

**Bioautographic fractionation of antibiotic natural products
from a marine-derived fungus**

by

Camryn R. Newlands

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Bachelor of Science with Honours in Biology

Supervisor: Christopher A. Gray, PhD, Department of Biological Sciences &
Department of Chemistry

THE UNIVERSITY OF NEW BRUNSWICK

April 2024

© Camryn R. Newlands, 2024

Abstract

The development of antibiotic resistance in recent decades is a major concern that has urged natural product researchers to investigate new biological sources of antimicrobial compounds. Fungi isolated from marine environments have proven to be a potent source of bioactive molecules. The crude extract of an unidentified seafoam-derived fungus, SC1-077G, demonstrated strong inhibition of *Staphylococcus aureus* in an antimicrobial activity screening in 2016. The objective of this research was to isolate the compound(s) responsible for the bioactivity of the crude extract using immersion bioautography guided fractionation. Flash chromatography and high-performance liquid chromatography generated an impure sample containing two putatively new natural products, which demonstrated strong inhibition of *S. aureus*, that could not be further purified chromatographically. Acetylation of the impure sample facilitated the isolation of two acetylated, putatively new natural product derivatives. Immersion bioautography proved to be an efficient method for directing the fractionation of bioactive natural products.

Dedication

To my dearest friend Kristin, for her unparalleled inspiration.

Acknowledgements

I would like to acknowledge first and foremost my supervisor, Dr. Christopher Gray, for his endless guidance and encouragement during this project, and for bringing me into the Natural Products Research Group early on in my undergraduate degree. These past two years have been incredibly educational and rewarding, and I will look back on my time in the NPRG with overwhelming gratitude.

Next, I would like to express my appreciation to Dr. Larry Calhoun from UNB Fredericton for obtaining several NMR spectra for this project, as well as Dr. Hebelin Correa and Josh Kelly at UPEI for acquiring HRESIMS data. This project would not have been possible without their gracious help.

Lastly, I would like to extend my gratitude to the graduate students of the NPRG: Matt Laprise, Janet Debly, Nick Morehouse, and Ally Bos. Their support, guidance, and friendship are treasured.

Statement of Research Contribution

The unidentified fungus SC1-077G was isolated from sea foam from the Bay of Fundy in 2016, and its crude extract demonstrated strong inhibition of *Staphylococcus aureus* in immersion bioautography. My research contributions involved the fractionation of the extract using flash chromatography, high performance liquid chromatography, and acetylation reactions that led to the isolation of two putatively new compounds, as well as a third compound bioactive against *S. aureus*.

Table of Contents

Abstract.....	ii
Dedication.....	iii
Acknowledgements.....	iv
Statement of Research Contribution.....	v
Table of Contents.....	vi
List of Figures.....	vii
List of Symbols, Nomenclature, and Abbreviations.....	viii
Introduction.....	9
Experimental.....	16
General experimental procedures.....	16
Liquid culture fermentation and extraction.....	17
Fractionation of extracts.....	17
Acetylation of CN2-025-02.....	19
Immersion bioautography.....	19
Results and Discussion.....	21
Conclusion.....	29
References.....	31
Appendix A: Immersion Bioautography Guided Fractionation.....	42

List of Figures

- Figure 1.** The chemical structures of (a) caffeine, (b) morphine, (c) quinine, and (d) penicillin G..... 10
- Figure 2.** The chemical structure of 2,4,6,9-tetrahydroxy-7-methyl-2-prenyl-1H-phenalene-1,3(2H)-dione. 14
- Figure 3.** Normal-phase thin-layer chromatography (TLC) immersion bioautography plate of the crude extract (CN2-001-01; 5 mg/mL) against *Staphylococcus aureus* (ATCC 29213) with positive control gentamicin (1.0 mg/mL). Purple regions on the plate indicate the presence of metabolizing cells, while yellow regions indicate the presence of metabolically inhibited cells (i.e., zones of inhibition)..... 20
- Figure 4.** (a) ¹H NMR spectrum (400 MHz, DMSO-d₆) of CN2-025-02 and (b) ¹³C NMR spectrum (400 MHz, DMSO-d₆) of CN2-025-02. Peaks in red boxes indicate proton resonances of low intensity, meaning there are impurities present in the sample. 22
- Figure 5.** Reversed-phase high performance liquid chromatography trace of (a) CN2-025-02 pre-acetylation, and (b) CN2-025-02 post-acetylation. The peaks overlapping in the 23-to-35-minute range of (a) indicated a poor separation of the sample, meaning the compounds in the sample could not be isolated chromatographically. Following acetylation of the sample, the peaks demonstrated far greater separation (b) than observed in (a), suggesting that acetylation of the impure sample allowed for the successful chromatographic isolation of its constituents Solvent system for both injections was 50 CH₃CN: 50 H₂O with an elution rate of 4 mL/min. Ultraviolet absorption was measured at 190 nm (traced in black) and 254 nm (traced in red)..... 25
- Figure 6.** ¹H NMR spectrum (400 MHz, DMSO-d₆) of (a) CN2-025-02 (pre-acetylation sample), (b) CN2-037-09 (post-acetylation fraction), and (c) CN2-037-12 (post-acetylation fraction). 27
- Figure A 1.** Normal-phase thin-layer chromatography (TLC) immersion bioautography of the reversed-phase flash column fractions of the crude extract (CN2-001-01; 5 mg/mL) against *Staphylococcus aureus* (ATCC 29213) with positive control gentamicin (1.0 mg/mL). The seventh fraction (CN2-019-07; 60 CH₃CN: 40 H₂O) demonstrated the largest zone of inhibition of *S. aureus* (encircled). 42
- Figure A 2.** Normal-phase thin-layer chromatography (TLC) immersion bioautography of the reversed-phase high performance liquid chromatography fractions of CN2-019-07 (5 mg/mL) against *Staphylococcus aureus* (ATCC 29213) with positive control gentamicin (1.0 mg/mL). The second fraction (CN2-025-02) demonstrated the largest zone of inhibition of *S. aureus* (encircled)..... 43
- Figure A 3.** Normal-phase thin-layer chromatography (TLC) immersion bioautography of the reversed-phase high performance liquid chromatography fractions of the acetylation reaction products of CN2-025-02 (5 mg/mL) against *Staphylococcus aureus* (ATCC 29213) with positive control gentamicin (1.0 mg/mL). The first fraction (CN2-037-01) demonstrated the only zone of inhibition of *S. aureus* (encircled). 44

List of Symbols, Nomenclature, and Abbreviations

^{13}C NMR	Carbon nuclear magnetic resonance spectroscopy
^1H NMR	Proton nuclear magnetic resonance spectroscopy
ACS	American Chemical Society
ATCC	American Type Culture Collection
CAMH	Mueller Hinton II cation adjusted
CH_3CN	Acetonitrile
δ	Chemical shift in ppm
DMSO- d_6	Deuterated dimethyl sulfoxide
EtOAc	Ethyl acetate
Hex	Hexanes
HPLC	High-performance liquid chromatography
HRESIMS	High-resolution electrospray ionization mass spectrometry
MeOH	Methanol
MHz	Megahertz
mins	Minutes
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
m/z	Mass to charge ratio
NP	Normal phase
NMR	Nuclear magnetic resonance spectroscopy
ppm	Parts per million
®	Registered trademark
RP	Reversed-phase
RPFC	Reversed-phase flash chromatography
RP-HPLC	Reversed-phase high-performance liquid chromatography
rpm	Rotations per minute
TFA	Trifluoroacetic acid
TLC	thin-layer chromatography
™	Trademark

Introduction

Compounds produced by living organisms can be classified as either primary or secondary metabolites (Croteau *et al.*, 2000; Fernie & Pichersky, 2015; Drew & Demain, 1977). Primary metabolites have essential roles in metabolism, making them requisite for the existence and development of life as we know it (Croteau *et al.*, 2000; Fernie & Pichersky, 2015; Drew & Demain, 1977). Conversely, biological compounds that are not required for the existence of life, but instead provide their producer some form of fitness advantage, are classified as secondary metabolites (Demain & Fang, 2000; Pavarini *et al.*, 2012; Croteau *et al.*, 2000; Drew & Demain, 1977). These compounds, often serving ambiguous or unknown roles for their producer (Croteau *et al.*, 2000), are evolutionary products that emerged in response to environmental challenges (Verdine, 1996; Maplestone *et al.*, 1992), and are characterized by their vast chemical diversity and potent biological properties (Atanasov *et al.*, 2021).

Humans have been using secondary metabolites, also known as natural products, as chemical tools for centuries, mainly as spices, dyes, waxes, and medicinal agents (Croteau *et al.*, 2000; Dias *et al.*, 2012). Over the years, the list of human-centred applications of natural products has only grown with their continuous discovery, and their impacts are felt world-wide in modern society (Atanasov *et al.*, 2021; Dias *et al.*, 2012). For example, the natural product caffeine (Figure 1a) has been estimated to be the second most heavily traded commodity in the world, providing jobs to over seventy-five million people globally (Pendergrast, 2009). Another example of an industry that has been heavily influenced by natural products is the agricultural industry; from the years 1997 to

2010, natural product research was responsible for nearly 70% of all new active ingredients added to western commercial pesticides (Cantrell *et al.*, 2012).

