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11-Chloro-Resistoflavin, An Active Antibiotic Against Methicillin-Resistant *Staphylococcus aureus*
(MRSA) Discovered from *Streptomyces* Collected on North Egyptian Coast

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Marine Biology

by

Zhifei Li

Committee in charge:

>Professor William Fenical, Chair
>Professor Bradley Moore
>Professor Maurizio Zanetti

2021

The thesis of Zhifei Li is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego
2021

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ABSTRACT OF THE THESIS

11-Chloro-resistoflavin, An Active Antibiotic Against Methicillin-Resistant *Staphylococcus aureus* (MRSA) Discovered from *Streptomyces* Collected on North Egyptian Coast

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Zhifei Li

Master of Science in Marine Biology

University of California San Diego, 2021

Professor William Fenical, Chair

Marine natural products play an important role in modern pharmaceutical studies; hundreds of antibacterial, antifungal and antitumor agents have been discovered from marine organisms such as bacteria, sponges, tunicates, etc. Our lab is collaborating with Egyptian scholars at Helwan University in Cairo to explore novel antibiotics produced by marine-derived *Streptomyce*s collected along the north Egyptian coast. In this thesis, a new chlorinated antibiotic that showed potent activities against Methicillin-Resistant *Staphylococcus aureus* (MRSA) is described. Analytical techniques including silica column chromatography, High Performance Liquid Chromatography (HPLC), Liquid

Chromatography-Mass Spectrometry (LCMS) and Nuclear Magnetic Resonance (NMR) were used to

elucidate the structure of the new molecule.

INTRODUCTION

Human beings have a long history of utilizing naturally available antibiotics in treating diseases and infections, but it was not until the nineteenth century that these compounds were purified. Since then, biologists and chemists started to explore therapeutic agents from microorganisms, animals and plants collected worldwide, marking the beginning of natural product chemistry.

The wide-scale use of antibiotics was accompanied by the growing resistance of pathogens such as multidrug-resistant bacteria that have posted severe threats on human health. Dealing with such challenges would require continuous discovery of novel antibiotics, which in turn requires new sources of natural products. One approach lies in exploring the ocean, where countless organisms await to be explored. Marine natural products have become a major and promising source of drug discovery in the pharmaceutical industry.

CHAPTER ONE

THE DEVELOPMENT OF ANTIBIOTICS: AN OVERVIEW

1.1 THE PRE-ANTIBIOTIC ERA

Selman A. Waksman defined the term "antibiotics" in the following way: "an antibiotic is a

chemical substance, produced by microorganisms, which has the capacity to inhibit the growth of and

even to destroy bacteria and other microorganisms" (Waksman, 1947). Clear evidence revealed the use of

antibiotics in ancient civilizations, where the naturally available antibiotic compounds were used to treat

infections (Gould, 2016). One example is the use of *Artemisia annua*, the sweet wormwood, in the

treatment of malaria by Chinese herbalists (Cui and Su, 2009). The extraction and purification of

Qinghaosu (artemisinin) from the leaves of *Artemisia annua* led to the 2015 Nobel Prize in Physiology or

Medicine for its potent activities against malaria. The discovery and herbological utilization of antibiotics

present in natural materials in the pre-antibiotic era were significant steps in enhancing human health.

Without sufficient knowledge and technologies in modern medical and pharmaceutical sciences, however,

these attempts were merely coarse practices and lacked potential for further development.

With the advancement in natural sciences in the 19th century, human beings were granted access

to purified antibiotic molecules and a more profound understanding of the biological nature of the

pathogenic bacteria. The modern antibiotic era was right around the corner.

1.2 THE MODERN ANTIBIOTIC ERA

Discussing the beginning of the modern antibiotic era cannot bypass two names: Paul Ehrlich and

Alexander Fleming. Ehrlich was a German physician, who, based on his observation that some synthetic

dyes, such as aniline, can dye some microbes but not others, proposed the concept of "magic bullet" that

illustrates the possibility of discovering certain compounds that target specifically on pathogenic microbes

while having minimal effects on host cells (Rustam, 2014). Motivated by his idea, Enrlich started a

large-scale screening program which eventually led to his discovery of salvarsan (dioxy-diamino-arsenobenzol-dihydrochloride), the first antibiotic that showed therapeutic effectiveness on the syphilis-causing microbe *Treponema pallidum*, in 1909 in cooperation with Alfred Bertheim (Rustam, 2014; Mohr, 2016). Since 1910, more than 12,000-14,000 ampoules of salvarsan were produced per day worldwide, by which two-thirds of the syphilis-infected patients could be cured (Mohr, 2016).

Since World War I, the funding and labor invested in antibiotic research has increased dramatically, inspired by the dreadful non-combat attrition caused by bacterial infection (Mohr, 2016). In this atmosphere, German chemist Josef Klarer, based on his preliminary experience in testing hundreds of azo dyes in pursuit of antibiotic compounds, came up with a productive idea to synthesize a sulfur-containing azo dye (referred to as KI-695) (Bentley, 2009). He then sent out this compound for in vivo testing to his colleague Gerhard Domagk, who confirmed the chemotherapeutic effectiveness of KI-695 in mice and worked further to synthesize KI-730, a derivative compound with more potent and more consistent effect on Streptococci in mice (Bentley, 2009). Known as sulfamidochrysoidine and its commercial name prontosil, KI-730 was marketed in 1935 followed by the mortality rate of pneumonia and childbed fever began dropping almost immediately (Mohr, 2016). Inspired by the great success and market profits of prontosil, the research and development of sulfa drugs flourished in the late 1930s (Mohr, 2016). Although sulfa drugs are infrequently used now as a consequence of pathogenic resistance and rather strong side effects, they were a milestone in the discovery and development of antibiotics (Mohr, 2016).

Another name that has been associated with the beginning of the antibiotic era is Alexander Fleming for his discovery of penicillin in 1929. Penicillin, the compound that has saved tens of millions of lives since its discovery and derivatives of which are still used in hospitals worldwide, originated from a blessed coincidence. Returning from vacation, Alexander Fleming noticed that one of his agar plates was contaminated by fungus and surprisingly, these invading fungus created a bacteria-free zone around its colony (Gaynes, 2017). He immediately realized the potential of the fungus and worked to obtain an extract that proved to have an antimicrobial effect on staphylococci and other gram-positive bacteria

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(Gaynes, 2017). Fleming named the active compound in the extract penicillin, after the genus name

Penicillium of the mold (Gaynes, 2017). The publication of penicillin in the *British Journal of*

Experimental Pathology did not bring Alexander Fleming much fame and gain, however, and it was not

until 1940 when Howard Florey and Ernst Chain elucidated the structure of penicillin and solved its

mass-production problem that finally brought penicillin to the attention of the scientific community (Tan

and Tatsumara, 2015). The discovery of salvarsan, prontosil and penicillin established paths to future

antibiotic research, followed by later researchers leading to the golden era of antibiotic study ((Rustam,

2014).

1.3 THE GOLDEN ERA OF ANTIBIOTICS & STRINGENT CHALLENGES

The numerous reports of novel antibiotics from microorganisms urged the microbiologist Selman

Waksman to initiate a systematic study of microbes (Hutchings *et al.,* 2019). His works included

confirming actinomycetes as prolific producers of antibiotic substances. His efforts led to a broader

investigation of these gram-positive bacteria, leading to the discovery of neomycin and streptomycin, the

first antibiotics active against tuberculosis. (Sebek, 1968; Hutchings *et al*., 2019). Waksman's

investigations opened a gate to the golden age of antibiotic research, when soil samples collected from all

corners of the earth were analyzed, looking for potential antimicrobial sources (Gould, 2016). This

world-wide campaign in antibiotic research soon paid off; vancomycin was discovered in 1958,

methicillin in 1959, ampicillin in 1961. Many other agents were discovered in the 1950s and 1960s that

dramatically decreased the mortality due to bacterial infection (Gould, 2016). In the meantime, drug

resistance of pathogenic bacteria had increased to a non-negligible level due to the abuse of antibiotics.

