UNIVERSITY OF GHANA

COLLEGE OF BASIC AND APPLIED SCIENCES

NOVEL POST-TRANSLATIONALLY MODIFIED NON-RIBOSOMAL PEPTIDES (NRPs) FROM THE GHANAIAN *STREPTOMYCES* SP. DE2E

BY

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DECLARATION

I, **Tetevi Gilbert Kwami Mawuli**, declare that the work contained in this thesis was undertaken solely by me under supervision and has neither wholly nor partially been presented elsewhere for another degree.

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ABSTRACT

Non-ribosomal peptides (NRPs) are currently receiving considerable interests for possible development as effective alternatives to clinically failing conventional small molecule antibiotics. Particularly, NRPs produced by the genus *Streptomyces* have attracted considerable attention given the antibiotic production history of the genus. In this current report, a novel Ghanaian *Streptomyces* sp. strain DE2E isolated from the high biodiversity rich wetlands of the Brong-Ahafo Region of Ghana was thoroughly investigated for its capacity to produce bioactive NRPs. The total crude extract (TCE) obtained after harvesting the extracts of *Streptomyces* sp. DE2E in tryptic soy broth (TSBY) culture media was analyzed by spectroscopic and spectrometric techniques. Screening and dereplication processes involving the use of HRESI/HPLC-MS^n data and AntiMarin database revealed the presence of large molecules which were found to be novel NRPs. A modified Kupchan solvent partitioning process of the TCE yielded four main fractions FH, FD, FM and WB amongst which the FD fraction was found to contain these large molecules. Sephadex LH-20 size exclusion chromatography fractionation produced the prioritized fraction SFB, which was subjected to HPLC to give five structurally related NRPs (peaks E–I). The proposed structure of the compound DE2E-TSBY-FD-SFB-I which was the largest among the five peptides isolated, was determined using 1D, 2D NMR interpretations and MS^n data analysis. This compound was found to be a post-translationally modified NRPs macrocyclic pentapeptide whose structural confirmation will facilitate the subsequent determination of the remaining four other peptides. Biological screening of the compound DE2E-TSBY-FD-SFB-I showed strong antibacterial and antiparasitic activity against *Shigella sonnei* (IC_{50} = 10.01μM) and *Trypanosoma brucei* (IC_{50} = 12.89μM) respectively.

DEDICATION

I dedicate this work to my beloved family; Mr. Tettevi Kwablah Raphael (late), Mrs. Tettevi Bridget Lawson, Tettevi Jennifer Edudzi and Tettevi Princess in appreciation for their prayers, love, provision and sacrifice throughout my life and education in particular my post graduate years. Thank you mum and dad for grooming me for this levels of success, prosperity and great achievements which will definitely materialize by the grace of the LORD Jesus Christ. May God richly bless you in this life and more in the life to come. I love you all deeply.

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To end with, I want to express my gratitude to my beloved family, loved ones and fellow post graduate colleagues for their continuous prayer, love and profound kindness. This would not have been possible without you. Best wishes to you all. Cheers!!!

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LIST OF ABBREVIATIONS

AceN	Acetonitrile
AIPs	Auto Inducing Peptides
AMPs	Antimicrobial Peptides
antiSMASH	Antibiotics and Secondary Metabolite Analysis Shell
ATP	Adenosine triphosphate
BGCs	Biosynthetic Gene Clusters
CYC	Cyclization
COSY	Correlation Spectroscopy
Dab	Diaminobutyric acid
DCM	Dichloromethane
Dha	Dehydroalanine
Dhb	Dehydrobutyrine
DNA	Deoxyribonucleic acid
FD	Dichloromethane Fraction
FH	Hexane Fraction
FM	50% Methanol Fraction
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence
HSQC-TOCSY	Heteronuclear Single Quantum Coherence- Total Correlation Spectroscopy
IAA	Indole-3-Acetic Acid

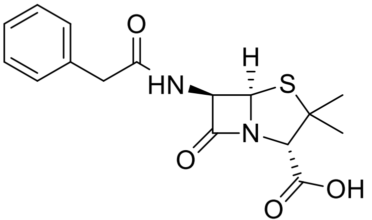
IC_{50}	50% Inhibitory Concentration
ISR	Induced Systemic Resistance
(ISP)2	International Streptomyces Protocol media 2
Lan	Lanthionine
Lantibiotics	Lanthionine- containing antibiotic peptides
LAPs	Linear Azoline Peptides
LC-MS	Liquid Chromatography- Mass Spectrometry
LPS	Lipopolysaccharides
MeLan	Methyllanthionine
MeOH	Methanol
NMR	Nuclear Magnetic Resonance Spectroscopy
NRPS	Non-Ribosomal Peptide Synthase
NRPs	Non-Ribosomal Peptides
m/z	mass-to-charge ratio
rRNA	Ribosomal Ribonucleic Acid
RPs	Ribosomal peptides
RiPPs	Ribosomally synthesized and Post-translationally modified Peptides
SF	Sephadex Fraction
TCE	Total Crude Extract
TSBY	Tryptic Soy Broth Yeast media
TOCSY	Total Correlation Spectroscopy
WB	Water/ Sec-butanol Fraction

CHAPTER ONE

1.0 INTRODUCTION

Current statistics provide evidence for the fact that, antimicrobial resistance continues to increase worldwide, while the prospective pipeline molecules for the development new antibiotics is rapidly depleting.[1] What is frightening is the fact that, antimicrobial resistance represents a phenomenon that is bound to continue for as long as antibiotics are deployed for the treatment of infections in both humans and animals. Bacterial infections that once were easily treated are becoming untreatable due to over- prescription of antibiotics, patient's inability to finish the entire prescribed course of antibiotics, overuse of antibiotics in livestock and fish farming, poor infection control in health care settings, poor hygiene and sanitation and absence of new antibiotics or the eminent prospects of rapid development of new ones.[2] The pharmaceutical giants are reluctant to invest heavily in antibiotic drug development due to dwindling economic incentives or rewards and current challenging regulatory requirements.[3,7]

Nevertheless, it appears that, the only long term solution to the problem of antibiotic resistance apart from the strict prescription regulations and usage is to keep the drug development pipeline full with molecules that can easily be further developed and called upon in the event of major infection outbreaks.[4] The philosophy behind this solution is that, different antibiotic scaffold structures have different timelines for the possible development of resistance after they are introduced in to the clinic.

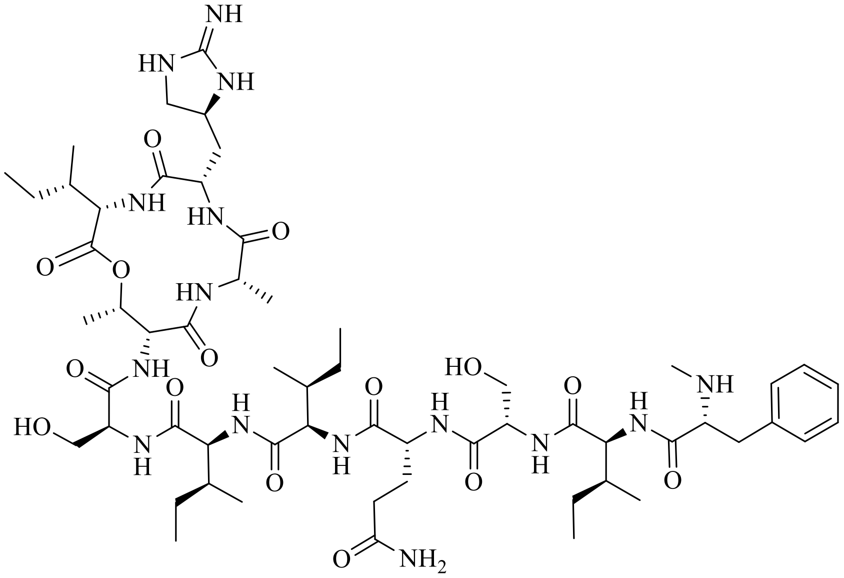


penicillin G
Penicillium chrysogenum

Figure 1: Structure of penicillin G

Penicillin was discovered by Alexander Fleming in 1928 as the first microbial derived antibiotic against gram positive bacteria, was accepted in clinical use in 1940, first incidence of resistance by *Staphylococcus aureus* was reported in 1942.[110]

For molecules like penicillin G, the appearance of resistance strains after introduction of this drug into the clinic was historically very rapid while molecules like teixobactin will most probably be in prescription for a longer time.[5]



Teixobactin
Elef theria terrae
Novel peptide antibiotic

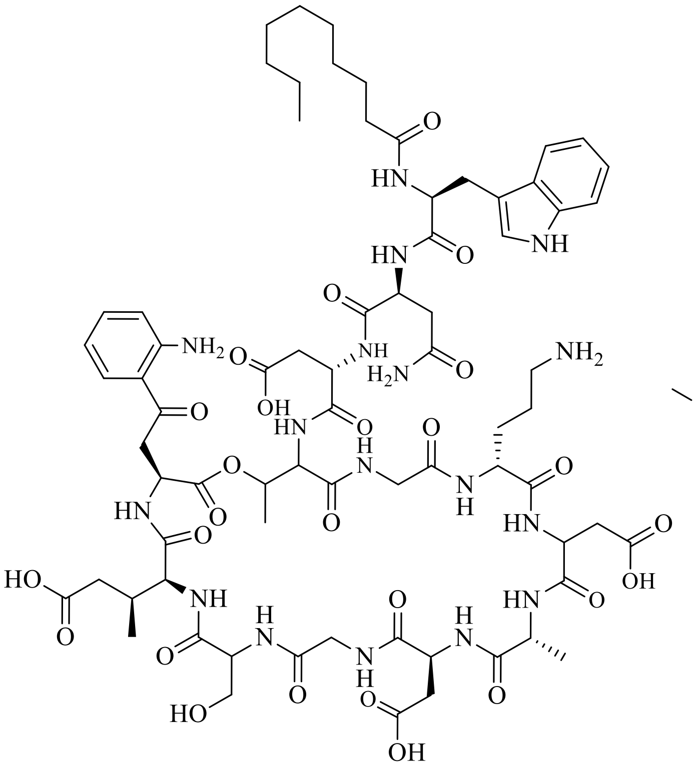
Most commonly-used small molecule antibiotics strongly inhibit single key steps in the growth and proliferation of pathogens and as a result, the disease agents are very quick to change or switch off this single target or in some instances, they proceed to activate efflux mechanisms that pump out the drugs, thereby resulting in the development of resistance to the drug.[6] Hence, the ideal antibiotic should be structurally large and heavily multifunctionalized in order to be able to inhibit multiple steps in the infection progression pathways, thereby, providing little room for the development of resistance. Such intelligent antibiotic drug design involving multiple less stringent inhibitory steps is beyond conventional synthetic drug design and requires natural evolutionary wisdom commonly found in bacteria.[6]

The concepts explained above are responsible for the current huge interests in deploying bacterial protein/peptide drugs which possess multiple domains as our future antibiotics compared to the small molecule compounds that are currently common.[6] These interests were initially hampered by the difficulty to detect and isolate protein/peptides from microbes and the eminent difficulty in solving their structures coupled with the unique physicochemical and pharmacokinetic properties they present.[7] However, current advances in mass spectrometry, HPLC chromatographic procedures, whole microbial genome sequencing, 1D and 2D-NMR techniques, bioinformatics and widespread databases provides the full potential to effectively explore microbial AMPs as future drugs.

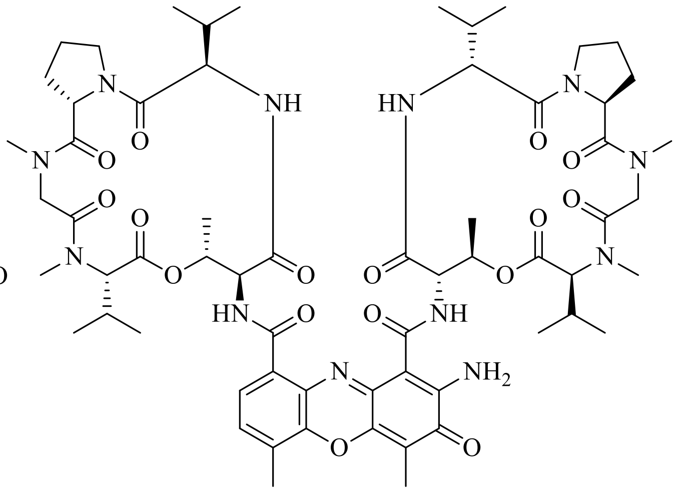
Antimicrobial peptides (AMPs) are also known as defense peptides due to their natural role as agents that are produced by microbes to kill pathogens. Generally, apart from protecting the producing bacteria, AMPs also serve as good arsenals for competing for substrates and space amongst microbes.[8] Against the backdrop of rapidly increasing resistance development to conventional antibiotics all over the world, attempts to bring AMPs into clinical use as more potent and effective antibiotics are accelerating. [8-12]

There are two main types of AMPs and these are ribosomal peptides (RPs) and non-ribosomal peptides (NRPs). The main difference between these two groups of peptides is that; the biosynthesis of ribosomal peptides draws on the twenty conventional amino acids and any post translational modifications thereof. However, the biosynthesis of non-ribosomal peptides, draws from an increasingly large reservoir of amino acids (>500) most of which are already modified and capable of further modification after translation.[13]

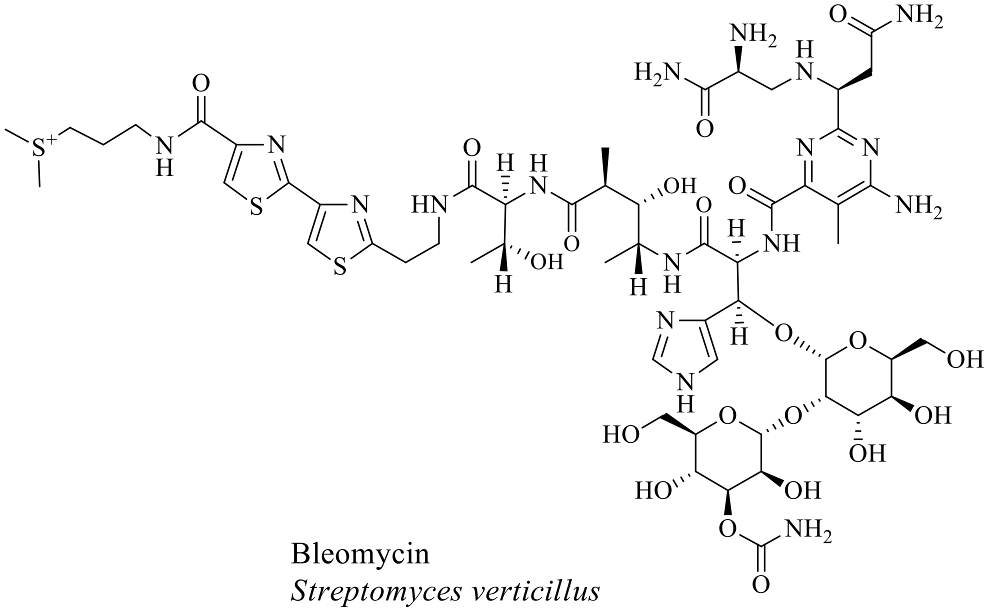
In our pursuit for possible future potent antibiotics, non-ribosomal peptides play a major role due to the fact that the genomes responsible for their biosynthesis have undergone millions of years of evolution that provides huge chemical diversity and multiple target specificity which is very well suited for future drugs.[9] Furthermore, in looking for pharmaceutically important non-ribosomal peptides, the genus *Streptomyces* has taken centre-stage due to the fact that; they produce over 80% of the current antibiotics in the clinic.[14] It is envisaged that, *Streptomyces* is ubiquitous environmentally as a result of their ability to produce antimicrobial peptides, most specifically, non-ribosomal peptides. Several non-ribosomal peptides possessing many different bioactivities including antibacterial, antiparasitic, antifungal, antiviral, immunosuppressant and anticancer have been isolated previously from the genus *Streptomyces.* Daptomycin, actinomycin D and bleomycin are classical examples of non-ribosomal peptides isolated from *Streptomyces* species with variant biological activities and are in clinical use as antibiotic, antitumor and anticancer agents respectively.[15, 16]



Daptomycin
Streptomyces roseosporus
Lipopeptide Antibiotic



Actinomycin D
Streptomyces parvullus
Anti tumor agent



Bleomycin
Streptomyces verticillus
Anti cancer agent

Figure 2: Some non-ribosomal peptides in clinical use produced by genus *Streptomyces*.
Bleomycin is used in treatment against testicular carcinoma and malignant lymphomas. Actinomycin D is administered intravenously and used in the treatment of gestational trophoblastic disease (GTD). Daptomycin is also used in treatment of systemic and life-threatening infections caused by Gram-positive bacteria.[90-92]

In this thesis, we isolated a novel Ghanaian *Streptomyces* sp. strain DE2E from the rhizosphere soils of a *Pterocarpus santalinoides* tree, growing in the wetland areas of the Digya National Park, in the Brong Ahafo Region. Using a sophisticated mixture of culture and growth, incubation conditions, followed by extraction of microbial metabolites with HP-20 resin, a modification of the Kupchan solvent partitioning process, Sephadex LH-20 size exclusion chromatography and HPLC, we isolated five non-ribosomal peptides from the DE2E strain. The structure determination of the largest member (*m/z*) of this group alongside its bioactivity is reported in this thesis.

1.1 Problem statement

The emergence of multi drug resistant pathogens has made conventional antibiotics ineffective in treating many infectious diseases at very alarming rates in the clinics globally, for the reason being that, small molecule antibiotics are rapidly getting susceptible to resistant bacteria pathogens.

Contrastingly, large molecules; structurally exemplified by Antimicrobial peptides (AMPs) are highly functionalized and therefore are able to inhibit bacteria pathogens effectively making them potent alternatives to conventional small molecule antibiotics.[8,12] Additionally, the genus *Streptomyces* has substantially produced over 80% of clinically useful antibiotics[14] and thus, the urgent quest for new and robust antibiotics to replace clinically failing conventional small molecule antibiotics, will be more rewarding if large molecules especially non-ribosomal peptides are discovered and characterised from the genus *Streptomyces* for possible development as effective and non-susceptible antibiotics against multi-drug resistant bacteria pathogens.

1.2 Overall goal

The current project aims to investigate the Ghanaian novel *Streptomyces* sp. strain DE2E for its capacity to biosynthesize interesting secondary metabolites such as NRPs, isolate novel non-ribosomal peptides produced by the strain, determine their structures using a combination of spectrometric and spectroscopic techniques and conduct a detailed study of the biological activity of the pure isolated compounds for possible antibiotic and antiparasitic activity.

1.3 Hypothesis

Since the genus *Streptomyces* has overwhelmingly produced over 80% of antibiotics used in the clinics[14] globally, it implies that, talented strains from this genus are more likely to be a viable source for the discovery of large molecules such NRPs which are considered to be more robust and effective against multi-drug resistant bacteria pathogens with low susceptibly rates for development as ideal future antibiotics urgently needed in the clinics.

1.4 Specific objectives

a) To collect soil sediments from different wetland locations in the Brong-Ahafo region along the River Volta.

b) To screen soil samples for novel strains of Actinomycetes bacteria using selective growth media conditions.

c) To perform a detailed study of the chemical profile of pure microbial strains by small scale culture followed by extraction of metabolites and subsequent spectrometric analysis and identification of novel metabolites.

d) To prepare large scale cultures of high priority strains using a sophisticated mixture of culture and growth, incubation conditions, in order to obtain isolable amounts of secondary metabolites.

e) To extract secondary metabolites produced and to subject the total crude extract to a modification of the Kupchan solvent partitioning process.

f) To investigate, track, isolate and purify novel compounds through thin-layer chromatography (TLC), column chromatography and high performance liquid chromatography (HPLC).

g) To characterize novel compounds using a combination of mass spectrometry, 1D and 2D NMR techniques.

h) To screen pure compounds for antibiotic and antiparasitic activity.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 A brief taxonomy of the genus *Streptomyces*.

Bacteria are believed to be the first forms of cellular life on Earth having existed about 3.5 billion years ago. Centuries of growth and evolution has led to an explosive bacterial biodiversity to this present day.[17] Bacterial taxonomic classification has also evolved over the years as new insights and knowledge into bacterial strains emerge with advancing scientific technologies. Currently, bacteria taxonomy is based on morphological classification, chemotaxonomic classification and molecular classification which incorporates genome sequencing, 16S rRNA gene analysis and DNA-DNA hybridization.[18-19] This bacterial taxonomic approach has enabled the detailed study and investigation of bacterial strains much easier. The domain bacteria with the kingdoms Archaebacteria and Eubacteria have about 30 different phyla established.[18] The phylum Actinobacteria is one of the largest taxonomic units recognized and thoroughly investigated within the Bacteria domain. The phylum Actinobacteria is subdivided based on its branching position in the 16S rRNA phylogeny but because rRNA sequences do not categorize appropriately between closely related species or genus sometimes creates ambiguity in their classifications.[18] The phylum Actinobacteria is subdivided into six classes namely; Thermoleophilia, Nitriliruptoria, Actinobacteria, Acidimicrobiia, Rubrobacteria and Coriobacteriia. The class Actinobacteria contains 16 orders, including Bifidobacteriales and Actinomycetales.[18,20] The order Actinomycetales is currently restricted to the members of the *Actinomycetaceae* family, however about 81% of the 53 families within the phylum Actinobacteria are designated to the class Actinobacteria with the remaining 10 families distributed among other five classes. The family *Actinomycetaceae* is very well dominated by the widely studied and researched genus, *Streptomyces*.[21-22]

2.1.1 Taxonomic classification of the genus *Streptomyces*

Domain:	Bacteria
Kingdom:	Archaebacteria/Eubacteria
Phylum:	Actinobacteria
Class:	Actinobacteria
Order:	Actinomycetales
Family:	Actinomycetaceae
Genus:	Streptomyces

2.2 A brief introduction to the genus *Streptomyces*

Streptomyces constitute the largest population of the class Actinobacteria and the family *Streptomycetaceae*. They largely dominate microbial soil populations, evident by the fact that this genus accounts for over 95% of all soil dwelling isolated Actinomycetales strains.**[17-18]** Apart from soil habitats, *Streptomyces* just as other Actinobacteria are free living micro-organisms that are variedly distributed in both terrestrial (soil and air) and aquatic (marine and fresh water) ecosystems.**[23]** However, *Streptomyces* are predominantly saprophytic soil dwelling organisms that spend the majority of their life cycles as semi-dormant spores. They are present both on the soil surfaces and at depths of about 2m and grow very well in alkaline soils rich in organic matter with population densities varying from $10^6 – 10^9$ cells per gram of soil.**[18]** The genus *Streptomyces* was introduced for the first time by Waksman and Henrici in 1943.**[23]** They are Gram-positive bacteria most widely reported and well investigated genus of the Actinomycetes with over 500 species of

Streptomyces have been classified to date. *Streptomyces* structurally resemble filamentous Fungi[14,23] but their morphological differentiation involves the formation of a layer of hyphae that can differentiate into a chain of spores which is unique among Gram-positives requiring a specialized and coordinated metabolism. Sporulation in *Streptomyces* is key to their survival in hostile environments. The sporulation pigments produced by the *Streptomyces* serves as toxins to kill or neutralise invading pathogens in their habitat, even sometimes organisms of the same genus.[14] *Streptomyces* are vital in soil ecology because they can bio-degrade nutrients; in particular is their ability to hydrolyze a variety of polysaccharides (cellulose, chitin, xylan, and agar) and other natural polymers.[25] The life cycle of the multicellular mycelial *Streptomyces* begins with the germination of a spore that grows out to form vegetative hyphae, after which a process of hyphal growth and branching results in an intricately branched vegetative mycelium under favourable environmental conditions. The DNA of *Streptomyces* comprises about 70% Guanine – Cytosine (GC) base pairs content compared with other bacteria such as *Escherichia coli* with 50% Guanine – Cytosine (GC) content.[14] *Streptomyces* are characterised by a complex secondary metabolism system which accounts for their most interesting property; that is their ability to produce bioactive secondary metabolites (natural products) with antibacterial, antifungal, antiviral, anti-hypertensive, immunosuppressant, anticancer properties and other applications such as bio-insecticides and bio-herbicides. These metabolites are inherently produced by *Streptomyces* as part of their defense mechanisms for survival against other invading micro-organisms.

2.3 Antibiotics production in *Streptomyces*

Streptomyces are among nature's most competent chemists highly capable of producing a stunning plethora of bioactive secondary metabolites which are of great interest in medicine, biotechnology and agriculture industries.[18,23] *Streptomyces* are of great significance to microbial natural product drug discovery because they are among the most numerous, most versatile and prolific antimicrobial producing micro-organisms with a high production rate. Many of their metabolites are potent antibiotics,[14] which has made the genus, the primary 'antibiotic producing factories' for pharmaceutical, veterinary and agricultural applications. *Streptomyces* are the main producers of clinical useful antibiotics and are accredited with over 80% of all antibiotics discovered from Actinobacteria. The firsts antibiotics discovered from Actinobacteria were actinomycin produced by *Streptomyces antibioticus* in 1940,[26] streptothricin from *Streptomyces lavendulae*[27] in 1942 and streptomycin from *Streptomyces griseus* in 1944.[28]

The stunning ability of *Streptomyces* to produce very unique, structurally diverse and potent antibiotics paved way for scientist to intensify their investigation into the genus for their antibiotic production. The most productive years of antibiotic discovery in the genus were between 1945 and 1960 known as the 'golden age' of the antibiotics discovery where a large repertoire of antibacterial agents in most chemical forms were discovered.[29,30]

Antibiotic production in *Streptomyces* is species specific, some strains are adapted to produce a single antibiotic whiles other can produce multiple antibiotics. The production of antibiotics is important for *Streptomyces* as this is key for strain survival in their habitat as they compete with other microbes they come in contact with[14], such that under adverse growth conditions, such as nutrient depletion, possibly causes *Streptomyces* vegetative mycelia to differentiate to form erected sporogenic aerial hyphae structures and thus, scientist are of the view that, it is this period in the life cycle of *Streptomyces* that most antibiotics are biosynthesized.[18]

This is well explained by the fact that, *Streptomyces* are mostly sessile organisms; when nutrient depletion sets in, their vegetative mycelia are programmed to autolytically degraded in a "cell death like" mechanism thereby enabling them to acquire the nutrient materials needed to erect a second mass of mycelium prompting the *Streptomyces* strains to accumulate amino acids, amino-sugars, nucleotides, and lipids they require. As a result, competition arises inevitably from attracted motile microbes in the habitat causing the *Streptomyces* strains to produce and secrete bioactive metabolites to protect the large pool of nutrients they have accumulated.**[18]** Tapping into this large reservoir of antimicrobial metabolites produced by *Streptomyces* has provide the foundation for most of the chemotherapeutic arsenals available to contemporary medicine.

Understanding the antibiotic production mechanisms of *Streptomyces* has greatly improved with current state of the art technologies available to scientist to underscore the biosynthetic machinery behind the splendid antibiotics production by *Streptomyces*. Modern and advancing technologies such as the whole genome sequencing and annotation has unveiled new insights into untapped reservoir of novel *Streptomyces* strains and putative/cryptic biosynthetic gene clusters that may lead to novel and robust antibiotics production to augment urgent clinical demands.**[31-33]** Researchers have wondered for years at the ability of single *Streptomyces* strains to produce a plethora of different bioactive compounds. For example, a prominent *Streptomyces* strain, *Streptomyces coelicolor* produces several bioactive compounds including actinorhodin, undecylprodigiosin, and methylenomycin. However, genomic data from *Streptomyces coelicolor* (used for microbial genome studies) revealed over twenty biosynthetic gene clusters encoding some bioactive compounds, reinstating that the strains inherent potential as a producer of bioactive compounds has been underestimated.**[14,23]**

Antibiotic producing capabilities of *Streptomyces* species conclusively is the norm rather than the exception. Some *Streptomyces* sp. are believed to be harbouring more than fifty different biosynthetic gene clusters**[14]** encoding different bioactive compounds. It therefore appears that the capacity of this genus for prospective novel antibiotics discovery and development is much greater than originally anticipated. Antibiotics from *Streptomyces* can be grouped into four major classes namely; peptides, polyketides terpenes and oligosaccharides.

2.4 Antimicrobial peptides (AMPs) produced by *Streptomyces*

Streptomyces produce a wide spectrum of antibiotics with different chemical forms, structural diversity and mechanisms of action. Among these unique scaffolds of antibiotics are Antimicrobial peptides (AMPs) which are considered as endogenous peptides[10] with molecular size less than 10 kDa.[34] They are produced by several organisms as an essential component of their innate immune response. These peptides can have broad activity to directly kill bacteria, yeasts, fungi, viruses and even cancer cells but primarily deployed as antibiotics to defend against potential pathogenic microbes, however, some microbes also produce AMPs to defend their environmental niche.[10,11] AMPs are biological molecules which usually function through a defined high-affinity antimicrobial target which makes them induce less resistance in micro-organisms unlike conventional antibiotics.

AMPs have been found to exert multiple antimicrobial activities that might provide a strategy to prevent bacteria from developing resistance.[35] Apart from directly attacking microbes, AMPs can confer protection by alternative mechanisms, such as maintenance of normal gut homeostasis and modulation of host inflammatory responses[36] such that these peptide molecules are currently receiving considerable attention from researchers in diverse aspect of science ranging from rational drug design to nanomaterials.[37] As viable antibiotic producers, *Streptomyces* species are able to biosynthesise highly functionalized and potent peptides, being able to integrate both proteinogenic and non-proteinogenic amino acids for their AMPs biosynthesis. These peptide molecules are also able to undergo bio-transformations such as post-translational modification to enhance structural diversity which greatly impacts their biological activities thereof.[38] Peptide compounds produced by *Streptomyces* are primarily of two origins; ribosomally synthesized and post-translationally modified peptides (RiPPs) or non-ribosomal peptides (NRPs). The biosynthetic machinery of both ribosomal peptides (RPs) and non-ribosomal peptides, including their sources, structural complexity and elucidation and reported bioactivities are discussed in the subsequent sections.

2.5.0 Ribosomally synthesized and post-translationally modified peptides (RiPPs)

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a large group of structurally diverse natural products. Unlike non-ribosomal peptides (NRPs) the ribosomal peptide (RP) natural products cannot integrate amino acids beyond the 20 classical proteinogenic L - amino acids, limiting diversity in their structures to some extent. [39-41] However, they can undergo comprehensive post-translational modifications that brings excellent structural features to their biosynthesised products with resemblance to NRPs.[39] These structural modifications enable ribosomal peptides to harness an expanded chemical space mostly available to NRPs, thereby equipping ribosomal peptides with unique structural features that increases their capacity to exhibit diverse biological activities such as antifungal, antibacterial and antiviral activities. Collectively, RiPPs represent an untapped source of chemical moieties with a vast potential for possible medicinal/pharmaceutical applications.[41] For example, research conducted on RiPPs over the past decade has led to the identification of novel chemical scaffolds for possible drug development and the characterization of unique biochemical transformations that are potentially useful for chemo-enzymatic applications.[40] Ribosomal peptide biosynthesis commences with the ribosomal production of a precursor peptide usually containing an N-terminal leader-peptide sequence and a

C-terminal core region harboring the different post-translational modification sites.[41] In some cases, a C-terminal recognition sequence is needed for biosynthesis. The biosynthetic machinery as illustrated in **Figure 3** recognizes the leader peptide and installs a myriad of different post-translational modifications in the core region.