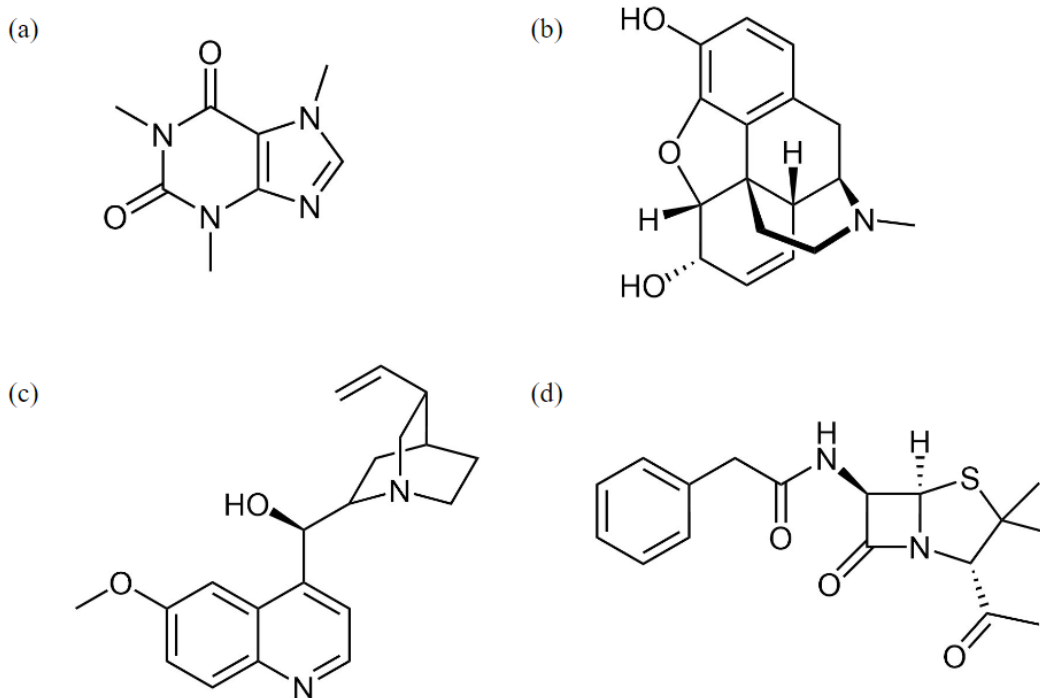


Figure 1. The chemical structures of (a) caffeine, (b) morphine, (c) quinine, and (d) penicillin G.

However, it can be argued that the greatest impact had by natural products on humanity has been through their medicinal applications. Historically, natural products have been administered to treat a wide variety of ailments through the use of medicinal plants (Dias *et al.*, 2012). The development of a more empirical approach to chemistry in the 19th century facilitated rigorous investigation of the therapeutic potential of plants (Beutler, 2009), leading to the discovery of various natural product drugs such as morphine (Figure 1b), an analgesic (Lockermann, 1951), and quinine (Figure 1c), an antimalarial (Borchardt, 1996). Additionally, fungi became organisms of interest for

natural product research during this time, with various macromycetes found to produce bioactive compounds (Stadler & Hoffmeister, 2015). In 1928, the investigation of fungal natural products was intensified with Fleming's discovery of the antibiotic natural product penicillin G (Figure 1d), produced by fungi of the *Penicillium* genus, which launched humanity into a new medicinal age: the age of antibiotics (Lobanovska & Pilla, 2017; Marek & Timmons, 2019).

Antibiotics have revolutionized the landscape of healthcare and are largely responsible for the 40% increase in life expectancy seen from the 19th century to present day (Adedeji, 2016). To date, there have been over 200 different antibiotics and antifungals approved for prescription by the FDA (Newman & Cragg, 2020), and during the period of 1981 to 2020, 49% of all approved antibacterials and antifungals were natural products, natural product derivatives, or synthetics inspired by natural product structures (Newman & Cragg, 2020).

However, the injudicious prescription of antibiotics is a prevalent issue in society (Belongia & Schwartz, 1998; Nyquist *et al.*, 1998; Schwartz *et al.*, 1998). Over a fifth of all antibiotic prescriptions in the US are written for upper respiratory tract illnesses, the vast majority of which are viral infections and therefore cannot be successfully treated with antibiotics (Nyquist *et al.*, 1998). These findings are consistent with more recent studies, which found that 25% of antibiotic prescriptions in developed countries are written to treat illnesses that will not benefit from the prescribed treatment (Meropol & Votruba, 2015; Hicks *et al.*, 2015; Vaz *et al.*, 2014).

A concerning increase in the prevalence of antibiotic resistance has resulted from the overprescription of antibiotics (Schwartz *et al.*, 1998; Hersh *et al.*, 2013; Institute of

Medicine, 2010). In the early 1940s, penicillin prescriptions were remarkably successful in treating staphylococcal infections (Lobanovska & Pilla, 2017; Ligon, 2004), but penicillin-resistant strains were identified in communities within two years of the public introduction of the drug (Rammelkamp & Maxon, 1942). By 1970, over 80% of all staphylococcal infections were resistant to treatment by penicillin (Lowy, 2003).

Presently, the planet is described as entering a global antibiotic-resistance crisis (Aslam *et al.*, 2023; Podolsky, 2018; Shallcross *et al.*, 2015), and mortality rates caused by infections have been projected to reach levels similar to those before antibiotics were ever mass produced (Shallcross *et al.*, 2015). Select developing countries have taken initiatives to curb this threat, such as designing comprehensive plans to contain the national spread of antibiotic resistant pathogens (Aslam *et al.*, 2023; Landers & Kavanagh, 2016) and investing in research for therapeutic alternatives (Mitea *et al.*, 2023; Luepke & Mohr, 2017), but antibiotic resistance is a worldwide problem, and action on a global scale has yet to be taken (Aslam *et al.*, 2023).

Various research strategies have emerged with the common goal of boosting drug discovery and development. Modern synthetic techniques include computational and combinatorial chemistries, which have produced more drug leads in a time and cost-efficient manner than ever before (Leelananda & Lindert, 2016; Liu *et al.*, 2017). Alternatively, natural product chemists are largely focusing on investigating new ecological niches that foster a rich diversity of bioactive natural products (Che, 2011; Sanchez *et al.*, 2012; Sasso *et al.*, 2012; Swift *et al.*, 2021; Debbab *et al.*, 2011; Shang *et al.*, 2012). Among these natural reservoirs of therapeutic leads are endophytic and marine-derived fungi (Che, 2011; Debbab *et al.*, 2011; Shang *et al.*, 2012; Bugni &

Ireland, 2003). Endophytes are organisms that spend at least part of their life cycle within the tissues of plants (Stone *et al.*, 2004), while marine-derived fungi are those that have been isolated from a marine environment (Bugni & Ireland, 2003).

Endophytic and marine-derived fungi have proven to be promising sources of bioactive natural products. (Luo *et al.*, 2017; Bhardwaj *et al.*, 2023; Che, 2011; Debbab *et al.*, 2011; Shang *et al.*, 2012; Bugni & Ireland, 2003). As such, the Natural Products Research Group (NPRG) has been investigating endophytic and marine-derived fungal extracts for the production of new natural products (Clark, 2019; Morehouse *et al.*, 2023a; Morehouse *et al.*, 2023b). Using nuclear magnetic resonance (NMR) and high-resolution electrospray ionization mass spectrometry (HRESIMS) prioritization and fractionation techniques, numerous new natural products have been discovered (Clark, 2019; Morehouse *et al.*, 2023a; Morehouse *et al.*, 2023b; Morehouse *et al.*, 2023c). Extracts produced from the NPRG's fungal library have also been subjected to bioactivity screening assays against a suite of infectious microorganisms in order to gauge the therapeutic capabilities of the extracts (Cox, 2016; Ellsworth *et al.*, 2013), and bioassay-guided fractionation techniques have led to the isolation of new natural products with potent bioactivities (Morehouse *et al.*, 2023d; Morehouse *et al.*, 2020; Flewelling *et al.*, 2015).

In 2016, 72 distinct fungi were isolated from sea foam from the Bay of Fundy (Cox, 2016). Various studies have demonstrated the rich diversity of sea foam-derived fungi (Hyde & Jones, 1989; Koehn, 1982; Nakagiri, 1989; Kirk, 1983), but few have investigated the therapeutic potential of their natural products (Oppong-Danquah *et al.*, 2020; Cox, 2016; Overy *et al.*, 2014). Of the 72 extracts generated from these isolates, 14

demonstrated antimicrobial properties (Cox, 2016), and further investigation of one of these extracts led to the isolation of a new antimicrobial phenalenone derivative, 2,4,6,9-tetrahydroxy-7-methyl-2-prenyl-1*H*-phenalene-1,3(2*H*)-dione (Figure 2) (Morehouse *et al.*, 2023d).

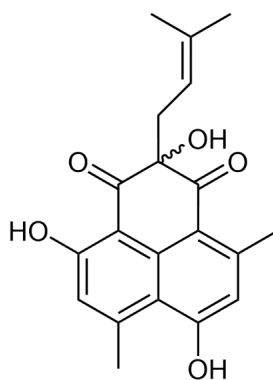


Figure 2. The chemical structure of 2,4,6,9-tetrahydroxy-7-methyl-2-prenyl-1*H*-phenalene-1,3(2*H*)-dione.

The crude extract of SC1-077G, another fungus isolated from sea foam (Cox, 2016), demonstrated strong inhibition of *Staphylococcus aureus* in thin-layer chromatography (TLC) immersion bioautography. Bioautography is an antimicrobial activity detection technique that can be used to rapidly screen for the presence of bioactive compounds in an extract (Dewanjee *et al.*, 2015; Choma & Grzelak, 2011). TLC immersion bioautography works by submerging a TLC plate spotted with a sample into an agar that has been inoculated with a microorganism. If there are compounds present in the sample that are bioactive against the microorganism, then zones of inhibition will form where the bioactive compounds are located on the TLC plate. Bioautography is less commonly used than microplate bioassays to assess the bioactivity of natural extracts (Sun *et al.*, 2018). However, its rapid, simple, and inexpensive nature

(Dewanjee *et al.*, 2015; Choma & Grzelak, 2011) makes it an ideal antimicrobial analysis technique to direct the isolation steps of biologically active natural products.

Therefore, the objective of this research was to isolate the compound(s) responsible for the *S. aureus* bioactivity of the crude extract of SC1-077G using TLC immersion bioautography guided fractionation.