Charles Darwin described the mechanism of evolution by natural selection in his book *On the*

Origin of Species, as organisms with habitable traits that favor survival, tend to leave more offspring

which means the favorable traits (genes) shall be possessed by more individuals. Similarly, bacterial cells

with genes that help them survive antibiotics shall pass down such genes to their offspring, and unlike

multicellular organisms, transport these genes to other bacterial cells by means of horizontal gene transfer

(transformation, transduction and conjugation). These mechanisms allow genes that counteract antimicrobials to be shared by the entire microbial community in a short period of time, thereby explaining the rapid rise of bacterial resistance since the early treatment with antibiotics. Rustam I. Aminov summarized 7 mechanisms of bacterial resistance, which can be classified as target or bullet-oriented:

> Target-Oriented:
> (i) protected by modifications, such as mutations in RNA polymerase conferring resistant to rifampin; (ii) modified by an enzyme, such as methylation of an adenine residue in 23S rRNA making it insensitive to macrolides; (iii) replaced, such as ribosomal protection proteins conferring resistance to tetracyclines and (iv) protected at cellular or population levels, such as formation of a protective barrier by secretion.
>
> Bullet-Oriented:
> (i) modified so the efficiency is lost, as in the case of acetylation of aminoglycosides; (ii) destroyed, as the β-lactam antibiotics by the action of β-lactamases; (iii) pumped out from the cell as in efflux pump mechanisms of resistance. (Rustam, 2014).

Once an antibiotic agent was approved for clinical use, target bacteria soon evolved to counteract the agent and gain resistance.

Among all virulent and wide-spreading drug-resistant strains, methicillin-resistant *Staphylococcus aureus* (MRSA) is particularly difficult to treat. Benzylpenicillin was introduced to clinical use in the early 1940s to treat Staphylococcal infections; in the following decade, however, the wide-scale use of benzylpenicillin favored the survival of *Staphylococcus* that produces □-lactamase, an enzyme that grants bacteria resistance to almost all antibiotics available at that time, including penicillin, streptomycin, tetracycline, inter alia. (Brumfitt and Hamilton-Miller, 1989). The situation was remedied in 1960s, when several semisynthetic antibiotics that are not subjects of □-lactamase such as oxacillin, methicillin and floxacillin were approved for clinical use (Rolinson *et al.*, 1960). Unfortunately, the temporary relief did not last long before new challenges arose. In 1961, only 2 years after the clinical use of methicillin, a new strain of *S. aureus* resistant not only to □-lactam antibiotics, but also to methicillin and aminoglycosides such as gentamicin and amikacin, was detected in London, according to the data published on the *British Medical Journal* by Professor Patricia Jevons (Moellering, 2012). The clone of this strain is later named methicillin-resistant *Staphylococcus aureus* (MRSA); since then, MRSA has spread across the world and

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posed threats on hospitalized patients and their caregivers. According to the Centers for Diseases Control and Prevention (CDC), approximately 33% of people in the US and 5% of patients in US hospitals carry MRSA in their nose or on their skin; although these usually do not lead to illness, MRSA can cause severe symptoms including pneumonia and blood infections in healthcare settings (CDC, 2019). MRSA, together with many other multidrug-resistant bacteria, threaten human health notoriously, and effective ways to overcome the challenges they bring are to discover new antibiotics.

While human beings are in urgent need, the discovery rate of new antibiotics dropped significantly in the past few decades. This antibiotic paradox has been examined by several scientific and press articles (Conly, 2005). The decline in the discovery rate of novel antibiotics can be explained from two aspects. First, after almost 90 years of exploration since the discovery of penicillin, most potent and easy-to-extract antimicrobial agents have been discovered; the remaining natural antibiotics either exist in very low concentrations or are vulnerable to decomposition, both of which hampered the accumulation of enough quantity for analytical purposes. Therefore, research and development projects of these compounds consume more time and money, discouraging the enthusiasm of pharmaceutical companies on investing in antibiotic research. The World Health Organization (WHO) reported that only five of the major pharmaceutical companies, GlaxoSmithKline, Novartis, AstraZeneca, Merck and Pfizer still have development programs for antibiotics in 2008 (Braine, 2011). Every year, more than 63,000 patients die from hospital-acquired bacterial infections in the United States, giving critical significance to new sources of antibiotics (Rustam, 2014). However, lack of funding for industrial and academic researchers crippled the development of new antibiotic agents, which then drove away investments to other more profitable industries. This paradox seems unsolvable, but there are hopes. Current efforts in confronting bacterial resistance include multidrug therapy (MDT), modification of existing antimicrobial agents to enhance their therapeutic effectiveness and exploring new environmental and taxonomic sources of antibiotic producers. Here, the approach of exploring new antibiotics from marine bacteria is discussed.

About three decades ago, many microbiologists believed that marine bacteria were simply terrestrial strains rinsed to the ocean, and therefore not worth studying (Fenical, 2020). Consequently, the

significance of marine natural products was underestimated and myriads of potential research on marine invertebrates and microbes had not been undertaken (Fenical, 2020). The status quo was challenged by some curious and adventurous researchers, such as Paul Jensen and William Fenical, who discovered that as they sampled deeper ocean sediments, the typical terrestrial strains of streptomycetes were replaced by diverse actinomycete taxa; Moreover, some bacteria collected from marine sediments grew better on seawater-based media, suggesting adaptation to growth in the sea. (Fenical, 2020). Jensen and Fenical were among the first to realize that many marine-derived bacteria were unique from their terrestrial strains and to design programs to explore their secondary metabolites. Since then, thanks to all researchers, mainly academics, who are devoted to the discovery of new marine natural products, hundreds of novel antimicrobial and antitumor agents have been discovered. Pestalone, salinosporamide, anthracimycin, all of the marine-bacteria-derived therapeutic agents have proved the potential of marine organisms in pharmaceutical studies (Cueto *et al*., 2001; Feling *et al*., 2003; Jang *et al*., 2013). Moreover, at least 27 molecules are currently in clinical trials (Alex Mayer, 2021). Given the fact that bacterial resistance has become a major threat to human health, while the discovery rate of new antibiotics from terrestrial microbes is dropping, the study of marine natural products is more significant than ever.

In this project, my goal was to learn the procedures of marine microbial drug discovery and to isolate and describe a new antibiotic. To achieve these goals, I cooperated with researchers at Helwan University in Cairo, Egypt to discover novel antibiotics from marine actinomycetes. Dr. Mohamed Saleh Abdelfattah's team collected 51 unique strains of actinomycetes along the North Egyptian coast and named them EG1 to EG51. These strains were sent to our lab at SIO for further analysis. In the thesis, a chlorinated antibiotic molecule discovered from *Streptomyces* strain EG32 will be described.

CHAPTER TWO

MATERIALS AND METHODS

2.1 FERMENTATION OF EG32

Fifty strains of actinomycetes that were collected along the Northern Egyptian Coast, named EG1 to EG50, and sent to SIO were capable of regrowing under lab conditions. The following procedures were performed in a biosafety hood. Frozen EG32 bacterial strain in a cryovial was resuspended in sterile solution and applied on a Waksman media agar plate (glucose 20g/L, beef extract 5g/L, peptone 5g/L, dried yeast (yeast extract) 3g/L, NaCl 5g/L, $CaCO_3$ 3g/L, 50% filtered seawater, 50% DI water, 1.8% agar) using the streaking technique. The bacterium was allowed to grow at 26 ℃ for 7 days. After the purity of the strain was confirmed by dilution streaking, a small piece of the agar plate containing a bacterial colony was cut and transferred to a glass flask containing 25 mL of Waksman media. The flask was placed on the shaker (190 rpm) for 5 days. After that, 15 mL of the culture was transferred to 1L of Waksman media and an inoculation loop was used to apply the remaining culture on a Waksman agar plate. The 1L media was placed on the shaker at 190 rpm for 5 days, followed by a purity check by observing growth by a small aliquot on an agar plate. Fifteen mL of new EG32 culture was added to 1 L (per bottle) of Waksman media and fermented for 7 days. Ten bottles of media were seeded each day for 4 days, giving a total volume of 40 L.

2.2 EXTRACTION OF THE SECONDARY METABOLITES

Twenty grams of XAD-7 resin were added to the 1L culture which was then placed on the shaker table for 3 hours. The resin was collected and extracted with 2 X 500 mL of acetone, and the solvent was reduced to dryness using a rotary evaporator. The remaining residue was extracted with ethyl acetate twice and the ethyl acetate layers were collected and evaporated under vacuum, giving 6.12 g of organic extract.