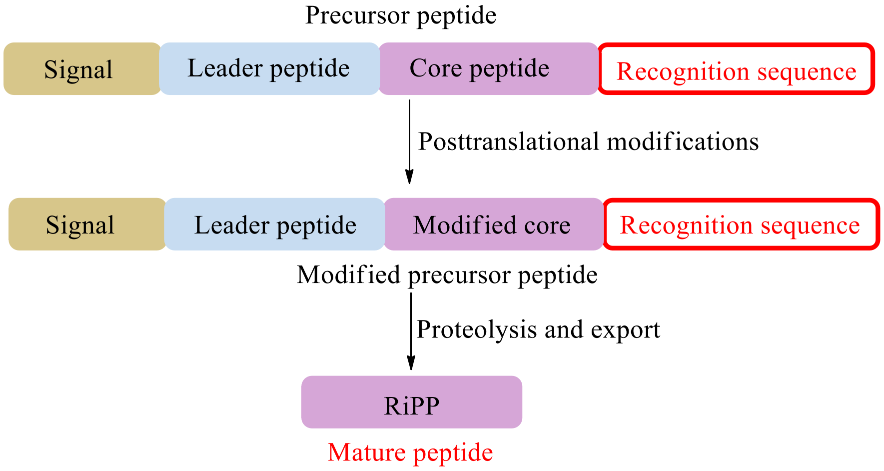


Figure 3: General biosynthetic pathway for RiPPs
The precursor peptide contains a core region that is transformed into the mature product. Many of the posttranslational modifications are guided by leader peptide and the recognition sequences. for subclasses of RiPPs. Products of eukaryotic origin also often contain an N-terminal signal peptide that directs the peptide to specialized compartments for modification and secretion.[41]

Finally, the leader peptide is removed by one or more peptidases, sometimes concomitant with N to C macrocyclization to yield the mature active peptide compound. However, in some cases, additional post-translational modifications may even occur after the removal of the leader peptide.[41]

Ribosomal peptides are grouped into different subfamilies/classes based on their biosynthetic machinery and structural features. Irrespective of their subfamily, they share several common grounds within their biosynthetic pathways. Some RiPPs subfamilies comprise; Lasso peptides, Lanthipeptides, Thiopeptides, Linaridins, Bottromycins, Sactipeptides, Cyanobactins, Amatoxins and Phallotoxins, Cyclotides, Orbitides, Conopeptides, Microcins, Glycocins, Methanobactin, Streptide and Thioamides. These RiPPs class of compounds are not limited in production to

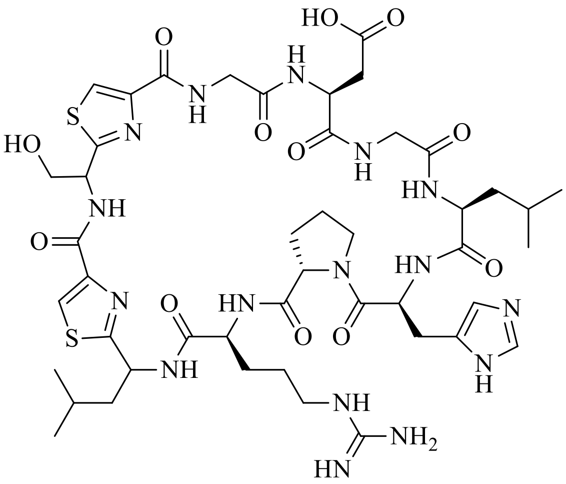
Archaea bacteria such as *Streptomyces* only but are also excellently produced by eukaryotes such as mollucs, plants and fungi.[39,40]

2.5.1 Sources of ribosomal peptides (RPs)

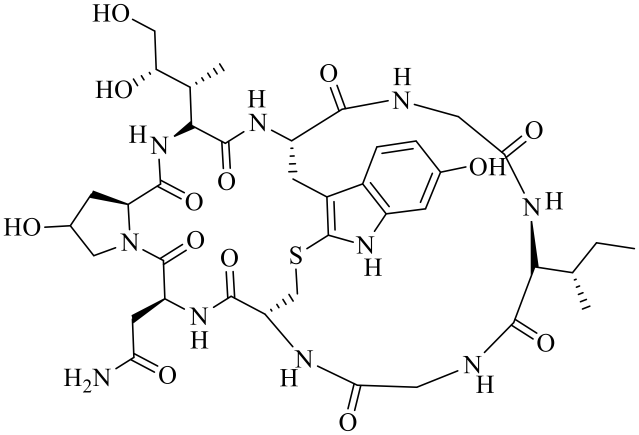
As reported earlier, ribosomal peptides are grouped into subfamilies based on their biosynthetic machinery and structural features. The biosynthetic gene clusters (BGC) encoding RPs are clustered in the organism's genome[40,42], such that, though some RPs biosynthetic gene clusters are variedly distributed among several organisms, there are some BGCs that are limited to a specific group of organisms, thus species specific. Hence, the biosynthesis of RPs can be correlated specifically to the groups of organisms producing them especially the RiPPs class of compounds. Several organisms from bacteria to eukaryotes existing in diverse habitats such as terrestrial, fresh water and marine ecosystems have been reported to produce modified RPs[42] amongst which archaea bacteria are the major producers of RiPPs subfamily compounds lanthipeptides.[41,43] Genome mining database highlights that the BGCs for lanthipeptides biosynthesis are highly distributed in microbes but are predominant in Actinobacteria and Firmicutes whose genus; *Micromonospora*, *Actinobacteria*, *Bacillus* and *Streptomyces* have been investigated to produce bacteriocins or lantibiotics. Linaridins are also mostly produced by Actinobacteria whiles cyanobactins are produced specifically by Cyanobacteria and Ascidians.[41] Actinobacteria are classical producers of thiopeptides, however the genus *Staphylococcus* and *Bacillus* have also been reported to heavily produce several scaffolds of thiopeptides. The bottromycins are original isolated from *Streptomyces bottropensis*, and the Microcins are modified ribosomal peptides produced the class Enterobacteriaceae predominantly *E. coli*. Lasso peptides are overwhelmingly produced by *Streptomyces* and *Rhodococcus* species even though some have also been isolated in

Proteobacteria such as *Escherichia* and *Burkholderia* species. An example is Capistruin; a 19 amino acids residue macrocyclic peptide produced by *Burkholderia thailandensis* E264.**[41]** The RiPPs molecules Microviridins have been isolated from bloom forming freshwater Cyanobacteria such as genus *Planktothrix* and *Microcystis* with the prototypic compound of this class, Microviridin A, isolated from *Microcystis viridis*. Linear azoline peptides (LAPs) are significantly produced by the *Streptococcus pyogenes* but genomic data and other chemical investigations revealed that some strains of the phylum Firmicutes have BGCs responsible for LAPs biosynthesis comprising microbes such as *Clostridium botulinum* and *Listeria monocytogenes*.**[40-41]**

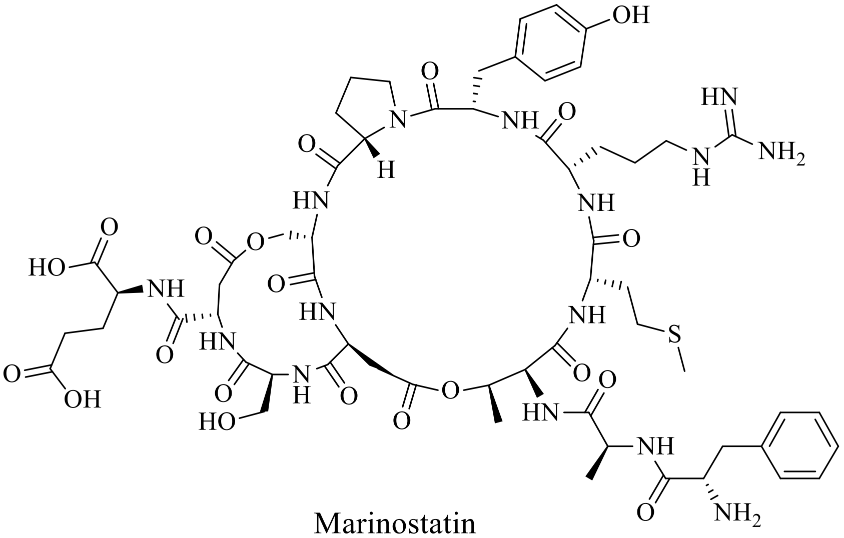
The RiPPs compounds Phallotoxins and Amatoxins are produced by Fungi (mushrooms), mostly among the genera *Conocybe*, *Lepiota*, *Galerina* and *Amanita*. Cyclotides are classes of ribosomal peptides produced by plants whose structures are dominated by a head to tail cyclisation backbone with a cysteine knot sub-structure made of three aligned disulfide bonds. A representative cyclotide ribosomal peptide is Kalata B1 produced *Oldenlandia affinis* (Rubiaceae) which comprises a total 29 amino acids monomers with an iconic six aligned cysteine subunits. Contrastingly, Orbitides are also RiPPs of plant origin but lacks disulfide bonds. They are biosynthesised by at least nine individual plant families including the Annonaceae, Caryophyllaceae, Euphorbiaceae, Lamiaceae, Linaceae, Phytolaccaceae, Rutaceae, Schizandraceae, and Verbenaceae.**[41]** Orbitides typically have sizes ranging from five to twelve amino acid residues. The RiPPs subfamily Conopeptides are derived from the venoms of the predatory marine snails such as those of the genus *Conus* (cone snails). The venom peptides from the Turridae and Clathurellidae families are known to be Turripeptides and Clathurellipeptides respectively. Contulakin G and Conotokin G are bioactive conopeptides isolated from the venom of *Conus geographus* which successfully reached clinical trials for pain and epilepsy treatments.**[41]**



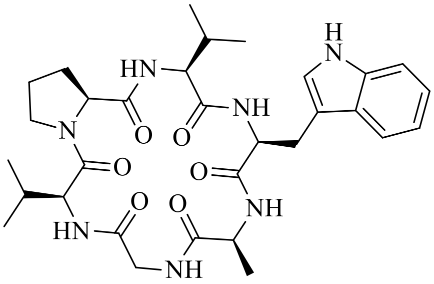
Trichamide
Cyanobacteria sp.
Cyanobactin



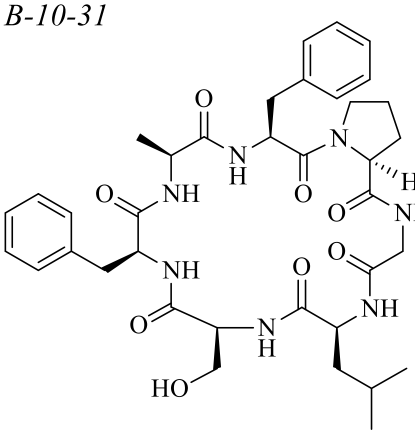
Amanitin
Amanita sp.
Amatoxin and Phallotoxin



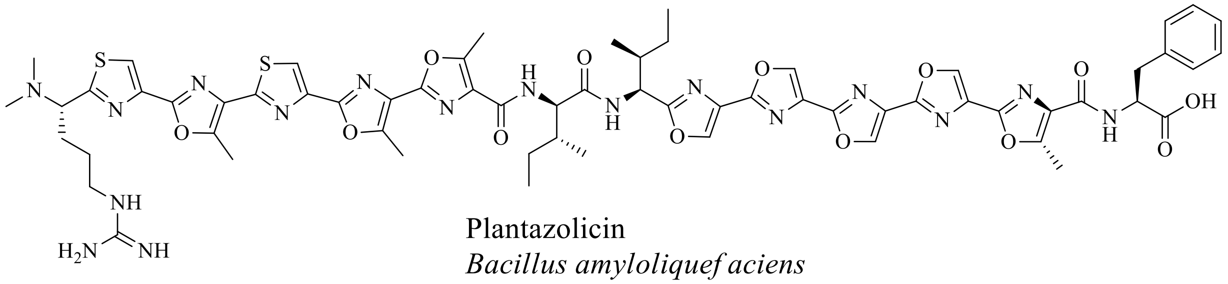
Marinostatin
Alteromonas sp. B-10-31
Microviridin



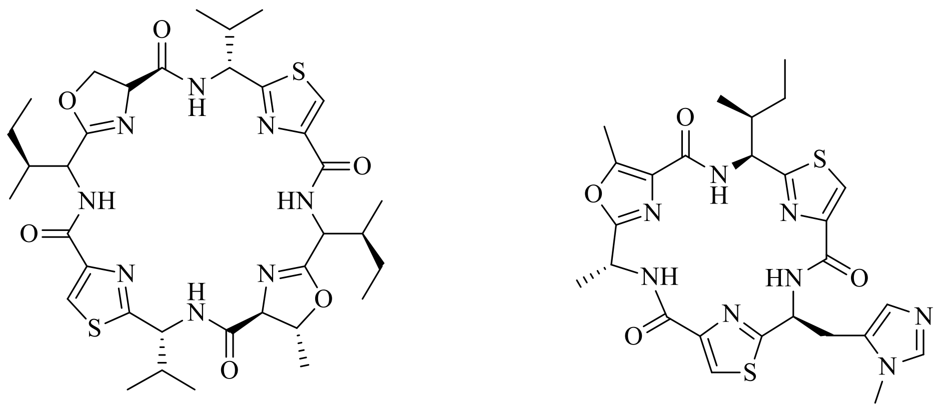
Segetalin A
Vaccaria segetalis (Caryophyllaceae)
Orbitide



Segetalin D
Vaccaria segetalis (Caryophyllaceae).
Orbitide

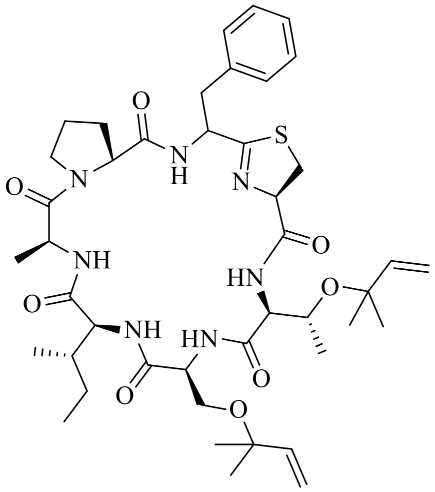


Plantazolicin
Bacillus amyloliquefaciens
Linear azoline peptide



Patellamide A
Cyanobacteria sp.
Cyanobactin

Microcyclamide
Cyanobacteria sp.
Cyanobactin



Trunkamide
Cyanobacteria sp.
Cyanobactin

Figure 4: Representative structures of some RiPPs classes of molecules
Illustrated structures highlights the huge structure complexity and diversity of RiPPs compounds being cyclic or linear with multiple functionalized subunits impacting their biological activities. Their structure complexity is as a result of the integral post-translational modifications during their biosynthesis.[40-41]

2.5.2 Structure complexity of ribosomal peptides (RPs)

Ribosomal peptides biosynthesis incorporates only the 20 classical proteinogenic amino acids which are primarily required for protein synthesis during translational process involving t-RNA molecules or aminoacyl t-RNA synthetase and ribosomes in the cytoplasm or the endoplasmic reticulum.[39,42] Proteinogenic amino acids incorporated for RiPPs biosynthesis are mostly the isomeric L amino acids, and thus the incorporation of just these 20 amino acids for ribosomal peptides biosynthesis may seem a little too simple. However, a critical look at some of the structures of RiPPs compounds illustrated in **Figure 4** are very far from assuming simplicity but rather exude extended levels of structural complexity. Structural diversity and complexity in ribosomal peptides is achieved through a series of extensive post-translational modifications.[39-41] As indicated earlier, the biosynthesis of RiPPs involves the core region of the precursor peptide in the peptide synthesis to be modified through enzymatic transformations some of which are analogous to non-ribosomal synthesis. Ribosomal peptides have unique modifications that enables them to explore an extensive chemical and biological diversity in a manner of resemblance to Non-ribosomal peptides.[39] These modifications also induce rigid conformations that enhances their structural stabilities. Structural modifications in RiPPs can be considered as either side chain or main chain modifications. Side chain modifications include dehydration of cysteine and threonine residues, lanthionine modifications, heterocyclization of cysteine, threonine and serine residues, prenylations, epimerization, amino acid α modifications, formation of disulfide bonds and oxidations either though halogenations or oxygen. Main chain modifications by RiPPs also include proteolysis, macrocyclizations, formylation, nucleotide base incorporation, lactone formation and glycosylations. Some modifications which induces structural diversity/complexity in ribosomal peptides/RiPPs compounds are further discussed and illustrated in the sections below.

2.5.3 Side chain modifications

2.5.3.1 Dehydration of serine and threonine residues

The hydroxyl-bearing amino acids threonine and serine are hydrated by enzymes to give a (*Z*)-2,3-didehydrobutyrine and 2,3 didehydroalanine respectively[46] resulting in an unsaturation at the α-carbon. Dehydrations occurs in many RiPPs including all classes of lantibiotics.[47-50] The dehydration of these amino acids residues makes their olefinic intermediate reactive towards sulfhydryl or amine side groups intra-molecularly to yield other sub-structures in RiPPs chemical scaffolds such as lanthionine, lysinoalanine or aminovinylcysteine [46] as illustrated below.

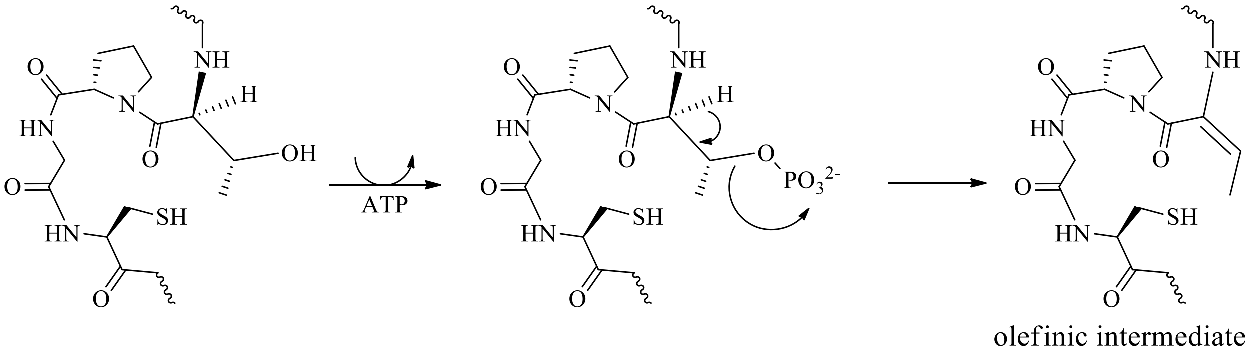


Figure 5: Dehydration pathway of a serine residue to yield the olefinic intermediate

For example, in the biosynthesis of lanthipeptides Subtilin and Nisin, the olefinic intermediate is proposed to react inter-molecularly with thiols sub-units on bacteria membranes.[51] The olefinic product from the dehydration in some cases is post-translationally hydrolyzed such as in synthesis of Lactocin S and Pep5.[52-53] Others involve a hydrolytic deamination of the N-terminus dehydrated threonine or serine to yield 2-oxobutyryl or 2-oxopropionyl groups on the peptides respectively.[39]

2.5.3.2 Lanthionine synthesis

RiPPs that have the lanthionine sub-structure are very common and have been described in several reviews.[46] Precursors for lanthionine synthesis are dehydrated amino acids; didehydroalanine or didehydrobutyrine and a thiol unit originating from the cysteine. This reaction mechanism has been very well demonstrated in the biosynthesis of lantibiotics in which the enzyme regulated attack of the cysteine sulfhydryl unit takes place in a 100% stereo-selective manner as illustrated in **Figure 6** below, producing a (2*S*,6*R*)-lanthionine or (2*S*,3*S*,6*R*)-3-methyllanthionine via serine or threonine residues pathways respectively.[46] Lanthionine bonds contributes to the rigidity of peptides in addition to their resistance proteases which essentially impacts the biological activity of some lantibiotics such as Nisin, Pep5, Lacticin 3147 and Mutacin II.[54-55]

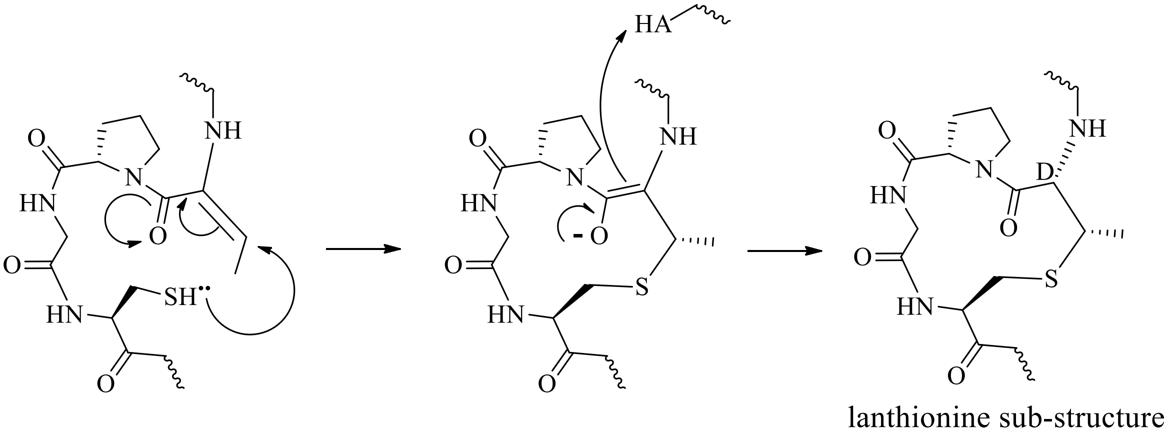
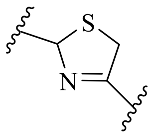
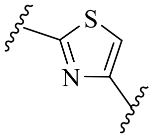


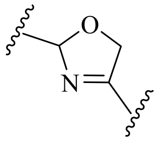
Figure 6: Formation of lanthionine sub-structure via the intermolecular cysteine sulfur attack

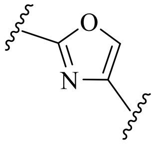
2.5.3.3 Heterocyclization of cysteine, threonine and serine residues

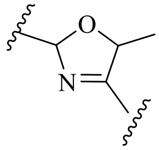
Cysteine, serine and threonine amino acid residues may undergo cyclization evident in many ribosomal peptides and also non-ribosomal peptides. Heterocyclic natural products have gained considerable attention in drug design and development.[56-58] These heterocycles are generated when the thiol units of a cysteine residue or hydroxyl units of serine and threonine reacts with the carbonyl functionality of the nearby amino acid resulting in a thiazoline, oxazoline or methyloxazoline sub-structures respectively. Further oxidation on the α–β connectivity which occurs in some biosynthesis results in a thiazole, oxazole or methyloxazole.[39]

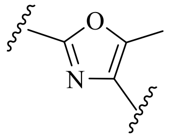

Thiazoline


Thiazole

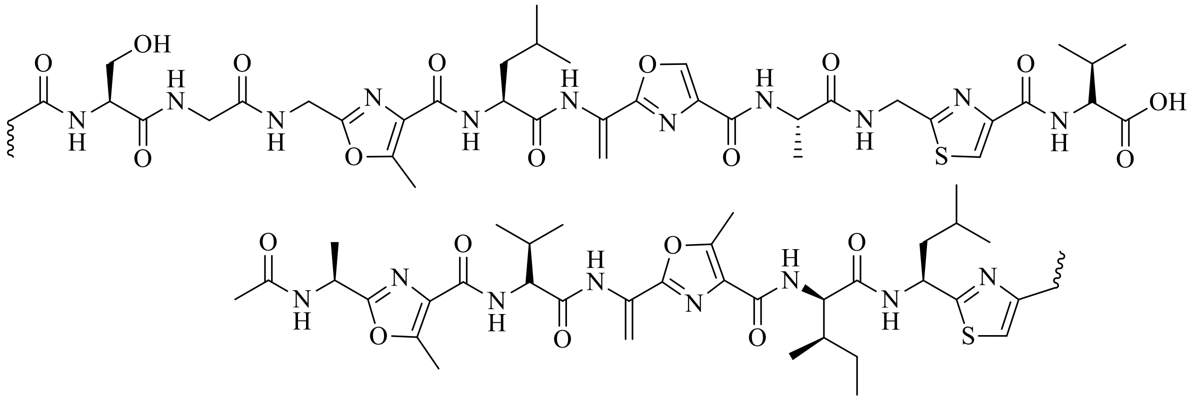

Oxazoline


Oxazole


5-methyloxazoline


5-methyloxazole

Heterocyclization is believed to induce a number of benefits on the ribosomal peptides. First, the making of heterocyclic rings restrains the peptide backbone conformationally.[59-62] Heterocycles also represent unique pharmacophores with interesting interactions between heterocyclic natural products and membrane nucleic acids/proteins.[56] Thiazoles in particular are common in many siderophores which offer the possibility that certain heterocycle-containing RiPPs might serve as natural metal scavengers.[62-64] Oxidation of thiazoline and oxazoline to thiazole and oxazole rings respectively improves structure conformation in the peptide that results in increased biological activities. Heterocyclic RiPPs include; Microcin B, Cyanobactin, Goadsporin, and Streptolysin S.


Goadsporin
Linear azoline peptide
Streptomyces sp.

2.5.3.4 Prenylation

Prenylation of ribosomal peptides enhances their lipophilicity for cellular proteins membrane targeting, inducing target specificity in such molecules. Prenylation involves the addition of isoprene subunits to a core peptide molecule. This structural modification occurs predominantly in peptide natural products. Two categories of prenylation of ribosomal peptides are widely known in literature with the first groups containing the signaling peptides ComX produced by *Bacillus subtilis*.**[65]** This compound comprise a highly conserved tryptophan residue which is farnesylated resulting in the modified prenylated peptides.**[67]** This mechanism illustrated in **Figure 7**, produces a bond between C-1 of the isoprenyl pyrophosphate and C-3 of Tryptophan with an additionally bond formed between the tryptophan N and tryptophan C-2 to form a third ring on the tryptophan residue.**[67,68]**

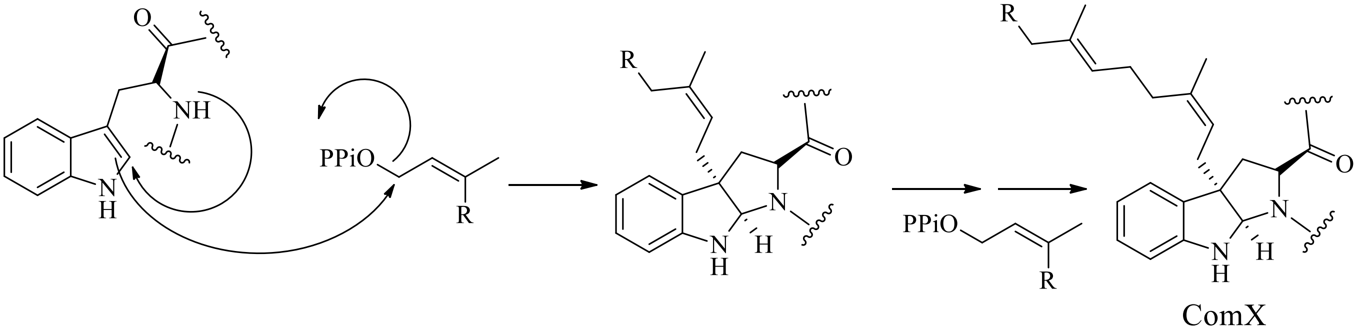
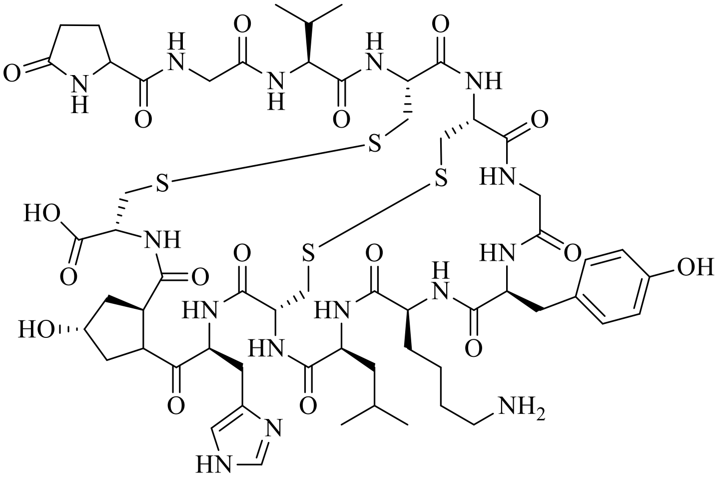


Figure 7: A summarized mechanism showing prenylation in a tryptophan residue to form ComX
ComX sub-structure identified in some ribosomally synthesized and post-translational peptides (RiPPs) is believed to induce cell signaling by binding to, ComP, a cell membrane bound receptor.[67]

The second category includes modification very common to cyanobactin peptides produced by *Prochloron* sp. a Cyanobacteria strain which involves a dimethylallylpyrophosphate pathway to yield a covalent ether connectivity using a serine or threonine hydroxyl groups.**[68]**

2.5.3.5 Disulfide Bridges

Disulfides bridges are arguably the most common structural modifications in peptides. They are predominantly involved in constraining small peptide sequences into rigid potent configurations in ribosomal peptides.**[69]** For example, multiple disulfide bridges occur in RiPPs subfamily conopeptides which often are small peptides but the introduction of disulfide conformations in their structures enhances their exerted biological activities.[70] This is also observed in some class II bacteriocins typified by Enterocin A and cyanobactins including Ulithiacylcamide.[71-72] XEN-2174 produced by venomous mollucs (cono snails) illustrated below is an example of RiPPs molecules with disulfide linkages.