Experimental

General experimental procedures

Solvents used for fractionation and purification procedures were ACS certified or high-performance liquid chromatography (HPLC) grade and were purchased from Fisher Scientific (Ottawa, Ontario, Canada). Deuterated solvents used for nuclear magnetic resonance (NMR) were purchased from Sigma-Aldrich® (Oakville, Ontario, Canada). NMR spectra were recorded using a Bruker AVII 400 instrument and processed using MNova® 14 software (Mestrelab Research® S.L.). Chemical shifts are expressed as ppm and referenced to residual protonated solvent resonances (DMSO-d₆: ¹H, 2.500 ppm; ¹³C, 39.52 ppm). Normal-phase thin-layer chromatography (TLC) was performed using Ultra-Pure Silica Gel on aluminium backing (Silicycle Chemical Division, Quebec City, Quebec, Canada). Reversed-phase flash column chromatography (RPFC) was conducted by eluting acetonitrile and H₂O through a Biotage Flash+ chromatography system with a C18 Silicycle cartridge (40-60 µm, 60 Å, 25 g; Silicycle, Quebec). Reversed-phase high performance liquid chromatography (RP-HPLC) was performed using a Waters 600 pump, a Waters 2487 dual wavelength absorbance detector (measuring absorbance at 190 and 254 nm), and a Phenomenex Luna C18 column (10 µm, 10-0 Å, 250 x 10 mm). Acetonitrile (CH₃CN) and H₂O were eluted at a flow rate of 4 mL/min. Reagent grade acetic anhydride purchased from Sigma-Aldrich® was used in acetylation reactions. High-resolution electrospray ionization mass spectrometry (HRESIMS) data of samples was acquired using a Thermo LTQ Exactive instrument and processed using Thermo Scientific™ Xcalibur™ software.

Liquid culture fermentation and extraction

Segments of a culture of SC1-077G (5 mm x 5 mm) were placed in flasks containing 1.2% Difco™ potato dextrose broth (VWR, Mississauga, Ontario, Canada) (100 mL per flask, 50 flasks total). The flasks were shaken at 150 rpm in the dark for two weeks in a temperature-controlled room (21°C ± 2°C). Following the fermentation period, the flasks were sonicated for 30 seconds, and the spent broth and cellular debris were then filtered through cotton wool, combining the filtrate (5 L). The filtrate was extracted with three washes of ethyl acetate (EtOAc; 1.6 L EtOAc per wash), and the ethyl acetate washes were combined and concentrated *in vacuo* to produce a crude extract (CN2-001-01; 388 mg). ¹H NMR and HRESIMS data were obtained for the extract.

Four 5 L fermentations were conducted in total, giving extracts of 388 mg, 322 mg, 273 mg, and 306 mg (CN2-001-01, CN2-001-01.2, CN2-001-01.3, and CN2-001-01.4, respectively). The extract (CN2-001-01) was tested against *Staphylococcus aureus* (ATCC 29213) using immersion bioautography to verify its bioactivity as originally discovered.

Fractionation of extracts

The crude extract (CN2-001-01) was loaded onto C18 silica (2.0 g) and subjected to reversed-phase flash chromatography (RPFC) employing a stepwise gradient of 100% water (H₂O) to 100% acetonitrile (CH₃CN) (10% increments, 150 mL elutions) to separate the extract into eleven fractions. The column was then washed with methanol (MeOH) followed by EtOAc (150 mL elutions), generating 13 fractions in total that were concentrated *in vacuo*. Reversed-phase flash chromatography was performed under the same conditions for all four extracts (CN2-001-01, CN2-001-01.2, CN2-001-01.3, and

CN2-001-01.4). *Staphylococcus aureus* immersion bioautography was conducted for each fraction generated by RPFC in addition to ¹H NMR and HRESIMS acquisition. Fraction seven (60 CH₃CN: 40 H₂O, 655 mg total) demonstrated the largest zone of inhibition in immersion bioautography (see Appendix A, Figure A1). A portion of fraction seven (47 mg) was purified using isocratic reversed-phase HPLC (50 CH₃CN: 50 H₂O), producing five fractions. ¹H NMR spectra and HRESIMS data were acquired for each fraction. Immersion bioautography against *S. aureus* was also conducted for each fraction. Fraction two (CN2-025-02; 31 mg) produced the largest zone of inhibition in *S. aureus* immersion bioautography (see Appendix A, Figure A2).

The ¹H NMR spectrum of CN2-025-02 indicated that the sample was impure. To purify CN2-025-02, various HPLC test injections were attempted at the following conditions: reversed-phase (RP), isocratic, 85 H₂O: 15 CH₃CN; RP, isocratic, 50 H₂O: 50 MeOH; RP, isocratic, 85 H₂O: 15 MeOH; RP, isocratic, 50 H₂O: 50 CH₃CN (0.05% trifluoroacetic acid [TFA]); RP, isocratic, 50 H₂O: 50 MeOH (0.05% TFA); RP, gradient, 90 H₂O: 10 CH₃CN to 100% CH₃CN (linear slope), 25 mins; RP, gradient, 90 H₂O: 10 CH₃CN to 100% CH₃CN (linear slope), 40 mins; RP, gradient, 90 H₂O: 10 CH₃CN to 100% CH₃CN (linear slope), 65 mins; normal-phase (NP), isocratic, 50 EtOAc: 50 hexanes (Hex); NP, isocratic, 25 EtOAc: 75 Hex. However, all traces showed insufficient peak separation, meaning the sample could not be purified chromatographically.

A final attempt to purify a portion (10 mg) of CN2-025-02 was conducted by acetylating the sample. The reaction mixture underwent reversed-phase column chromatography, generating three fractions. Fraction 2 (CN2-033-02; 8 mg) was then subjected to isocratic RP-HPLC (50 H₂O: 50 CH₃CN), producing sixteen fractions that

were concentrated *in vacuo*. ¹H NMR spectra and HRESIMS data were obtained for all fractions. Immersion bioautography against *S. aureus* was also conducted for all fractions.

Acetylation of CN2-025-02

CN2-025-02 (10 mg) was dissolved in pyridine (250 µL) and treated with acetic anhydride (36 µL, [0.38 mmol]) for six hours at 20°C with stirring before MeOH (100 µL) was added and the mixture stirred for a further 30 minutes. The reaction mixture was subjected to reversed-phase column chromatography (1.0 g of C18 silica), which employed a stepwise solvent gradient from 100% H₂O to 100% CH₃CN (50% increments, 2 mL elutions), followed by a MeOH wash (2 mL elution), collecting the fractions in separate vials. Fraction 2 (50 H₂O: 50 CH₃CN) and fraction 3 (100% CH₃CN) were then combined based on TLC profiles and concentrated *in vacuo* (CN2-033-02; 8 mg).

Immersion bioautography

Antimicrobial activity of the crude extract (CN2-001-01), as well as subsequent RPFC and RP-HPLC fractions, were qualitatively assessed against *Staphylococcus aureus* (ATCC 29213) using thin-layer chromatography (TLC) immersion bioautography. To prepare for immersion bioautography, samples (5 mg/mL) were spotted on a normal-phase TLC plate (2 cm x 8 cm; spots were 1 cm from bottom of plates), which was then developed in a closed solvent chamber (90 MeOH: 10 H₂O). After air-drying, a stock solution of positive control gentamicin (1.0 mg/mL; Sigma-Aldrich®, Oakville, Ontario, Canada) was spotted on the bottom left corner of the developed TLC plate. The TLC plate was then placed face-up in a sterile square Petri

dish (10 cm x 10 cm). 83 μ L of a 1.0 McFarland standard of *S. aureus* was transferred to an Erlenmeyer flask containing liquefied 2.2% Difco™ Mueller Hinton II cation adjusted (CAMH) agar (25 mL) and swirled. The inoculated agar was then poured onto the TLC plate in the Petri dish. Once the agar solidified, the Petri dish was sealed with Parafilm M® and incubated for 24 hours (37°C). Following the incubation period, the surface of the agar was sprayed with a light, even coating of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL). The plate was then incubated for an additional four hours. The yellow MTT solution turns purple in the presence of metabolizing cells during incubation, allowing areas of dead or inhibited cells (i.e., zones of inhibition) to be observed visually (Dewanjee *et al.*, 2015) (Figure 3). Following the 4-hour incubation period of the MTT-coated plate, zones of inhibition were observed and recorded.

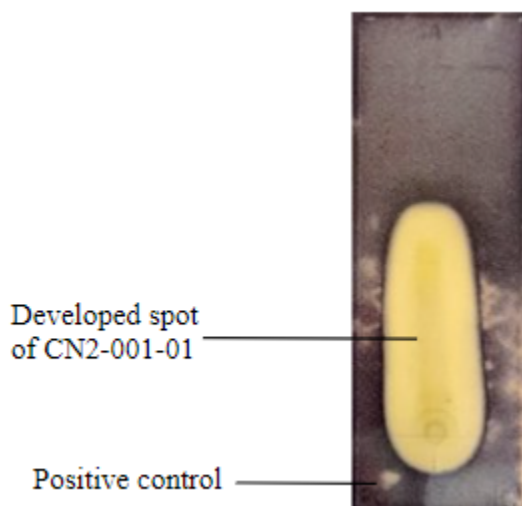


Figure 3. Normal-phase thin-layer chromatography (TLC) immersion bioautography plate of the crude extract (CN2-001-01; 5 mg/mL) against *Staphylococcus aureus* (ATCC 29213) with positive control gentamicin (1.0 mg/mL). Purple regions on the plate indicate the presence of metabolizing cells, while yellow regions indicate the presence of metabolically inhibited cells (i.e., zones of inhibition).

Results and Discussion

The crude extract of the unidentified fungus SC1-077G underwent reversed-phase flash chromatography (RPFC) to isolate the compounds responsible for the bioactivity against *Staphylococcus aureus*, which generated thirteen fractions that were then tested against *S. aureus* using thin-layer chromatography (TLC) immersion bioautography (see appendix A; Figure A1). Fraction seven (CN2-019-07; 60 CH₃CN: 40 H₂O; 655 mg) demonstrated the largest zone of inhibition against *S. aureus* (Figure A1). Reversed-phase high performance liquid chromatography (RP-HPLC) of CN2-019-07 produced five fractions. The second fraction (CN2-025-02; 31 mg) demonstrated the largest zone of inhibition against *S. aureus* (see Appendix A; Figure A2).

The ¹H NMR spectrum of CN2-025-02 contained several low intensity proton resonances (δ_{H} 1.45, 1.57, 1.75, 1.90, 2.08, 2.26, 3.17, 3.51, 4.75, 5.17, 6.16, 6.28, 6.57, 6.98, 7.11, 7.24, and 9.17 ppm), suggesting the sample was impure (Figure 4a). High resolution electrospray ionization mass spectrometry (HRESIMS) data revealed more than one molecular ion peak, with M+H⁺ peaks at 517.1155 and 519.1289 *m/z* being the most abundant components of the sample. This provides further evidence of the impurity of the sample.