2.3 ANTIBACTERIAL TESTING

Antimicrobial susceptibility was tested by the agar disk diffusion method on Waksman Media (WM) agar plate. Negative control (methanol) and 0.4 mg of sample dissolved in methanol were applied on 6 mm blank paper discs and solvent removed. The positive control was performed with a pre-prepared, ciprofloxacin-applied paper disc. These discs were placed on an WM agar plate evenly inoculated with MRSA in 3 different directions. After that, the plate was incubated at 26 ℃ for 24 h. The diameter of the bacteria-free zone around the disks were measured and recorded to the nearest whole millimeter. The antimicrobial susceptibility 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.

The Minimum Inhibitory Concentration (MIC) broth-dilution bioassay was devised using the following method. The methicillin-resistant *Staphylococcus aureus* (MRSA) strain TCH1516 (ATCC BAA-1717) and *E.coli* strain LptD4213 were used. The minimum inhibitory concentration (MIC) of the test subject was evaluated by the broth microdilution method according to CLSI guidelines. Briefly, a few colonies of a pure culture of TCH1516 on agar were put into cation adjusted Mueller-Hinton broth (CAMHB). The inoculum was diluted in CAMHB to be equivalent to a 0.5 McFarland standard. Two-fold serial dilutions of the test subject were dispensed in a microtiter plate as well as the inoculum. The overall volume in each well was 200 μL. The plate was incubated at 37 °C in air for 20 h, then the optical density (OD) at 650 nm was read using a plate reader (EmaxPrecision Microplate Reader by Molecular Devices). Ciprofloxacin HCl hydrate was used as a positive control for both MRSA and *E.coli*. Negative control and quality control were carried out in replicate analyses.

2.4 ISOLATION OF COMPOUNDS

Three grams of the crude extract were subjected to the silica gel chromatography, eluting with hexane, ethyl acetate and methanol (ratios: 100:0:0, 50:50:0, 20:80:0, 0:100:0, 0:90:10, 0:66:34, 0:50:50, 0:0:100) to afford 8 fractions, named EG32-S1 to EG32-S8. The activities of each fraction obtained from the antibacterial assay were used to guide the isolation of fractions. Fractions were subjected to C-18

reversed-phase HPLC (Phenomenex Luna C-18 column, 10 × 250 mm, 5 μm column; 3 mL/min flow rate, UV detection at 254 nm) with 50% to 100% acetonitrile/water over 30 min with the same solvent eluting 10 more min to obtain pure compounds.

2.5 SPECTRAL DATA OF THE COMPOUND

The LC-MS data were obtained on an Agilent 6530 Accurate-Mass Q-TOF that was coupled to an Agilent 1260 LC system with a Phenomenex Luna C18 column (4.6×100 mm, 5μm, flow rate 0.7 mL/min). The 1D (^{1}H, ^{13}C) and 2D (HSQC, HMBC, COSY) NMR spectral data were obtained on a JEOL 500 NMR spectrometer. The UV spectra were measured with a Beckman Coulter DU800 spectrophotometer with a path length of 1 cm, and IR spectra were acquired on a JASCO FTIR-4100 spectrometer. The optical rotation was measured on a P-2000 Jasco polarimeter.

2.6 THE 16S rRNA SEQUENCING

A colony grown on solid medium was scraped, resuspended, and extracted in DMSO. The microbial DNA was then subjected to be amplified by Polymerase Chain Reaction (PCR). FC27 was used as the forward primer and RC1492 was used as the reverse primer. The DNA sample was then sent to Eton Bioscience Inc. for sequencing.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 LOCATING THE TARGET COMPOUNDS

Forty liters of bacterial culture were extracted in 4 days, giving 4 parts of crude extracts, which were named EGD1, EGD2, EGD3 and EGD4.

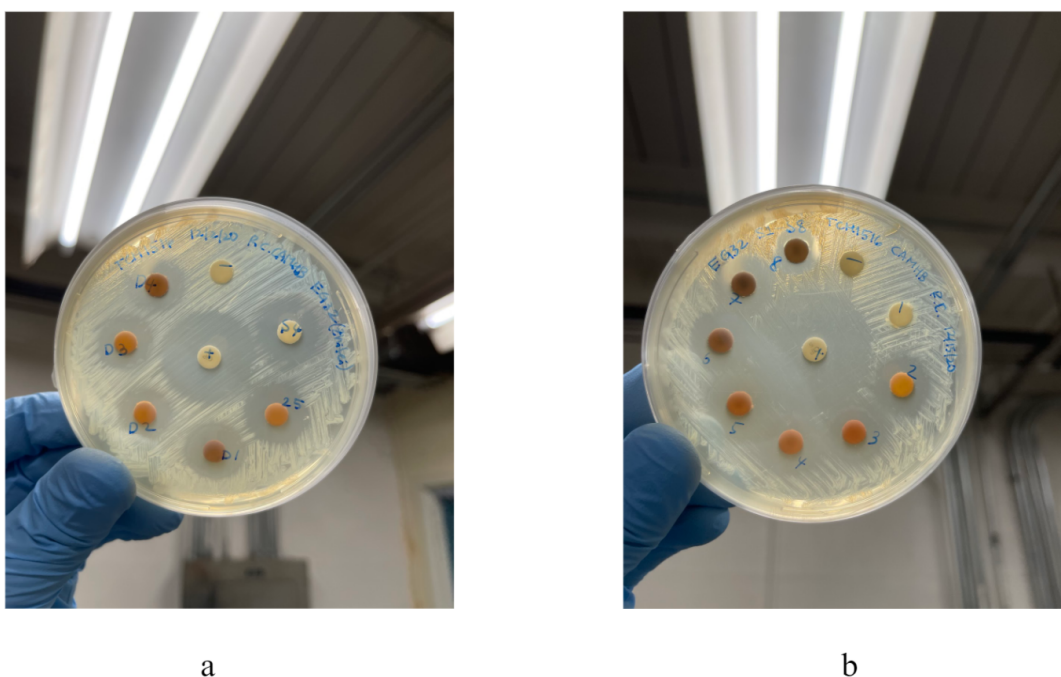a   b

Figure 1(a): the agar plate showing the antibacterial susceptibility of EGD1~EGD4. All extracts showing the inhibition diameter > 15 mm (P.PP). Figure 1(b): the agar plate showing the antibacterial susceptibility of fraction EG32-S1~EG32-S8. Fractions EG32-S1 to EG32-S7 have the inhibition diameter > 15 mm (P.P.P) while EG32-S8 has the inhibition diameter > 10 mm but < 15 mm (P.P).

The antimicrobial susceptibility test by disk diffusion method showed P.P.P for all 4 fractions and therefore they were combined to give a total mass of 6.12 g. Three grams of the crude extract were subjected to the silica gel chromatography, affording 8 fractions which were named EG32-S1~EG32-S8.

The antimicrobial susceptibility test by disk diffusion method showed P.P.P for S1 to 7, and P.P for S8.

Since fraction S2 had significantly more quantity (2000 mg) than other fractions, it was further purified

by C-18 reversed-phase HPLC, obtaining 9 compounds. Compound 1 (2.3 mg, Rt 13.06), 2 (2.5 mg, Rt

16.11), 3 (1.4 mg, Rt 19.02), 4 (0.5 mg, Rt 23.30), 5 (15.1 mg, Rt 23.84), 6 (0.5 mg, Rt 25.00), 7 (5.8 mg,

Rt 26.73), 8 (5 mg, Rt 32.44), and 9 (1.5 mg, Rt 40.12).