XEN-2174
Conopeptide

Examples of peptides containing disulfide linkages are Sublancin and Plantaricin W. The interruption of disulfide bridges via processes like mutagenesis can reduce their biological activities especially at elevated temperatures which re-emphasizes the primary role of disulfide bonds in most RiPPs is to induce conformational rigidity in the structure of these peptides.[73]

2.5.3.6 Oxidation via halogens or oxygen

Some modifications are rare within the bacterial ribosomal peptides. These include oxidation via oxygen or halogen atoms. Oxidation to substructure carbon atoms have been observed either being induced by attached hydroxyl groups or halogen atoms especially chlorine or bromine. For example, the lantibiotic Microbiospora was recently characterized and its structure revealed oxidative modifications such as mono and di-hydroxyproline, as well as a halogenation on main structure indicated as a 5-chlorotryptophan.[74]

2.5.4 Main chain modification

2.5.4.1 Macrocyclization

Macrocyclization is predominant in ribosomal peptides of bacteria origin just as most eukaryote-derived RPs. For eukaryotes, peptide macrocyclization occurs via an N to C terminal, whereas in bacterial RiPPs amide bonds may be formed either between the N and C termini sometimes with nucleophilic side chain residues.[35] N to C cyclized ribosomal peptides from bacteria exhibit extreme variability in molecular size. For example, known cyanobactins are 6 – 12 amino acids in length while larger bacteriocins such as Uberolysin, Butyrivibriocin AR10, Gassericin A, Circularin A, and AS-48280 are up to 70 amino acids long. Contrastingly, the compounds Microcin J25 and Capstruin are cyclized from their N-termini to the side chain carboxylic acid which is typical of many non-ribosomal peptides.[75]

The cyclic depsipeptides known as Microviridins were found to be ribosomally synthesized and post-translationally modified with side-chain cyclization, containing two ester bonds and one amide bond formed via threonine - asparagine, serine – glutamine and lysine - glutamine crosslinks, respectively. Macrocyclic peptides have been identified to be very rigid and highly stable even in solution such that they only adopt a single or two set conformations predominantly. It is envisaged that the induced macrocyclic rigidity enables these peptides to bind more selectively to their cellular targets and also the fact that macrocyclization may improve therapeutic properties such as being resistant to proteases enzymes.[76]

2.5.4.2 Lactone rings

Ribosomal peptides can be post-translationally modified by forming lactone rings which adds to the structural complexity of peptide compounds. Lactones substructures are usually found among peptides isolated from Gram-positive bacteria such as *Staphylococcus* spp.[77] The lactone ring containing peptides includes the variants of Auto-inducing peptides (AIPs) which are commonly characterized with a 5 to 9 amino acid thiolactone cyclization. The lactone rings are biosynthetically constructed through a cysteine residue from a C terminus attacking a terminating carboxylic unit using its nucleophilic thiol residue, that yields the thiolactone ring. However, serine residues at similar positions to the C terminus performing similar nucleophilic attacks produces lactones rather than thiolactones. The lactone substructures have been identified to essentially exert tremendous biological activity in RiPPs.[78]

2.5.4.3 Glycosylation

The addition of a sugar moiety to peptide is occurs insignificantly among eukaryotes and prokaryotes. A recently discovered example of a ribosomally synthesized glycosylated lantibiotics is the huge 84-amino acid residue antibiotic Microcin E492m.[79] Attached to its C-terminus is a modified serine residue linked to β-D-glucose.

A very unique advantage of the ribosomally synthesized and post-translationally modified peptides (RiPPs) compared to their counterpart non-ribosomal peptide modification is that the RiPPs sequences may be modified by simple manipulation of a few codons to produce novel bioactive compounds compared to the laborious and very extensive genetic engineering that must be applied in non-ribosomal peptide synthesis.

2.5.5 RiPPs subfamilies produced by *Streptomyces* sp. and their reported biological activities

2.5.5.1 Lanthipeptides

Lanthipeptides are a class of RiPPs that are characterized by the presence of the thioether amino acids lanthionine (Lan) or 3-methyllanthionine (MeLan). The first Lanthipeptide, Nisin was discovered as early as 1928 but its structure was elucidated in 1971.**[40,41]** Lanthipeptides possessing antimicrobial activity are known as lantibiotics. The ribosomal origin of lanthipeptides was first acknowledged in 1970 but biosynthetically verified in 1988 when the BGCs for the lantibiotic Epidermin was fully sequenced. Subsequently, the precursor BGCs for the lanthipeptides Subtilin and Nisin were also identified.**[41]**

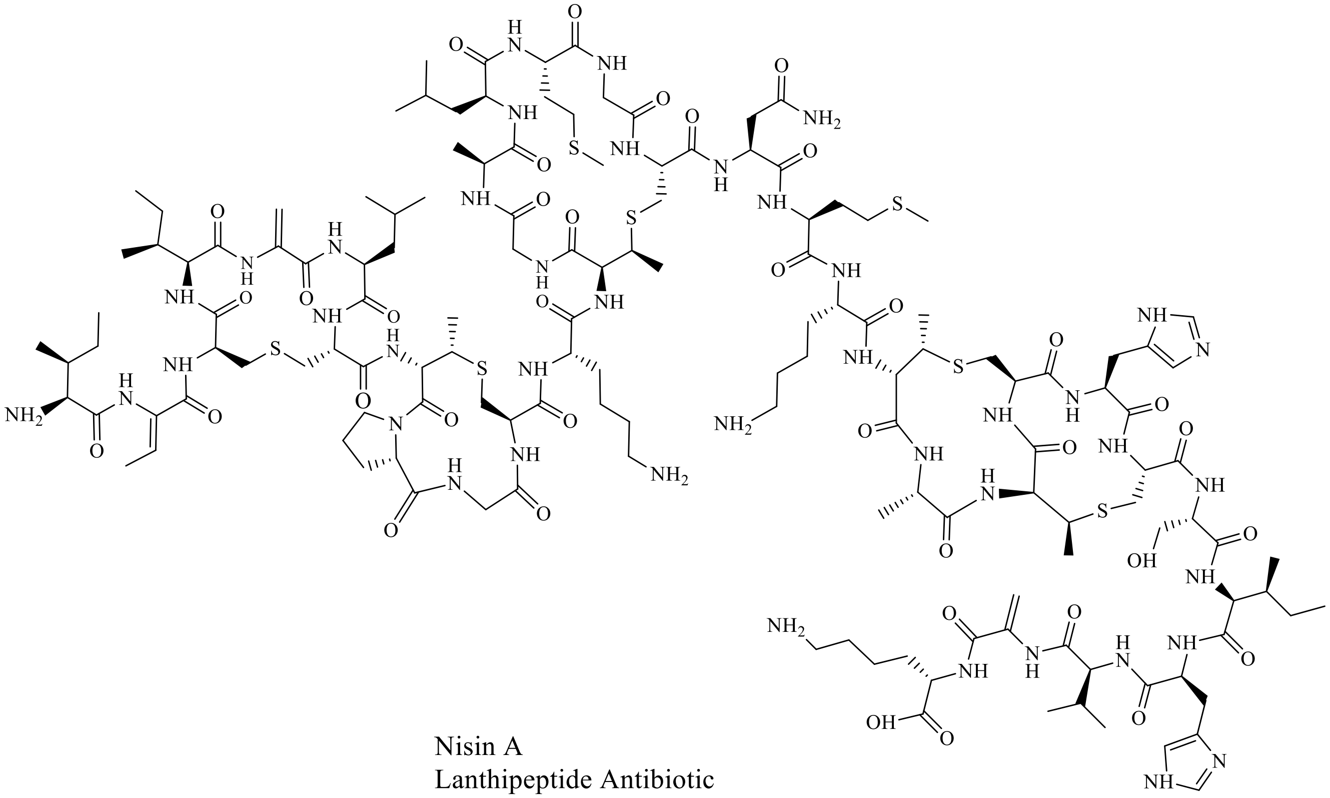


Figure 8: Structure of the lantibiotic Nisin
Nisin sub-structures comprise a meso-lanthionine and 3-methyllanthionine units that defines the classical lanthipeptides substructure (for lanthionine-containing peptides) class of molecules. Nisin is actively used in the food industry as a preservative. Its structure is predominant with aliphatic amino acid residues containing the lanthionine substructure.

Lanthionine consists of two alanine monomers cross-linked through a thioether linkage that connects their β-carbons but 3-methyllanthionine has one additional methyl group to the lanthionine substructure. The lanthionine and methyllanthionine units are introduced in a two-step post-translational modification processes. The first step involves a serine and threonine residues in the precursor peptide which are dehydrated to give a dehydroalanine and dehydrobutyrine units respectively from a phosphorylated intermediate.[40-41] The thioether crosslinks are generated through a Michael-type addition mechanism by cysteine residues onto the dehydrated amino acids. Currently, lanthipeptides are grouped into four main classes; lanthipeptide I - IV depending on the enzymes engaged in their biosynthesis to install the lanthionine/methyllanthionine units and the formation of thioether rings. Lanthipeptides produced by *Streptomyces* species include Ancovenin (*Streptomyces* sp.), Cinnamycin (*Streptomyces cinnamoneus*), Duramycin (*Streptomyces cinnamoneus*), SapB morphogen (*Streptomyces coelicolor*),[80] Avermipeptin (*Streptomyces avermitilis* DSM 46492) and Streptocollin (*Streptomyces collinus* Tü 365).[41,43]

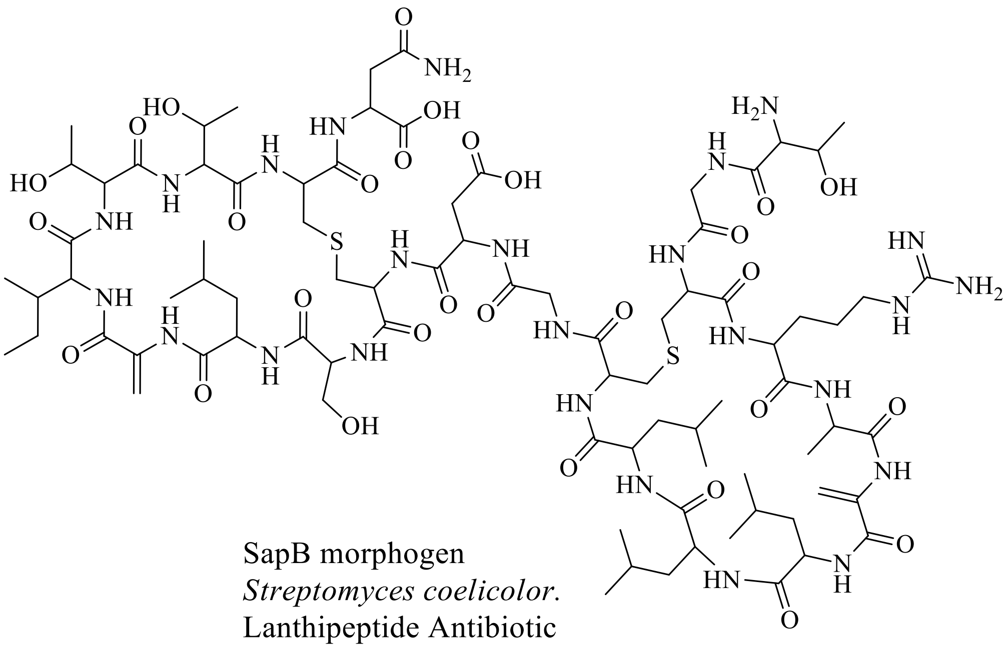
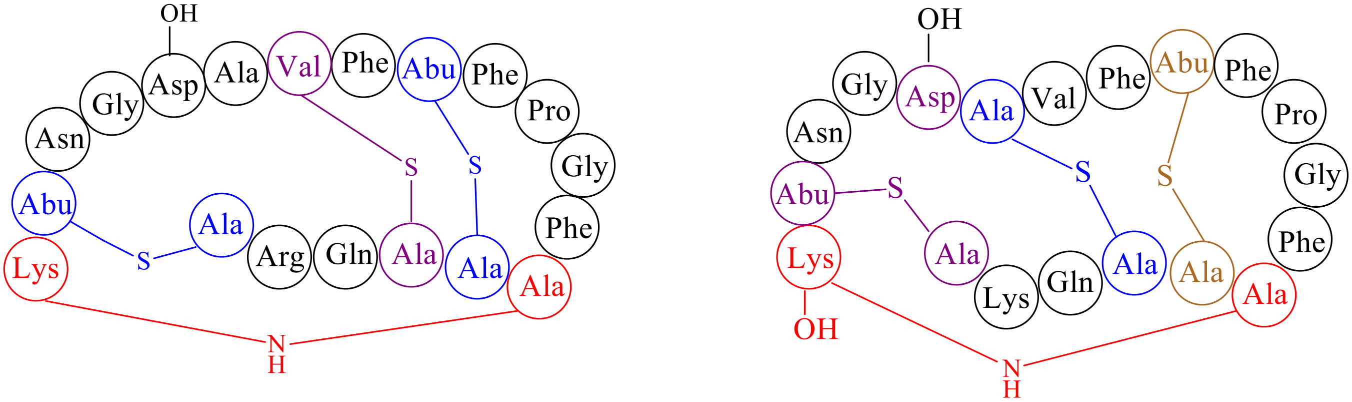


Figure 9: Structure of SapB morphogen

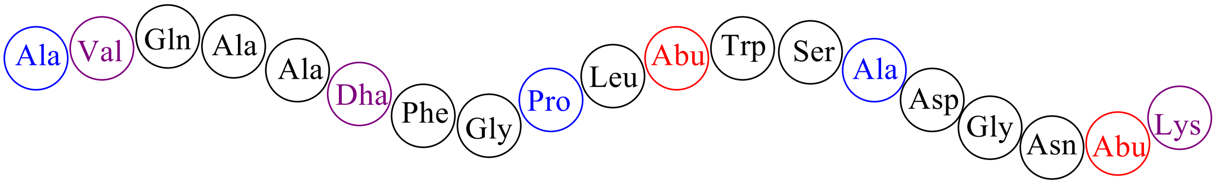
SapB morphogen belongs to the lanthionine-containing morphogen family. The structure highlights the complete aliphatic nature of this compound with no aromatic residues present. SapB morphogen peptide is crucial in aerial mycelia formation in *Streptomyces coelicolor.*[80]

Cinnamycin, duramycin and ancovenin form a group of natural variants that present other biological activities in addition to a weak antimicrobial action. These peptides exhibit a similar globular structure with one lanthionine, two methyllanthionine and an unusual lysinoalanine bridge between lysine and serine. They also exhibit a modification in position 15, an aspartate hydroxylation yielding the erythro-3-hydroxy-aspartic acid. Ancovenin is the most different variant, neither presenting the aspartate 15 modification nor the lysine–alanine bridge.[41,43]



Cinnamycin
Streptomyces cinnamoneus
Lantibiotic

Duramycin
Streptomyces cinnamoneus
Lantibiotic



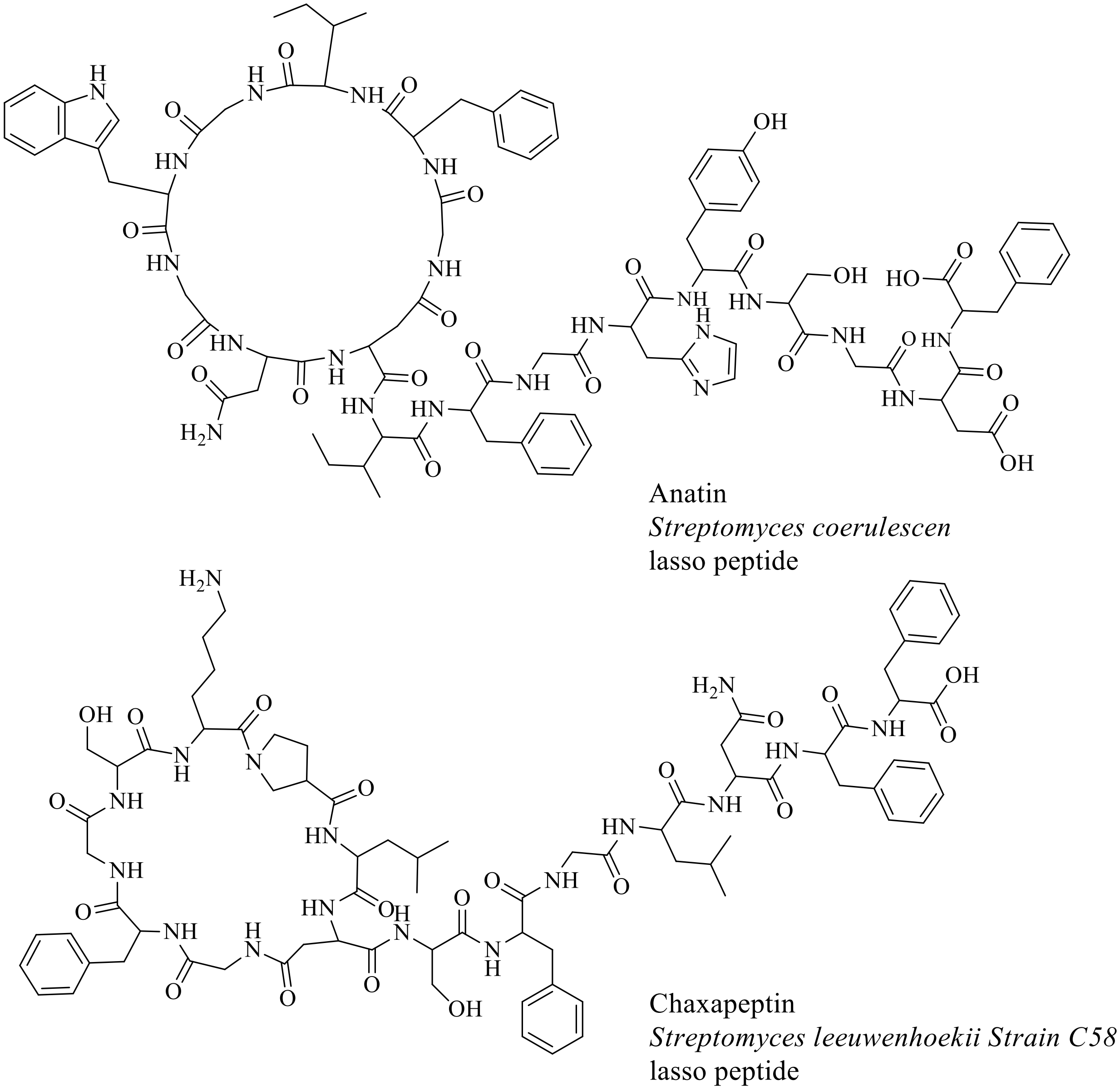
Ancovenin
Streptomyces sp.
Lantibiotic

Figure 10: Modular structures of the lantibiotics; cinnamycin, duramycin and ancovenin
Cinnamycin, duramycin and ancovenin have almost the same amino acid composition but differs in about six amino acid residues. Even by observation of their structures, Cinnamycin and duramycin can be considered variants of each other. Duramycin and cinnamycin are all 19 amino acids residue in length comprising one lanthionine and two methyl lanthionine rings in very conserved positions.[111]

Cinnamycin is produced exclusively by *Streptomyces* even though it has also been reported in *Streptoverticillium* species. Cinnamycin inhibits the growth of *Bacillus subtilis*, anaerobic bacteria, fungi and yeasts. This lanthibiotic has also been reported to inhibit the proliferation of herpes simplex virus (HSV) type 1.[43] Duramycin has an inhibitory action against *Rhodococcus fascians*, that infects a wide range of plants (phytopathogen) causing a leafy gall formation. Ancovenin and cinnamycin inhibit the activity of the angiotensin-converting enzyme, presenting a potential application in blood pressure regulation. Cinnamycin and duramycin both act as phospholipase A2 (PLA2) inhibitors by binding at a 1:1 ratio and with high affinity and exclusive specificity to the substrate of phospholipase A2.[81] This binding alters the operation of ion channels a feature that is exploited by the pharmaceutical industry for cystic fibrosis treatment. The inhibition of phospholipase A2 by the lantibiotics has also been explored in treatment of diseases such as atherosclerosis, diabetes and cancer. Duramycin is in phase II of clinical trials to be used in the treatment of cystic fibrosis.[82]

2.5.5.2 Lasso peptides

Lasso peptides are a unique class of bioactive ribosomally synthesized and post-translationally modified bacterial peptides that are characterized with the iconic lasso substructure. They are mostly biosynthesised by Actinobacteria including *Streptomyces* and *Rhodococcus*. These peptides consist of 16 to 21 amino acid residues, having an N-terminal macrolactam ring produced from the reaction of the N-terminal amino group with a side chain carboxylate of a glutamate or aspartate occurring at positions 8 or 9.[41] The lactam ring formation irreversibly connects the C-terminal end within the macrocycle resulting in a highly stable and rigid structure that has proven very difficult to obtain by conventional peptide synthesis. Their stable structures accounts for their significantly resistance to enzymatic degradation (proteases) and denaturing agents which correlates to the strong biological activities lasso peptides exhibit. Some lasso peptides produced by *Streptomyces* sp. include Aborycin (*Streptomyces griseoflavus* Tu472), Siamycin I and Siamycin II (*Streptomyces* sp. MS-271), Anantin (*Streptomyces coerulescen*), Sviceucin (*Streptomyces sviceus* ATCC 29083), RES-701-1 (*Streptomyces* sp. RE-701), Sungsanpin (*Streptomyces* sp. SNJ013) and Chaxapeptin, (*Streptomyces leeuwenhoekii* Strain C58).[83]



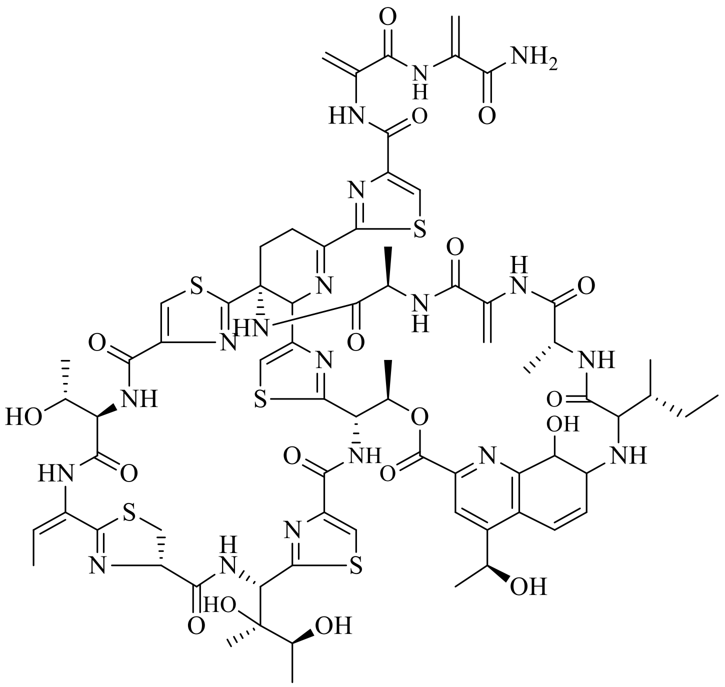
Anatin
Streptomyces coerulescen
lasso peptide

Chaxapeptin
Streptomyces leeuwenhoekii Strain C58
lasso peptide

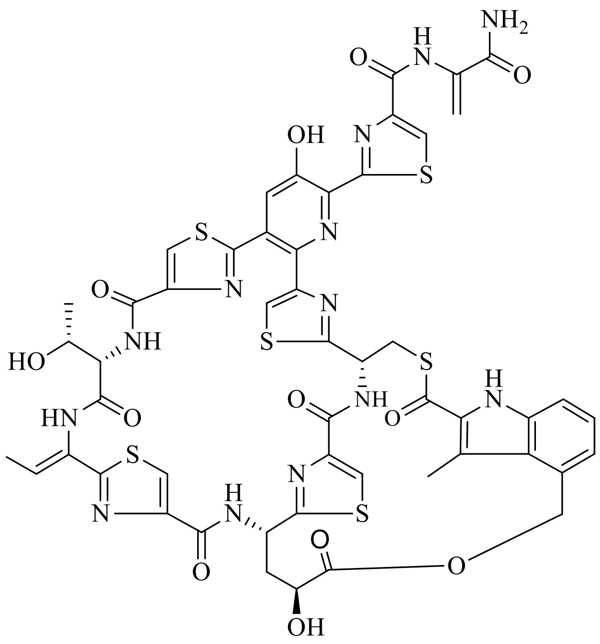
Figure 11: Representative structures of lasso peptides produced by *Streptomyces* sp.
Anantin and chaxapeptin are prominent lasso peptides. Their structures display their rigid lasso backbone which accounts for their rigidity/stability.

2.5.5.3 Thiopeptides

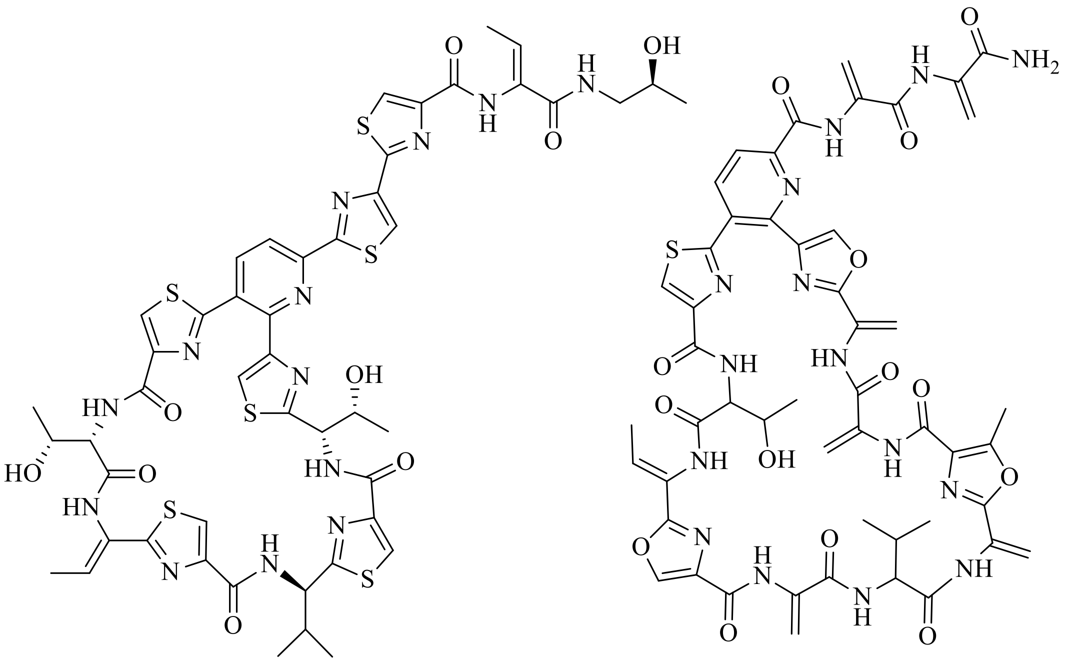
A common feature of a thiopeptide macrocycle is the presence of a six membered nitrogenous ring occuring in three varying oxidation states such as a piperidine, dehydropiperidine, or pyridine. Additional structure complexity in some thiopeptides is achieved by the addition of a second ring that integrates a tryptophan derived quinaldic acid/indolic acid residue exemplified in the structures of thiostrepton A and nosiheptide[41] (structures below, **Figure 12**). Sixty years pass the discovery of the thiopeptide micrococcin, it was still unclear whether thiopeptides were biosynthesised ribosomally having the extensive post-translational modification to synthesized precursor peptides or if their polypeptide sub-structures were the products of non-ribosomal peptide Synthetase. In 2009, scientist discovered the BGCs of five thiopeptides, leading to the formal recognition that thiopeptides are indeed ribosomal peptides (RPs).[41]



Thiostrepton A
Streptomyces sp.
Thiopeptide



Nosiheptide
Streptomyces actuosus
Thiopetide



Micrococcin P1
Micrococcus sp.
Thiopeptide

Val-Geninthiocin
Streptomyces sp. RSF18
Thiopeptide

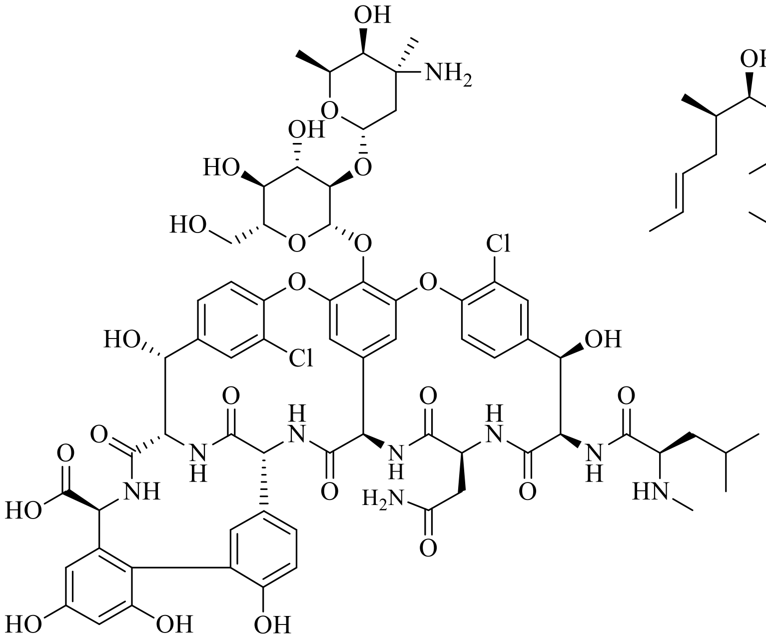
Figure 12: Representative structures of thiopeptides produced by *Streptomyces*
Micrococcin P1 is prototypic thiopeptide isolated from a *Micrococcus* strains in late 1940s which are well known to produce Microcins. Val-Geninthiocin was recently discovered from a model *Streptomyces* strain RS18 with very interesting biological activities.[41]

Thiopeptides are produced by diverse bacteria species including *Streptomyces*, *Bacillus*, and *Micrococcus* with some reported antibacterial activities. They can be structurally grouped into five series depending on the oxidation state and substitution on the central nitrogen containing ring. **Series A** thiopeptides, are much represented by thiopeptins, which has the main heterocyclic ring as a fully saturated piperidine. **Series B** have thiostrepton A as its prototypic peptide with dehydropiperidine ring as its core heterocycle. **Series C** currently have only one peptide; compound Sch 40832 whose nitrogen heterocycle contains an imidazo-piperidine ring. The tri-substituted pyridine ring observed in micrococcin P1 exemplifies the **series D** which are the most frequently discovered scaffolds among thiopeptides. The **series E** has a pyridine and a hydroxyl unit at position 5 of the ring as displayed in the structure of nosiheptide**[41]** in **Figure 12**.

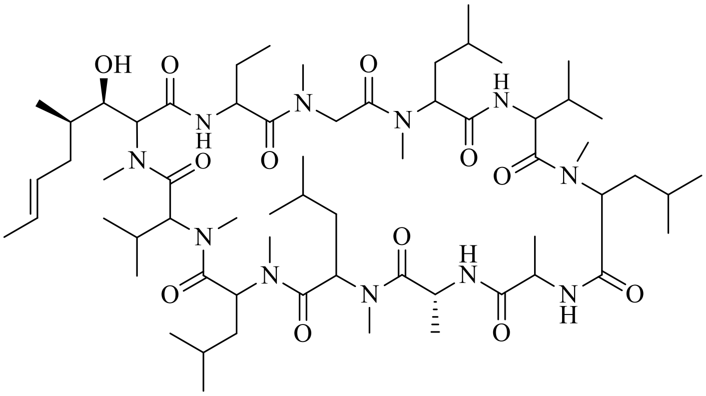
Thiopeptides have been used as antibacterial agents against Gram-positive bacteria but exert weak antibacterial effect against Gram-negative bacteria. This exhibited antibacterial activity can be explained by the inability of thiopeptides to penetrate the outer membrane of Gram-negative pathogens.[84] Most of the thiopeptide antibiotics inhibit protein synthesis in bacteria pathogens and thus share this mode of antibacterial action. Thiostrepton, whose antibiotic activity is best understood, acts by binding tightly to the prokaryotic ribosome and thus inhibits translation.[85] Apart from their well-known antibacterial properties, certain thiopeptides have potent antimalarial and anticancer activities. For example, thiostrepton A inhibits two opposing aspects of protein homeostasis in the malaria parasite *Plasmodium falciparum*, precisely the proteasome and protein synthesis in the apicoplast organelle of the parasite.[86] For cancer cell lines, thiostrepton A induces apoptosis by an interplay of proteasome inhibition and directly interfering with the FOXM1 transcription factor.[87] A recently discovered thiopeptide is Val-Geninthiocin isolated from of *Streptomyces* sp. RSF18 exhibiting potent antibacterial activities against the gram positive bacteria *Bacillus subtilis*, *Staphylococcus aureus* and *Streptomyces viridochromogenes* and also antifungal activities against *Mucor miehei* and *Candida albicans*.[88]

2.6.0 Non-ribosomal peptides (NRPs)

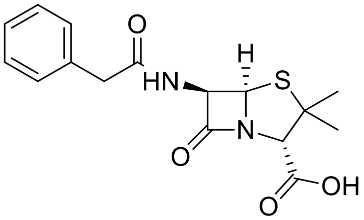
Non-ribosomal peptides are an important group of secondary metabolites from bacterial and fungal origin[89] comprising several diverse scaffolds peptides such as β – lactams, glycopeptides, cyclosporins and bacitracins.[90] Currently, NRPs are receiving considerable levels of attention in the microbial drug discovery over the past decade mainly because of their very significant biological activities demonstrated against pathogens. Their proteolytic stability and highly selective biological activities have made them very attractive to both the pharmaceutical and agricultural industries as viable lead compounds for drug development.[89] Therapeutic agents with NRPs origin include well known antibiotics (daptomycin, penicillin, gramicidin, vancomycin.), antifungals (bacillomycin) and antivirals (luzopeptin).