The ¹³C NMR spectrum of CN2-025-02 (Figure 4b) contained fourteen carbon resonances. This data, coupled with the HRESIMS data, informed the non-protonated chemical formulas of the most abundant molecular ions to be C₁₄H₁₂N₁₆O₇ and C₁₄H₁₄N₁₆O₇. These are putatively new natural products, returning zero results when either chemical formula was searched in the Natural Products Atlas database (van Santen *et al.*, 2022), CAS Scifinderⁿ or Google Scholar.

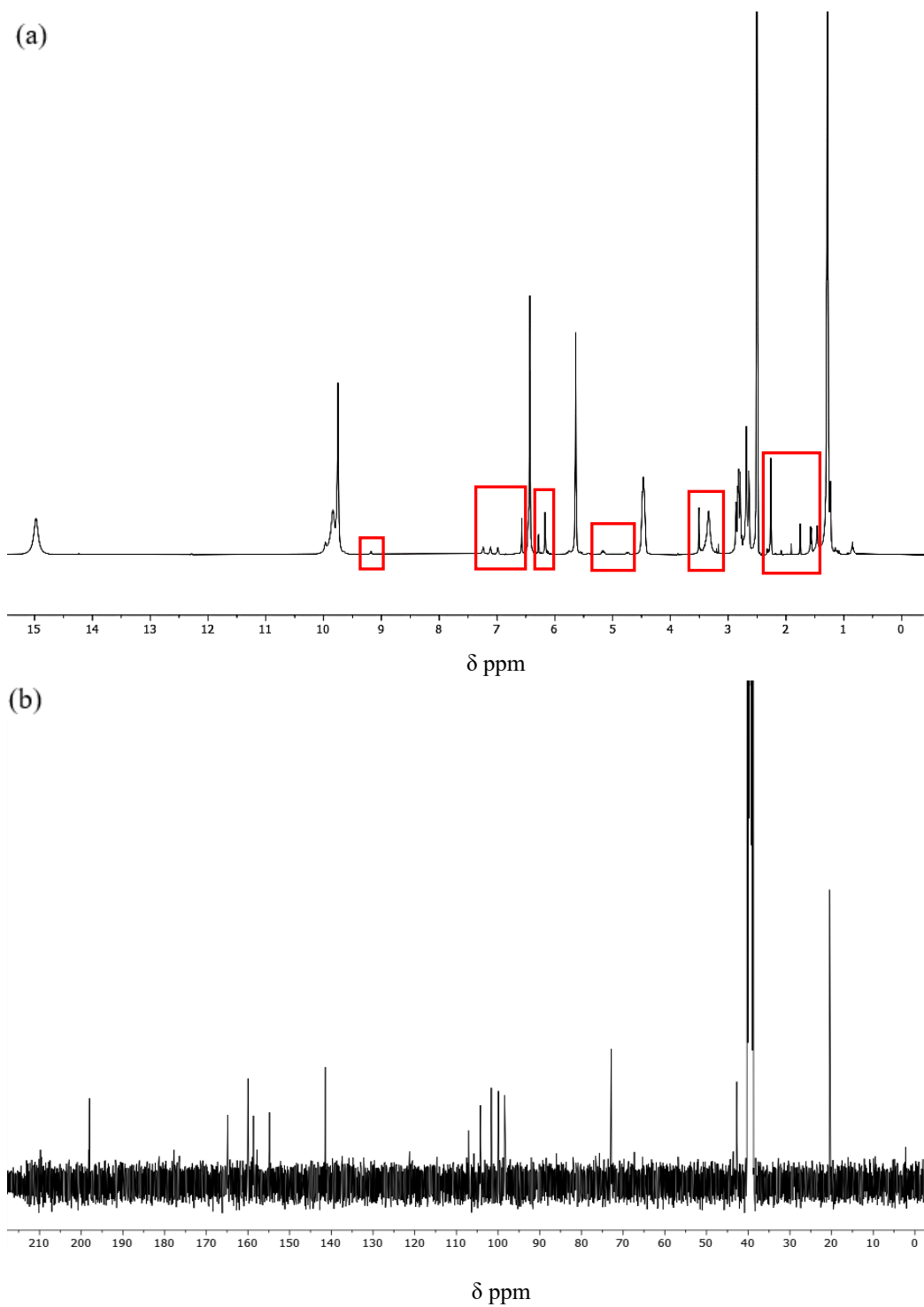


Figure 4. (a) ^1H NMR spectrum (400 MHz, DMSO- d_6) of CN2-025-02 and (b) ^{13}C NMR spectrum (400 MHz, DMSO- d_6) of CN2-025-02. Peaks in red boxes indicate proton resonances of low intensity, meaning there are impurities present in the sample.

Various attempts were made to purify CN2-02-025-02 using HPLC. First, the sample was injected into a reverse-phase column with a more polar solvent system (isocratic, 85 H₂O: 15 CH₃CN), as an increased polarity of the mobile phase in RP-HPLC will generally slow down the elution of the compounds in the sample, increasing their separation. However, this yielded a trace very similar to that produced by the original HPLC conditions used when injecting CN2-019-07 (50 H₂O: 50 CH₃CN), meaning it would not suffice to isolate the constituents of the sample.

Next, two isocratic injections were attempted using the reversed-phase column and H₂O and MeOH as the solvents (50 H₂O: 50 MeOH and 85 H₂O: 15 MeOH). By replacing CH₃CN with a more polar solvent (i.e., MeOH), the polarity of the solvent system is thereby increased, which may alter the interactions of the compounds in the sample with the column, changing their elution times. Both injections, however, produced traces with insufficient separation of peaks.

Injections were then attempted on the reversed-phase column using H₂O and CH₃CN or MeOH with 0.05% trifluoroacetic acid (TFA). The addition of TFA to the solvent systems functions to protonate any basic and acidic functional groups of the compounds in the sample, which may alter their interactions with the column. However, these injections yielded similar results. Reversed-phase gradient injections of varying durations were then attempted (90 H₂O: 10 CH₃CN to 100% CH₃CN [linear slope] over 25 minutes, 40 minutes, and 65 minutes), as gradient solvent systems can be useful in isolating compounds that do not demonstrate sufficient separation in isocratic conditions, but insufficient traces were produced once again.

Lastly, injections of CN2-025-02 were attempted on a normal-phase column using EtOAc and Hex as the solvents (50 EtOAc: 50 Hex and 25 EtOAc: 75 Hex). Normal-phase HPLC, unlike reversed-phase HPLC, employs a polar chromatography column and a non-polar solvent system, and therefore can be successful in isolating compounds that cannot be isolated using reversed-phase methods. However, the traces from these injections again demonstrated poor peak separation.

An acetylation reaction was then attempted on a portion (10 mg) of CN2-025-02 as a last resort to isolate the compounds in the sample. The ^1H NMR spectrum of CN2-025-02 displayed proton resonances (δ_{H} 9.71 and 9.88 ppm) characteristic of de-shielded hydroxyl groups (e.g., phenolic hydroxyl groups; Figure 4a). Reacting the hydroxyl groups with acetic anhydride would form acetate groups. These acetate groups were hypothesized to change the polarity of the compound(s) containing the hydroxyl groups and/or replace chromatographically labile protons, altering how the compounds interact with the chromatography column. These changes would in turn allow for the chromatographic isolation of the compounds in the sample. The acetylated sample was injected into the HPLC at the same conditions (isocratic, 50 CH_3CN : 50 H_2O) as the non-acetylated starting material, and the stark difference in the chromatographic separation of the two samples can be observed in Figure 5.