The HPLC chromatogram of the S2 fraction of EG32 revealed 9 compounds, named

EG32-S2-1~EG32-S2-9. The UV absorption spectra and LCMS spectra of these 9 compounds were
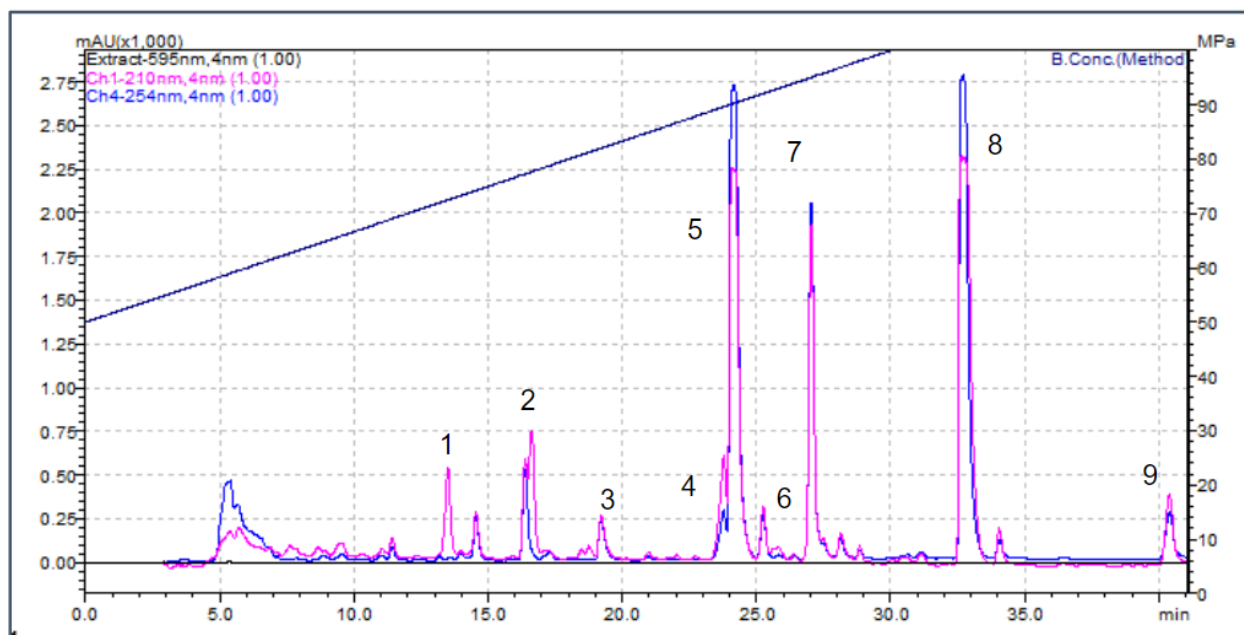
acquired.

Figure 2: The C-18 silica chromatogram of fraction S2 of the EG32 strain extract. Nine compounds are

shown and numbered.

The UV spectra obtained from each peak of the fraction indicated that compounds EG32-S2-5, EG32-S2-7 and EG32-S2-8 have similar chromophores, with EG32-S2-5 and EG32-S2-7 being almost identical.
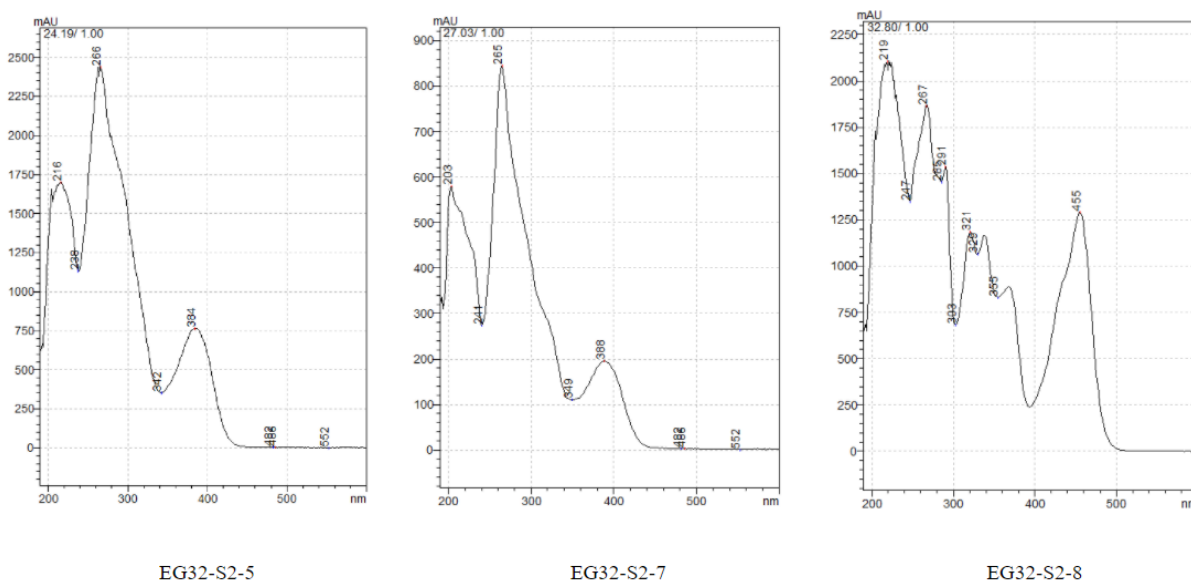

Figure 3: The UV absorption spectra of the 3 compounds. Compounds EG32-S2-5 and EG32-S2-7 share similar absorption maxima at 385 nm, while EG32-S2-8 is unique with a longer wavelength absorption at 455 nm.

The LCMS spectral data indicated a molecular ion for S2-5, S2-7, S2-8 to be at *m/z* 392.71, 426.09 and 376.54, respectively. Based on the characteristic UV and molecular weights, it was concluded that EG32-S2-5 was likely resistoflavin and EG32-S2-8 was likely resistomycin, two cytotoxic pentacyclic polyketides reported to be produced by *Streptomyces* spp (Koch *et al.*, 2005). No matching data for EG32-S2-7 were found, indicating that it was likely a new metabolite related to the resistoflavin class. Furthermore, the LCMS spectrum of EG32-S2-7 showed two peaks separated by 1 and 2 amu from the major peak, indicative of the two chlorine isotopes. The major peak is composed of ^{12}C and ^{35}Cl, while the second peak contains ^{13}C and ^{35}Cl, which is one amu heavier than the first peak. The third peak

contains ^{12}C and ^{37}Cl, which explains the difference of 2 amu. In order to elucidate the structure of

EG32-S2-7 and confirm its structure, comprehensive 1D and 2D NMR spectra were acquired.
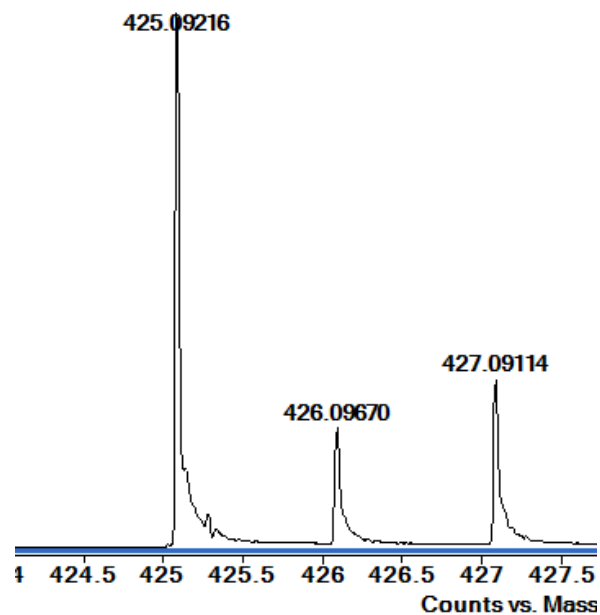

Figure 4: The negative-mode LCMS spectrum of peak EG32-S2-7. The molecular mass of the compound

was calculated as $425.09 + 1 = 426.09$. The peak of 425.09 is the molecular ion containing ^{12}C and ^{35}Cl.