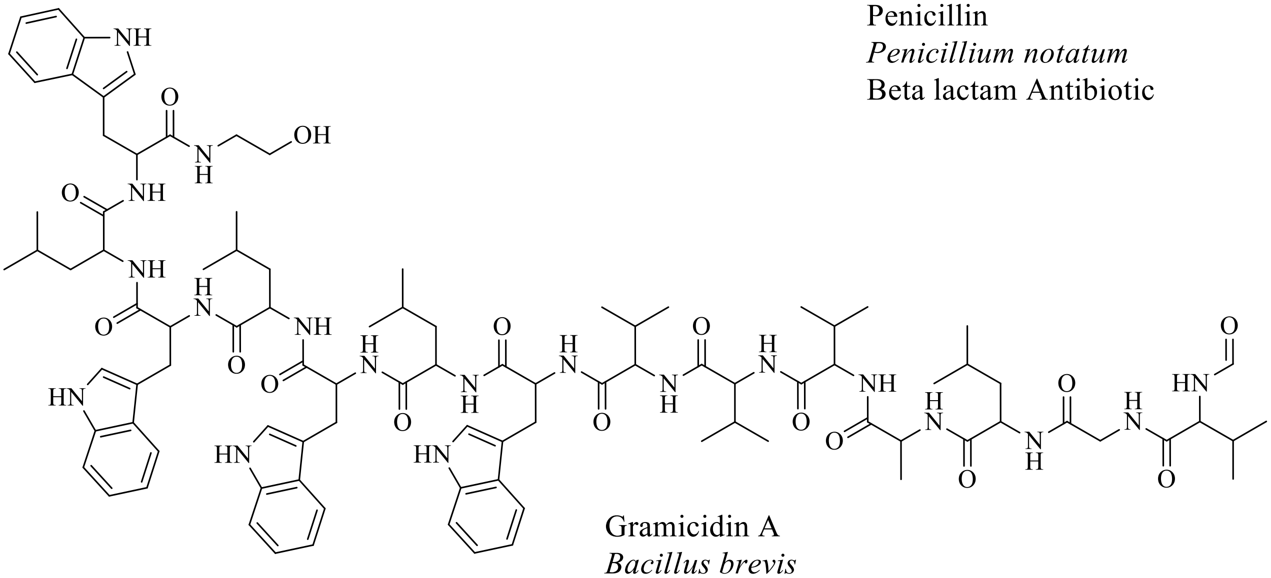
Vancomycin
Amycolatopsis orientalis
Glycopeptide Antibiotic



Cyclosporin A
Trichoderma polysporum
Immunosuppressant



Penicillin
Penicillium notatum
Beta lactam Antibiotic



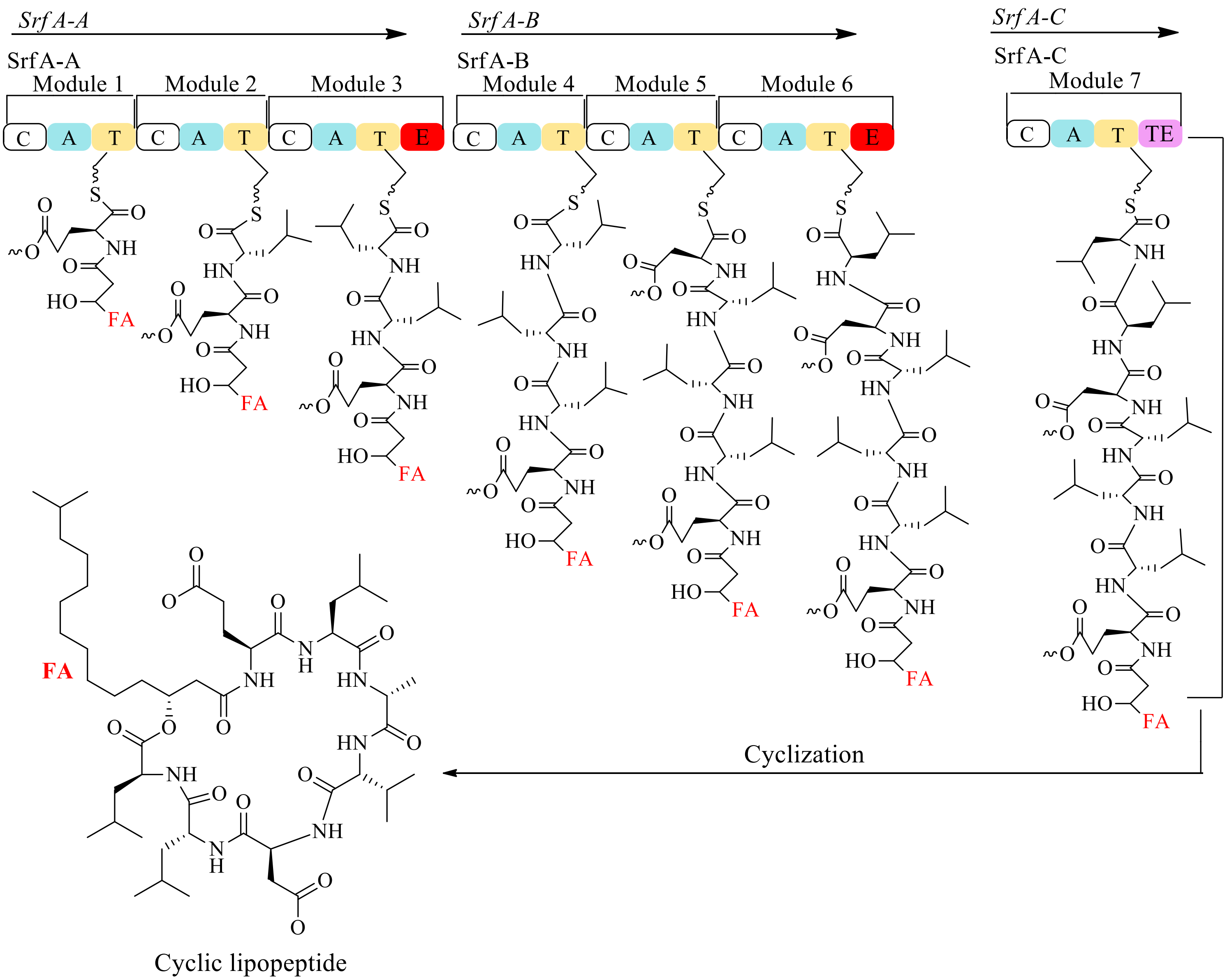
Gramicidin A
Bacillus brevis
Antibiotic

Figure 13: Structures of some clinically useful NRPs

Additionally, antitumor agents (actinomycin D), immunosuppressants (cyclosporine A), anticancer agents (bleomycin), toxins (cyanotoxins) anti-inflammatory compounds and a host of others have been discoverred as non-ribosomal peptides. Industrially, they have been employed as siderophores (enterobactin, vibriobactin) and biosurfactants.**[90-93]**

Non-ribosomal peptides (NRPs) are encoded by large multimodular enzymes called non-ribosomal peptide synthases (NRPS) that utilize complex regiospecific and stereospecific reactions to assemble structurally and highly functionalized peptides through a thiotemplate mechanism.**[91,93]** The isolation of the cyclic decapeptide gramicidin S in 1968 from the cell extracts of *Bacillus brevis* even in the presence RNase or ribosome inhibitors in the culture coupled with further biochemical analyses demonstrated that gramicidin S biosynthesis did not engage t-RNA molecules or aminoacyl t-RNA synthetases required for ribosomal peptide synthesis.**[90]** These findings by researchers (Gevers et al.,1968) actually led to the discovery of the NRPS enzymes. Typical non-ribosomal peptide synthetases (NRPS) show a modular structure and each module is responsible for the condensation of one amino acid. A module is usually composed of three domains, condensation (C) domain, adenylation (A) domain, and thiolation (T) domain.**[94]** The T domain is a carrier protein containing a serine residue that is phosphopantetheinylated; an amino acid or a growing peptide is covalently attached to the phosphopantetheine arm via a thioester bond.**[94]**

The A domain is responsible for amino acid activation/selection and catalyzes the formation of a thioester bond between the phosphopantetheine arm of the T domain and an amino acid substrate by consuming ATP. The C domain is responsible for the peptide bond formation between an amino acid attached to one T domain and the growing peptide (an amino acid in the initial condensation) attached to another T domain. Sometimes a module contains accessory enzyme domains such as



C —C domain catalyses the peptide bond formation between an amino attached to a T domain and a growing peptide chain

A —A domain activates and selects amino acids and catalyses the formation of the thioester linkage

T —T domain is a carrier protein containing a serine residue on which an amino acid or a growing peptide is covalently attached

TE —TE domain catalyses the simple hydrolysis or macrocycle formation to terminate the peptide synthesis

Figure 14: Representative biosynthetic pathway of NRPs regulated by multi-modular NRPS enzymes
Above is the proposed biosynthetic of the biosurfactant NRPs molecule surfactin. The biosynthetic gene cluster involves several genes at respective modules (SrfA-A to SrfA-C genes) that encodes enzymes for the regulate the biotransformation at each stage of the biosynthesis. The successive precursor amino acids condensation elongates the peptide chain which finally ends with a cyclization domain that yields the cyclic lipopeptide. The proposed biosynthesis suggests that a long fatty acid chain (FA) is incorporated at the early stage of the biosynthesis which is unique among NRPs.[112]

methyltransferase (MT) domain and epimerization (E) domain to modify the growing peptide or amino acid before condensation. In some cases, the C domain is substituted by the heterocyclization (CYC) domain catalyzing formation of the oxazoline or thiazoline rings. The synthesized peptide is cleaved off from the enzyme by the thioesterase (TE) domain which catalyzes simple hydrolysis or macrocycle formation as illustrated in **Figure 14**. This modular structure enables the engineering of NRPS to produce various novel bioactive peptides by substituting A domain which is responsible for amino acid activation/selection.[94]

2.6.1 Sources of non-ribosomal peptides (NRPs)

Genetically, NRPs are expressed by organisms that bare putative non-ribosomal peptide synthases biosynthetic gene clusters (BGC) in their genome. Genome mining and bio-informatics tools have revealed NRPS to be genetically expressed by some species of Archaea bacteria and Fungi.[89-91] Compared to their counterpart ribosomally synthesized and post-translationally modified peptides (RiPPs) which has origins in eukaryotes such as plants, sponges, mollucs and mushrooms, NRPs currently can be restricted to only have bacterial and fungal origins. Among Archaea bacteria, *Streptomyces* species are outstanding producers of NRPs compounds.

2.6.2 The Norine database

Norine is the first database entirely dedicated to non-ribosomal peptides (NRPs). It provides a complete computational tool for systematic study of NRPs in numerous species. This platform provides information to equip researchers with a better knowledge of these natural products (NRPs) and their underlying biological mechanisms. The overall goal of the norine database is to contribute to the redesigning of natural products in order to obtain new bioactive compounds for drug discovery. Norine currently contains over 1180 NRPs compounds and numerous publications to that effect.[89,95]

2.6.3 Structural complexity of non-ribosomal peptides (NRPs)

NRPs compounds occupy a huge area of chemical space and diversity. Unlike the ribosomally synthesized peptides limited to the classical 20 proteinogenic amino acids only, non-ribosomal peptide synthases modular enzymes can utilize not only the available 20 proteinogenic amino acids but also use hundreds (>500) of different acyl monomer substrates including non-proteinogenic amino acids, fatty acids, hydroxyl and aromatic acids as building blocks for NRPs.[91] The Norine database shows that proteinogenic L-amino acids form only 40% of the total number of NRPs monomers. Most of the NRP monomers are of non-proteinogenic origin such as D-amino acids, non-canonical amino acids (3-methylglutamic acid, 3-hydroxyasparagine, 3-phosphohydroxyasparagine and 4-hydroxyphenylglycine), lipids, sugars, and polyketide building blocks. Bacterial NRPs often contain D-serine, D-alanine, 2,4-diaminobutyric acid, and 4-hydroxyphenylglycine as non-proteinogenic building blocks, which are rarely observed in fungal NRPs.[89-93]

In addition to high molecular diversity at the monomer level, extra diversification of NRPs occurs during chain assembly on the NRPS multi-enzyme complex such as on-template heterocyclization which significantly increases the rigidity of NRPs and protects them against proteolytic degradation. Chain termination is another diversification step during NRP biosynthesis. For example, full-length NRPs can be cleaved from NRPS enzymes as linear peptides, as peptide amides, as peptide alcohols, and as cyclic or branched-cyclic macrocycles such as macrolactones, macrolactams, and cycloimines.[96] Finally, another diversification step is achieved by post assembly line tailoring reactions which are analogous to post-translational modifications observed in ribosomally synthesized peptides as described in **section 2.5.5**.

Just as post-translational modifications are chemical modifications to precursor peptides after the release from the ribosome, post assembly line tailoring occurs after the cleavage of NRPs from the NRPS assembly line. In contrast to post-translational modifications as the only structural complexity-generating tool for ribosomally synthesized peptides (RiPPs), non-proteinogenic building blocks and post assembly line tailoring generate structural diversity and complexity which substantially contributes to the observed biological activity in non-ribosomal peptides.[94]

2.7 Structural elucidation of ribosomal peptides/RiPPs and non-ribosomal peptides

The structural complexity of RiPPs and NRPs coupled with their very frequent high molecular weight averaging about 1-5 kDa normally makes the structure elucidation of RiPPs/NRPs very intriguing and highly time-consuming.[97] Conventional small molecule metabolites structure elucidation would often involve basic spectroscopic tools (UV-Vis, FTIR, 1D and 2D NMR techniques) and spectrometry tools (mass spectrometry) to establish their structures, however the very often complex and diverse structures of RiPPs/NRPs would mean that researchers would have to involve further modern and sophisticated structure elucidation techniques for the complete structural characterization of these metabolites. In the absence of such sophisticated structure elucidation tools, the chemical characterization of RiPPs/NRPs can be very difficult and take a much longer time. For example, the lantibiotic Nisin was identified in 1928 but took nearly 50 years (1971) for its complete structure to be established.[40,41] Also discovery of the polytheonamides which were reported in 1994 as cytotoxic constituents of the Japanese sponge

Theonella swinhoei, which harbours a large diversity of symbiotic bacteria took more than 10 years for the same research group to establish the precise structure of these compounds. [97]

Even though 1D and 2D NMR techniques (^{13}C, HSQC, ^{1}H- ^{1}H COSY, HMBC, gHSQC-TOCSY, 1D- TOCSY/COSY irradiations) have been significantly involved in the structure elucidation of most organic molecules, such analysis used for RiPPs/NRPs metabolites often yield highly complicated spectra with extremely overlapping NMR signals.[98] However, the increasing availability and low cost of modern analytical techniques such as sequence tag mass spectrometry/tandem mass spectrometry (MS/MS), X-ray crystallography (protein crystallography) and genomics (genome mining) has facilitated the easy characterization of peptide molecules.

Structure elucidation of very large peptides is made somewhat easier by fragmenting the peptide molecule into its amino acid residues and determining their relative sequences. This is the technique employed in protein mass spectrometry such as sequence tag mass spectrometry/tandem mass spectrometry (MS/MS). Mass analysis of peptides is a popular method of peptide characterization since cheaper instrument designs are currently available for identification of peptides and their post-translational modifications. The two primary methods used for the peptide ionization in protein mass spectrometry are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). [99,100] These ionization techniques are used in conjunction with mass analyzers such as tandem mass spectrometry. Tandem mass spectrometry (MS/MS) is used to measure fragmentation spectra and identify proteins at high speed and accuracy whiles collision-induced dissociation is used in mainstream applications to generate a set of fragments from a specific peptide ion. The fragmentation process primarily gives rise to cleavages along peptide bonds and thereby releasing shorter fragments (amino acids) whose masses (*m/z*) can be correlated with a database of predicted masses for one of many given peptide sequences. The most widely used instrument for peptide mass analysis is the MALDI-TOF (*time of flight*) MS.[99]

X-ray crystallography is a powerful tool for studying the three dimensional structure and supramolecular assembly of peptides. Crystallography is arguably the most widely used technique for studying the structures of proteins and nucleic acids at atomic resolution. Advances in the tools of protein crystallography coupled with an increases in computer processing power, advances in charge-coupled device (CCD) area detectors and access to synchrotron X-ray radiation sources now make it possible to rapidly determine crystal structures of RiPPs/NRPs.[101] Commercially available crystal screening kits and high-throughput screening robotics have greatly reduced the time required to discover appropriate crystal growing conditions while reducing the quantities of material needed for growing crystals. Precise diffraction data collection and powerful crystallographic software have allowed the development of multiple phasing techniques, such as anomalous diffraction and molecular replacement, to rapidly determine crystallographic structures of peptides within days, however, the only setback is that X-ray crystallography cannot be used to analyze peptides that do not crystalize.[102]

Lastly, the used of genomics tools can been employed to obtain and interpret genomic information for structure elucidation by determining the biosynthetic gene clusters (BGC) that are responsible for the biosynthesis of secondary metabolites such as RiPPs/NRPs. This facilitates the correlation of enzymatic pathway in the biosynthesis to confirm the proposed structures of peptides. A number of genomic-driven databases and bioinformatics tools such as antiSMASH (Antibiotics and Secondary Metabolite Analysis Shell) currently exist for a comprehensive BGC identification and interpretation that is intended to provide a broad biosynthetic survey of genomes to predict the structures of metabolites they encode including RiPPs/NRPs.[103]

2.8 Clinically or industrially useful compounds produced by *Streptomyces*.

Over 22,500 bioactive secondary metabolites have been isolated from microbes. Actinobacteria are estimated to produce about 45% of all isolated bioactive microbial secondary metabolites which is largely contributed by *Streptomyces* producing close to 7600 bioactive compounds. *Streptomyces* are well known for their production of antibiotics as described in **section 2.3.** Some of these antibiotics (**listed in the table 1**) are currently used as the only defense against multi-drug resistant pathogens. The major classes of clinical antibiotics produced by *Streptomyces* include the following; aminoglycosides (neomycin, kanamycin, streptomycin), angucyclines (Auricin, landomycin and moromycin), ansamycins (rifamycin, geldanamycin), anthracyclines (primarily antitumor agents; daunorubicin) lactams (cephamycins and also the important lactamase inhibitors clavulanic acid and chloramphenicol), glutarimides (cycloheximide), glycopeptides (vancomycin), lipopeptides (daptomycin) , lantibiotics (mersacidin, actagardine), macrolides (clarythromycin, erythromycin, tylosin, clarithromycin), oxazolidinones (cycloserine) and tetracyclines.[18]

Table 1: Clinically useful antibiotics produced by *Streptomyces*

Antibiotic	Producer strain	Discovery year
Platensimycin	*Streptomyces platensis*	2006
Daptomycin	*Streptomyces roseosporus*	2003
Ribostamycin	*Streptomyces ribosidificus*	1970
Fosfomycin	*Streptomyces fradiae*	1969
Kanamycin	*Streptomyces kanamyceticus*	1957
Noviobiocin	*Streptomyces niveus*	1956
Vancomycin	*Streptomyces orientalis*	1956
Cycloserine	*Streptomyces garyphalus*	1955

Lincomycin	*Streptomyces lincolnensis*	1952
Virginiamycin	*Streptomyces pristinaespiralis*	1952
Viomycin	*Streptomyces vinaceus, S. capreolus*	1951
Nystatin	*Streptomyces noursei*	1950
Tetracycline	*Streptomyces aureofaciens*	1950
Neomycin	*Streptomyces fradiae*	1949
Chloramphenicol	*Streptomyces venezuelae*	1949
Cephalosporins	*Streptomyces clavuligerus*	1945
Streptomycin	*Streptomyces griseus*	1944
Actinomycins	*Streptomyces anulatus*	1944
Amphomycin	*Streptomyces canus*	1955
Hygromycin	*Streptomyces hygroscopicus*	1953
Oleandomycin	*Streptomyces antibioticus*	1950
Oxytetracycline	*Streptomyces rimosus*	1949
Pristinamycin	*Streptomyces* spp.	1972
Rifamycin	*Streptomyces mediterranei*	1959
Spiramycin	*Streptomyces ambofaciens*	1952
Streptothricin	*Streptomyces lavendulae*	1942
Staphylomycin	*Streptomyces virginiae*	1958

Besides antibiotics, *Streptomyces* also produce a wide variety of other secondary metabolites with activity as herbicides, antifungals, antitumor or immunosuppressant drugs, and anthelmintic agents. Some examples are listed in the subsequent tables below.[18]

Table 2: Antifungal agents produced by *Streptomyces*

Antifungal agent	**Producer strain**	**Discovery year**
Natamycin	*Streptomyces natalensis*	1954
Actinomycins	*Streptomyces anulatus*	1944

Oligomycin	*Streptomyces diastatochromogenes*	1953
Resistomycin	*Streptomyces canus*	1971
Amphotericin B	*Streptomyces nodosus*	1955
Blasticidin	Streptomyces griseochromogenes	1950
Candicidin	*Streptomyces griseus*	1952
Kasugamycin	*Streptomyces kasugaensis*	1965
Nikkomycin	*Streptomyces tendae*	1970
Polyoxin B	*Streptomyces cacaoi*	1963
Validamycin	*Streptomyces hygroscopicus*	1970
Tetracenomycin	*Streptomyces canus*	1997
Kitamycin	*Streptomyces* spp.	1998

Table 3: Antiparasitic agents produced by *Streptomyces*

Antiparasitic agent	**Producer strain**	**Discovery year**
Avermectins	*Streptomyces avermitilis*	1975
Trioxacarcin	*Streptomyces bottropensis*	2004

Table 4: Antiviral agents produced by *Streptomyces*

Antiviral agent	**Producer strain**	**Discovery year**
Hygromycin	*Streptomyces hygroscopicus*	1953
Virantmycin	*Streptomyces* spp.	1980

Table 5: Antitumorr agents produced by *Streptomyces*

Antitumor agent	**Producer strain**	**Discovery year**
Borrelidine	*Streptomyces* spp.	1949
Doxorubicin (adriamycin)	*Streptomyces peucetius*	1970

Daunorubicin (daunomycin)	*Streptomyces peucetius*	1960

Table 6: Immunosuppressive agents produced by *Streptomyces*

Immunosuppressive agent	**Producer strain**	**Discovery year**
Hygromycin	*Streptomyces filipinensis*	1980
Pentalenolactone	*Streptomyces filipinensis*	1992

Table 7:Bioherbicide/Biopesticide agent produced by *Streptomyces*

Bioherbicide/Biopesticide agent	**Producer strain**	**Discovery year**
Herbimycin	*Streptomyces hygroscopicus*	1970
Ivermectin	*Streptomyces avermitilis*	1975
Prasinons	*Streptomyces prasinus*	1973

2.9 Shigellosis

2.9.1 Pathogenicity, mode of transmission and symptoms of shigellosis

Shigellosis is a type of diarrhea caused by a bacterial infection from *Shigella*.[104] The genus *Shigella* is distributed worldwide and is the main cause of inflammatory dysentery, responsible for 5 to 10% of diarrheal illness in many parts of the world. *Shigella* has four classified species; *Shigella* dysenteriae, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei*. *Shigella sonnei* together with *Shigella flexneri* are responsible for 90% of shigellosis cases. Shigellosis is typically transmitted by exposure to infected fecal material through contaminated food, water, or hands. Contamination may spread easily by vector flies or when changing diapers.[104]

Shigella sp. are relatively resistant to gastric acid, therefore ingestion of as few as 10 to 100 organisms can cause the disease. Epidemics occur most frequently in overcrowded populations with inadequate sanitation. The infection is particularly common among younger children living in endemic areas, however it is less severe in adults.[105] Symptoms generally start one to two days after exposure and include diarrhea, fever, abdominal pain, and pain accompanied with passing bloody stools. Symptoms typically last five to seven days, however complications can include post infectious arthritis, sepsis, seizures, and hemolytic uremic syndrome. This infections incidence is approximately 165 million reported cases globally with about 1 million deaths recorded worldwide every year.[105]

2.9.2 Treatment of shigellosis

Treatment consists mainly of replacing fluids and salts lost because of diarrhea. Oral fluids replacement is satisfactory for most people, but some may need to receive fluids intravenously.[106] Antibiotics are used in severe cases or for certain populations with mild symptoms (elderly, immunocompromised, food service industry workers, child care workers). For Shigella-associated diarrhea, antibiotics shorten the length of infection, but they are usually avoided in mild cases because many Shigella strains are becoming resistant to common antibiotics. Antibiotics, such as trimethoprim-sulfamethoxazole, ciprofloxacin are sometimes given to very young or very old patients when the disease is severe or when there is a high risk of the infection spreading quickly to other people.[106] The antibiotic ampicillin has been approved for Shigellosis treatment and has been somewhat effective in treating this disease, however due to the increasing incidence of resistance, the first choice of Shigellosis treatment drug is now the antibiotic Pivmecillinam.[106] Furthermore, effective medications are often in short supply in developing countries, which carry the majority of the disease burden from Shigella indicating the urgent needs for more robust and effective antibiotic alternatives.[107]

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Soil Sample Collection from Brong Ahafo

Soil samples were collected from areas in the Brong Ahafo region of Ghana situated in and around the Digya National Park, a forest reserve which is home to a wide variety of organisms. The selection of sampling sites was based on the level of human activities that existed in a prospective area to be sampled. The areas chosen for sampling were noted for the predominance of many undisturbed habitats which represents one of the most bio-diverse environments in the country.

Many soils were sampled with the most intensive sampling done at the core areas of the Digya Park where samples were collected 100 m apart. The GPS coordinates of the sampling sites for the microbes described in this thesis was (coordinates: 7031'44.85"N and 0036'48.15"W). All GPS coordinates showing sampling locations are recorded and stored in Google Earth. A geographical map of the sampling sites in the Brong Ahafo region is been shown in **Figure 15**. Soils were collected by clearing the humus or topsoil with shovel to a depth of between 70-80 cm after which the soils were scooped directly into 50 ml sterile centrifuge or falcon tubes and covered. A total of nine samples were collected and labeled DE2E, DE2SH, DE2, DE2A, DE21A, DE21B, DE2B, DE21CA and DC2.

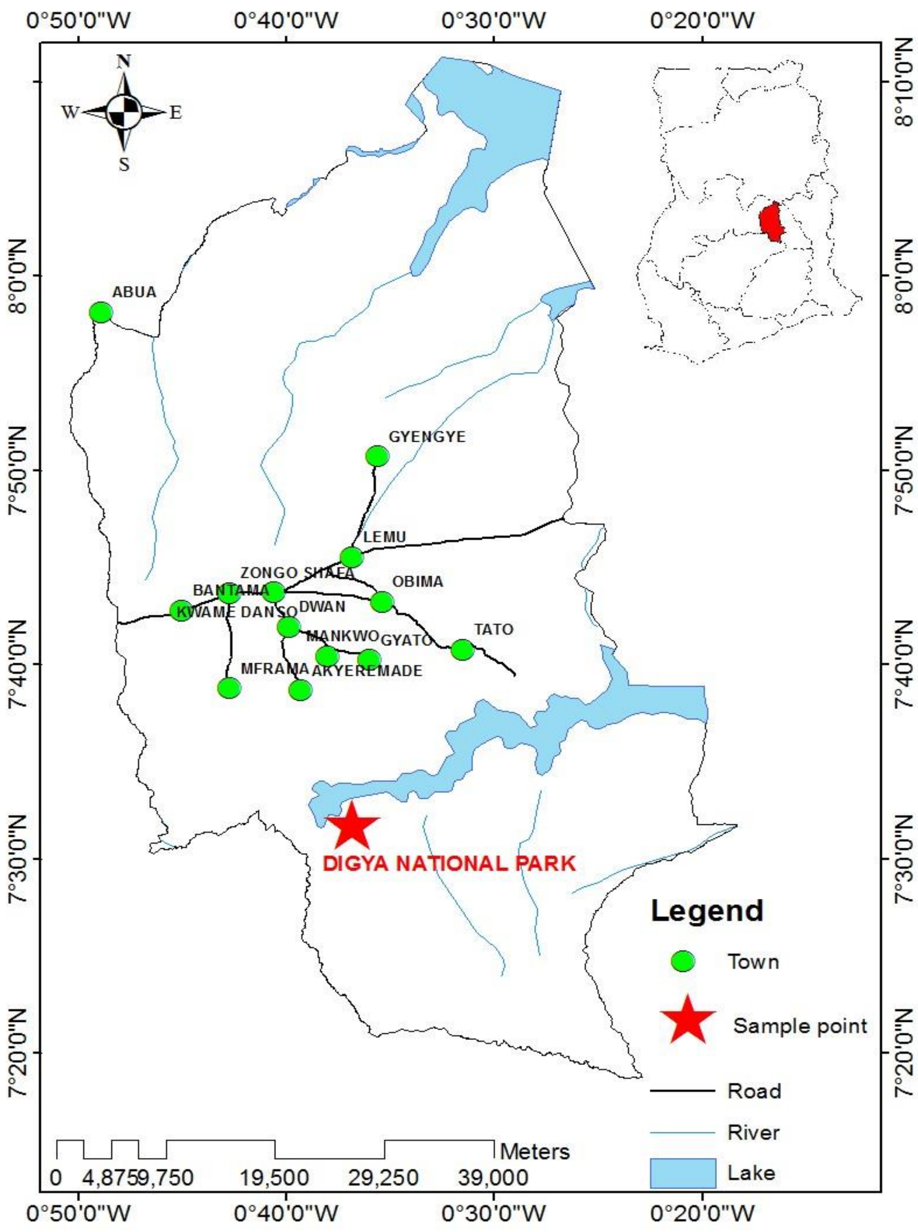


Figure 15: A geographical map of Sene West district in the Brong Ahafo Region showing the sampling area.