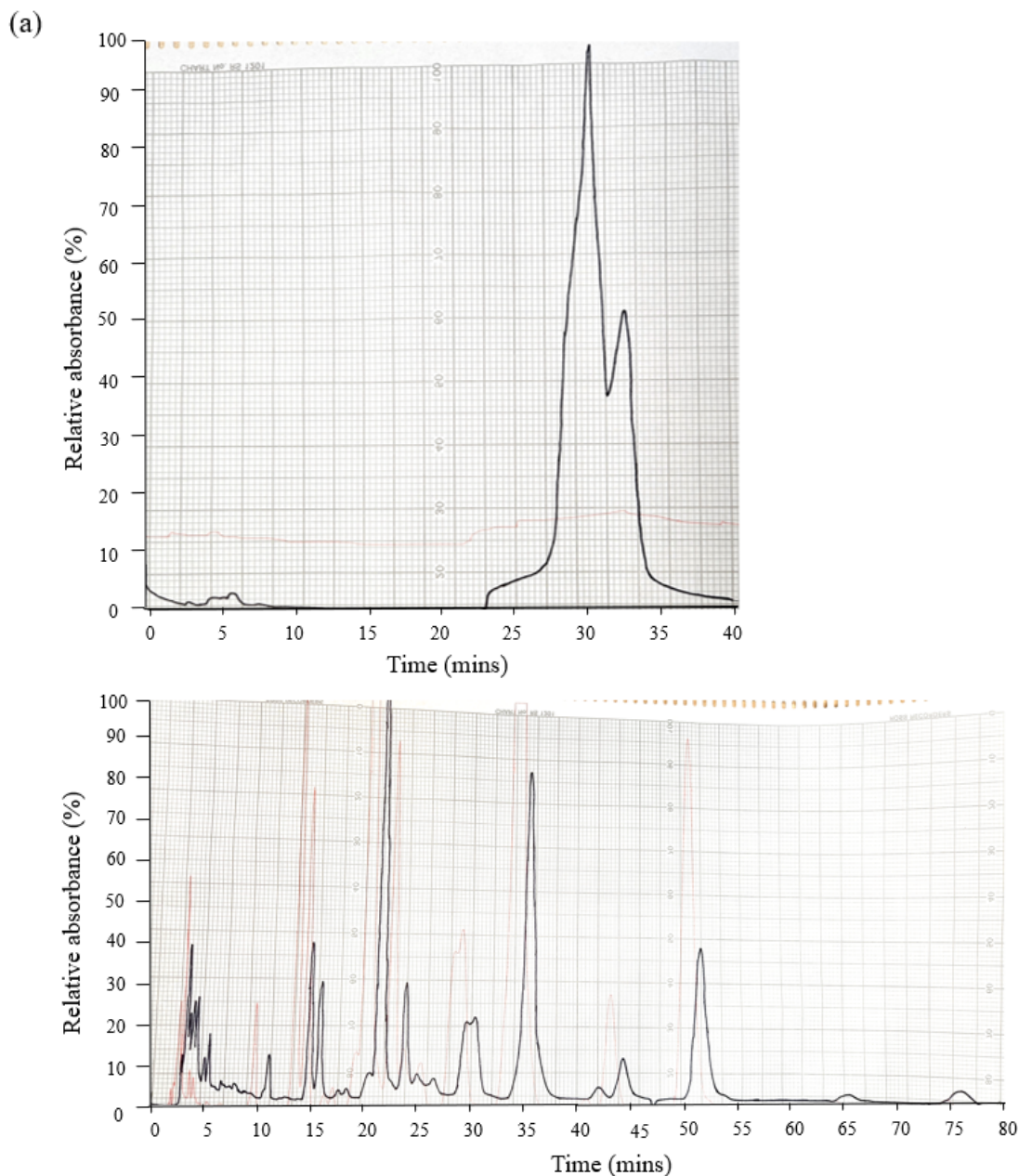


Figure 5. Reversed-phase high performance liquid chromatography trace of (a) CN2-025-02 pre-acetylation, and (b) CN2-025-02 post-acetylation. The peaks overlapping in the 23-to-35-minute range of (a) indicated a poor separation of the sample, meaning the compounds in the sample could not be isolated chromatographically. Following acetylation of the sample, the peaks demonstrated far greater separation (b) than observed in (a), suggesting that acetylation of the impure sample allowed for the successful chromatographic isolation of its constituents. Solvent system for both injections was 50 CH₃CN: 50 H₂O with an elution rate of 4 mL/min. Ultraviolet absorption was measured at 190 nm (traced in black) and 254 nm (traced in red).

The peaks in Figure 5b were collected and concentrated *in vacuo*, giving sixteen fractions. Fraction 9 (CN2-037-09; 1 mg) and fraction 12 (CN2-037-12; 1 mg) contained the most material and exhibited ^1H NMR spectra with similar proton resonances to that of CN2-025-02 (CN2-025-02: δ_{H} 1.28, 2.67, 2.82, 4.45, 5.63, 6.43 ppm; CN2-037-09: δ_{H} 1.31, 3.51, 4.59, 6.31, and 7.37 ppm; CN2-037-12: δ_{H} 1.31, 2.70, 2.95, 3.50, 4.61, 6.36, and 7.34 ppm; Figure 6). However, the hydroxyl resonances apparent in the ^1H NMR spectrum of CN2-025-02 (δ_{H} 9.71 and 9.88 ppm) are notably absent in the ^1H NMR spectra of CN2-037-09 and CN2-037-12 (Figure 6b, 6c). There are also two additional proton resonances present in the spectra of CN2-037-09 and CN2-037-12 (CN2-037-09: δ_{H} 1.93 and 2.45 ppm; CN2-037-12: 1.92 and 2.45 ppm) that are characteristic of acetyl methyl groups (Figure 6b, 6c). This suggests that the hydroxyl groups of the compounds in CN2-025-02 were successfully acetylated. Moreover, the presence of similar proton resonances between 0 ppm and 5 ppm in the spectra of CN2-037-09 and CN2-037-12 suggest that these are two compounds with similar chemical structures. In other words, CN2-037-09 and CN2-037-12 are likely two natural products derived from the same metabolic precursor. This makes sense considering both compounds eluted at the same time in the HPLC of CN2-025-02 ($t = 21$ mins, Figure 5a), and were not able to be separated chromatographically through the other various HPLC methods attempted. Compounds with very similar chemical structures can have nearly identical chromatographic properties, meaning they cannot be isolated chromatographically. By acetylating both compounds, the changes in their chemical structures generated a change in their polarities and/or possession of chromatographically labile protons that then allowed for their chromatographic isolation by HPLC.

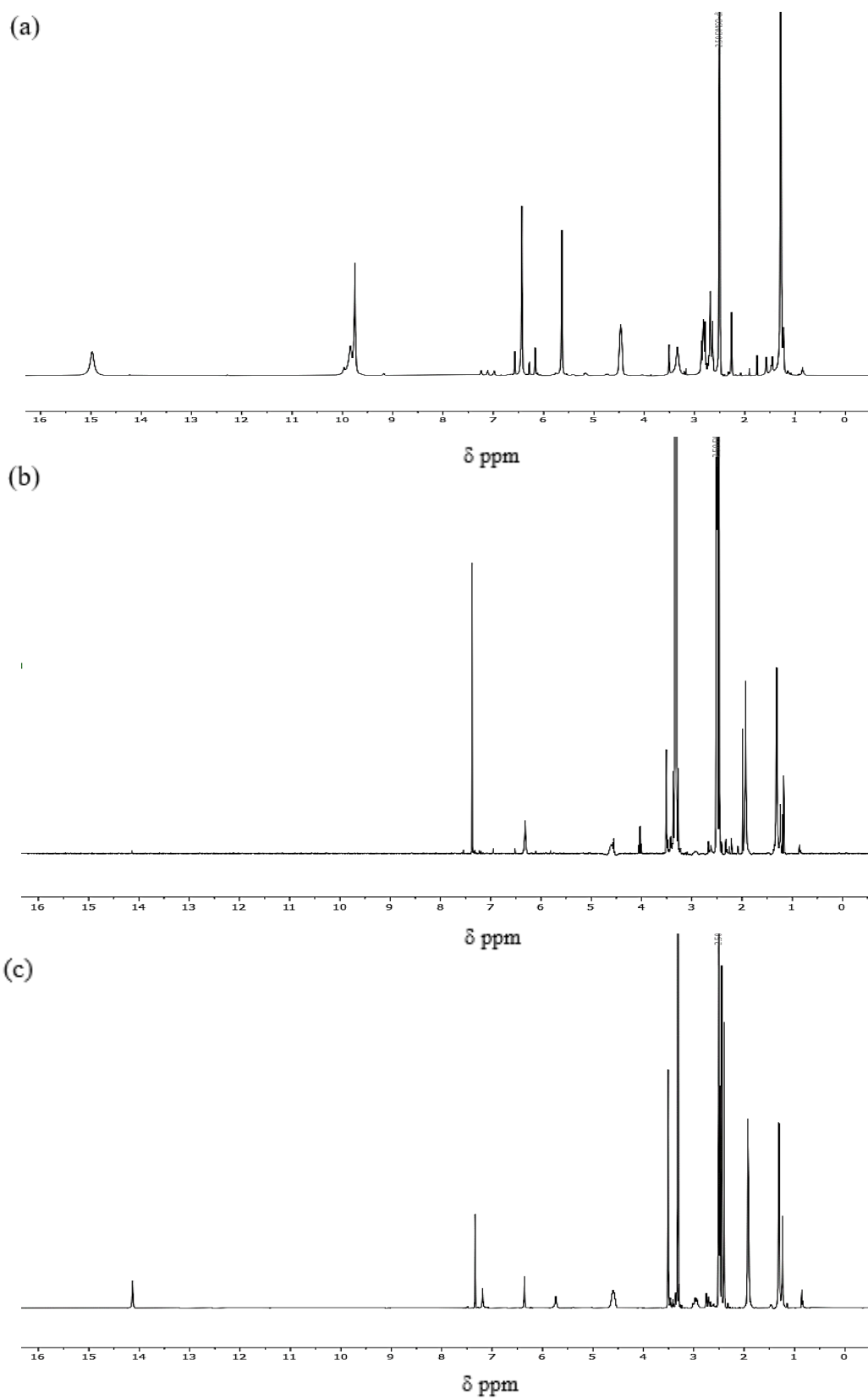


Figure 6. ^1H NMR spectrum (400 MHz, DMSO- d_6) of (a) CN2-025-02 (pre-acetylation sample), (b) CN2-037-09 (post-acetylation fraction), and (c) CN2-037-12 (post-acetylation fraction).

The sixteen fractions generated by the HPLC of the acetylated sample were tested against *S. aureus* in immersion bioautography (see Appendix A, Figure A3). The first fraction (CN2-037-01; 0.2 mg) was the only fraction to produce a zone of inhibition (Figure A3). The hydroxyl groups of the primary constituents of CN2-025-02 may have been part of the pharmacophore that caused the bioactivity against *S. aureus*, in which case acetylating these groups would have changed the chemical structure of the pharmacophore, possibly rendering the compound(s) inactive against *S. aureus*; this would explain why CN2-037-09 and CN2-037-12 did not demonstrate bioactivity against *S. aureus* (Figure A3). The acetylation reaction may have also generated side products and/or introduced leftover reagents into the sample that are bioactive against *S. aureus*, which would explain the bioactivity demonstrated by CN2-037-01 (Figure A3).

Alternatively, the bioactivity of CN2-025-02 against *S. aureus* may not have been caused by the primary constituents of the sample (i.e., the non-acetylated structures of CN2-037-09 and CN2-037-12), but rather one of the more scarce constituents responsible for the impurity peaks seen in Figure 6a. Acetylating the sample allowed for the chromatographic separation of the scarce constituents of CN2-025-02 from the primary constituents, thus isolating the bioactive component from the non-bioactive components. However, the chemical composition of CN2-037-01 was not possible to determine using NMR spectroscopy due to the sub-milligram quantity of the sample; further acetylation of CN2-025-02 followed by HPLC is required to determine the chemical structure of CN2-037-01.

Conclusion

Immersion bioautography is a bioassay technique that is typically used as a quick and simple alternative to microplate bioassays when screening complex natural product extracts for the presence of bioactive compounds (Dewanjee *et al.*, 2015; Choma & Grzelak, 2011). The use of TLC-immersion bioautography as the guide for the isolation of natural products in this project led to the purification of two putatively new natural products, as well as a compound bioactive against *S. aureus*. This research demonstrates that bioautography techniques, though historically less common than microplate bioassay techniques (Sun *et al.*, 2018), can facilitate an efficient fractionation of bioactive natural products. Furthermore, while several studies using TLC-bioautography to screen their extracts have been met with success in detecting the presence of bioactive natural products (Sun *et al.*, 2018; Grzelak *et al.*, 2016; Jesionek *et al.*, 2015; Móricz *et al.*, 2017; Legerská *et al.*, 2022; Móricz *et al.*, 2012; Galindo-Cuspinera & Rankin, 2005), this project is among the first to use TLC immersion bioautography to inform each purification step of natural product isolation (Xu *et al.*, 2024).