The peak of 426.09 contains ^{13}C and ^{35}Cl. The peak of 427.09 contains ^{12}C and ^{37}Cl.
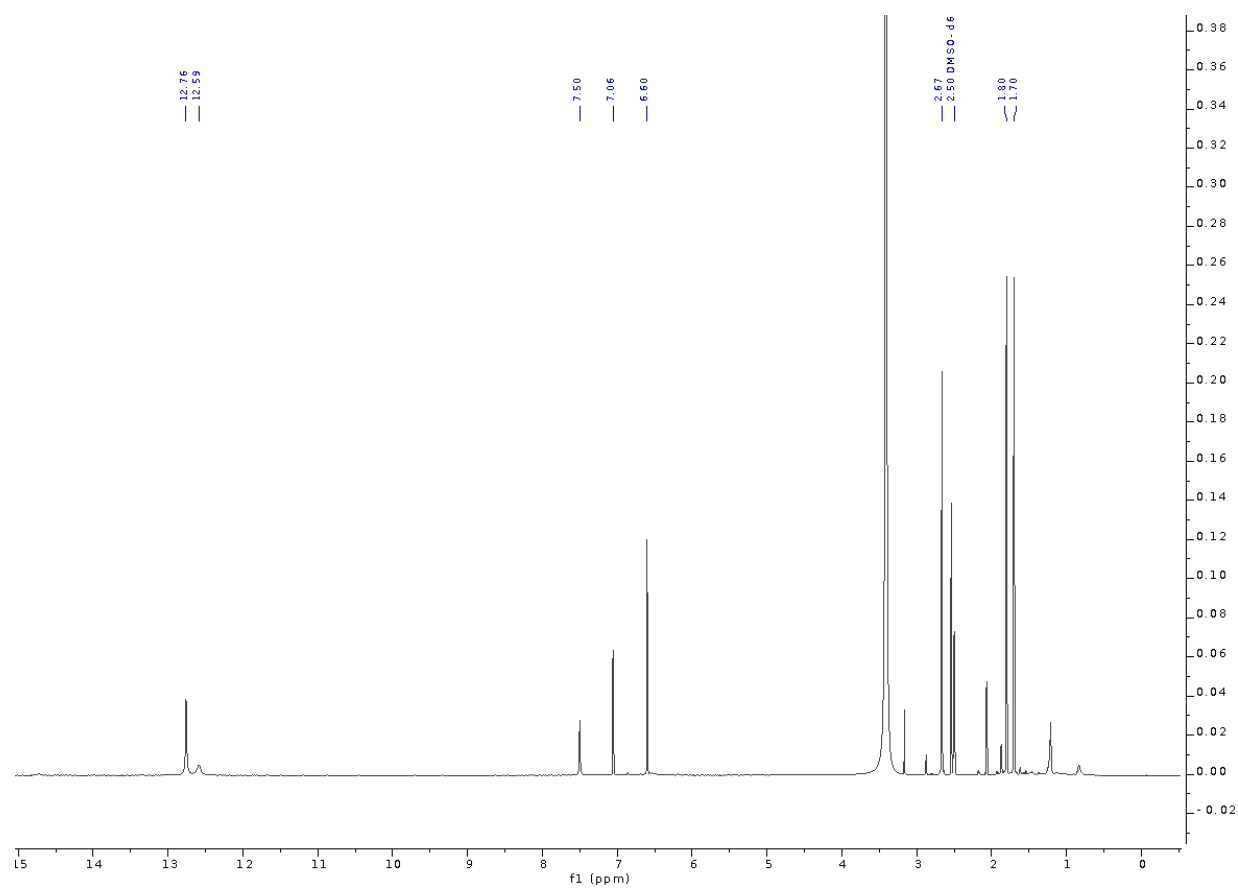
3.2 1D AND 2D NMR SPECTRAL DATA OF COMPOUND EG32-S2-7

Figure 5: The ^{1}H NMR spectrum of compound EG32-S2-7 in DMSO-d_6.

The ^{1}H NMR spectrum of compound EG32-S2-7 showed 2 aromatic methine signals at δ_H 6.60 and 7.06, 3 methyl signals at δ_H 1.70, 1.80 and 2.67, and 2 hydroxy signals at δ_H 7.50 and 12.76.
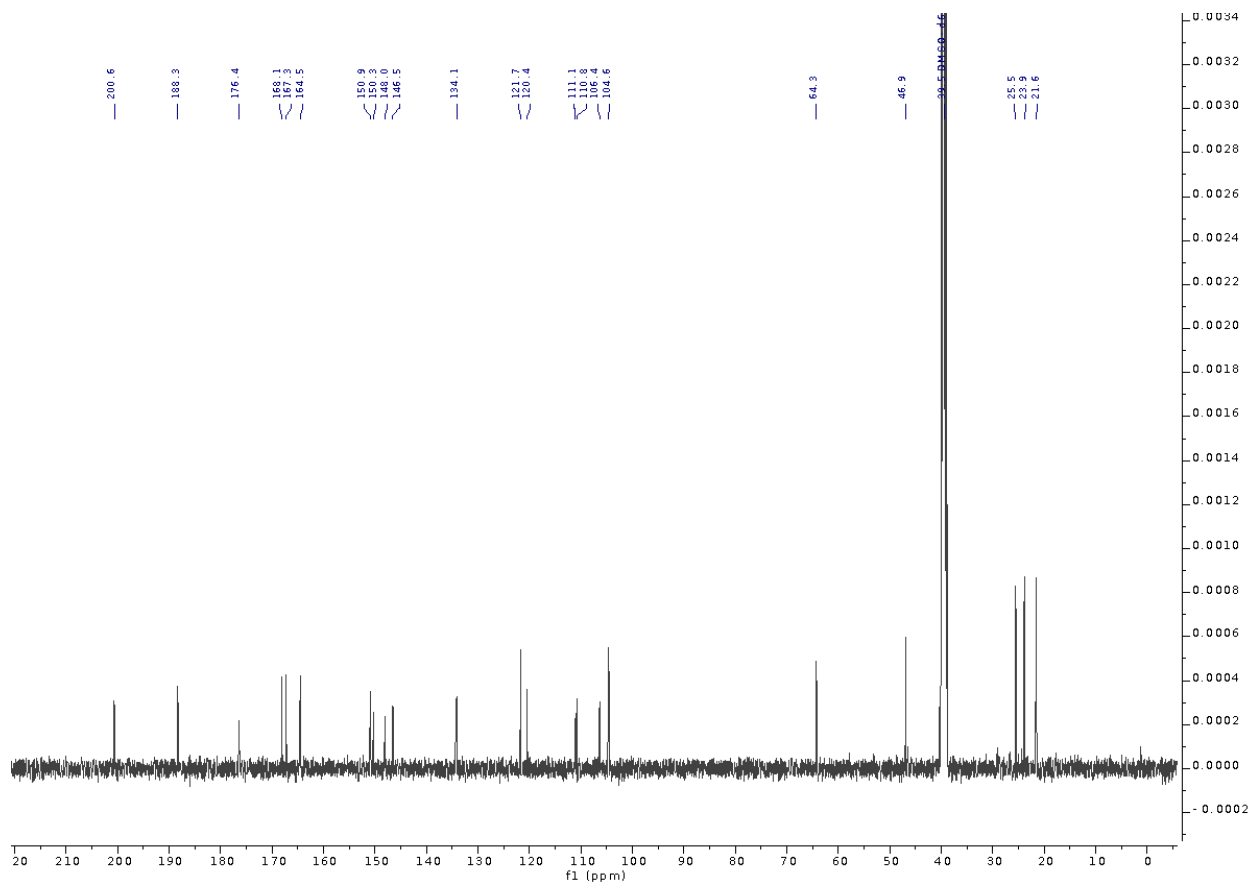
Figure 6: The ^{13}C NMR spectrum of compound EG32-S2-7 in DMSO-d_6, showing 22 carbon atoms, many of which were in the aromatic region.

