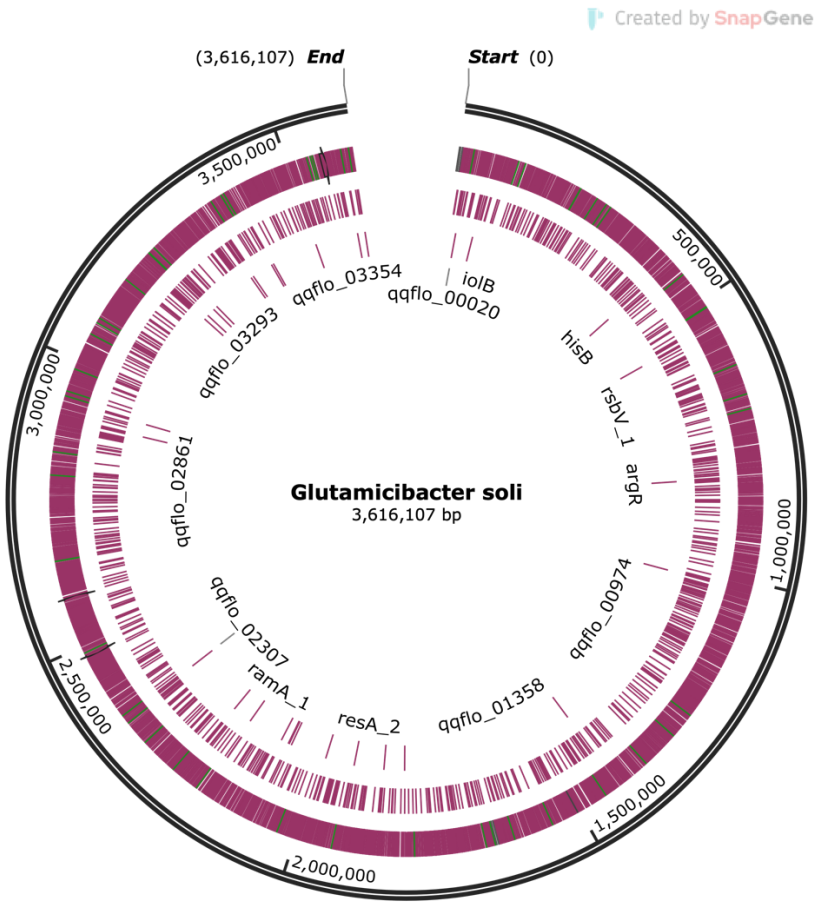

Figure 13. Annotated genome of *Glutamicibacter soli* strain McD-86.

Chapter 5 DISCUSSION

Scientists are currently engaged in an extensive analysis of the bacterial diversity in hard corals to better understand their susceptibility against Stony Coral Tissue Loss Disease. Despite intensive research efforts, there is no known cause for this devastating disease, although some clues suggest a potential bacterial origin. Researchers are focused on finding the causative agent, while also observing resilient coral communities in hopes of identifying symbiotic bacterial strains that may provide protective mechanisms against this disease. The primary focus of this study was to investigate the antibiotic activity exhibited by symbiotic strains against coral pathogens. The results of my testing highlighted an intriguing observation in the disk-diffusion method, revealing a 'hazy' zone of inhibition, specifically for *Vibrio coralliilyticus*. Nonetheless, it is important to be realistic in the interpretation of these results, as a 'complete cell death' compared to the positive control (ciprofloxacin) suggests distinct complexities. The question remains regarding the specific impact of secondary metabolites produced by these strains on coral pathogens. Several hypotheses may shed light on the outcomes observed. It is reasonable that initial pathogen growth might lead to the activation of secondary metabolite biosynthesis. Alternatively, compounds could be potentially disrupting critical pathways that are essential to the pathogen's survival, but through non-antibiotic mechanisms yet to be fully interpreted.

Predominantly, this study emphasizes the complicated interaction between coral symbiotic bacteria and pathogens, implying the intricate interaction of biochemical processes underlying coral health and disease resilience. This project aimed to prove that the four coral-associated strains; because of their antibiotic production, would provide corals with the ability to evade pathogens. Metabolites with mild antibiotic activity were observed, however, there was no significant evidence to support the hypothesis. Future exploration and in-depth analyses are

likely to solve the full spectrum of these complex dynamics and their implications for coral reef ecosystems.