Immersion bioautography also proved to be a rapid and effective means of analyzing the bioactivity profiles of the acetylation reaction products. Though commonly used in natural product isolation, the use of TLC-bioautography in synthetic chemistry has only been reported once (Yüce & Morlock, 2021). The successful use of TLC-bioautography to analyze reaction results in this project is indicative of the pivotal role that TLC-bioautography could play in synthetic drug development.

Future work following this project should focus on: elucidating the structures of CN2-037-09 and CN2-037-12 using two-dimensional (2D) NMR spectroscopy and

HRESIMS; elucidating the structure of CN2-037-01 using 2D NMR spectroscopy and HRESIMS to help inform the source of the bioactivity demonstrated by CN2-025-02; and developing a reaction method to hydrolyze the acetyl groups of CN2-037-09 and CN2-037-12 and screening their bioactivities against *S. aureus*.

References

- Adedeji, W. A. (2016). The treasure called antibiotics. *Annals of Ibadan Postgraduate Medicine*, 14(2), 56–57.
- Aslam, B., Wang, W., Arshad, M. I., Khurshid, M., Muzammil, S., Rasool, M. H., Nisar, M. A., Alvi, R. F., Aslam, M. A., Qamar, M. U., Salamat, M. K. F., & Baloch, Z. (2018). Antibiotic resistance: A rundown of a global crisis. *Infection and Drug Resistance*, 11, 1645–1658. <https://doi.org/10.2147/IDR.S173867>
- Barone, J. J., & Roberts, H. R. (1996). Caffeine consumption. *Food and Chemical Toxicology*, 34(1), 119–129. [https://doi.org/10.1016/0278-6915\(95\)00093-3](https://doi.org/10.1016/0278-6915(95)00093-3)
- Belongia, E. A., & Schwartz, B. (1998). Strategies for promoting judicious use of antibiotics by doctors and patients. *BMJ*, 317(7159), 668–671. <https://doi.org/10.1136/bmj.317.7159.668>
- Beutler, J. A. (2009). Natural Products as a Foundation for Drug Discovery. *Current Protocols in Pharmacology / Editorial Board, S.J. Enna (Editor-in-Chief) ... [et Al.]*, 46, 9.11.1-9.11.21. <https://doi.org/10.1002/0471141755.ph0911s46>
- Bhardwaj, S., Abbas, N. S., Kaula, B. C., & Prakash, A. Diversity of fungal endophytes at different maturity levels of *Cryptolepis buchanani* leaves. *Current Botany* 2023, 14, 24-31.
- Borchardt, J.K. (1996). A short history of quinine. *Drug News & Persp*, 9, 116–120.
- Cantrell, C. L., Dayan, F. E., & Duke, S. O. (2012). Natural Products As Sources for New Pesticides. *Journal of Natural Products*, 75(6), 1231–1242. <https://doi.org/10.1021/np300024u>

- Che, Y.-S. (2011). Discovery of new bioactive natural products from fungi of unique ecological niches. *Journal of International Pharmaceutical Research*, 12–27.
- Choma, I. M., & Grzelak, E. M. (2011). Bioautography detection in thin-layer chromatography. *Journal of Chromatography A*, 1218(19), 2684–2691.
<https://doi.org/10.1016/j.chroma.2010.12.069>
- Clark, T. N. (2019). The application of NMR and LC-HRMS based prioritization strategies for the discovery of natural products by endophytic fungi from medicinal plants. <https://unbscholar.lib.unb.ca/handle/1882/13531>
- Cox, S. (2016). Sea foam as a source of fungi for natural product discovery.
- Debbab, A., Aly, A. H., & Proksch, P. (2011). Bioactive secondary metabolites from endophytes and associated marine derived fungi. *Fungal Diversity*, 49(1), 1–12.
<https://doi.org/10.1007/s13225-011-0114-0>
- Dewanjee, S., Gangopadhyay, M., Bhattacharya, N., Khanra, R., & Dua, T. K. (2015). Bioautography and its scope in the field of natural product chemistry. *Journal of Pharmaceutical Analysis*, 5(2), 75–84. <https://doi.org/10.1016/j.jpha.2014.06.002>
- Ellsworth, K. T., Clark, T. N., Gray, C. A., & Johnson, J. A. (2013). Isolation and bioassay screening of medicinal plant endophytes from eastern Canada. *Canadian Journal of Microbiology*, 59(11), 761–765. <https://doi.org/10.1139/cjm-2013-0639>
- Fernie, A. R., & Pichersky, E. (2015). Focus Issue on Metabolism: Metabolites, Metabolites Everywhere. *Plant Physiology*, 169(3), 1421–1423.
<https://doi.org/10.1104/pp.15.01499>

- Flewelling, A. J., Bishop, A. L., Johnson, J. A., & Gray, C. A. (2015). Polyketides from an Endophytic *Aspergillus fumigatus* Isolate Inhibit the Growth of *Mycobacterium tuberculosis* and MRSA. *Natural Product Communications*, 10(10), 1934578X1501001009. <https://doi.org/10.1177/1934578X1501001009>
- Galindo-Cuspinera, V., & Rankin, S. A. (2005). Bioautography and Chemical Characterization of Antimicrobial Compound(s) in Commercial Water-Soluble Annatto Extracts. *Journal of Agricultural and Food Chemistry*, 53(7), 2524–2529. <https://doi.org/10.1021/jf048056q>
- Grzelak, E. M., Hwang, C., Cai, G., Nam, J.-W., Choules, M. P., Gao, W., Lankin, D. C., McAlpine, J. B., Mulugeta, S. G., Napolitano, J. G., Suh, J.-W., Yang, S. H., Cheng, J., Lee, H., Kim, J.-Y., Cho, S.-H., Pauli, G. F., Franzblau, S. G., & Jaki, B. U. (2016). Bioautography with TLC-MS/NMR for Rapid Discovery of Anti-tuberculosis Lead Compounds from Natural Sources. *ACS Infectious Diseases*, 2(4), 294–301. <https://doi.org/10.1021/acsinfecdis.5b00150>
- Hersh, A. L., Jackson, M. A., Hicks, L. A., & American Academy of Pediatrics Committee on Infectious Diseases. (2013). Principles of judicious antibiotic prescribing for upper respiratory tract infections in pediatrics. *Pediatrics*, 132(6), 1146–1154. <https://doi.org/10.1542/peds.2013-3260>
- Hicks, L. A., Bartoces, M. G., Roberts, R. M., Suda, K. J., Hunkler, R. J., Taylor, T. H. J., & Schrag, S. J. (2015). US outpatient antibiotic prescribing variation according to geography, patient population, and provider specialty in 2011. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, 60(9), 1308–1316. <https://doi.org/10.1093/cid/civ076>

- Hyde, K.D., & Jones, E. B. G. (1989). Ecological observations on marine fungi from the Seychelles. *Botanical Journal of the Linnean Society*, 100(3), 237–254.
<https://doi.org/10.1111/j.1095-8339.1989.tb01720.x>
- Jesionek, W., Móricz, Á. M., Alberti, Á., Ott, P. G., Kocsis, B., Horváth, G., & Choma, I. M. (2015). TLC-Direct Bioautography as a Bioassay Guided Method for Investigation of Antibacterial Compounds in *Hypericum perforatum* L. *Journal of AOAC INTERNATIONAL*, 98(4), 1013–1020. <https://doi.org/10.5740/jaoacint.14-233>
- Kirk, P. W. (1983). Direct Enumeration of Marine Arenicolous Fungi. *Mycologia*, 75(4), 670–682. <https://doi.org/10.1080/00275514.1983.12023736>
- Koehn, R. D. (1982). Fungi Isolated from Sea Foam Collected at North Padre Island Beaches. *The Southwestern Naturalist*, 27(1), 17–21.
<https://doi.org/10.2307/3671402>
- Landers, T., & Kavanagh, K. T. (2016). Is the Presidential Advisory Council on Combating Antibiotic Resistance missing opportunities? *American Journal of Infection Control*, 44(11), 1356–1359. <https://doi.org/10.1016/j.ajic.2016.07.008>
- Leelananda, S. P., & Lindert, S. (2016). Computational methods in drug discovery. *Beilstein Journal of Organic Chemistry*, 12(1), 2694–2718.
<https://doi.org/10.3762/bjoc.12.267>
- Legerská, B., Chmelová, D., Ondrejovič, M., & Miertuš, S. (2022). The TLC-Bioautography as a Tool for Rapid Enzyme Inhibitors detection—A Review.

Critical Reviews in Analytical Chemistry, 52(2), 275–293.

<https://doi.org/10.1080/10408347.2020.1797467>

Ligon, B. L. (2004). Penicillin: Its discovery and early development. *Seminars in Pediatric Infectious Diseases*, 15(1), 52–57.

<https://doi.org/10.1053/j.spid.2004.02.001>

Liu, R., Li, X., & Lam, K. S. (2017). Combinatorial chemistry in drug discovery. *Current Opinion in Chemical Biology*, 38, 117–126.

<https://doi.org/10.1016/j.cbpa.2017.03.017>

Lobanovska, M., & Pilla, G. (2017). Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future? *The Yale Journal of Biology and Medicine*, 90(1), 135–145.

Lockermann, G. (1951). Friedrich Wilhelm Serturmer, the discoverer of morphine.

Journal of Chemical Education, 28(5), 277. <https://doi.org/10.1021/ed028p277>

Lowy, F. D. (2003). Antimicrobial resistance: The example of *Staphylococcus aureus*. *The Journal of Clinical Investigation*, 111(9), 1265–1273.

<https://doi.org/10.1172/JCI18535>

Luepke, K. H., & Mohr, J. F. (2017). The antibiotic pipeline: Reviving research and development and speeding drugs to market. *Expert Review of Anti-Infective Therapy*, 15(5), 425–433. <https://doi.org/10.1080/14787210.2017.1308251>

Luo, X., Zhou, X., Lin, X., Qin, X., Zhang, T., Wang, J., Tu, Z., Yang, B., Liao, S., Tian, Y., Pang, X., Kaliyaperumal, K., Li, J. L., Tao, H., & Liu, Y. (2017). Antituberculosis compounds from a deep-sea-derived fungus *Aspergillus* sp.