The ^{13}C NMR spectrum of EG32-S2-7 displayed 22 carbon signals consisting of 3 carbonyl signals (δ_C 200.6, 188.3 and 176.4), 10 quaternary carbon signals (δ_C 46.9, 110.8, 64.3, 106.4, 111.1, 146.5, 150.9, 120.4, 150.3 and 148.0), 2 aromatic methine carbon signals (δ_C 104.6, 121.7), 3 methyl signals (δ_C 21.6, 25.5 and 23.9) and 4 hydroxy-attached carbon signals (δ_C 168.1, 167.3, 164.5, and 148.0).
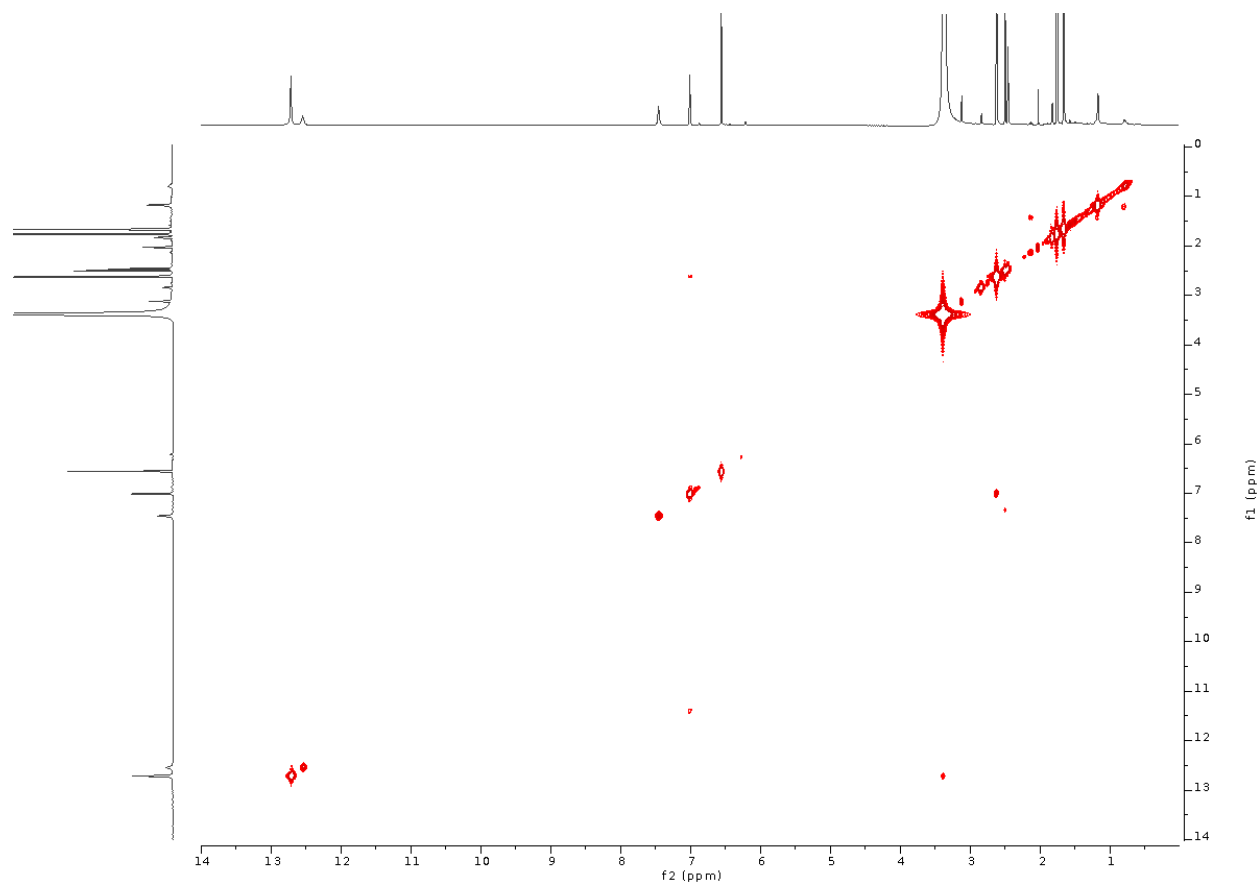
Figure 7: The 2D COSY NMR spectrum of compound EG32-S2-7 in DMSO-d_6.

The ^{1}H-^{1}H COSY NMR spectrum displayed correlations of δ_H 7.06 (H-8) and δ_H 2.67 (H-9-Me) on both sides of the diagonal, therefore this correlation was confirmed. Correlations between δ_H 3.3 and δ_H 12,7, as well as between δ_H 7.01 and δ_H 11.20, however, were only observed on the lower side of the diagonal, therefore they are not correlations of the molecule, but more likely to be caused by impurities.
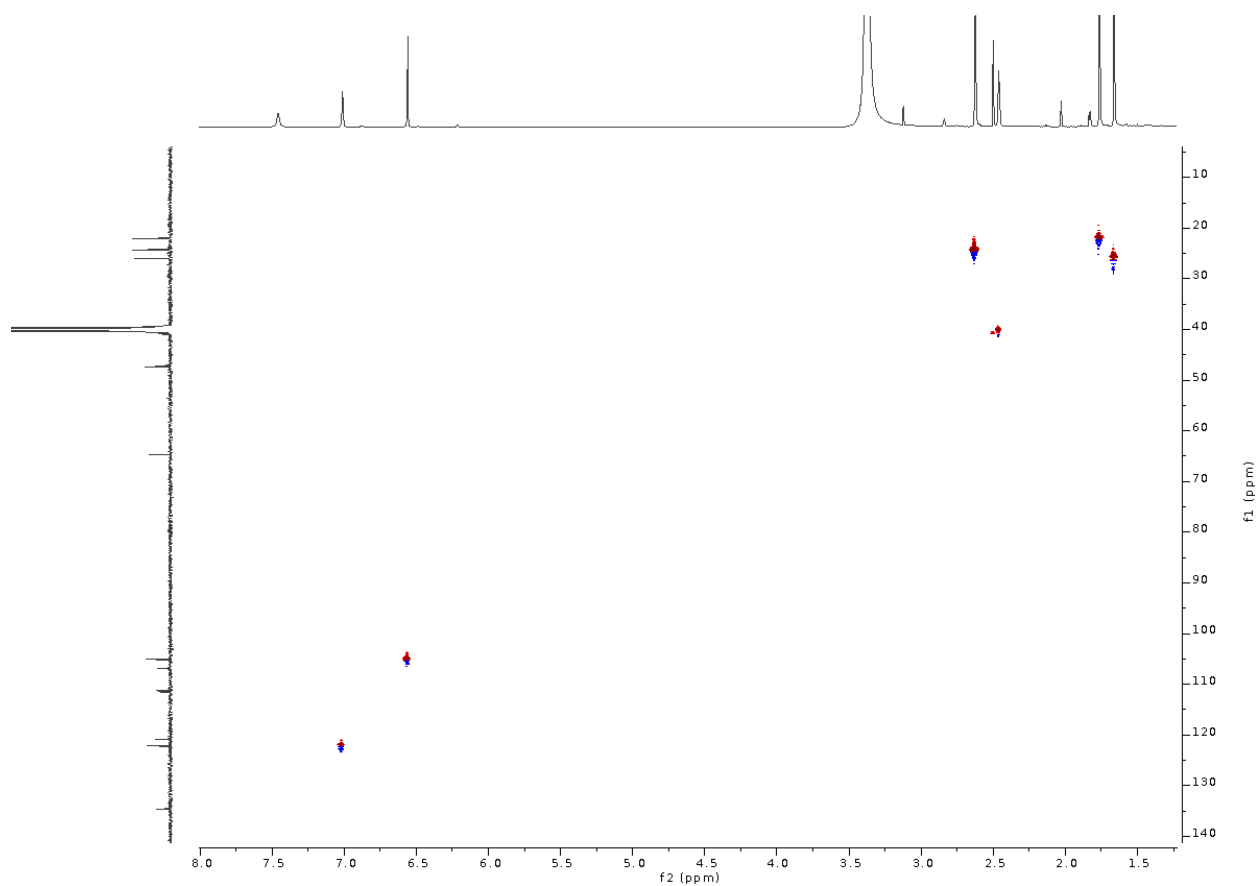
Figure 8: The 2D HSQC NMR Spectrum of compound EG32-S2-7 in DMSO-d_6.