3.2 Bacteria media preparation

3.2.1 Materials

Agar powder (Oxoid), Yeast exract, Malt extract, D-glusoce, Tryptone, Phytone, Sodium chloride, Disodium phosphate, Glucose, Water, 1L autoclave bottle with cap (Pyrex).

3.2.2 Preparation of International Streptomyces Protocol (ISP)2 solid agar media

Ten grams of malt extract, 4g each of yeast extract and D-glucose and 15g of Oxoid agar was placed in a 1L beaker and 900mL of tap water was added. With the help of a magnetic stirrer and a magnetic plate, the contents of the beaker were thoroughly mixed for about 10 minutes after which it was transferred into a 1L autoclave bottle. Media was then autoclaved at 121ºC for about 1 hour and about 50 ml emptied into petri dishes in a clean bench to about two- thirds the volume of the petri dishes. The agar media was then allowed to set under sterile conditions after which they were sealed, para-filmed and stored at 4ºC for future use.

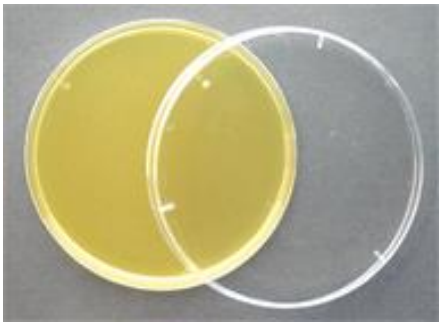


Figure 16: A picture of ISP 2 agar plate on which the microbes were cultured.

3.2.3 Preparation of International Streptomyces Protocol (ISP)2 liquid media

10g of malt extract, 4g each of yeast extract and D-glucose was placed in a 1L beaker and 900mL of tap water was added. With the help of a magnetic stirrer and a magnetic plate, the contents of the beaker were thoroughly mixed for about 10 minutes after which it was transferred into a 1L autoclave bottle, plugged with non-absorbent cotton wool and autoclaved using a set program. The sterilized liquid media was labeled and stored until it was needed.

3.2.4 Preparation of Tryptic soy (TSBY) media

Tryptone (17g), 3g of phytone, 5g of sodium chloride and 2.5g each of disodium phosphate and glucose were placed in a 1L beaker and 900mL of tap water was added. Content was thoroughly mixed as described in **section 3.2.3**, the pH of the media was adjusted to 7.3 and transferred into an autoclave bottle. Autoclave bottle was plugged with non-absorbent cotton wool and autoclaved using a set program after which the sterilized liquid media was labeled and stored for future use.

3.3 Culture, seeding and preservation of bacteria strains from soil samples

3.3.1 Materials

Soil samples, (ISP)2 agar media, (ISP)2 liquid media, TSBY liquid media, sterile inoculation loops (Sigma Aldrich), sterile 60% glycerol stock solution, 50ml falcon tubes (Greiner), sterile Eppendorf tubes (Fischer), 250ml Erlenmeyer flaks (Fischer), 2ml screw capped cryo-preserved tubes (Fischer), water bath.

3.3.2 Culture and isolation of novel bacteria strains from soil samples

The soil sample with code DE2E was prepared for the isolation of bacteria strains by placing 5g of soil sample into a new sterile 50 mL falcon tube. 10 mL of sterilized tap water was then added and the falcon tube was capped under sterile conditions. The sample was subsequently placed in a hot water bath for an hour at 55°C to prevent the growth of fast growing gram-negative bacteria and fungi. The soil sample was then allowed to cool and placed in a clean bench which was initially sterilized with 70% ethanol followed by exposure to ultraviolet (UV) light for an hour. About 10 ml of sterilized tap water was added to the sample and 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions were prepared. This was done by pipetting 1ml of the stock 10ml suspension into a new sterile 50ml falcon tube and diluted with 9ml of sterilized tap water after which it was shaken thoroughly to mix to afford the 10^{-1} dilution. 1ml of the 10^{-1} suspension was then pipetted into a new 50ml centrifuge tube and subsequently diluted with 9ml of sterilized tap water as described earlier to afford the 10^{-2} dilution. The process was repeated to afford the other dilutions after which pre-sterilized inoculation loops were used to smear evenly the different concentrations of the soil sample on pre-modified (ISP)2 agar plates that had been supplemented with 25 µg/ml each of nalidixic acid and nystatin antibiotic. The antibiotics prevented the growth of other bacteria apart from those of the order *Actinomycetales*. The plates were then para-filmed and incubated at 28ºC for 7 days to allow the growth of bacteria. After the seven-day period, the agar plates were taken out of the incubator and observed in a clean bench. Single colonies that had different phenotypes were re-plated on new agar plates using sterilized inoculation loops in the clean bench to obtain pure strains. Parent plates that had fungal contamination on them or did not have single individual colonies to pick were discarded to prevent contamination of the lot. The rest were para-filmed and placed in the incubator to allow for continued bacteria growth. Pure strains that had been transferred onto new agar plates were also para-filmed and placed in the incubator for periodic observation. All the colonies initially sub-cultured from the parent or master plates were subsequently sub-cultured until very pure strains were obtained.

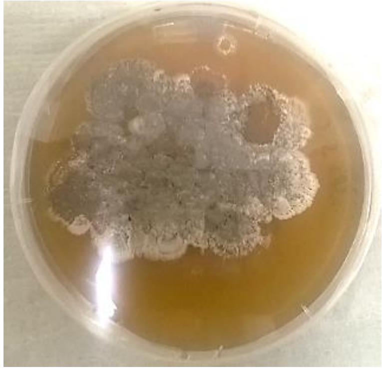


Figure 17: Agar plate showing the pure colonies of the isolated novel *Streptomyces* sp. strain DE2E

3.3.3 Small scale fermentation of pure strains

A small scale fermentation of pure strains which were obtained from the culture and isolation process described in **section 3.3.2** was prepared. Single colonies of the strain DE2E were transferred from the pure culture plate and used to inoculate a 250 mL Erlenmeyer flask containing about 100 mL of (ISP)2 liquid media. The inoculated flask was labelled and placed in a shaker at a temperature of 28 °C and rotating at 165 rotations per minute (rpm) for 14 days to allow for the growth of bacteria. After the 14-day period, the culture broth was subjected to suction filtration to separate bacteria mycelia from the broth. The filtrate was then extracted with about 100ml of ethyl acetate and the mycelia extracted repeatedly and alternatively with methanol and dichloromethane (500 ml each). The ethyl acetate, methanol and dichloromethane extracts were dried under vacuum at a temperature of 50 °C and subsequently combined to give a total crude extract (TCE).

Figure 18: Pictures of small scale cultures of isolated bacteria strains

3.3.4 Cryopreservation of bacteria species

After the small scale culture of bacteria as described in **section 3.3.3**, 1 mL of the culture was pipetted into a 2 mL cryo-screwed tube and 400 µL of 60 % glycerol stock solution was added to it and shaken. They were then labelled for long term storage in a freezer at -80 °C. This was carried out in duplicate and the glycerol solution is to serve as a cryo-protectant by forming a hibernation wall around the bacteria thereby providing an indefinite longevity to cells.

Figure 19: Pictures showing preserved bacteria strains in duplicates (left) at preparation stage and when in storage in -80 °C freezer (right).

3.4 De-replication of crude extracts from small scale culture

About 1ml of the total crude extract (TCE) obtained as detailed **section 3.3.3** was made-up to obtain 1.0mg/ml concentration using 100% methanol (CH_3OH) as solvent and sent to the University of Aberdeen in Scotland, UK for HRESI/HPLC-DAD-MS^n analysis. Data from LC-MS measurements ascertained that the strain DE2E produces very interesting secondary metabolites. This deduction was also confirmed by ^{1}H-NMR of the crude extract in deuterated chloroform ($CDCl_3$) which gave very interesting NMR signals. Prior to the large scale culture, a decision was made to also culture the strain in TSBY and to compare the type of metabolites the species produces with those observed for the (ISP)2 media.

3.5 Culture, isolation, small scale fermentation and de-replication of crude extracts from small scale culture in tryptic soy (TSBY) media

The processes described from **section 3.3** through to **3.4** were in same fashion carried out on the soil sample labelled DE2E but this time round, cultured in tryptic soy broth supplemented with yeast extract (TSBY) media prior to large scale fermentation of the strain DE2E. Results from the LC-MS measurements, suggested that the strain biosynthesized much more interesting secondary metabolites compared to those observed in the (ISP)2 culture (**section 3.4)**. The detection of more metabolites in greater amounts from the LC-MS data suggested the strain was more prolific biosynthetically when cultured in TSBY compared to (ISP)2 media. ^{1}H-NMR acquired for the TSBY crude extract revealed several interesting peaks hence a large scale culture was carried out in TSBY media but also replicated for (ISP)2 media.

3.6 Large scale fermentation of strain DE2E in TSBY and (ISP)2 liquid media

A large scale fermentation of the strain DE2E was carried out in tryptic soy broth (TSBY) media and replicated also for the (ISP)2 liquid media. The strain DE2E was obtained from the -80 ^oC freezer and allowed to thaw after which it was re-plated and incubated at 28^{o}C for a week. This was done to ensure that the strain was still pure and had not been contaminated. Culturing and fermentation procedures were carried out as described in **section 3.3.3.** This included a seed culture which was made from the pure colonies of the strain DE2E for approximately one week. After the 7-day period, 2L of sterilized TSBY liquid media was prepared and equally distributed in nine 1L conical flasks which were further inoculated with 30ml of the liquid seed culture (total volume ≈ 250 ml volume per flask). The nine culture broths were then incubated in a shaker at a temperature of 28°C and rotating at 165 rpm. The cultures were incubated for 14 days.

3.7 Extraction and isolation of secondary metabolites produced by *Streptomyces* sp. DE2E

3.7.1 Materials

Sterilized Dion HP20 resin, Dichloromethane (DCM), Methanol (MeOH), acetonitrile (AceN), Hexane, sec- Butanol, Distilled H_2O, Sephadex LH-20, fractionating columns and cotton wool.

3.7.2 Extraction

After the 14-day culture/incubation period, 25g of the HP-20 resin was added to each of the cultured broths and allowed to shake for an additional 2 days after which the cultures were harvested. The addition of the HP-20 resin was to extract secondary metabolites produced by the strain. This large scale cultured broths were then filtered using a Büchner funnel and glass wool. The filtrate was extracted once with 250 ml of ethyl acetate in separatory funnels whiles the mycelia was repeatedly and alternatively extracted with methanol and dichloromethane. The ethyl acetate, methanol and dichloromethane extracts were dried under vacuum and subsequently combined to give a total crude extract or TCE (1.357g).

3.8 Isolation and purification of compounds of interest from the total crude extract (TCE)

3.8.1 Kupchan solvent partitioning

The total crude extract (TCE) obtained from the TSBY media large scale fermentation of DE2E as detailed in **section 3.6** was then subjected to a modification of Kupchan's solvent partitioning process to separate the crude extracts into fractions of well-defined polarities using a combination of solvent systems with polarities ranging from the relatively non-polar hexane to the extremely polar water solvent such as methanol. The total crude extract (TCE) was first suspended in about 200ml of de-ionized water in a 1L separatory funnel. This mixture was then extracted three times with 200ml portions of DCM (1:1). The three DCM organic fractions were combined and dried under vacuum. The remaining aqueous layer was extracted once with 200ml *sec*-Butanol. The *sec*-butanol layer was dried under vacuum and to give the fraction labelled **WB** (275.678mg) whiles the remaining water layer was discarded. The DCM extract was suspended in 200ml of a MeOH : H_2O (9:1) / 90% mixture which was then extracted three times with 200ml of Hexane to obtain the Hexane fraction **FH** (206.543mg). The 90% MeOH : H_2O layer was adjusted to 50% with H_2O. This 50% MeOH : H_2O layer was further extracted three times with 200ml of DCM to give the fraction labelled **FD** (544.490mg). The 50% MeOH : H_2O layer remaining was dried under vacuum and to give the fraction labelled **FM** (147.378mg). The framework of this solvent partition process is summarized in **Figure 20** below.

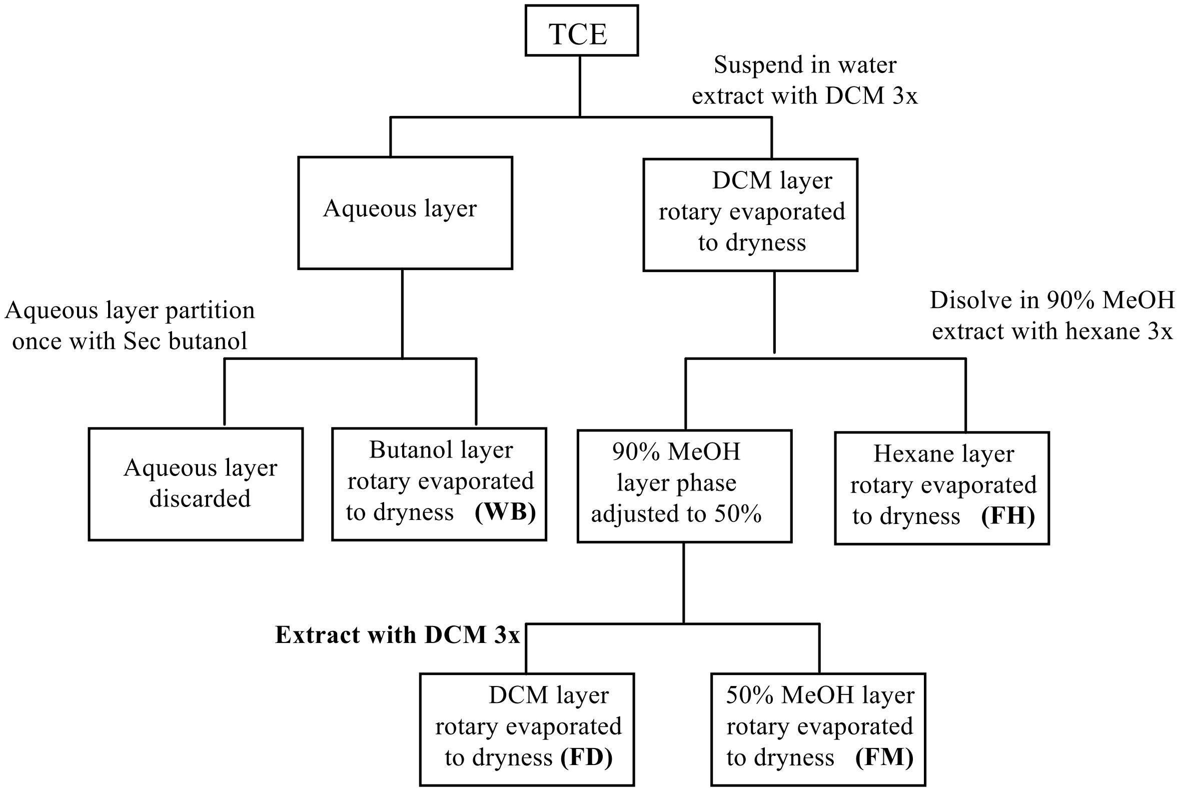


Figure 20: A flowchart of the modified Kupchan Solvent Partition process

This solvent partition module enables some levels of separation of metabolites from the total crude extract (TCE) based on their relative affinities/solubility in the separation solvents used. The Kupchan solvent partitioning (modified) is able to produce for distinct semi pure fractions as labelled in the chart based on their respective polarities. The butanol fraction (WB) is the most polar and hexane fraction (FH) is the least polar. The FD and FM fractions are of middle-range polarities.

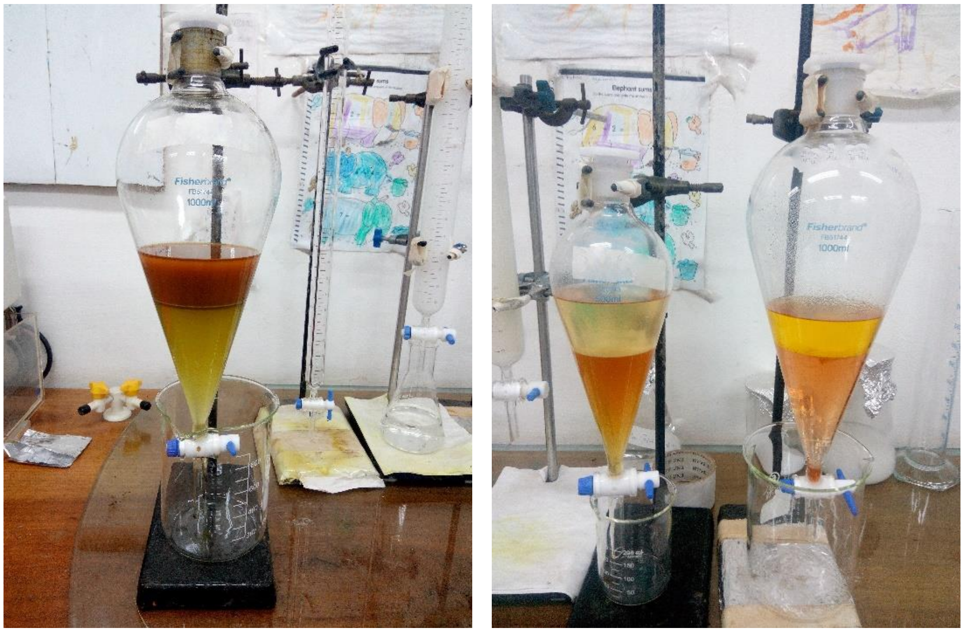


Figure 21: Pictures of the modified Kupchan solvent partitioning process

3.8.2 HRESI/HPLC-DAD-MS^n

High resolution mass spectrometric data was obtained for all Kupchan fractions using a Thermo Instrument MS system (LTQ XL/ LTQ Orbitrap Discovery, UK) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler and Accela pump). Conditions used in data acquisition were: capillary voltage of 45V, sheath gas flow rate of 40- 50 arbitrary units, mass range of 100- 2000 amu (maximum resolution of 30,000), capillary temperature of 320ºC, spray voltage of 4.5 kV and an auxiliary gas flow rate between 10-20 arbitrary units. Separations on HPLC were done using a Phenomenex reversed- phase (C-18, 250 6 10 mm, L × i. d) column which was connected to an Agilent 1200 series binary pump. An Agilent photodiode array was used in monitoring and detection was carried out at a wavelength of 227 nm.

3.8.3 1D and 2D NMR analysis

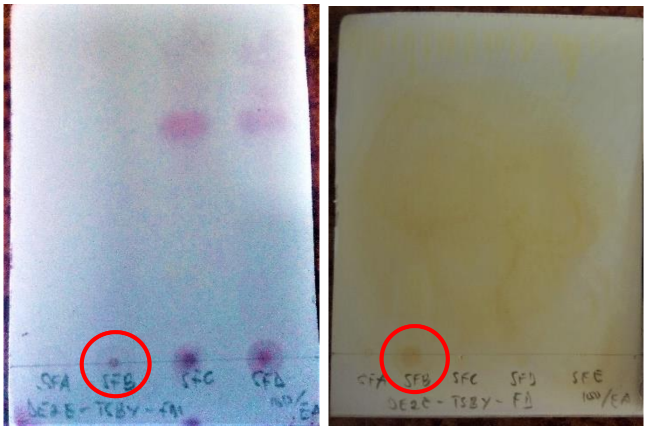
1D and 2D NMR data were acquired on a 500 MHz Bruker spectrometer with $CDCl_3$/DMSO-d_6 as solvents for both fraction and pure isolated compounds. ^{1}H, ^{13}C, HSQC, ^{1}H- ^{1}H COSY, HMBC, gHSQC- TOCSY, 1D- TOCSY experiments and several 1D selective irradiations were among the NMR data acquired on a Bruker Ascend 500 MHz NMR spectrometer in the Department of Chemistry, University of Ghana.

3.8.4 Sephadex LH-20 column chromatography, TLC and phytochemical Analysis

All Kupchan fractions were subjected to HRESI/HPLC-DAD-MS^n analysis and ^{1}H-NMR acquired in deuterated chloroform ($CDCl_3$) or methanol (CD_3OD) based on each fractions level of polarity. Upon careful analysis of the LC-MS and ^{1}H-NMR experimental data acquired for all Kupchan fractions, the FD Kupchan fraction was selected for further purification to enable the isolation and characterization of the compounds of interest. The FD fraction presented a unique chemistry from the analysis of the MS and ^{1}H-NMR experiments coupled with the fact that this fraction had the largest weight (544.4902mg) compared to the other Kupchan fractions. Thus, the compounds of interest from the FD fraction should also be in isolable amounts. Additionally, thin layer chromatography (TLC) followed by some phytochemical screening of the Kupchan fractions was also a decider in choosing the Kupchan fraction FD. Since our interest was to isolate peptide compounds, such phytochemical analysis could easily detect the presence of peptide/alkaloid molecules with relevant phytochemical tests as observed in the case of the fraction FD.

The prioritized fraction, labelled DE2E-TSBY-FD was therefore subjected to size-exclusion chromatography using Sephadex LH-20 as the stationary phase, eluted with a solvent mixture (mobile phase) of methanol:acetonitrile (1:1) to yield seven distinct fractions labeled; SFA

(15.114mg), SFB (164.079mg), SFC (128.462mg), SFD (117.934mg) SFE (84.726mg), SFF (63.395mg) and SFG (42.356mg). These fractions were dried under vacuum, transferred into their respective labelled vials and were further investigated using ^{1}H-NMR, thin layer chromatography (TLC) and phytochemical screening. The phytochemical analysis adopted were ninhydrin to detect the presence of free nitrogens (1° amines/amides) and Dragendorff to determine trap nitrogens (2°/3° amines/amides) which both are very common in peptide molecules. The TLC runs were carried out on aluminum foil coated with silica (normal phase) as stationary phase and eluted with the solvent mixture (mobile phase) of methanol:ethyl acetate in a 1:1 ratio. Sephadex fractions SFB, SFC and SFD all gave positive test for ninhydrin after TLC run and TLC plate development with ninhydrin. However, the Sephadex fraction SFB also tested positive for the Dragendorff to indicate the presence of 3° amines/amides in the SFB (**Figure 22**). The careful TLC profile and ^{1}H-NMR spectra analysis of these three fractions led to the prioritization of the Sephadex fraction SFB to contain predominantly peptide/alkaloid molecules and with less complexity in terms of purity to afford the easy isolation of targeted peptide compounds compared to fractions SFC/SFD.



(A) **(B)**

Figure 22: Developed TLC plates of the Sephadex LH-20 column fractions for DE2E-TSBY-FD fraction

A= TLC plate of sample DE2E-TSBY-FD developed with ninhydrin reagent

B= TLC plate of sample DE2E-TSBY-FD developed with Dragendorff reagent

Fraction SFB tested positive for both developing reagents indicating peptide/alkaloid metabolites present in the fraction but more interestingly, fraction SFB was observed to be much less complex in terms of content/purity for easy and possible isolation of targeted peptide metabolites.

3.8.5 Isolation of peptide metabolites from fraction DE2E-TSBY-FD-SFB on HPLC.

The fraction labelled DE2E-TSBY-FD-SFB was prioritized and subsequently purified further to isolate targeted compounds using a Waters 1525 HPLC series with a binary pump and Waters 2998 photodiode array detector with column heater and an in-line degasser. A Phenomenex Luna reversed-phase C18 column (C18 250 × 10 mm, L × i.d.) was the fitted HPLC column used. The purification was achieved using the stated C-18 column as the stationary phase and the sample eluted with the solvent systems; A= 80/20% (milliQ-H_2O/CH_3CN) and B= 100% (CH_3CN), 30 minutes apart. HPLC Column flow rates was set at 1.5 mL/min and this chromatographic run led to the distinct separation of nine fractions (A to I).

Interestingly, the group of fractions E to I eluted very late in the chromatographic run between retention times (R_T) 45 – 100 minutes, thus taking almost 55 minutes for this group of compounds to elute out of the column. All isolated HPLC fractions were dried under vacuum, dissolved in deuterated chloroform ($CDCl_3$) for ^{1}H-NMR acquisition. HPLC fractions E – I after vacuum drying were observed to be white powders but somewhat crystalizes in a dried chloroform or deuterated chloroform solution.

The ^{1}H-NMR acquisition revealed that HPLC fractions E – I (white powders) as pure isolates were possible secondary metabolites produced by the strain *Streptomyces* sp. DE2E. Our interest for peptide metabolites especially non-ribosomal peptides was amazingly reward when the ^{1}H-NMR revealed several purported protons attached to a heteroatom which was believed to be nitrogen as expected in peptide molecules. This also confirmed the initially phytochemical analysis for the fraction DE2E-TSBY-FD-SFB to contain molecules that have free nitrogens (1^o/2^o amines/amides) just as in peptides. The deduction therefore was that, these HPLC isolates, fraction E – I labelled respectively DE2E-TSBY-FD-SFB-(E/F/G/H/I) could be non-ribosomal peptides produced by the

Ghanaian *Streptomyces* sp. DE2E. The ^{13}C and full 2D NMR (HSQC, ^{1}H-^{1}H-COSY, gHSQC-TOCSY, NOESY and HMBC) data were acquired for all five compounds DE2E-TSBY-FD-SFB-(E/F/G/H/I). However, due to time constraints, NMR data for fraction DE2E-TSBY-FD-SFB-I (purest of all 5) only was processed to facilitate the complete structure elucidation of compound I which will afford us the opportunity to know the skeletal structure of this compound which is possibly related to the other four compounds (E, F, G and H).

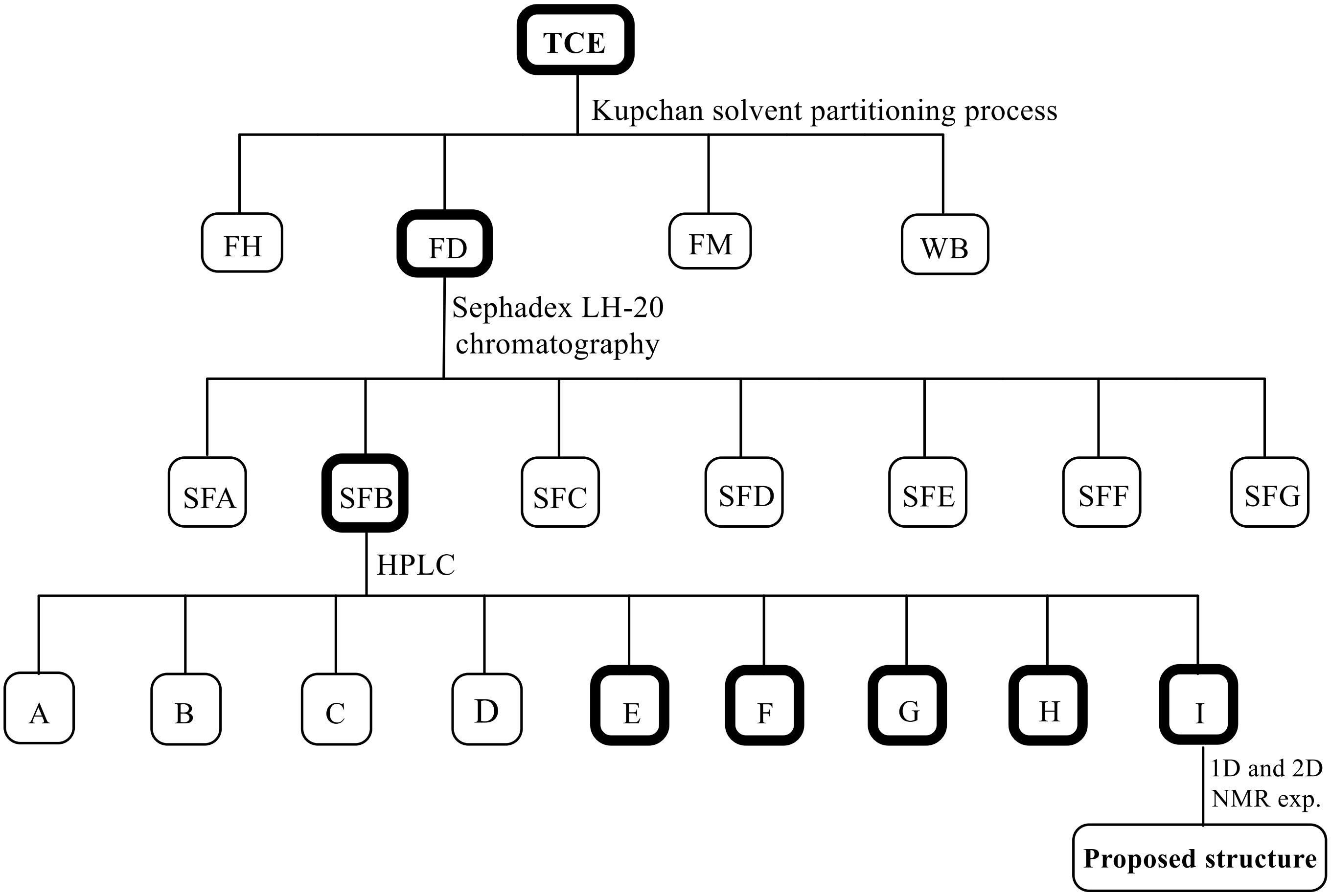


Figure 23: A flowchart showing the route for the purification and isolation of the compounds of interest from the DE2E-TSBY TCE.

3.9 Biological activity tests

3.9.1 Chemicals and reagents

RPMI-1640, IMDM, M-199, HEPES, YI-S, Foetal Bovine Serum (FBS), Adult Bovine Serum (ABS) Gentamycin, Penicillin-Streptomycin-L-Glutamine (PSG), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), Artesunate, Alamar dye, Dimethyl sulphoxide (DMSO), Sodium citrate, Adenine, Sodium bicarbonate ($NaHCO_3$), AlbuMax II, Sodium chloride (NaCl), Potassium chloride (KCl), Sodium Phosphate Dibasic (Na_2HPO_4), Sodium Phosphate Monobasic (KH_2PO_4), Sodium bicarbonate ($NaHCO_3$), Sodium hydroxide (NaOH), all bought from Sigma-Aldrich in the United States of America.

3.9.2 Preparation of compounds for bioactivity testing

A stock solution of 10 mM concentration of DE2E-TSBY-FD-SFB-I was prepared. The compound was first dried using nitrogen gas after which its mass was obtained by means of a mass balance. The compound was then dissolved in an appropriate volume of dimethyl sulfoxide (DMSO) to obtain the 10 mM concentration. This was followed by vortexing of solution and filter sterilization into vials through 0.45µm millipore filters under sterile conditions. The solution was then stored and at -20°C until used.