SCSIO Ind09F01. *Natural Product Research*, 31(16), 1958–1962.

<https://doi.org/10.1080/14786419.2016.1266353>

Marek, C. L., & Timmons, S. R. (2019). 9—Antimicrobials in Pediatric Dentistry. In A. J. Nowak, J. R. Christensen, T. R. Mabry, J. A. Townsend, & M. H. Wells (Eds.), *Pediatric Dentistry (Sixth Edition)* (pp. 128-141.e1). Elsevier.

<https://doi.org/10.1016/B978-0-323-60826-8.00009-2>

Mitea, G., Schröder, V., Iancu, I. M., Iancu, V., & Blebea, N. M. (2023). Therapeutic strategies against antibiotic resistance. *Technium BioChemMed*, 7(1), 118–126.

Morehouse, N. J., Clark, T. N., Kerr, R. G., Johnson, J. A., & Gray, C. A. (2023c).

Caryophyllene Sesquiterpenes from a *Chaetomium globosum* Endophyte of the Canadian Medicinal Plant *Empetrum nigrum*. *Journal of Natural Products*, 86(6), 1615–1619. <https://doi.org/10.1021/acs.jnatprod.2c01159>

Morehouse, N. J., Flewelling, A. J., Johnson, J. A., & Gray, C. A. (2020). Halogenated Bianthrone From *Penicillium roseopurpureum*: A Fungal Endophyte of the Marine Alga *Petalonia fascia*. *Natural Product Communications*, 15(1), 1934578X20901405. <https://doi.org/10.1177/1934578X20901405>

Morehouse, N. J., Flewelling, A. J., Liu, D. Y., Cavanagh, H., Linington, R. G., Johnson, J. A., & Gray, C. A. (2023a). Tolypocaiibols: Antibacterial Lipopeptaibols from a *Tolypocladium* sp. Endophyte of the Marine Macroalga *Spongomorpha arcta*. *Journal of Natural Products*, 86(6), 1529–1535.

<https://doi.org/10.1021/acs.jnatprod.3c00233>

- Morehouse, N. J., Graham, K. M., Cox, S. L., Johnson, J. A., & Gray, C. A. (2023d). Isolation of an antimicrobial racemic phenalenone derivative from a marine-derived *Penicillium* sp. Fungus. *Canadian Journal of Chemistry*.
<https://doi.org/10.1139/cjc-2023-0100>
- Morehouse, N. J., Johnson, J. A., & Gray, C. A. (2023b). Aureobasidols A and B: New C11-Polyketides From an Endophytic *Aureobasidium pullulans* Isolate. *Natural Product Communications*, 18(10), 1934578X231210086.
<https://doi.org/10.1177/1934578X231210086>
- Móricz, Á. M., & Ott, P. G. (2017). Screening and Characterization of Antimicrobial Components of Natural Products Using Planar Chromatography Coupled with Direct Bioautography, Spectroscopy and Mass Spectrometry: A Review. *Current Organic Chemistry*, 21(18), 1861–1874.
<https://doi.org/10.2174/1385272821666170127154900>
- Móricz, Á. M., Ott, P. G., Böszörményi, A., Lemberkovics, É., Mincsovcics, E., & Tyihák, E. (2012). Bioassay-Guided Isolation and Identification of Antimicrobial Compounds from Thyme Essential Oil by Means of Overpressured Layer Chromatography, Bioautography and GC–MS. *Chromatographia*, 75(17), 991–999. <https://doi.org/10.1007/s10337-012-2233-5>
- Nakagiri, A. (1989). Marine fungi in sea foam from Japanese coast. *Research communications*, 14, 52-79.
- Newman, D. J., & Cragg, G. M. (2020). Natural Products as Sources of New Drugs over the Nearly Four Decades from 01/1981 to 09/2019. *Journal of Natural Products*, 83(3), 770–803. <https://doi.org/10.1021/acs.jnatprod.9b01285>

- Nyquist, A.-C., Gonzales, R., Steiner, J. F., & Sande, M. A. (1998). Antibiotic Prescribing for Children With Colds, Upper Respiratory Tract Infections, and Bronchitis. *JAMA*, 279(11), 875–877. <https://doi.org/10.1001/jama.279.11.875>
- Oppong-Danquah, E., Passaretti, C., Chianese, O., Blümel, M., & Tasdemir, D. (2020). Mining the Metabolome and the Agricultural and Pharmaceutical Potential of Sea Foam-Derived Fungi. *Marine Drugs*, 18(2), Article 2. <https://doi.org/10.3390/md18020128>
- Overy, D. P., Berrue, F., Correa, H., Hanif, N., Hay, K., Lanteigne, M., Mquilian, K., Duffy, S., Boland, P., Jagannathan, R., Carr, G. S., Vansteeland, M., & Kerr, R. G. (2014). Sea foam as a source of fungal inoculum for the isolation of biologically active natural products. *Mycology*, 5(3), 130–144. <https://doi.org/10.1080/21501203.2014.931893>
- Pavarini, D. P., Pavarini, S. P., Niehues, M., & Lopes, N. P. (2012). Exogenous influences on plant secondary metabolite levels. *Animal Feed Science and Technology*, 176(1), 5–16. <https://doi.org/10.1016/j.anifeedsci.2012.07.002>
- Pendergrast, M. (2009). Coffee: Second to oil? *Tea Coffee Trade J*, 181, 38–41.
- Podolsky, S. H. (2018). The evolving response to antibiotic resistance (1945–2018). *Palgrave Communications*, 4(1), Article 1. <https://doi.org/10.1057/s41599-018-0181-x>
- Rammelkamp, C.H., & Maxon, T. (1942). Resistance of Staphylococcus aureus to the Action of Penicillin. *Proceedings of the Society for Experimental Biology and Medicine*, 1942;51(3):386-389. doi:10.3181/00379727-51-13986

- Sanchez, L. M., Wong, W. R., Riener, R. M., Schulze, C. J., & Linington, R. G. (2012). Examining the Fish Microbiome: Vertebrate-Derived Bacteria as an Environmental Niche for the Discovery of Unique Marine Natural Products. *PLOS ONE*, 7(5), e35398. <https://doi.org/10.1371/journal.pone.0035398>
- Sasso, S., Pohnert, G., Lohr, M., Mittag, M., & Hertweck, C. (2012). Microalgae in the postgenomic era: A blooming reservoir for new natural products. *FEMS Microbiology Reviews*, 36(4), 761–785. <https://doi.org/10.1111/j.1574-6976.2011.00304.x>
- S. Bugni, T., & M. Ireland, C. (2004). Marine-derived fungi: A chemically and biologically diverse group of microorganisms. *Natural Product Reports*, 21(1), 143–163. <https://doi.org/10.1039/B301926H>
- Shallcross, L. J., Howard, S. J., Fowler, T., & Davies, S. C. (2015). Tackling the threat of antimicrobial resistance: From policy to sustainable action. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 370(1670), 20140082. <https://doi.org/10.1098/rstb.2014.0082>
- Shang, Z., Li, X.-M., Li, C.-S., & Wang, B.-G. (2012). Diverse Secondary Metabolites Produced by Marine-Derived Fungus *Nigrospora* sp. MA75 on Various Culture Media. *Chemistry & Biodiversity*, 9(7), 1338–1348. <https://doi.org/10.1002/cbdv.201100216>
- Schwartz, B., Barden, L., Dowell, S., & Lackey, C. (1998). Why doctors over prescribe antibiotics: results of focus group discussions with pediatricians (Ped) and family physicians (FP) and application to a resistance prevention campaign (abstract).

Presented at the International Conference on Emerging Infectious Diseases, Atlanta. Atlanta, GA:Centers for Disease Control and Prevention, 1998:66.

Stadler, M., & Hoffmeister, D. (2015). Fungal natural products—The mushroom perspective. *Frontiers in Microbiology*, 6.

<https://www.frontiersin.org/articles/10.3389/fmicb.2015.00127>

Stone, J. K., Polishook, J. D., & White, J. F. (2004). Endophytic fungi. *Biodiversity of fungi: inventory and monitoring methods*, 241, 270.

Sun, Z.-L., Liu, T., Wang, S.-Y., Ji, X.-Y., & Mu, Q. (2019). TLC-bioautography directed isolation of antibacterial compounds from active fractionation of *Ferula feruloides*. *Natural Product Research*, 33(12), 1761–1764.

<https://doi.org/10.1080/14786419.2018.1431640>

Swift, C. L., Louie, K. B., Bowen, B. P., Olson, H. M., Purvine, S. O., Salamov, A., Mondo, S. J., Solomon, K. V., Wright, A. T., Northen, T. R., Grigoriev, I. V., Keller, N. P., & O'Malley, M. A. (2021). Anaerobic gut fungi are an untapped reservoir of natural products. *Proceedings of the National Academy of Sciences*, 118(18), e2019855118. <https://doi.org/10.1073/pnas.2019855118>

van Santen, J. A., Poynton, E. F., Iskakova, D., McMann, E., Alsup, T. A., Clark, T. N., Fergusson, C. H., Fewer, D. P., Hughes, A. H., McCadden, C. A., Parra, J., Soldatou, S., Rudolf, J. D., Janssen, E. M.-L., Duncan, K. R., & Linington, R. G. (2022). The Natural Products Atlas 2.0: A database of microbially-derived natural

products. *Nucleic Acids Research*, 50(D1), D1317–D1323.