The HSQC NMR spectrum displayed 5 carbon-proton connectivities including 3 methyl signals (δ_C21.6 to δ_H1.80, δ_C25.5 to δ_H1.70 and δ_C23.9 to δ_H2.67), and two aromatic methine signals (δ_C104.6 to δ_H6.60 and δ_C121.7 to δ_H7.06).
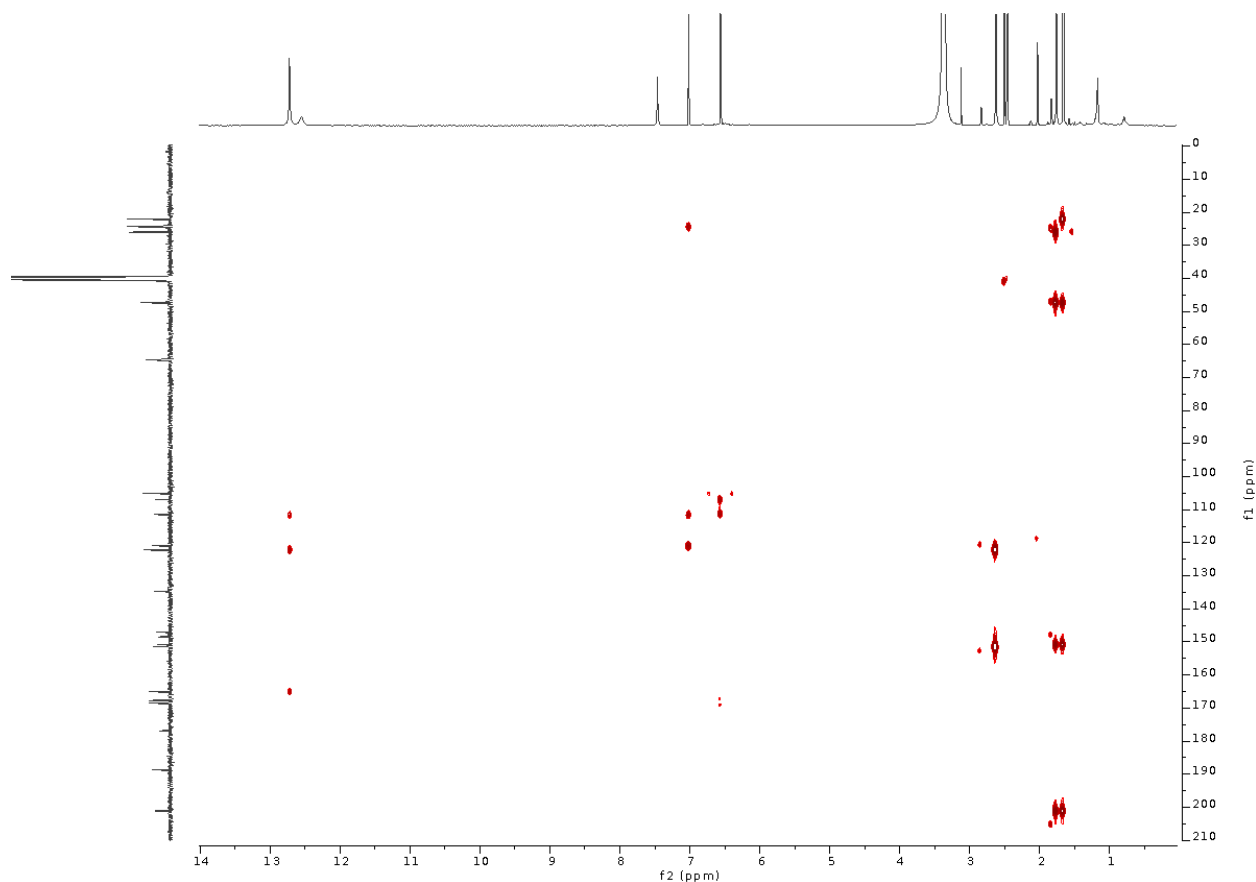
Figure 9: The 2D HMBC NMR spectrum of compound EG32-S2-7 in DMSO-d_6.

The HMBC spectrum displayed correlations from H-1-Me-1 to C-1-Me-2, C-1, C-2 and C-11a, H-1-Me-2 to C-1-Me-1, C-1, C-2 and C-11a, H-4 to C-2a, C-3, C-5 and C-5a, H-7 (OH) to C-6a, C-7 and C-8, H-8 to C-6a, C-9a and C-9-Me, and H-9-Me to C-8 and C-9.

The chlorination of EG32-S2-7 can take place by replacing the hydrogen in the aromatic methylene groups (C-4, C-8, C-11). The carbon-proton bondings of C-4 (δ_C 104.6) and C-8 (δ_C 121.7) were observed in the HSQC spectrum, while C-11 was missing, indicating that the hydrogen here was substituted by chlorine. To further support this assignment, the ^{1}H and ^{13}C spectra of EG32-S2-7 were compared with those of resistoflavin (Gorajana, 2007) as these two compounds have similar molecular structure. By comparison, protons in the methyl groups connected to C-1 experienced enhanced chemical shifts due to the deshielding effect caused by adjacent chlorine (resistoflavin: δ_H 1.54, δ_H 1.61; EG32-S2-7:

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δ_H1.70, 1.80). Similar trend was also observed on chlorine-attaching C-11 (resistoflavin: δ_C127.7;

EG32-S2-7: δ_C134.1). Based on the observations above, it was confirmed that the chlorine attaching site

was on C-11, and therefore EG32-S2-7 was named 11-chloro-resistoflavin.

3.3 PHYSICAL-CHEMICAL PROPERTIES OF 11-CHLORO-RESISTOFLAVIN

Compound 11-chloro-resistoflavin was isolated as a greenish-yellow powder and its molecular

formula was assigned as $C_{22}H_{15}ClO_7$ based on HR-ESI-MS data ([M-H] at *m/z* 426.0927. The specific

optical rotation of 11-chloro-resistoflavin is [a]$_D^{24}$ = -251, *c* = 0.25 in pyridine. The molecular structure

with key HMBC and COSY correlations was assigned as below.
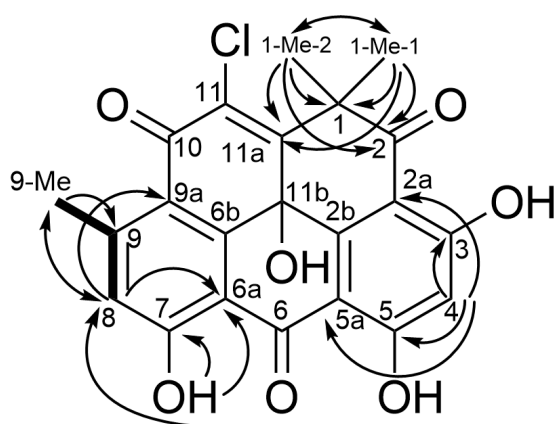

Figure 10: The molecular structure of 11-chloro-resistoflavin illustrating key HMBC and COSY

correlations. Arrows point from hydrogens to correlated carbons.

The antibacterial susceptibility of 11-chloro-resistoflavin measured by MIC values are shown in the table below.

Table 1: the minimum inhibitory concentration (MIC) values of 3 compounds against MRSA and *E.coli*. The MIC values against MRSA are much lower than those against *E.coli*. Resistomycin and resistofavin were purified from fraction EG32-S2 together with 11-chloro-resistoflavin.

MIC Values		
Compounds	MRSA	*E.coli*
11-Chloro-Resistoflavin	0.25 µg/mL	32 µg/mL
Resistomycin	0.125 µg/mL	32 µg/mL
Resistoflavin	0.25 µg/mL	64 µg/mL

Although the potency is lower than resistomycin, 11-chloro-resistoflavin exhibits a similar antibacterial activity to resistoflavin against MRSA, with the MIC value of 0.25 µg/mL. It has been demonstrated that these 3 pentacyclic polyketides are specialists against MRSA, with the MIC values 100-fold lower than those against *E.coli*.

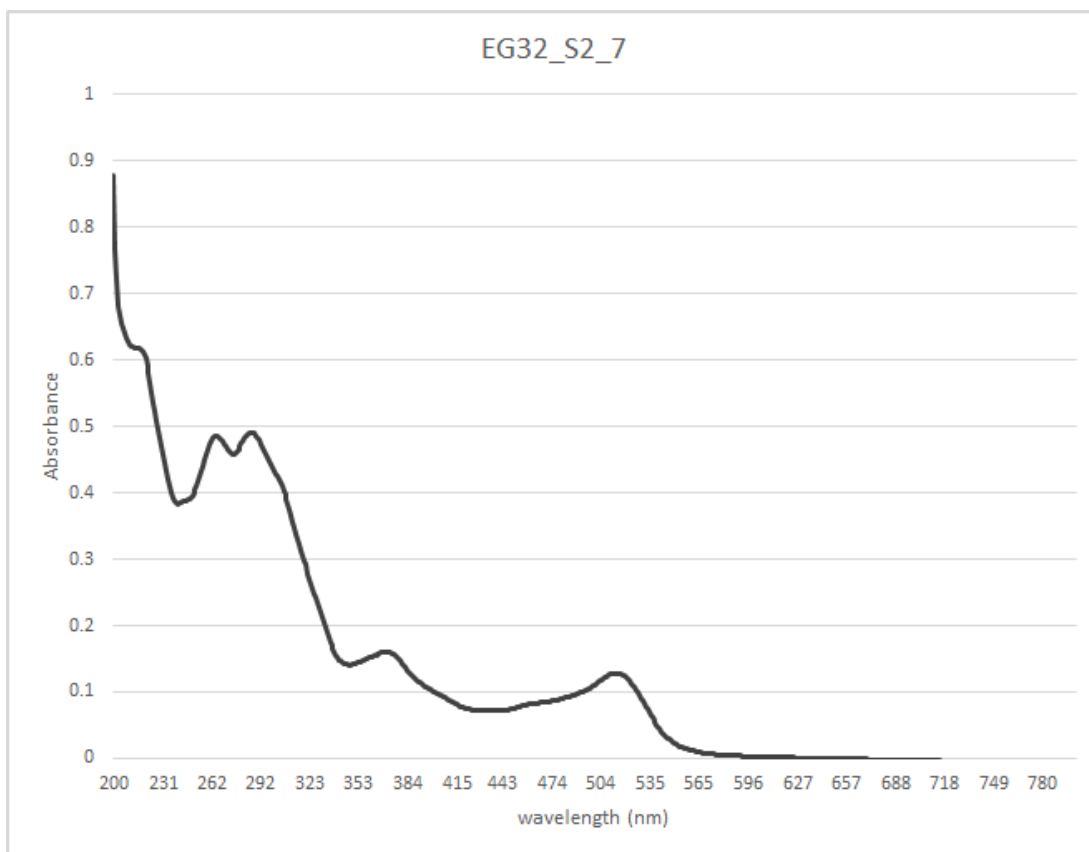The UV absorption spectrum of 11-chloro-resistoflavin is shown below.