This study illuminates the observation of cholic acid found in the cultivation of marine actinobacteria. To discuss the production of cholic acid, it is essential to describe where this molecule is commonly found. Cholic acid is a naturally occurring bile acid that represents the major component of the bile acid pool in humans. It is initially synthesized in the liver from cholesterol and then it is complexed into hundreds of different mainly amino acid derivatives. While it is traditionally associated with mammalians' digestive systems, the revelation of its presence in prokaryotic bacterial cultures highlights the significance of this discovery. Bacteria are known to produce and transform different types of steroids enzymatically (Maneerat, *et al.* 2004), although the production of bile acids by bacteria has been barely reported. Notably, this is not the first instance of cholic acid discovery in marine bacterial cultivations. The earliest documented report is from 2004, by Maneerat and collaborators, who extracted it from cultures of *Myroides* sp., a marine bacterium isolated from seawater, and cultured in Marine Broth. The authors also speculate on the potential presence of bile acids in the media, given that one of the ingredients present in MB is peptone, sourced from bovine and porcine origins. The presence in media components is supported by the fact that mammals typically produce bile acids, hinting at their potential appearance in the growth medium. Cholic acid may be present in bacterial extracts due to various factors. One highly plausible scenario involves the enzymatic activity of bile salt hydrolase, through which bacteria may deconjugate bile acid derivatives present in the media (Kim *et al.* 2007). While less likely, it's worth noting the potential for de novo biosynthesis of cholic acid by bacteria, previously described by Pheiffer and collaborators (2023).

The significance of identifying cholic acid lies in its potential to unveil the metabolic pathway responsible for its production. Such insight could revolutionize current pharmaceutical processes, potentially replacing the laborious synthesis of cholic acid with a more efficient biological approach. Presently, cholic acid and its derivatives are being administered as treatments for adults with bile acid synthesis disorders, among other diseases. Traditionally sourced from bovine bile concentrates, the industrial production of cholic acid faces sustainability and ethical concerns. Discovering the metabolic pathway for its production could pave the way for sustainable alternatives.

In conclusion, the discovery of cholic acid production by marine bacteria signifies a remarkable potential advancement in our understanding of microbial metabolism. This discovery expands our knowledge of the biosynthesis of bile acids and enlightens the metabolic versatility of bacteria in diverse environments. Furthermore, it highlights the importance of exploring natural sources for bioactive compounds, especially those with potential pharmaceutical or industrial applications. Moving forward, continued research in this area brings hope of uncovering novel microbial-derived compounds and their potential uses in various fields, from medicine to all forms of biotechnology. Specifically giving prominence to the capabilities of marine bacteria in addressing humanity’s needs.

Chapter 6 CONCLUSIONS

The analysis of four coral-derived strains led to the isolation of *N*-[2-(4-Hydroxyphenyl)ethyl]-3-methylbutanamide, an aromatic metabolite that appears to be derived from tyrosine, with modest antibiotic activity against putative coral pathogens related to SCTLD. Although in this study, insufficient evidence supported the hypothesis, this study paved the way for the future chemical analysis of coral-associated strains to protect corals against ongoing threats, specifically pathogenic bacteria.

Chapter 7 MATERIALS AND METHODS

7.1 BACTERIA GROWTH CONDITIONS

The four coral-associated strains provided by Dr. Valerie Paul from the Florida Keys corals were labeled as shown in Table 1. The strains were received in cryovials and the following procedures were performed in a biosafety hood. Bacterial strains were individually resuspended in natural seawater (Scripps Pier water source)-based Seawater Broth (SWB) medium (4 g/L tryptone, 2 g/L yeast extract, 100% seawater) and streaked into Seawater Agar (SWA) plates. Strains were allowed to grow for 7 days at 28 ºC. After microscopic confirmation of purity, multiple colonies from agar plates were cut and transferred to a glass flask containing 25 mL of SWB and shaken at 190 rpm for 5 days. Ten mL were transferred into 1L bottles with SWB, and an inoculation loop was used to apply on a SWA plate. The 1L media bottles were placed on the saker at 190 rpm for 7 days, followed again by a purity check.

For extraction purposes, 1L volumes of each strain were extracted individually in separatory funnels with EtOAc (2 x 500 mL). A total of 1L of the EtOAc layer was collected from each strain. Using a rotary evaporator, the solvent was removed and the resulting extract masses obtained are shown in Table 2.