3.9.3 Screening of compounds for antimicrobial activity

Nine different standard bacteria strains, 6 Gram negative, *Eschericha coli* (ATCC 25922), *Salmonella paratyphi ß (9150)*, *Shigella sonnei* (ATCC 12022), *Shigella dysenteriae* (13313), *Salmonella typhimurium* (14028) and *Salmonella enteritidis (*ATCC 13076) and 3 Gram-positive, *Staphylococcus aureus* (ATCC 29213), *Bacillus cereus* (ATCC 14579) and *Staphylococcus* *epidermidis* (ATCC 12228) were used in this study. Each stocked standard bacteria strain was incubated overnight at 37ºC on a Mueller-Hinton agar (Park Scientific Limited) plate at 37ºC before the antimicrobial assay. Three individual colonies from the bacteria plate were selected, transferred into media and incubated at 37ºC overnight for the bacteria to reach the log phase of growth. The log phase bacteria were diluted with sterile saline to achieve a turbidity of 0.5 McFarland standard, an approximate concentration of 2 x 10^8 CFU/ml. The bacteria were then diluted to the working concentration, which varied between bacteria. Log phase of bacteria at a concentration range of 1 x10^2 to 1x10^6 CFU/ml were incubated with different concentrations of the compounds (100 µM – 0 µM) and 10% Alamar Blue® reagent at 37ºC for 6-8 hrs. Absorbances were read at wavelengths of 540 nm for the samples and 595 nm for the reference standard using the TECAN Sunrise Wako spectrophotometer, AUSTRIA GmbH. IC_{50} values of compounds were calculated by linear regression. Ampicillin was used as positive control.

3.9.4 Screening of compounds for antitrypanosomal activity

3.9.4.1 Culturing of trypanosome parasites

In vitro culturing of parasites was carried out using previously established specifications[129] and in this study, a strain of the blood stream form of *T, brucei* called the GUTat 3.1 strain was employed. Upon reaching confluence, a concentration of 1×10^6 parasite/mL of the parasites was cultured and the concentrations estimated using the Neubauer counting chamber. This was followed by the dilution of parasites to a concentration of 3×10^5 parasites/ml with Iscove's Modified Eagle's Medium (IMEM) and this was used for the drug assay.

3.9.4.2 In vitro viability test for trypanosome parasites

The viability of both treated and untreated trypanosome parasites was estimated using the alamar blue assay performed in a 96-well-plate following the manufacturers with some modifications. Varying concentrations of the compound, ranging from 0 µM to 100 µM was used to seed a 1.5 x 10^4 concentration of trypanosome parasites and final concentrations of DMSO were kept at 0.1%. After a 24-hour period, 10% alamar blue was added to the seeded parasites which had been kept at 37ºC in 5% CO_2 and incubated with or without the compound. Subsequently, the parasites were incubated for another 24 hours without exposure to light. The plate was then read for absorbance at 540 nm using the Tecan Sunrise Wako spectrophotometer after the combined period of 48 hours. The trend curve was drawn to obtain a 50% inhibitory concentration (IC_{50}) of the compound.

3.9.5 Screening of compounds for anti-leishmania activity.

3.9.5.1 Culturing of leishmania parasites.

Working concentrations of 6×10^6 cells/ mL of M-119 growth medium were used in culturing *L-donovani* (D10) and *L*- major log phases. After parasites had reached confluency, they were used and estimation of its concentration was carried out using the Neubauer counting chamber. Diluted parasite concentration of 3×10^5 parasites/ml in M199 medium and were used for the drug assay.

3.9.5.2 In vitro viability test for leishmania parasites.

The viability of both treated and untreated leishmania parasites was estimated using the alamar blue assay performed in a 96-well-plate following the manufacturer's instructions while making slight modifications. Varying concentrations of the compound, ranging from 0 µM to 100 µM was used to seed a 1.5X10^4 concentration of leishmania parasites and final concentrations of DMSO were kept at 0.1%. After a 24-hour period, 10% alamar blue was added to the seeded parasites which had been kept at 37ºC in 5% CO_2 and incubated with or without the compound. Subsequently, the parasites were incubated for another 24 hours without exposure to light. The plate was then read for absorbance at 540 nm using the Tecan Sunrise Wako spectrophotometer after the combined period of 48 hours. The trend curve was drawn to obtain a 50% inhibitory concentration (IC_{50}) of the compound.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Initial chemical investigations of *Streptomyces* sp. DE2E

The initial chemical investigation of the Ghanaian novel bacteria strain *Streptomyces* sp. DE2E began with the isolation of the bacteria from sampled soils as described in **section 3.3.2** which was carefully undertaking to ensure the isolation and growth of the pure colonies of *Streptomyces* sp. DE2E void of any microbial contaminations. Under favourable laboratory, growth and culture conditions *Streptomyces* sp. DE2E was isolated on (ISP)2 agar plates and observed to be a highly sporulating bacteria as highlighted in **Figure 17** which is quite typical of *Streptomyces*. Based on careful analysis of the HRESI/HPLC-DAD-MS^n and ^{1}H-NMR data of the TCE of obtain in both (ISP)2 and TSBY small scale cultures as detailed in **sections 3.3 – 3.5**, the decision was taken to large scale culture the strain in the TSBY liquid media over the (ISP)2. However, a replica (ISP)2 media large scale culture was also done to afford a vivid comparison of the TCE from both (ISP)2 and TSBY large scale cultures of *Streptomyces* sp. DE2E. As detailed in **section 3.6**, the harvested cultures were exhaustively extracted for their produced secondary metabolites using the HP-20 resin followed by solvent extraction of the resin and broths alternatively to yield the combined total crude extracts (TCE). The Kupchan modified solvent partitioning performed by a combination of different solvent systems on the both (ISP)2 and TSBY culture TCE resulted in four distinct semi pure fractions; WB, FD, FM and FH with varying polarities after which their respective LC-MS and ^{1}H-NMR data were all acquired. The HRESI/HPLC-DAD-MS^n data was measured in positive mode and was analyzed using the software Xcalibur. The major deduction from the careful analysis of the LC-MS and ^{1}H-NMR data for all Kupchan fractions confirmed the initial idea that the strain *Streptomyces* sp. DE2E was more biosynthetically prolific in the TSBY media compared to the (ISP)2 liquid media. The careful examination of the LC-MS data for each corresponding Kupchan fraction in both media, clearly validates this assumption, illustrated by the presence of more metabolites in the TSBY Kupchan fractions compared to their corresponding (ISP)2 fractions. For example, a thorough overview of the LC-MS data for Kupchan FH fraction from the (ISP)2 culture labeled DE2E-FH (**Appendix 1a**) compared to the Kupchan FH fraction from the TSBY media labeled DE2E-TSBY-FH (**Appendix 1b**) shows that the latter fraction has more detected metabolites produced by the strain for possible isolation and characterization compared to the former. This phenomenon is also well observed in the other LC-MS data analysis for the rest of the Kupchan fractions as illustrated in **Appendix 2a – 4b**.

4.2 Isolation of targeted peptide metabolites produced *Streptomyces* sp. DE2E

Based on the deductions made in **section 4.1**, the aim of isolating any large molecule peptides could only come from the investigations of the DE2E-TSBY Kupchan fractions rather than the DE2E-(ISP)2 fractions. Among the DE2E-TSBY Kupchan fractions, it was observed that the Kupchan fraction FD was much interesting compared to the other Kupchan fractions after critically analyzing their LC-MS and ^{1}H-NMR data. Very interesting was the observed series of metabolites in the LC-MS data of the FD (**Figure 24/Appendix 2b**) with high molecular weights ($m/z \approx 800$ - 900) supported by its intriguing ^{1}H-NMR spectra highlighting very interesting NMR signals. However, it was the TLC followed with phytochemical analysis that suggested that, this detected metabolite in the DE2E-TSBY-FD fraction could be possible large peptide molecules. The adopted phytochemical screening; ninhydrin and Dragendorff all gave positive tests to indicate the presence of free nitrogens (1^o amines/amides) or trap nitrogens ($2^o/3^o$ amines/amides) which are predominant in peptide molecules. The prioritized Kupchan fraction DE2E-TSBY-FD was further purified by size-exclusion chromatography using Sephadex LH-20 with an isochratic elution

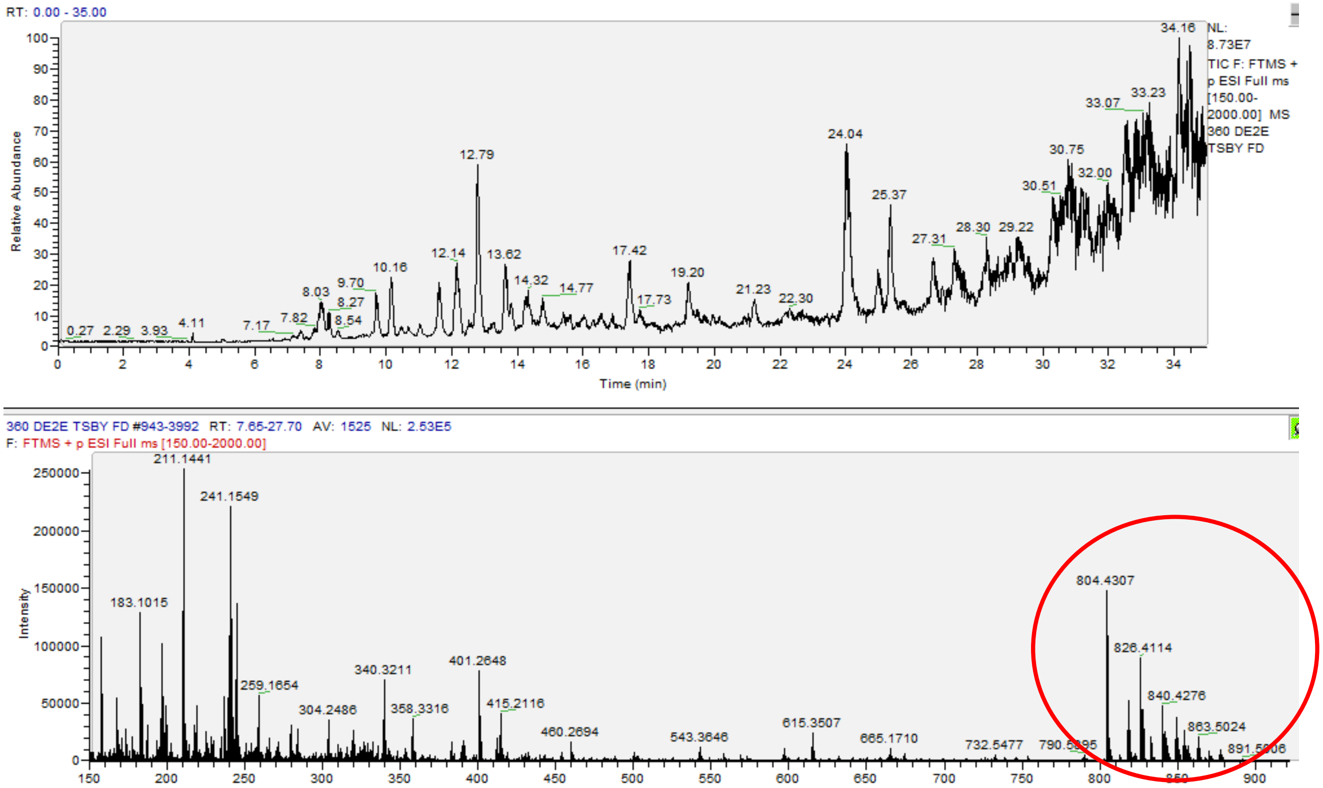
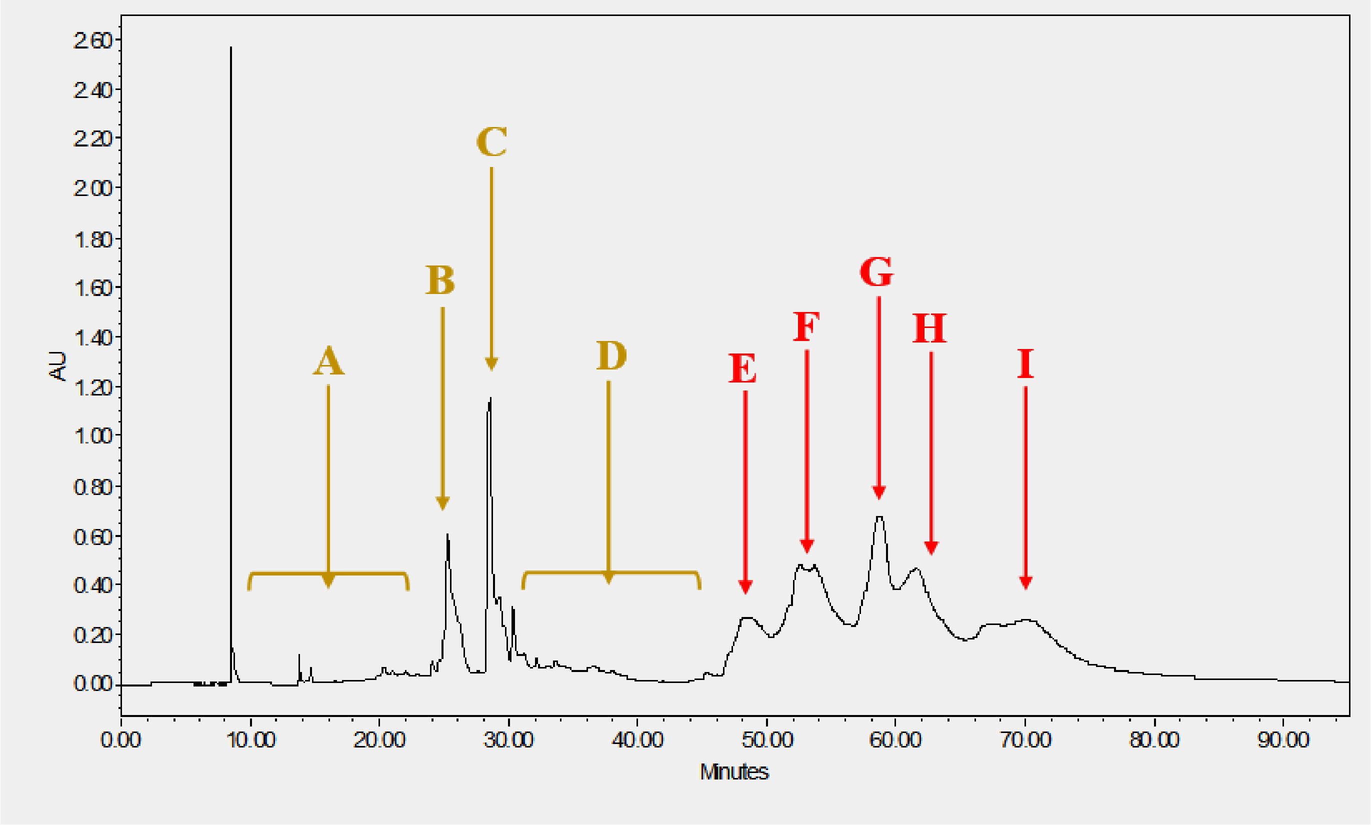


Figure 24: **HRESI-MS data for fraction DE2E-TSBY- FD indicating the targeted metabolites**

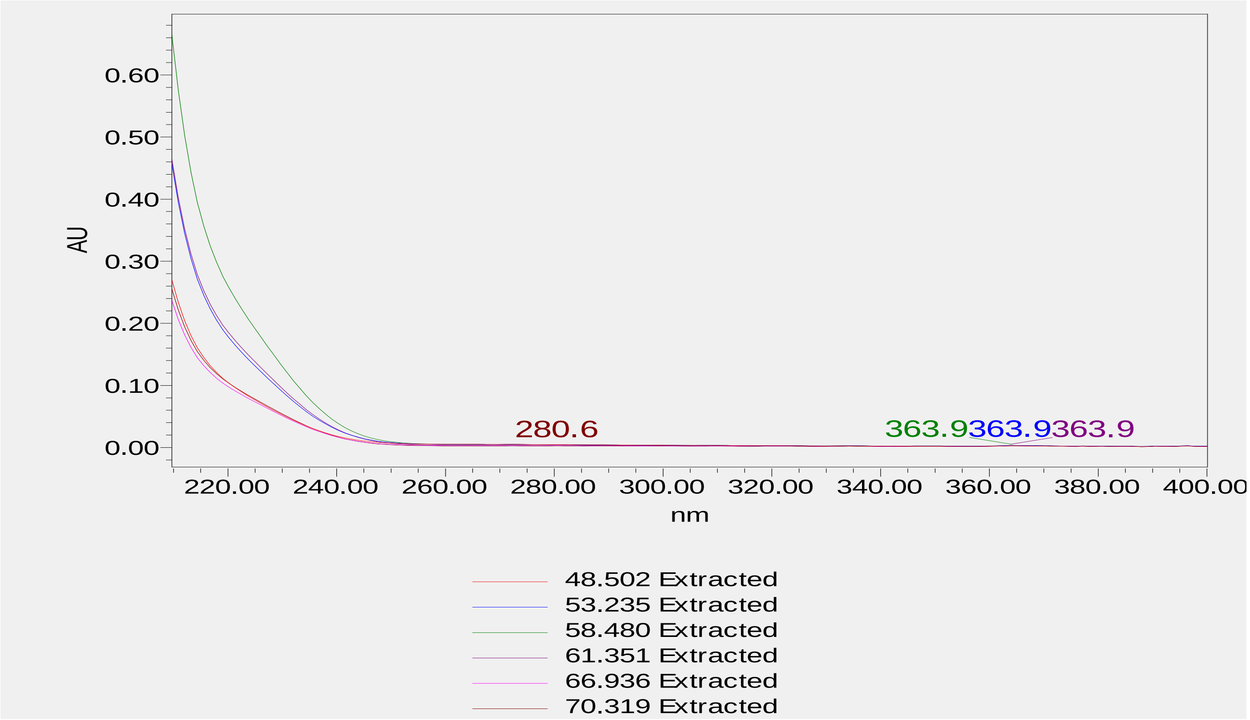
buffer made of 50:50 MeOH/AceN solvents. A total of seven fractions (SFA – SFG) were eluted from this fractionation as detailed in **section 3.8.4**. Subjecting all the Sephadex fractions to previously stated chemical profiling analysis, led to the prioritization of the Sephadex fraction B (SFB) to possibly contain the target peptide molecules. Aside the fraction SFB, fractions SFC and SFD through the analysis of their ^{1}H-NMR and relevant phytochemical screening also demonstrated the presence of peptide/alkaloid molecules (**Figure 22**), however the relative lesser complexity (in terms of purity) of the fraction SFB was identified to be more promising for the easy and quick isolation of the detected metabolites. Additionally, the fraction SFB recorded the highest weight of 164.079mg, coupled with the fact that it was the second fraction to elute from the size exclusion chromatography in which relatively large molecules due to their inability to interact with the pores of the stationary phase, glide around the matrix and quickly elute out from the column compared to relatively small molecules that interact greatly with the pores of this stationary phase (Sephadex LH-20) to spend much longer times on the column, indicating that the metabolites contained in the fraction SFB are possible large molecules.

The fraction SFB labelled as DE2E-TSBY-FD-SFB was therefore subjected to HPLC analysis to isolate the targeted metabolites as detailed in **section 3.8.5.** This was achieved by reconstituting half of the fraction DE2E-TSBY-FD-SFB in about 15mL of DCM and MeOH solution for the HPLC analysis. About 250µL of the sample solution with concentration 5.4mg/mL was injected with each chromatographic run taking about 100 minutes to complete, totaling a whopping 44 hours for 26 injections obtained. The HPLC analysis yielded nine fractions (A - I) as illustrated in **Figure 25**, however the more interesting fractions were fractions E to I eluting with retention times (R_T) 45 – 100 minutes, thus taking about 55 minutes for this group of compounds to elute out of the column. Per the analysis, for compounds to elute this very late on a reverse phase C18 column suggests that they must be lipophilic/aliphatic enough to greatly interact with the long aliphatic chains of the C18 stationary phase. The isolated fractions DE2E-TSBY-FD-SFB- (E, F, G, H and I) were all detected to lack any UV absorption (**Figure 25**) due to the absence of any chromophore or conjugate pi systems in their structures. They were also observed to be white powders that somewhat crystalizes well in a dried DCM or deuterated $CDCl_3$ solution.

The ^{1}H-NMR and ^{13}C NMR for this interesting isolates were acquired in deuterated chloroform ($CDCl_3$) which revealed the five metabolites (E - I) are possible peptide molecules produced by strain *Streptomyces* sp. DE2E. The closeness of their HPLC peaks explains the fact that the structures of these five compounds must be closely related. This was confirmed by the analysis of their NMR spectra exemplified by compounds DE2E-TSBY-FD-SFB-G and DE2E-TSBY-FD-SFB-I illustrated in **Figures 26** and **27** respectively with similar ^{1}H-NMR and ^{13}C NMR signals.



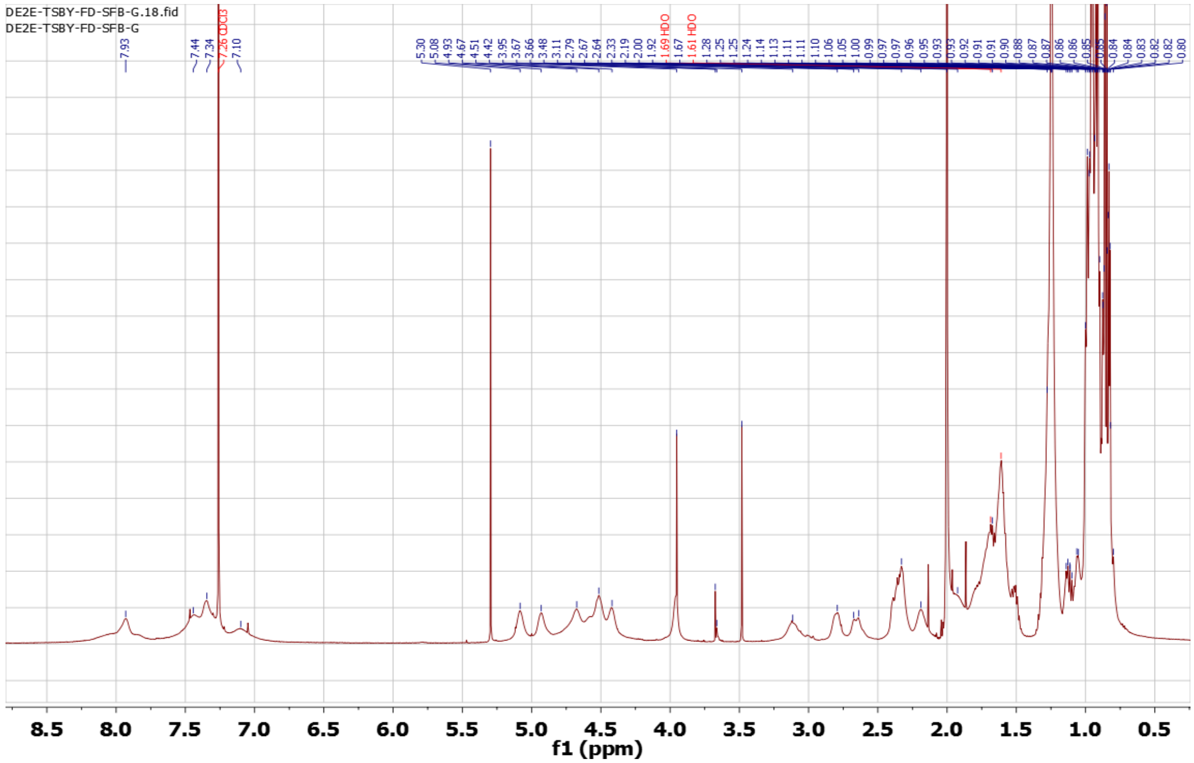
(a)



(b)

Figure 25:(a) HPLC chromatogram obtained for the fraction DE2E-TSBY-FD-SFB (b) UV absorption profile for isolated compounds E - I

(a)



(b)

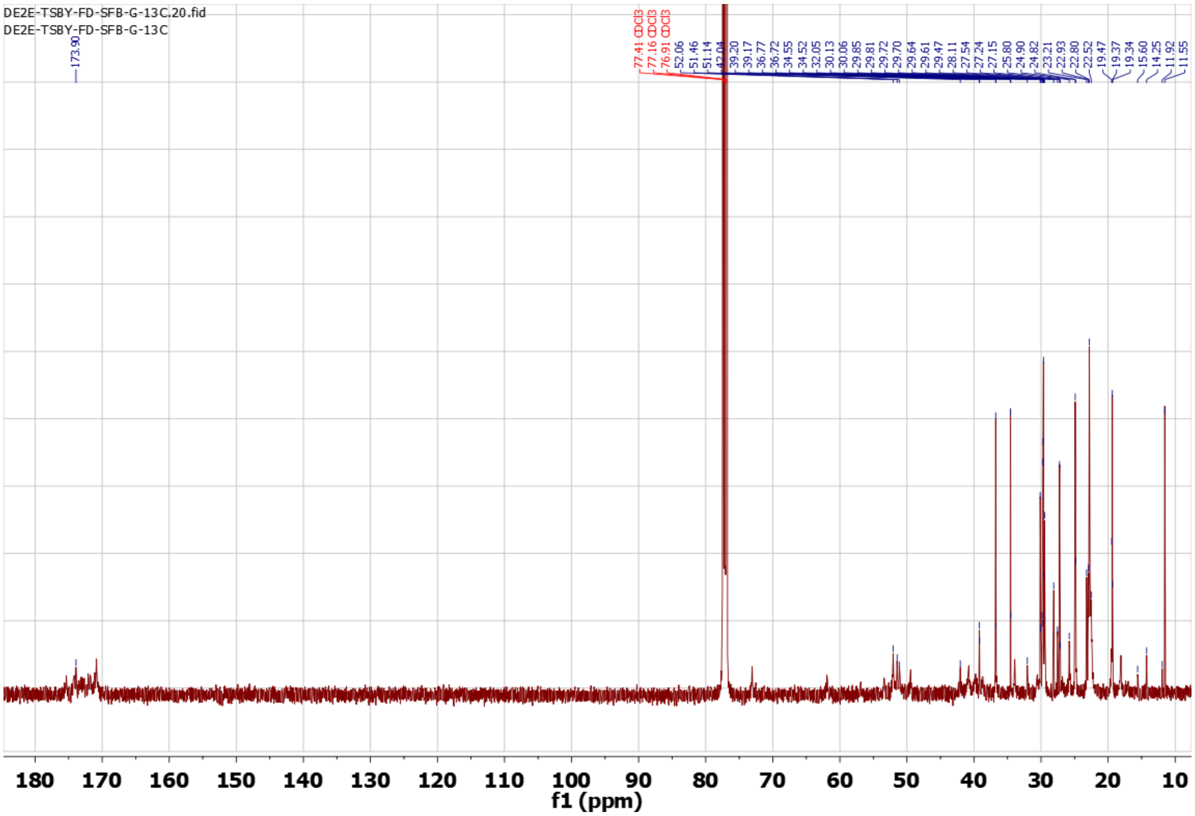


Figure 26: (a) ^{1}H-NMR and (b) ^{13}C NMR spectra for compound DE2E-TSBY-FD-SFB-G in **$CDCl_3$**

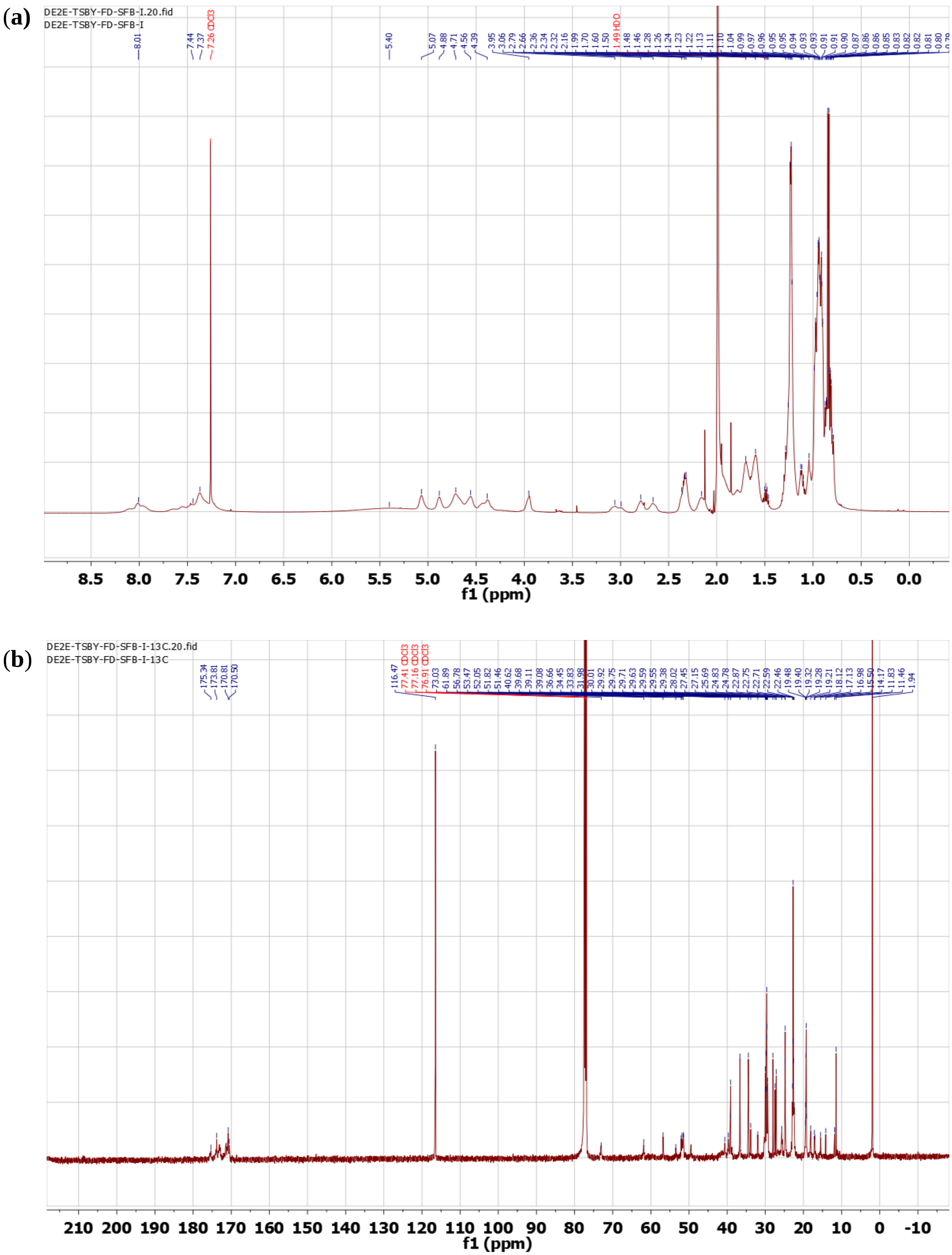


Figure 27: (a) ^{1}H-NMR and (b) ^{13}C NMR spectra for compound DE2E-TSBY-FD-SFB-I in **$CDCl_3$**

Based on a comprehensive examination of the ^{1}H NMR of the five isolated compounds, DE2E-TSBY-FD-SFB- (E, F, G, H and I), the compound DE2E-TSBY-FD-SFB-I was chosen for full NMR data acquisition and subsequent structure determination with the expectation that, its proposed structure will provide insights to the structures of the other four HPLC isolates (E – H). The ^{13}C, full 2D-NMR data (HSQC, ^{1}H-^{1}H-COSY, gHSQC-TOCSY, NOESY and HMBC) acquired in $CDCl_3$ (**Appendix 5a-10g)** and the HRESI/HPLC-DAD-MS^{n} data were all acquired for the compound DE2E-TSBY-FD-SFB-I to afford its complete structure elucidation.