Figure 11: The UV absorption spectrum of 11-chloro-resistoflavin. The spectrum shows five absorption maxima at 220 nm, 264 nm, 286 nm, 369 nm and 513 nm, respectively.

The UV absorption spectrum of 11-chloro-resistoflavin shows five absorption maxima. The highest peak A = 0.6017 is located at 220 nm, two middle peaks A = 0.4857 and A = 0.4908 are located close to each other at 264 nm and 286 nm. Two lower peaks A = 0.1607 and A = 0.1286 are located at 369 nm and 513 nm.

The IR spectrum of 11-chloro-resistoflavin is shown below.
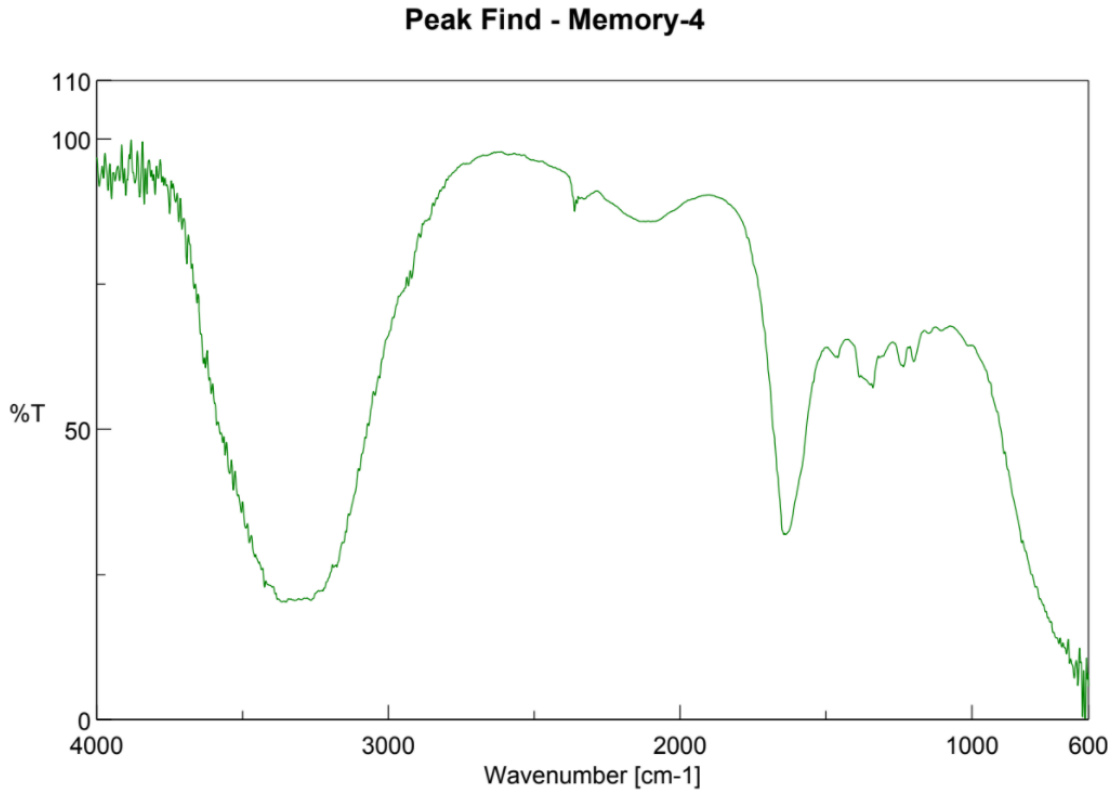

Peak Find - Memory-4

Figure 12: The infrared absorption spectrum of 11-chloro-resistoflavin. The spectrum shows a broad peak at around 3450 to 3200 cm^{-1} and a sharp peak at around 1600 cm^{-1}.

The infrared absorption spectrum of 11-chloro-resistoflavin shows a broad peak around 3450 to 3200 cm^{-1} , representing the hydroxyl groups. The spectrum also has a sharper peak at around 1600 cm^{-1} which represents the carbonyl groups.

3.5 THE 16S rRNA SEQUENCING

The nucleic acid sequences of strain EG32 is presented below:

CTCCCTCCCACAAGGGGTTGGGACCACCGGCTTCGGGTGTTACCGACTTTCGTGACGTGACG
GGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCG
ACTCCGACTTCATGGGGTCGAGTTGCAGACCCCAATCCGAACTGAGACCGGCTTTTTGAGATT
CGCTCCACCTCGCGGTATCGCAGCTCATTGTACCGGCCATTGTAGCACGTGTGCAGCCCAAGA

CATAAGGGGCATGATGACTTGACGTCGTCCCCACCTTCCTCCGAGTTGACCCCGGCGGTCTCC
CGTGAGTCCCCAGCACCACAAGGGCCTGCTGGCAACACGGGACAAGGGTTGCGCTCGTTGC
GGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCACCACCTGTACACCGA
CCACAAGGGGGGCACCATCTCTGATGCTTTCCGGTGTATGTCAAGCCTTGGTAAGGTTCTTCG
CGTTGCGTCGAATTAAGCCACATGCTCCGCCGCTTGTGCGGGCCCCGTCAATTCCTTTGAGT
TTTAGCCTTGCGGCCGTACTCCCCAGGCGGGGCACTTAATGCGTTAGCTGCGGCACGGACGAC
GTGGAATGTCGCCCACACCTAGTGCCCACCGTTTACGGCGTGGACTACCAGGGTATCTAATCC
TGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTATCGGCCCAGAGATCCGCCTTCGCCAC
CGGTGTTCCTCCTGATATCTGCGCATTTCACCGCTACACCAGGAATTCCGATCTCCCCTACCGA
ACTCTAGCCTGCCCGTATCGACTGCAGACCCGGGGTTAAGCCCCGGGCTTTCACAACCGACGT
GACAAGCCGCCTACGAGCTCTTTACGCCCAATAATTCCGGACAACGCTCGCGCCCTACGTATT
ACCGCGGCTGCTGGCACGTAGTTAGCCGGCGCTTCTTCTGCAGGTACCGTCACTTTCGCTTCT
TCCCTGCTGAAAGAGGTTTACAACCCGAAGGCCGTCATCCCTCACGCGGCGTCGCTGCATCA
GGCTTTCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCA
GTCCCAGTGTGGCCGGTCGCCCTCTCAGGCCGGCTACCCGTCGTCGCCTTGGTGAGCCATTAC
CTCACCAACAAGCTGATAGGCCGCGGGCTCATCCTGCACCGCCGGAGCTTTCGAACCGCTTG
GATGCCCAAGCGGGTCAGTATCCGGTATTAGACCCCGTTTCCAGGGCTTGTCCCAGAGTGCAG
GGCAGATTGCCCACGTGTTACTCACCCGTTCGCCACTAATCCCCTCCCGAAGGAGGTTCATCG
TTCGACTGC

The sequences were compared on the National Center for Biotechnology (NCBI) database for identification, indicating 100% query cover and 99.93% percent identity to *Streptomyces variabilis*, *S. griseorubens* and *S. griseoincamatus*. Therefore, even though the accuracy did not extend to species, it was confirmed that strain EG32 belongs to the genus of *Streptomyces*.

CHAPTER FOUR

CONCLUSION

A chlorinated resistoflavin homolog has been isolated and identified from the marine *Streptomyces strain* EG32. The presence of this new compound and its potent antibacterial properties against MRSA demonstrated the significance of marine bacteria as a new source for antibiotics. Compared to terrestrial strains, marine bacteria have abundant supplies of halogens from seawater, which may explain the halogenation often observed in marine-bacteria-derived compounds. Future research is required to reveal the gene cluster responsible for the halogenation of the secondary metabolites.

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