7.2 FERMENTATION AND EXTRACTION OF *GLUTAMICIBACTER SOLI* (MCD-86)

Given that the first experiment using the disk diffusion assay gave positive results only for *Glutamicibacter soli* a 20L cultivation was performed. The strain was grown in Marine Broth (Difco Marine Broth 2216, 34.7 g/L, 100% DI water). Marine Broth is composed of peptone (0.5%), yeast extract (0.1%), NaCl (1.9%), and a mixture of trace elements. From a 1L culture, 10 mL were transferred (per bottle) for a total of 20 flasks. The 20 culture flasks were placed in a

shaker at 190 rpm for 7 days at 28 ºC. After 7 days of cultivation, 20 grams of sterilized XAD-7 resin were added to each 1L flask to absorb the organic products and placed back on the shaker table for 3 hours. Subsequently, the resin and cell mass were filtered through cheesecloth using a porcelain Buchner funnel, and the resin was collected and extracted with 2 X 1L of acetone. Using a rotary evaporator, the acetone was removed, and the remaining aqueous layer was extracted twice using EtOAc (2 x 500 mL) in a separatory funnel. The ethyl acetate layers were collected and dried once again in a rotary evaporator, resulting in a total of 704 mg of organic extract.

7.3 ANTIMICROBIAL TESTING

To determine antimicrobial activity against coral pathogens, the agar-based disk diffusion assay was executed on SWA plates.. Negative control (MeOH) and 0.4 mg of samples dissolved in MeOH were applied to 6 mm blank paper discs, and the solvent was allowed to evaporate. The positive control was performed with a pre-prepared ciprofloxacin-applied paper disc. The disks were placed on SWA plates evenly inoculated with the three coral pathogens in different plates each. Plates were incubated at 28 ºC for 24 hours. The diameter of inhibition, or bacteria-free zone around the disks was measured and recorded to the nearest whole millimeter. The antimicrobial activity was noted based on the following criteria: P if inhibition diameter is less than 10 mm; P.P if more than 10 mm but less than 15 mm; P.P.P. if more than 15 mm and N.C.I. for “not complete inhibition” as indicated by a ‘hazy’ incomplete inhibition zone.

7.4 ISOLATION AND CHARACTERIZATION OF CHOLIC ACID AND *N*-[2-(4-HYDROXYPHENYL)ETHYL]-3-METHYLBUTANAMIDE

The organic extract of *Glutamicibacter soli,* strain McD-86 (704 mg) was subjected to silica gel vacuum flash chromatography, eluting with methylene chloride (DCM) and methanol (MeOH) (100:0, 50:1, 10:1, 5:1, 1:1, 0:100) to afford six fractions. Fraction 6 (38 mg) was further purified by repeated C-18 reversed-phase HPLC (Luna, 250 x 10 mm, 10µm column; 3 mL/min flow rate, UV detection at 210 and 254 nm) with 30% to 100% acetonitrile/H_2O over 20 min with the same solvent eluting an additional 5 min to obtain 20 additional fractions: FR0-FR20. For NMR analysis, samples were dissolved in MeOH-d_4, and 1D (1H) NMR spectral data were obtained using a JEOL 500 NMR spectrometer. This led to the discovery of cholic acid in FR3, with a total mass of 6 mg. Further MS analysis of this fraction was carried out on an LC-MS instrument consisting of an Agilent 1260 LC system with a Phenomenex Luna C18 column coupled with an Agilent 6530 Accurate-Mass Q-TOF spectrometer. Specific spectral data 1D (1H, 13C) and 2D (HSQC, HMBC, COSY) NMR data were obtained and interpreted.

The NMR data from FR3, illustrated that the sample was not completely pure. Undescribed aromatic metabolites seemed to be present which could explain the weak antibacterial activity observed. To isolate this aromatic molecule (s), a new 20L culture was extracted and purification of the ethyl acetate extract was performed using a silica-based Teledyne ISCO CombiFlash Rf+ Lumen System in the normal phase using DCM-MeOH. In the antibacterial test, it was clear that fraction 29 had weak inhibition activity. Further purification of fraction 29 on HPLC resulted in 4 different fractions labeled FR0, FR1, FR2, and FR3. From NMR analysis and repeated antibacterial testing, FR3 contained the molecule *N*-[2-(4-Hydroxyphenyl)ethyl]-3-methylbutanamide, with a total mass of 3 mg.

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