4.3 Structure elucidation of compound DE2E-TSBY-FD-SFB-I

DE2E-TSBY-FD-SFB-I was isolated from the Ghanaian novel *Streptomyces* sp. DE2E as a white sparkly powder and odorless compound, very soluble in DCM and somewhat crystalizes in a dried solution of $CDCl_3$. The UV spectrum of this compound showed no prominent absorption maxima indicating the absence of a principal chromophore or conjugated pi systems in the compound. The high resolution mass spectrometry analysis of the compound gave molecular ion m/z = 864.6347 $[M+H]^{+}$ (**Figure 28**) representing a molecular formula of $C_{47}H_{85}N_5O_9$ with error margin, $\Delta = \pm 0.3$ ppm having 8 degrees of unsaturation. The ^{13}C NMR of this compound gave 47 carbons comprising 7 quaternaries, 11 methines, 19 methylenes and 10 methyls. Due to the rather large size of this molecule, a different approach to the analysis of the 1D and 2D-NMR was adopted which involved solving the structure of its component amino acids one at a time and using the HRESI-MS data to verify proposed structures in such a way that the substructures obtained can be adequately pieced together.

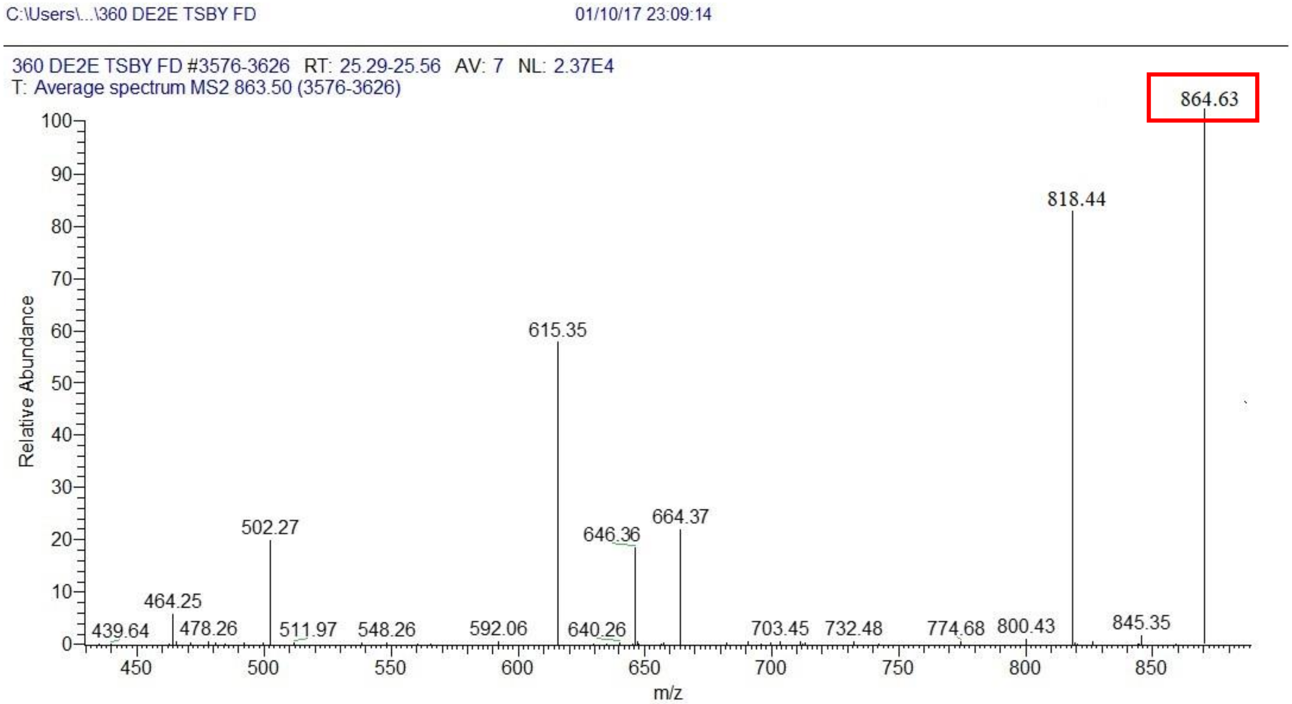


Figure 28: HRESI/HPLC-DAD-MS^n spectra for compound DE2E-TSBY-FD-SFB-I indicating the molecular ion 864.63 [M+H]$^+$ and its various fragment ions.

4.3.1 Leucine residues

Two leucine residues were detected in the 1D and 2D NMR data of this peptide and their presence were confirmed by the appearance of δ_H 0.95 (6H, ov., H-**M**.), δ_H 0.96 (6H, ov., H-**L**.), 1.59 (1H, ov., H-**P**), δ_H 1.72 (1H, ov., H-**P'**), δ_H 1.66 (1H, ov., H-**J.**), δ_H 2.82 (1H, ov., H-**Q**), δ_H 2.32, (1H, ov., H-**Q'**),δ_H 4.55 (1H, s., H-**K**) and δ_H 4.72 (1H, s., H-**N**) with corresponding carbons at C-**M.** (δ_C 22.5), C-**L.** (δ_C 22.7), C-**P** (δ_C 19.3), C-**J.** (δ_C 24.7), C-**Q** (δ_C 39.6), C-**K** (δ_C 53.4), and C-**N** (δ_C 51.4) respectively. The full 1D and 2D-NMR correlations for the first and second leucine residues are summarized **Tables 8** and **9** and further illustrated in **Figures 29** and **30** respectively.

#	δ ^{13}C (ppm)	^{13}C mult	δ ^{1}H (ppm)	Mult (Hz)	^{1}H-^{1}H COSY	^{1}H-^{1}H TOCSY	HMBC
E	170.8	C					
K	53.4	CH	4.55	s	p, p'	p, l•	
P	40.5	CH_2	1.59 1.72	m m	k	k, l•	l•
J•	24.7	CH	1.66	ov.		k, l•	l•
L•	22.7	CH_3	0.96	ov.		j• k, p	j•, p

Table 8: 1D and 2D-NMR data for the first Leucine residue acquired in $CDCl_3$

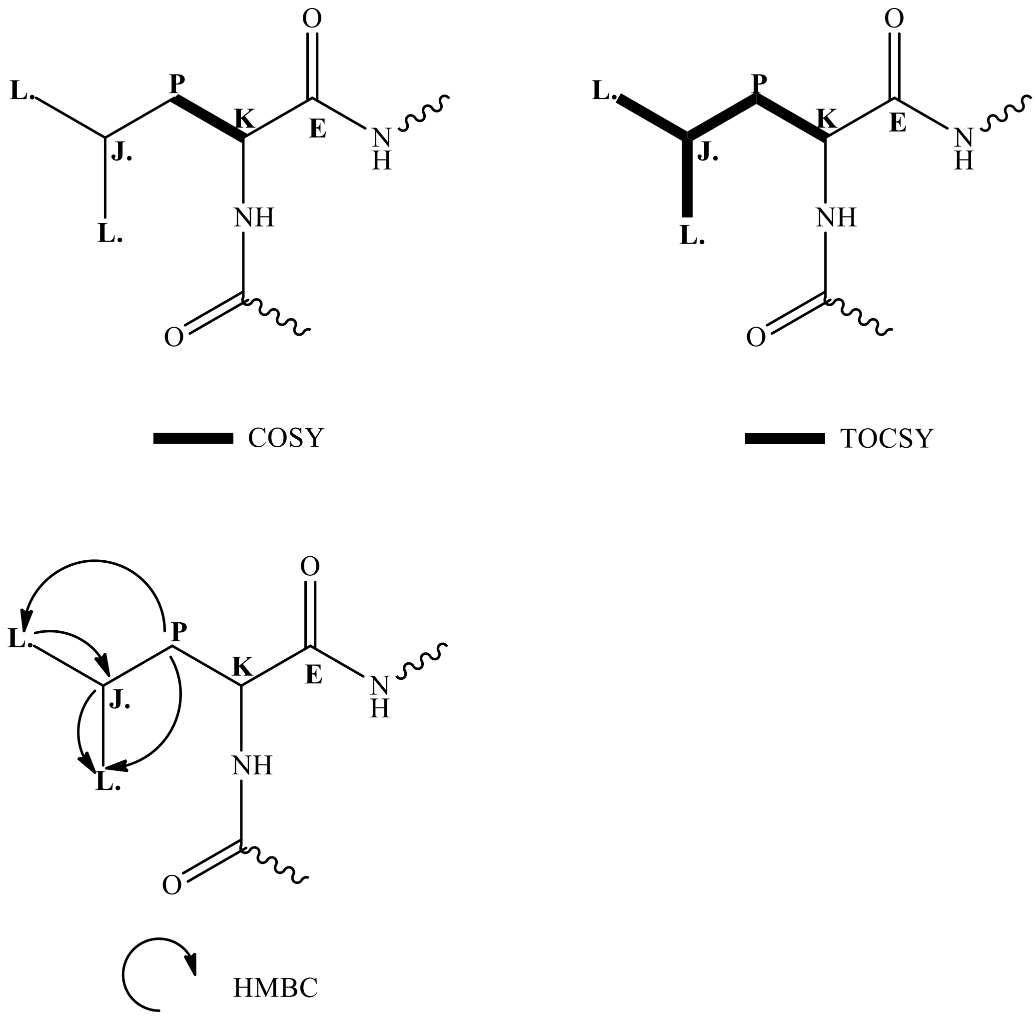


Figure 29: Substructure for the first Leucine residue showing TOCSY, COSY, and HMBC correlations

#	δ ^{13}C (ppm)	^{13}C mult	δ ^{1}H (ppm)	Mult (Hz)	^{1}H-^{1}H COSY	^{1}H-^{1}H TOCSY	HMBC
1NH					n	n	
E	170.8	C					
N	51.4	CH	4.72	s	j, 1NH	j., q', 1NH	
Q	39.6	CH$_2$	2.35 2.82	ov ov		m., n	
J.	24.7	CH	1.66	ov.	n	n,	m.
M.	22.5	CH$_3$	0.95	ov.		n, q	j.

Table 9: 1D and 2D-NMR data for second Leucine residue acquired in CDCl$_3$

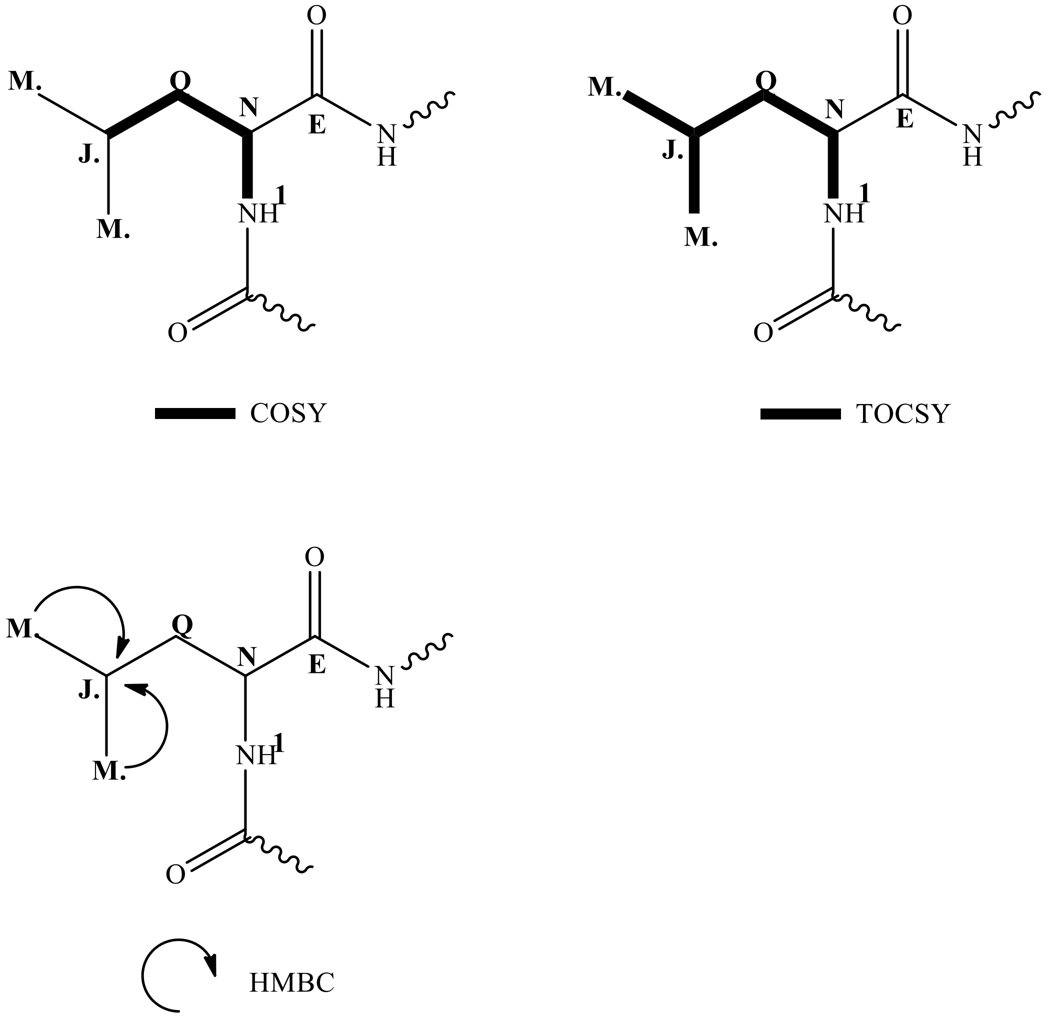


Figure 30: Substructure for the second Leucine residue showing TOCSY, COSY, and HMBC correlations

4.3.2 Valine residue

One valine residue was also identified in this peptide sequence by analysis of the 1D and 2D NMR data. This substructure was evident from the δ_{H} 0.82 (3H, ov., H-**R.**), δ_{H} 0.88 (3H, ov., H-**S.**), δ_{H} 2.16 (1H, ov., H-**V**) and δ_{H} 4.39 (1H, s, H-**J**) with corresponding carbons at C-**R.** (δ_{C} 19.19.3), C-**S.** (δ_{C} 19.2), C-**V** (δ_{C} 31.7) and C-**J** (δ_{C} 56.7) respectively. The crucial correlations in this spin systems are illustrated in **Figure 31** with a summary of the data given in **Table 10**.

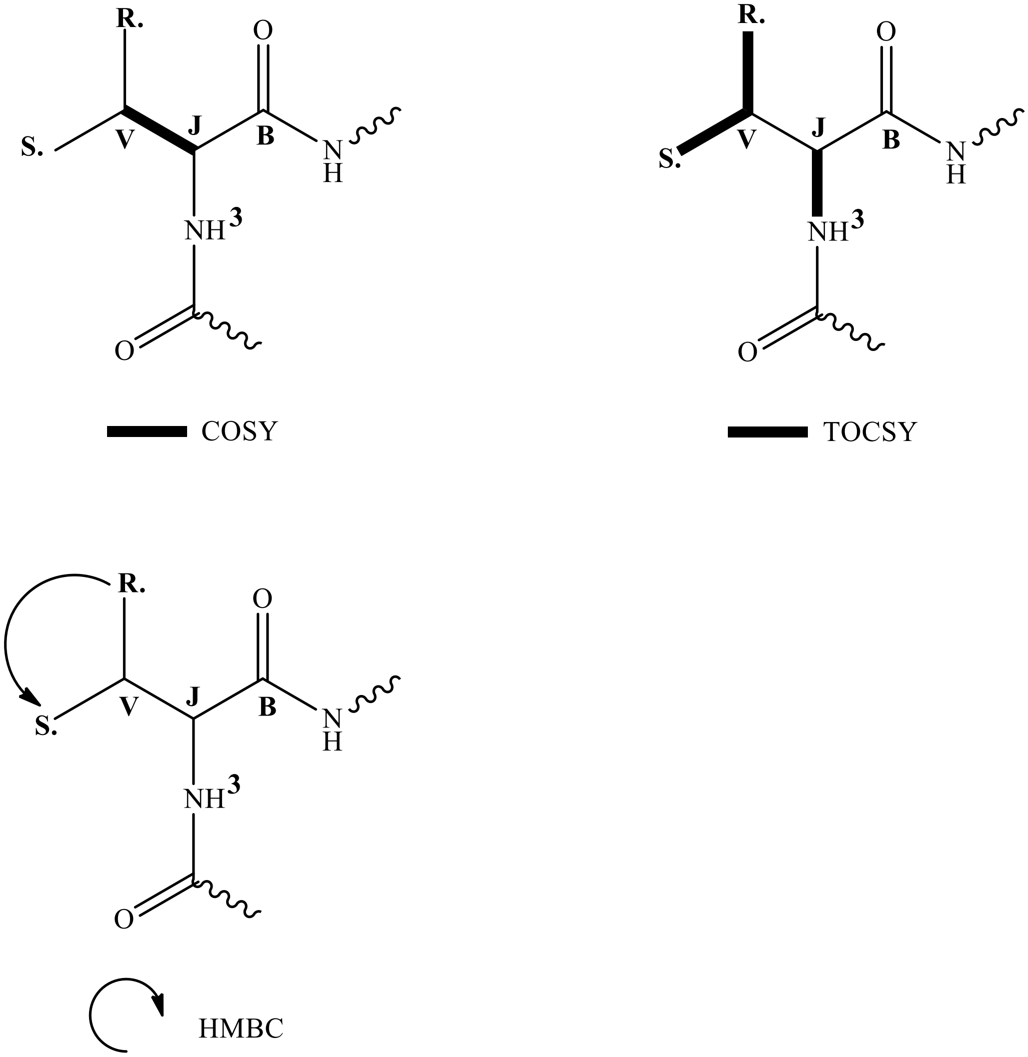


Figure 31: Substructure for the Valine residue showing TOCSY, COSY, and HMBC correlations

#	δ ^{13}C (ppm)	^{13}C mult	δ ^{1}H (ppm)	Mult (Hz)	^{1}H-^{1}H COSY	^{1}H-^{1}H TOCSY	HMBC
3NH						j	
B	173.8	C					
J	56.7	CH	4.39	s	v	3NH	
V	31.7	CH	2.16	ov.	j	r., s.	
R.	19.3	CH_3	0.82	ov.		v	s.
S.	19.2	CH_3	0.88	ov.		v	r.

Table 10: 1D and 2D-NMR data for the Valine residue acquired in $CDCl_3$

4.2.3 Aspartic acid residue

Furthermore, one aspartic acid residue was also identified in the structure of this novel peptide. The presence of this amino acid was evident by the observation of protons δ_H 2.68 (1H, m, H-**S**), δ_H 3.08 (1H, m., H-**S**') and δ_H 4.92 (1H, s, H-**O**) with corresponding carbons at C-**S** (δ_C 36.5) and C-**O** (δ_C 49.4) respectively. The characteristic correlations of this substructure are shown in **Figure 32** with a summary of the data listed in **Table 11**.

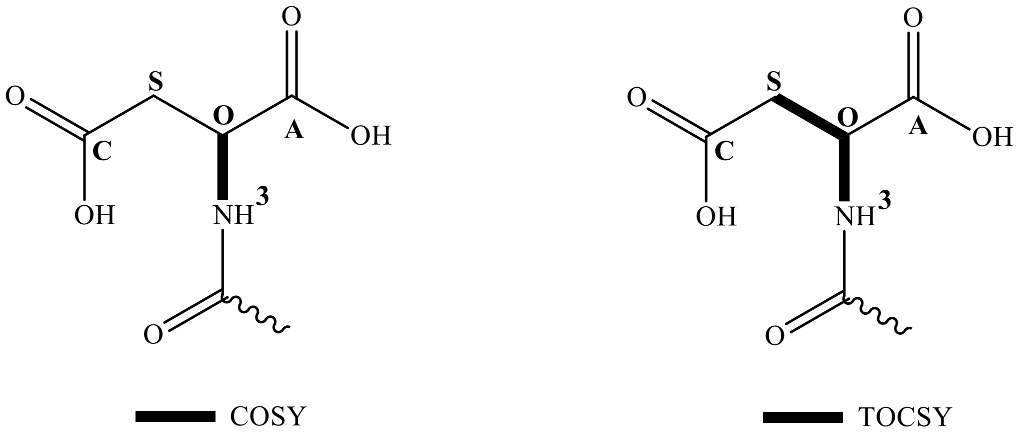


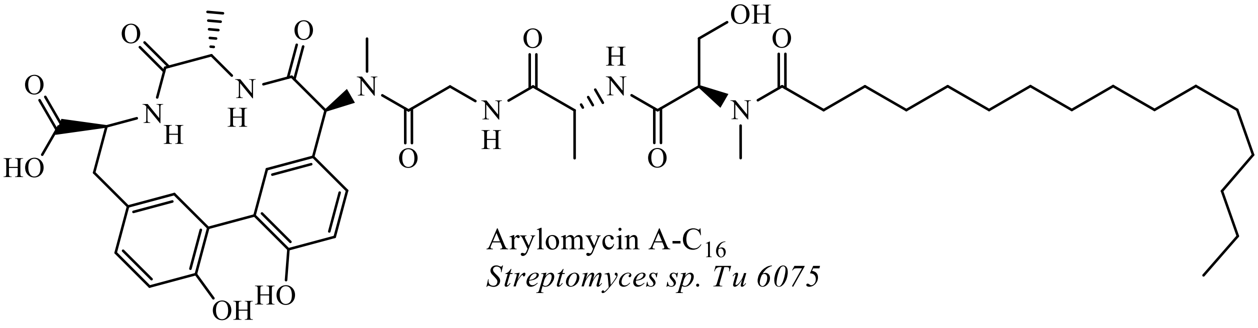
Figure 32: Substructure for Aspartic acid residue showing TOCSY and COSY correlations

#	δ ^{13}C (ppm)	^{13}C mult	δ ^{1}H (ppm)	Mult (Hz)	^{1}H-^{1}H COSY	^{1}H-^{1}H TOCSY
3NH						o, s
A	175.2	C				
C	173.0	C				
O	49.4	CH	4.92	s	3NH	3NH, s, s'
S	36.5	CH_2	2.68 3.08	m m		s', o, 3NH

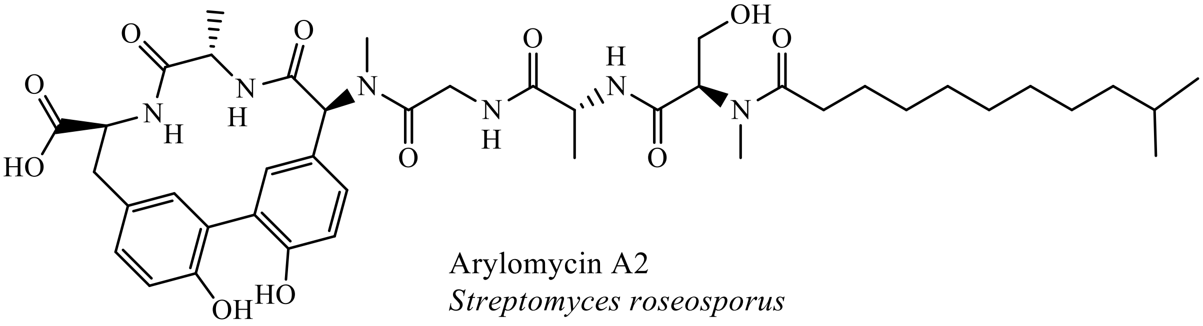
Table 11: 1D and 2D-NMR data for the Aspartic acid residue acquired in $CDCl_3$

Apart from the proteinogenic amino acids whose structures have been determined for this novel peptide, there were some post-translationally modified amino acids detected as well. The structures of these were determined by interpretation of the current experimental data in comparison to literature data for such peptide compounds with very intriguing post-translational modifications produced by some *Streptomyces* sp. As ardently elaborated in **sections 2.5** and **2.6**, both ribosomal peptides and non-ribosomal peptides can be heavily post-translationally modified, however more interesting is the presence of non proteinogenic amino acids and the incorporation of polyketides and fatty acids moieties in the biosynthesis of non-ribosomal peptides which results in their excellent structural complexity and diversity (multi-functionalized). A very common integration to the peptide chains of NRPs are prolong aliphatic C-C units especially in saturated/lipophilic NRPs produced by *Streptomyces* sp. For example, the unique scaffolds of the potent antibiotics; Arylomycin class of compounds (lipoglycopeptides) are biosynthesised by the NRPS machinery that constitute a recently discovered class of natural product antibiotics, displays very extended aliphatic carbon chain moieties exemplified in the structures of Arylomycin A2 and Arylomycin A-C_{16} (**Figure 33**) defined by the integration of different fatty acid lipid tails.[108] The Arylomycins exhibit a broad-spectrum antibacterial activity that are needed to treat multi-drug resistant pathogens by mostly inhibiting the type I signal peptidases (SPases) of both Gram-positive and

Gram-negative bacteria which can be alluded to the presence of aliphatic/lipophilic long fatty acid tails that enables them to actively interact with receptor membranes.[109]



Arylomycin A-C_{16}
Streptomyces sp. Tu 6075



Arylomycin A2
Streptomyces roseosporus

Figure 33: Representative structures of the Arylomycin class of compounds (lipopeptides)

The structure elucidation process for the isolated compound DE2E-TSBY-FD-SFB-I detected a non proteinogenic acid (2/α)-aminobutanoic acid (AABA/homoalanine) and a **C_{20}** prenylated or fatty acid tail unit as post-translational modifications integrated in the peptide structure of the compound. The structure elucidation of these two residues are discussed in the sections below.

4.2.4 α-aminobutanoic acid (AABA) residue

A (2/α)-aminobutanoic acid residue was found in the structure of this peptide confirmed by significant 1D and 2D NMR correlations within this substructure. The characteristic correlations data of this substructure are summarised in **Table 12** and illustrated in **Figure 34** below.

#	δ ^{13}C (ppm)	^{13}C mult	δ ^{1}H (ppm)	Mult (Hz)	^{1}H-^{1}H COSY	^{1}H-^{1}H TOCSY	HMBC
D	171.3	C					
I	61.8	CH	3.95	s	e•	e•, t•	t•
E•	29.3	CH_2	2.19	ov.	i	i	
T•	18.08	CH_3	0.94	ov.		i, e•	i

Table 12: 1D and 2D-NMR data for the 2-aminobutanoic acid acquired in $CDCl_3$

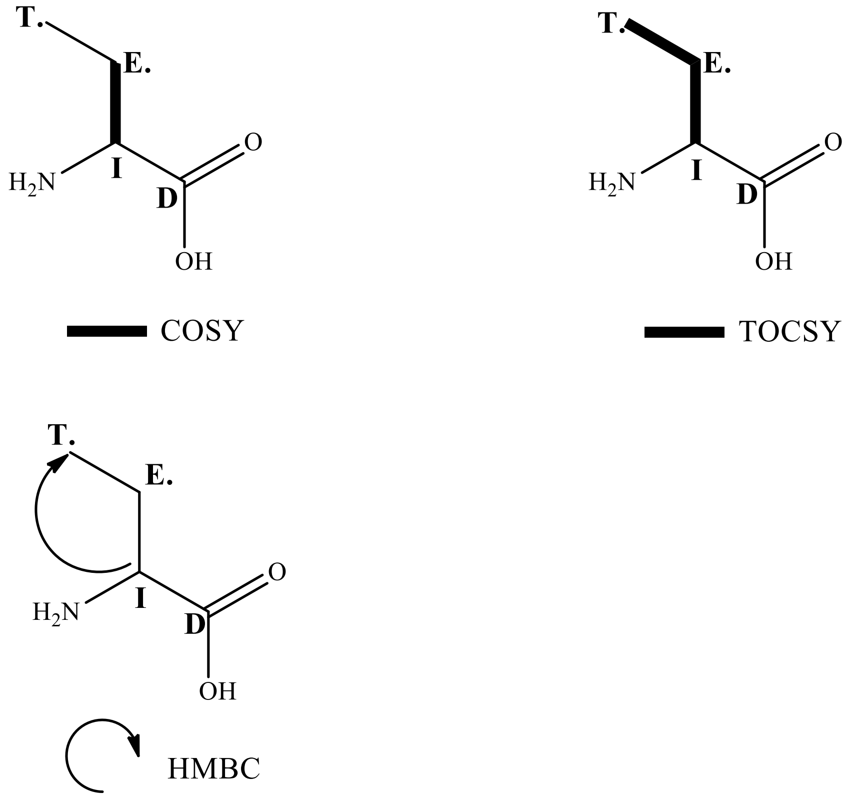


Figure 34: Substructure for 2-aminobutanoic acid residue showing TOCSY, COSY, and HMBC correlations

4.2.5 Non-amino acid Prenylation or fatty acid residue

One non-amino acid prenylation or fatty acid substructure was also obvious from the analysis of the NMR data. Interestingly, this substructure was also seen in other Streptomyces-derived Non-ribsomal peptides that prenylated or integrate fatty acids in their biosynthesis observed in the case of the Arylomycins discussed in the previous section. It’s characteristic and significant correlations are shown in **Figure 35** and the data summarized in **Table 13**.