<https://doi.org/10.1093/nar/gkab941>

Vaz, L. E., Kleinman, K. P., Raebel, M. A., Nordin, J. D., Lakoma, M. D., Dutta-Linn, M. M., & Finkelstein, J. A. (2014). Recent Trends in Outpatient Antibiotic Use in Children. *Pediatrics*, 133(3), 375–385. <https://doi.org/10.1542/peds.2013-2903>

Xu, W., Shi, D., Chen, K., & Popovich, D. G. (2024). TLC-Bioautography-Guided Isolation and Assessment of Antibacterial Compounds from Manuka (*Leptospermum scoparium*) Leaf and Branch Extracts. *Molecules*, 29(3), Article 3. <https://doi.org/10.3390/molecules29030717>

Yüce, I., & Morlock, G. E. (2021). Nanomole-scaled high-throughput chemistry plus direct bioautography on the same chromatography plate for drug discovery. *Analytica Chimica Acta*, 1182, 338950. <https://doi.org/10.1016/j.aca.2021.338950>

Appendix A: Immersion Bioautography Guided Fractionation

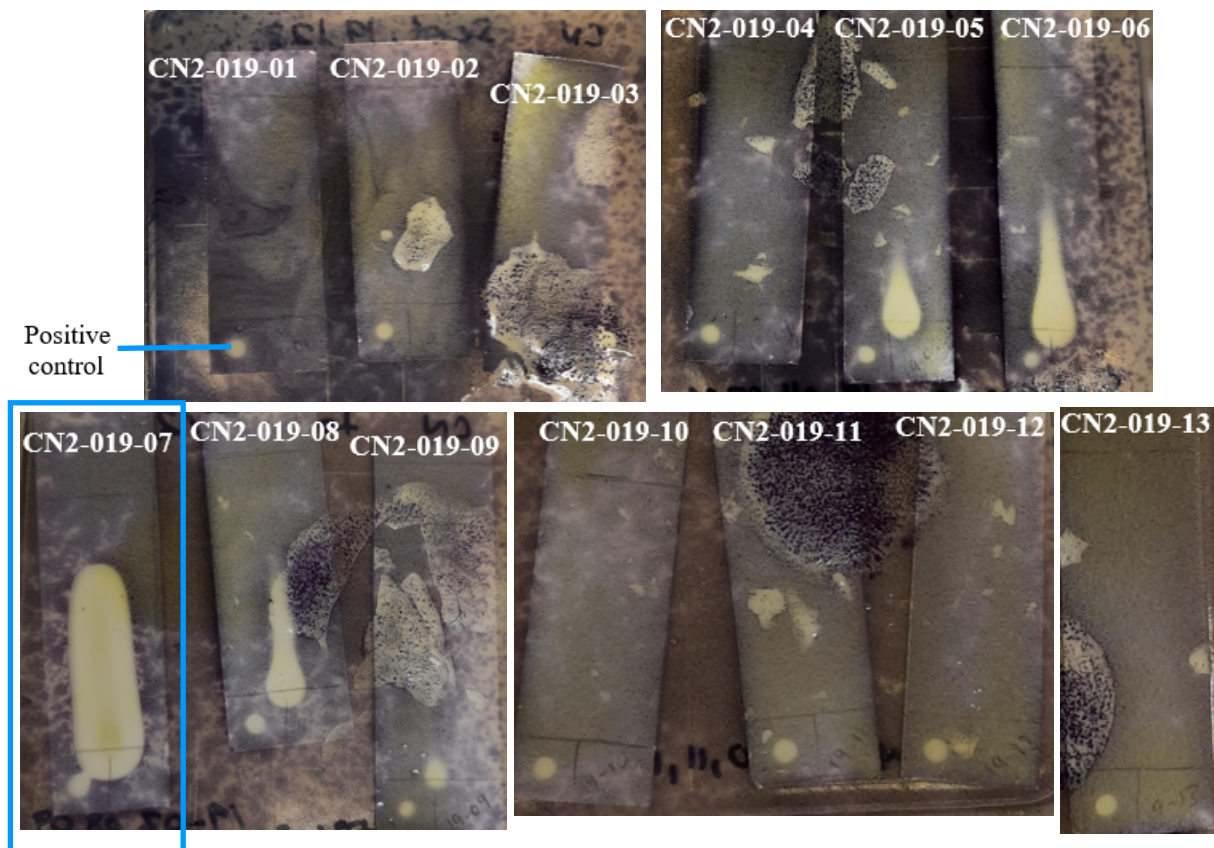


Figure A 1. Normal-phase thin-layer chromatography (TLC) immersion bioautography of the reversed-phase flash column fractions of the crude extract (CN2-001-01; 5 mg/mL) against *Staphylococcus aureus* (ATCC 29213) with positive control gentamicin (1.0 mg/mL). The seventh fraction (CN2-019-07; 60 CH₃CN: 40 H₂O) demonstrated the largest zone of inhibition of *S. aureus* (encircled).

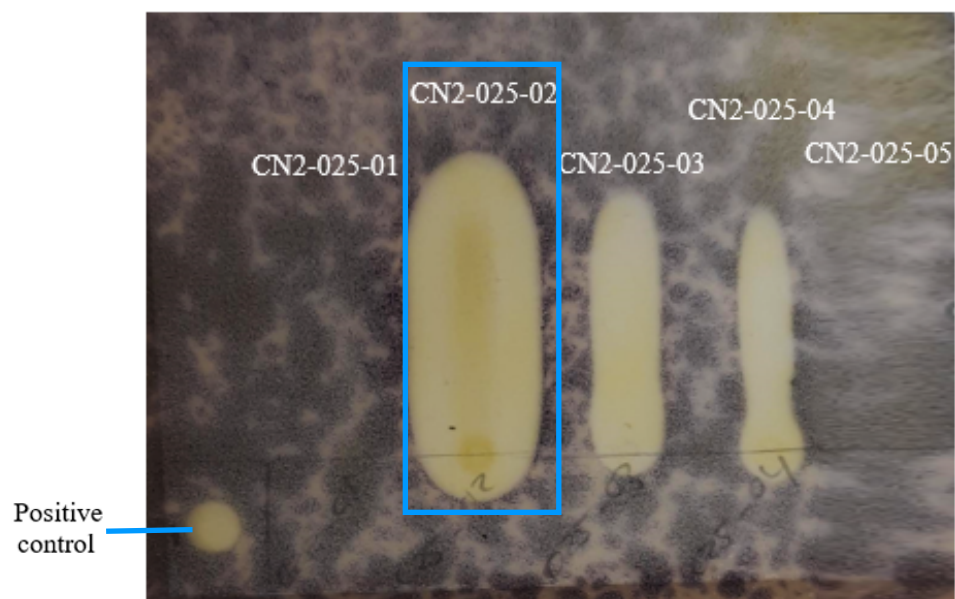


Figure A 2. Normal-phase thin-layer chromatography (TLC) immersion bioautography of the reversed-phase high performance liquid chromatography fractions of CN2-019-07 (5 mg/mL) against *Staphylococcus aureus* (ATCC 29213) with positive control gentamicin (1.0 mg/mL). The second fraction (CN2-025-02) demonstrated the largest zone of inhibition of *S. aureus* (encircled).

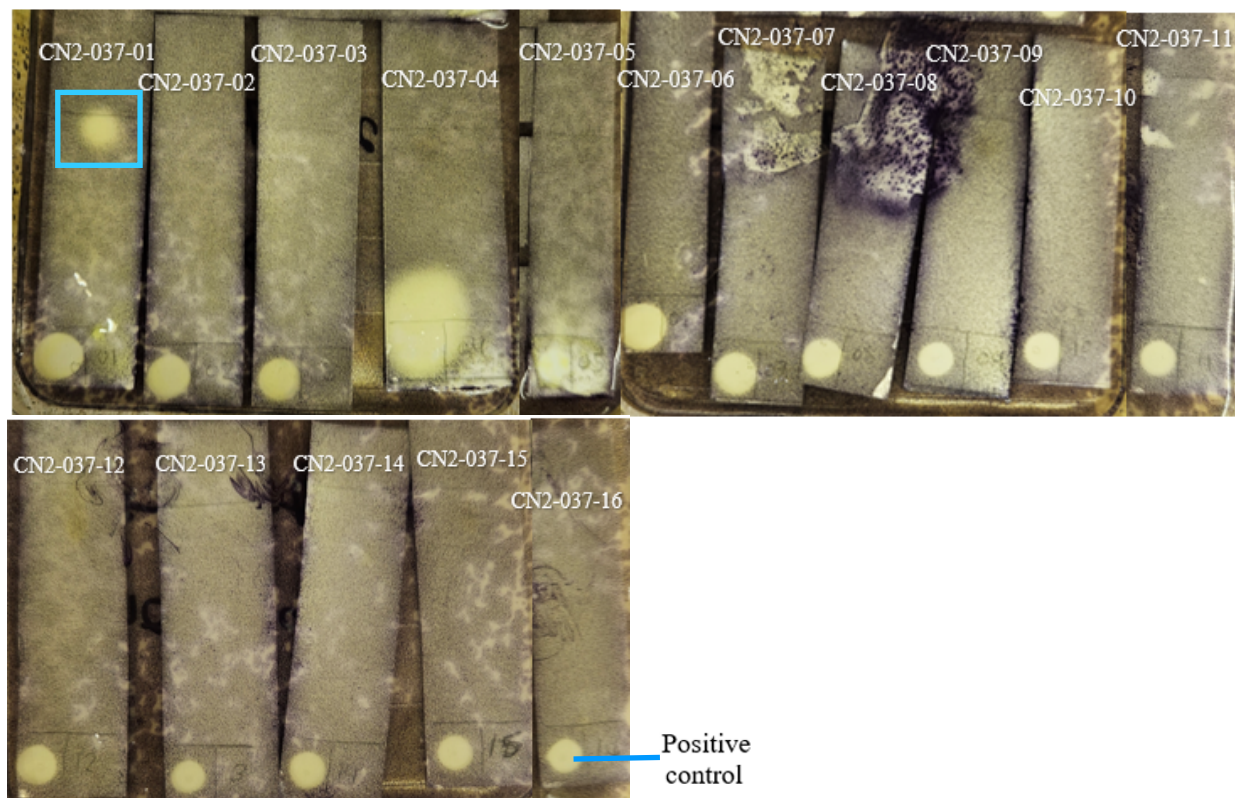


Figure A 3. Normal-phase thin-layer chromatography (TLC) immersion bioautography of the reversed-phase high performance liquid chromatography fractions of the acetylation reaction products of CN2-025-02 (5 mg/mL) against *Staphylococcus aureus* (ATCC 29213) with positive control gentamicin (1.0 mg/mL). The first fraction (CN2-037-01) demonstrated the only zone of inhibition of *S. aureus* (encircled).