#	δ ^{13}C (ppm)	^{13}C mult	δ ^{1}H (ppm)	Mult (Hz)	^{1}H-^{1}H COSY	^{1}H-^{1}H TOCSY	HMBC
F	170.5	C					
H	73.0	CH	5.08	q, q'	u, q		
Q	39.6	CH$_2$	2.81 2.34	ov. ov.	h, q'		
R	39.0	CH$_2$	1.13	m	f•	f•, t, g•, m•	f•
C••	36.6	CH$_2$	1.26 1.06	m m		l•, b•,	
T	34.4	CH	1.29	ov.		r, l•, i•	
U	33.7	CH$_2$	1.92 1.61	ov. ov.	h		
B•	29.6	CH$_2$	1.22	s		c••, i•	
C•	29.5	CH$_2$	1.26	ov.			
F•	28.0	CH	1.50	hept, J= 7.0, 6.6	r	r, m•	h•, m•
G•	27.1	CH$_2$	1.29	ov.		r	
H•	27.1	CH$_2$	1.25	ov.			f•
I•	25.6	CH$_2$	1.29	ov.		t, b•	
L•	22.7	CH$_3$	0.93	ov.		c••	
M•	22.5	CH$_3$	0.91	ov.		f•, r	f•

Table 13: 1D and 2D-NMR data for the non-amino acid prenylation or fatty acid residue acquired in CDCl$_3$

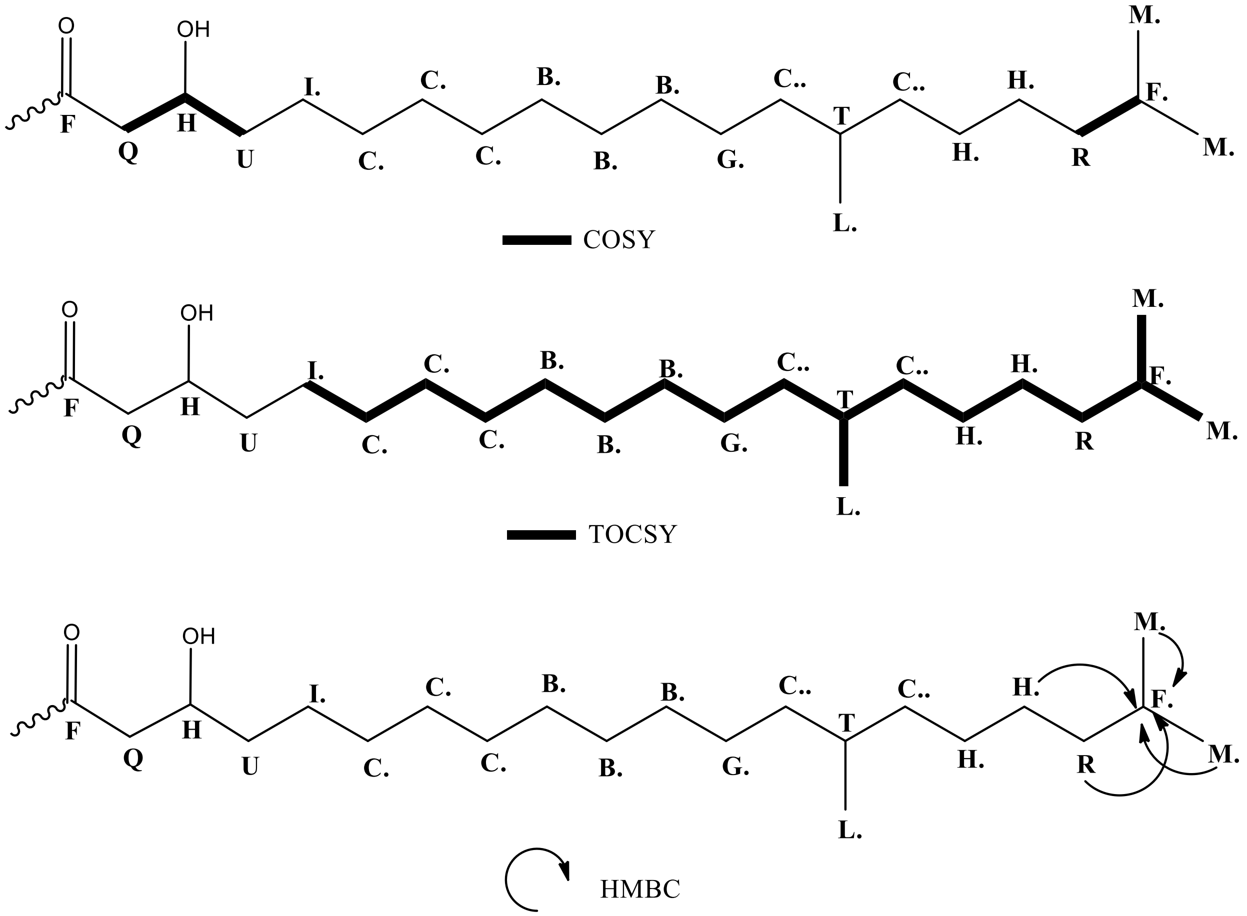
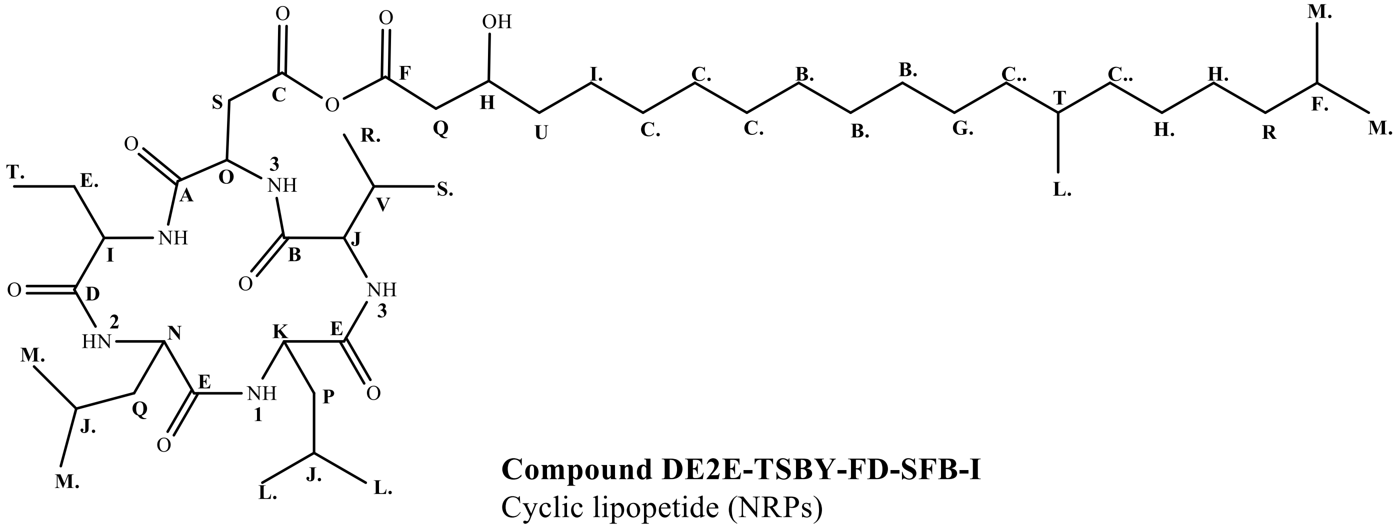


Figure 35: Substructure for the non-amino acid prenylation or fatty acid residue showing TOCSY, COSY, and HMBC correlations

The various substructure generated up to this point were joined together using the ROESY correlations such as **k-1NH**, **p-1NH**, **P-3NH**, **o-3NH**, **s-3NH**, **s-3NH**, **I-2NH** and **e-2NH**. Based on this correlations, the proposed structure of this compound was found to be a five amino acid cyclic peptide (pentapeptide) comprising one valine, two leucine, one aspartic acid and a non proteinogenic α-aminobutanoic acid (AABA) residue and a long aliphatic side chain (prenylated/fatty acid tail) attached to the aspartic acid residue. We therefore proposed the structure of compound DE2E-TSBY-FD-SFB-I as post-translationally modified NRPs.



Compound DE2E-TSBY-FD-SFB-I
Cyclic lipopetide (NRPs)
Streptomyces sp. DE2E
(proposed structure)

In order to fully confirm this structure, a further interpretation of the mass spectrometry data was done with structural proposals for the different mass fragments detected in the LC-MS is shown in **Figure 36**. The high resolution mass spectrometry analysis of the compound gave molecular ion with *m/z* = 864.6347 $[M+H]^+$ (**Figure 28**) with its fragment ions, provided information for the proposed fragmentation pattern of the compound. This proposed analysis assisted in the confirmation of the final structure of compound DE2E-TSBY-FD-SFB-I shown above. However, the lack of enough spin systems in the NMR data acquired in deuterated $CDCl_3$ due to the extensive NMR signal overlaps commonly exhibited by large peptide molecules implies that, advanced structural techniques such as the sequence tag mass spectrometry/tandem mass spec., X-ray crystallography and bioinformatics data must also be obtained to finally confirm this proposed structure of the compound DE2E-TSBY-FD-SFB-I.

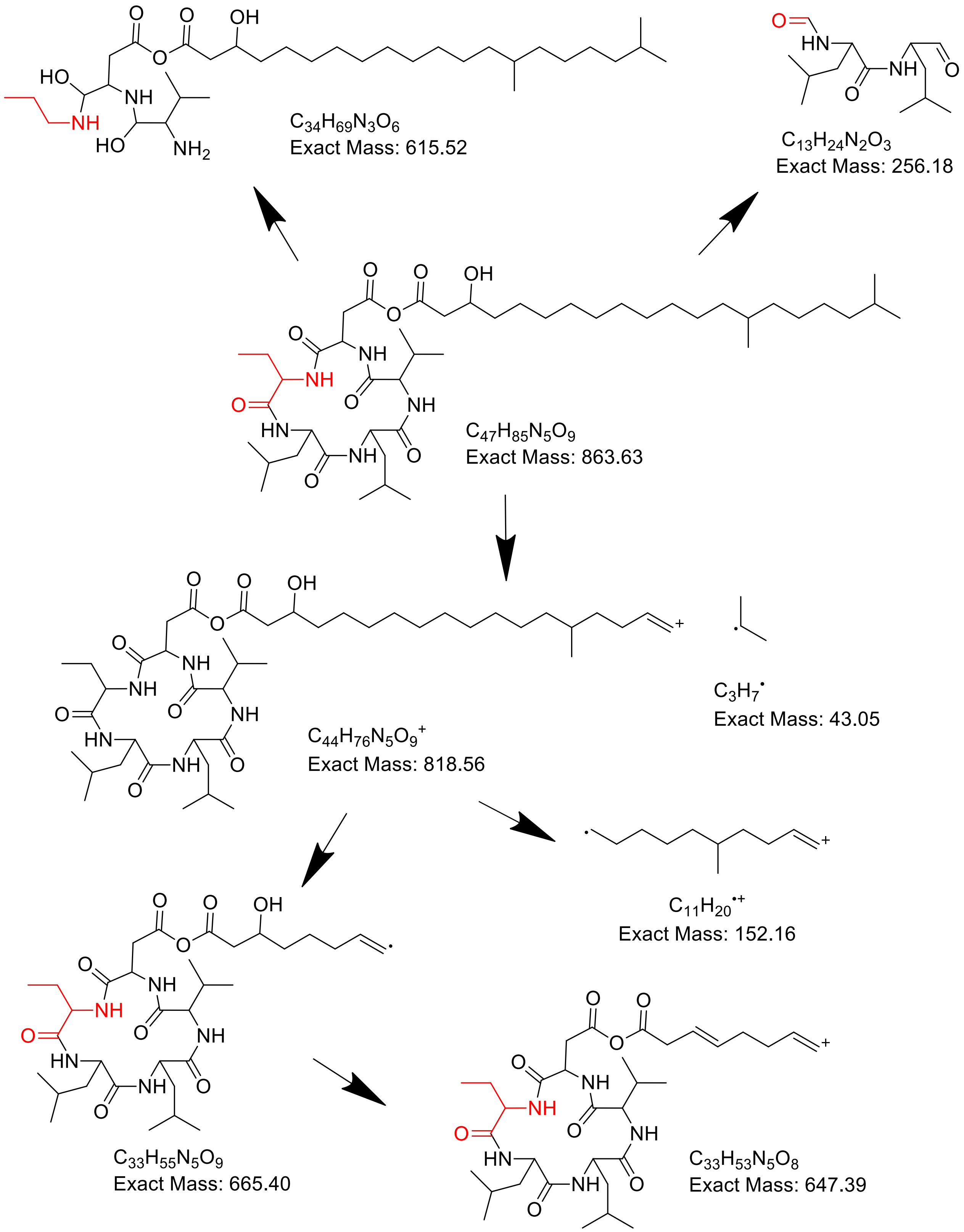


Figure 36: Proposed fragmentation pattern of the molecular ion of the compound DE2E-TSBY-FD-SFB-I through the electron impact ionization analysis.

4.4 Biological activity tests

For biological activity, the compound DE2E-TSBY-FD-SFB-I was assayed for anti-microbial and antiparasitic (anti-leishmania and anti-trypanosome) activities. Tests were carried out on nine different standard bacteria strains comprising 6 Gram negative, *Eschericha coli* (ATCC 25922), *Salmonella paratyphi ß (9150), Shigella sonnei* (ATCC 12022), *Shigella dysenteriae* (13313), *Salmonella typhimurium* (14028) and *Salmonella enteritidis (*ATCC 13076), also 3 Gram-positives, *Staphylococcus aureus*(ATCC 29213), *Bacillus cereus* (ATCC 14579) and *Staphylococcus epidermidis* (ATCC 12228) were used for the antimicrobial potency of the compound using Ampicillin as the antibiotic control. The highest antibacterial activity exerted by the compound was against the *Shigella sonnei* (ATCC 12022) and *Shigella dysenteriae* (13313) with significant IC_{50} values of 10.01µM and 29.72µM respectively compared to the current antibiotic used in treatment of Shigella infections(shigellosis) Pivmecillinam with IC_{50} = 0.05µM and IC_{50} = 0.1µM by the antibiotic Ampicillin.

However, the compound expressed a relatively weak antibacterial activity against *Eschericha coli* (ATCC 25922) and *Salmonella enteritidis (*ATCC 13076) with IC_{50} >100 µM. The antimicrobial biological test results for compound DE2E-TSBY-FD-SFB-I are detailed in the **Table 14** below.

Compound DE2E-TSBY-FD-SFB-I	
Tested Bacteria strain	**IC_{50} (µM)**
Eschericha coli	>100
Salmonella paratyphi ß	47.90
Shigella sonnei	**10.01**
Shigella dysenteriae	**29.72**

Salmonella typhimurium	54.27
Salmonella enteritidis	>100
Staphylococcus aureus	54.76
Bacillus cereus	48.35
Staphylococcus epidermidis	>100

Table 14: Antimicrobial test results for compound DE2E-TSBY-FD-SFB-I

However, for the antiparasitic assay, the compound DE2E-TSBY-FD-SFB-I was tested against *Leishmania. donovani* for the anti-leishmania activity using Amphotericin B as a positive control. Anti-trypanosome activity test was carried out on *Trypanosome brucei* while using Metronidazole as positive control. The cell growth-inhibition potency of the compound was expressed as IC_{50} values. The compound showed strong anti-trypanosome activity against *Trypanosome brucei* with a significant IC_{50} of 12.89µM. However, the compound also showed moderate activity compared to Amphotericin B (IC_{50} 0.31µM) which is used in the treatment of leishmaniasis in some parts of the world recording an IC_{50} value of 30 µM against *L. donovani*. Biological activity tests for other possible antiparasitic and antibiotic activity of compound DE2E-TSBY-FD-SFB-I are currently on going. Biological activity results for the other isolated peptides are also listed in **Appendix 11a** and **11b**.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The incidence of multi-drug resistance is increasing sporadically, which suggests that a long term solution to this crisis is to constantly fill the drug discovery pipeline with active molecules for possible antibiotic development to augment clinical demands. Currently, Antimicrobial peptides (AMPs) are receiving enormous considerations for development as alternatives to conventional small molecule antibiotics considered non susceptible to resistant pathogens especially those produced by *Streptomyces*, since this genus is well known for its antibiotics production.

This current project was aimed at investigating the novel Ghanaian *Streptomyces* sp. strain DE2E isolated from an extreme biodiverse environment (Digya National Park) for its ability to biosynthesize novel AMPs especially non-ribosomal peptides (NRPs) noted for their significant antibacterial activities demonstrated by their huge structural diversity. The detailed chemical investigation of this *Streptomyces* strain led to the prioritization of the Kupchan fraction FD (DE2E-TSBY-FD) to contain series of large molecules with $m/z \approx 800 – 900$ purported to be peptides. A very efficient HRESI/HPLC-DAD-MS^n measurements and appropriate de-replication techniques coupled with the relevant phytochemical analysis were employed in identifying and tracking these targeted molecules. The size exclusion chromatography of the DE2E-TSBY-FD fraction yielded Sephadex fraction SFB whose further purification on HPLC led to the isolation of five chemically related peptides in fractions E – I based on the analysis of their ^{1}H-NMR and ^{13}C NMR spectra. The relevant full 2D NMR data for fraction I, labelled DE2E-TSBY-FD-SFB-I were acquired and processed to facilitate the structure elucidation of this compound. The HRESI/HPLC-DAD-MS^n analysis of this pure isolate led to the confirmation of the proposed structure of compound DE2E-TSBY-FD-SFB-I in this thesis as post-translationally modified NRPs using the available spin systems generated from 1D and 2D NMR experiments. However, due to the lack of enough spin systems and proton multiplicities generated in deuterated $CDCl_3$ considering the rather large size of this molecule, we hope to acquire the full 1D and 2D NMR data for this compound in a much better solvent such as deuterated DMSO-d_6 coupled with relevant spectroscopic measurements such as sequence tag MS/tandem MS and bioinformatics which are mostly employed to elucidate the structures of this large peptide molecules. The precise confirmation of the structure of this compound will definitely provide structural information for the elucidation of the other four isolates (peptides).

Compound DE2E-TSBY-FD-SFB-I was screened for antimicrobial activity using 9 bacteria strains comprising 6 Gram-negatives and 3 Gram-positives. The compound exerted its strongest antibacterial activity against *Shigella sonnei* with IC_{50} = 10.01µM. The compound also exerted a moderate antiparasitic activity against *Trypanosome brucei* with a significant IC_{50} = 12.89µM. The biological activities demonstrated by this NRPs produced by the Ghanaian *Streptomyces* sp. DE2E re-emphasizes the key role NRPs can play in looking for possible potent and robust alternatives to the conventional small molecule antibiotics in addressing the multi-drug resistant crisis.

5.2 Recommendations

The isolation and characterization of this novel post-translationally modified NRPs in this current work from the Ghanaian *Streptomyces* sp. DE2E with some degrees of antibacterial activity reaffirms the concept for developing microbial derived NRPs as antibiotics. Therefore, more Ghanaian *Streptomyces* should be chemically investigated for the production of novel antibacterial NRPs for possible development as future antibiotics. The other sub-fractions from this current investigation which were identified to be interesting, containing purported peptide molecules in isolable amounts should be thoroughly investigated with the aim of isolating more of these bio-active NRPs. Pure compounds should be tested in a wide range of bio-assays to ascertain their effectiveness against pathogens.

REFERENCES

1. Milshteyn, A., Schneider, J. S., & Brady, S. F. (2014). Mining the metabiome: Identifying novel natural products from microbial communities. *Chemistry and Biology*, *21*(9), 1211–1223. https://doi.org/10.1016/j.chembiol.2014.08.006
2. Ventola, C. L. (2015). The antibiotic resistance crisis: part 1: causes and threats. *P & T : A Peer-Reviewed Journal for Formulary Management (2015)*, *40*(4), 277–83. https://doi.org/Article
3. Fleming, A., & Chain, E. B. (2015). BenchMarks A New Golden Age of Natural Products Drug Discovery. *Cell*, *163*(6), 1297–1300. https://doi.org/10.1016/j.cell.2015.11.031
4. Uchil, R. R., Kohli, G. S., Katekhaye, V. M., & Swami, O. C. (2014). Strategies to combat antimicrobial resistance. *Journal of Clinical and Diagnostic Research*, *8*(7), 8–11. https://doi.org/10.7860/JCDR/2014/8925.4529
5. Arias, C., & Murray, B. (2015). A new antibiotic and the evolution of resistance. *New England Journal of Medicine*, *372*(12), 1168–1170. https://doi.org/10.1056/NEJMcibr1500292.A
6. Avner, B. S., Fialho, A. M., & Chakrabarty, A. M. (2012). Overcoming drug resistance in multi-drug resistant cancers and microorganisms: A conceptual framework. *Bioengineered*, *3*(5), 262–270. https://doi.org/10.4161/bioe.21130
7. Fair, R. J., & Tor, Y. (2014). Perspectives in Medicinal Chemistry Antibiotics and Bacterial Resistance in the 21st Century. *Perspectives in Medicinal Chemistry*, 25–64. https://doi.org/10.4137/PMC.S14459.
8. Mahlapuu, M., Håkansson, J., Ringstad, L., & Björn, C. (2016). Antimicrobial Peptides : An Emerging Category of Therapeutic Agents, 6(December), 1–12.

https://doi.org/10.3389/fcimb.2016.00194

9. Wang, S., Zeng, X., Yang, Q., & Qiao, S. (2016). Antimicrobial Peptides as Potential Alternatives to Antibiotics in Food Animal Industry. https://doi.org/10.3390/ijms17050603
10. Seo, M., Won, H., Kim, J., Mishig-ochir, T., & Lee, B. (2012). Antimicrobial Peptides for Therapeutic Applications: A Review, 12276–12286. https://doi.org/10.3390/molecules171012276
11. Zhang, L., & Gallo, R. L. (2016). peptides. CURBIO, 26(1), R14–R19. https://doi.org/10.1016/j.cub.2015.11.017
12. Pushpanathan, M., Gunasekaran, P., & Rajendhran, J. (2013). Antimicrobial peptides: versatile biological properties. *International journal of peptides*, *2013*.
13. Grünewald, J., & Marahiel, M. A. (2013). *Nonribosomal Peptide Synthesis*. *Handbook of Biologically Active Peptides* (Second Edi). Elsevier Inc. https://doi.org/10.1016/B978-0-12-385095-9.00021-X
14. de Lima Procópio, R. E., da Silva, I. R., Martins, M. K., de Azevedo, J. L., & de Araújo, J. M. (2012). Antibiotics produced by Streptomyces. *Brazilian Journal of Infectious Diseases*, *16*(5), 466–471. https://doi.org/10.1016/j.bjid.2012.08.014

15. Strieker, M., & Tanovic, A. (2010). Nonribosomal peptide synthetases : structures and dynamics ´ and Mohamed A Marahiel. https://doi.org/10.1016/j.sbi.2010.01.009
16. Reimer, J. M., Haque, A. S., Tarry, M. J., & Schmeing, T. M. (2018). ScienceDirect Piecing together nonribosomal peptide synthesis. *Current Opinion in Structural Biology*, *49*, 104–113. https://doi.org/10.1016/j.sbi.2018.01.011

17. Chater, K. F. (2006). Streptomyces inside-out : a new perspective on the bacteria that provide us with antibiotics, (February), 761–768. https://doi.org/10.1098/rstb.2005.1758
18. Barka, E. A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H.-P., … van Wezel, G. P. (2016). Taxonomy, Physiology, and Natural Products of Actinobacteria. Microbiology and Molecular Biology Reviews, 80(1), 1–43. https://doi.org/10.1128/MMBR.00019-15
19. Buchanan, R. E. (1917). Studies in the nomenclature and classification of the bacteria: II. The primary subdivisions of the schizomycetes. *Journal of bacteriology*, *2*(2), 155.
20. Ludwig, W., Euzéby, J., Schumann, P., Busse, H. J., Trujillo, M. E., Kämpfer, P., & Whitman, W. B. (2012). Road map of the phylum Actinobacteria. In *Bergey's manual® of systematic bacteriology* (pp. 1-28). Springer, New York, NY.
21. Gao, B., & Gupta, R. S. (2012). Phylogenetic framework and molecular signatures for the main clades of the phylum Actinobacteria. *Microbiology and Molecular Biology Reviews*, *76*(1), 66-112.
22. Kämpfer, P. (2012). *Bergey's Manual of Systematic Bacteriology: The Actinobacteria*. M. Goodfellow, H. J. Busse, M. E. Trujillo, K. I. Suzuki, W. Ludwig, & W. B. Whitman (Eds.). New York: Springer.
23. Hasani, A., Kariminik, A., & Issazadeh, K. (2014). Streptomycetes: characteristics and their antimicrobial activities. *International Journal of Advanced Biological and Biomedical Research*, *2*(1), 63-75.
24. Williams , S., Goodfellow, M., Alderson, G., Wellington, E., Sneath , P., & Sackin, M. (1983). Numerical classification of Streptomyces and related genera. Journal of General Microbiology,, 129, 1743-1813.

25. Chater, K. F., Biro, S., Lee, K. J., Palmer, T., & Schrempf, H. (2010). The complex extracellular biology of Streptomyces. *FEMS microbiology reviews*, *34*(2), 171-198.
26. Waksman, S. A., & Woodruff, H. B. (1940). Bacteriostatic and bactericidal substances produced by a soil Actinomyces. *Proceedings of the society for Experimental Biology and Medicine*, *45*(2), 609-614.
27. Waksman, S. A., & Woodruff, H. B. (1942). Selective antibiotic action of various substances of microbial origin. *Journal of bacteriology*, *44*(3), 373.
28. Schatz, A., & Waksman, S. A. (1944). Effect of Streptomycin and Other Antibiotic Substances upon Mycobacterium tuberculosis and Related Organisms. *Proceedings of the Society for Experimental Biology and Medicine*, *57*(2), 244-248.
29. Bérdy, J. (2012). Thoughts and facts about antibiotics: where we are now and where we are heading. *The Journal of antibiotics*, *65*(8), 385.
30. Berdy, J. (2005). Bioactive microbial metabolites. *The Journal of antibiotics*, *58*(1), 1.
31. Li, L., Jiang, W., & Lu, Y. (2017). New strategies and approaches for engineering biosynthetic gene clusters of microbial natural products. Biotechnology Advances, 35(8), 936–949. https://doi.org/10.1016/j.biotechadv.2017.03.007
32. Jensen, P. R. (2016). Natural Products and the Gene Cluster Revolution. Trends in Microbiology, 24(12), 968–977. https://doi.org/10.1016/j.tim.2016.07.006
33. Niu, G. (2018). Genomics-Driven Natural Product Discovery in Actinomycetes. *Trends in biotechnology*, *36*(3), 238-241.
34. Lai, Y., & Gallo, R. L. (2009). AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends in immunology*, *30*(3), 131-141.
35. Peschel, A., & Sahl, H. G. (2006). The co-evolution of host cationic antimicrobial peptides

and microbial resistance. *Nature Reviews Microbiology*, *4*(7), 529.

36. Wang, S., A Thacker, P., Watford, M., & Qiao, S. (2015). Functions of antimicrobial peptides in gut homeostasis. *Current Protein and Peptide Science*, *16*(7), 582-591.
37. Mohammadipanah, F., Matasyoh, J., Hamedi, J., Klenk, H. P., & Laatsch, H. (2012). Persipeptides A and B, two cyclic peptides from Streptomyces sp. UTMC 1154. *Bioorganic & medicinal chemistry*, *20*(1), 335-339.
38. Walsh, G., & Jefferis, R. (2006). Post-translational modifications in the context of therapeutic proteins. *Nature biotechnology*, *24*(10), 1241.
39. McIntosh, J. A., Donia, M. S., & Schmidt, E. W. (2009). Ribosomal peptide natural products: Bridging the ribosomal and nonribosomal worlds. Natural Product Reports, 26(4), 537–559. https://doi.org/10.1039/b714132g
40. Ortega, M. A., & Donk, W. A. Van Der. (2016). Review New Insights into the Biosynthetic Logic of Ribosomally Synthesized and Post-translationally Modified Peptide Natural Products. Cell Chemical Biology, 23(1), 31–44. https://doi.org/10.1016/j.chembiol.2015.11.012
41. Arnison, P. G., Bibb, M. J., Bierbaum, G., Bowers, A. A., Bugni, T. S., Bulaj, G., ... & Cotter, P. D. (2013). Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature. *Natural product reports*, *30*(1), 108-160.
42. Schmidt, E. W. (2010). The hidden diversity of ribosomal peptide natural products. BMC Biology, 8, 2–5. https://doi.org/10.1186/1741-7007-8-83
43. Gomes, K. M., Duarte, R. S., & Bastos, M. do C. de F. (2017). Lantibiotics produced by Actinobacteria and their potential applications (A review). Microbiology (United

Kingdom), 163(2), 109–121. https://doi.org/10.1099/mic.0.000397

44. Donia, M. S., Hathaway, B. J., Sudek, S., Haygood, M. G., Rosovitz, M. J., Ravel, J., & Schmidt, E. W. (2006). Natural combinatorial peptide libraries in cyanobacterial symbionts of marine ascidians. *Nature chemical biology*, *2*(12), 729.
45. Donia, M. S., Ravel, J., & Schmidt, E. W. (2008). A global assembly line for cyanobactins. *Nature chemical biology*, *4*(6), 341.
46. Willey, J. M., & Van Der Donk, W. A. (2007). Lantibiotics: peptides of diverse structure and function. *Annu. Rev. Microbiol.*, *61*, 477-501.
47. Givot, I. L., Smith, T. A., & Abeles, R. H. (1969). Studies on the mechanism of action and the structure of the electrophilic center of histidine ammonia lyase. *Journal of Biological Chemistry*, *244*(23), 6341-6353.
48. Wickner, R. B. (1969). Dehydroalanine in histidine ammonia lyase. *Journal of Biological Chemistry*, *244*(23), 6550-6552.
49. Langer, M., Reck, G., Reed, J., & Retey, J. (1994). Identification of serine-143 as the most likely precursor of dehydroalanine in the active site of histidine ammonia-lyase. A study of the overexpressed enzyme by site-directed mutagenesis. *Biochemistry*, *33*(21), 6462-6467.
50. Schuster, B., & Retey, J. (1995). The mechanism of action of phenylalanine ammonia-lyase: the role of prosthetic dehydroalanine. *Proceedings of the National Academy of Sciences*, *92*(18), 8433-8437.
51. Morris, S. L., Walsh, R. C., & Hansen, J. N. (1984). Identification and characterization of some bacterial membrane sulfhydryl groups which are targets of bacteriostatic and antibiotic action. *Journal of Biological Chemistry*, *259*(21), 13590-13594.

52. Skaugen, M., Nissen-Meyer, J., Jung, G., StevanoVic, S., Sletten, K., Inger, C., ... & Nes, I. F. (1994). In vivo conversion of L-serine to D-alanine in a ribosomally synthesized polypeptide. *Journal of Biological Chemistry*, *269*(44), 27183-27185.
53. Kellner, R., Jung, G., Josten, M., Kaletta, C., Entian, K. D., & Sahl, H. G. (1989). Pep5: structure elucidation of a large lantibiotic. *Angewandte Chemie International Edition in English*, *28*(5), 616-619.
54. Cotter, P. D., Deegan, L. H., Lawton, E. M., Draper, L. A., O'connor, P. M., Hill, C., & Ross, R. P. (2006). Complete alanine scanning of the two-component lantibiotic lacticin 3147: generating a blueprint for rational drug design. *Molecular microbiology*, *62*(3), 735-747.
55. Chen, P., Novak, J., Kirk, M., Barnes, S., Qi, F., & Caufield, P. W. (1998). Structure-activity study of the lantibiotic mutacin II from Streptococcus mutans T8 by a gene replacement strategy. *Applied and environmental microbiology*, *64*(7), 2335-2340.
56. Roy, R. S., Gehring, A. M., Milne, J. C., Belshaw, P. J., & Walsh, C. T. (1999). Thiazole and oxazole peptides: biosynthesis and molecular machinery. *Natural product reports*, *16*(2), 249-263.
57. Jin, Z. (2005). Muscarine, imidazole, oxazole and thiazole alkaloids. *Natural product reports*, *22*(2), 196-229.
58. Matsunaga, S. (2006). Trisoxazole macrolides from Hexabranchus nudibranchs and other marine invertebrates. In *Molluscs* (pp. 241-260). Springer, Berlin, Heidelberg.
59. Abbenante, G., Fairlie, D. P., Gahan, L. R., Hanson, G. R., Pierens, G. K., & Van Den Brenk, A. L. (1996). Conformational control by thiazole and oxazoline rings in cyclic octapeptides of marine origin. Novel macrocyclic chair and boat conformations. *Journal*

of the American Chemical Society, *118*(43), 10384-10388..

60. Bernhardt, P. V., Comba, P., Fairlie, D. P., Gahan, L. R., Hanson, G. R., & Lötzbeyer, L. (2002). Synthesis and structural properties of patellamide a derivatives and their copper (II) compounds. *Chemistry–A European Journal*, *8*(7), 1527-1536.
61. Cusack, R. M., Grøndahl, L., Fairlie, D. P., Hanson, G. R., & Gahan, L. R. (2003). Studies of the interaction of potassium (I), calcium (II), magnesium (II), and copper (II) with cyclosporin A. *Journal of inorganic biochemistry*, *97*(2), 191-198.
62. Walsh, C. T., & Nolan, E. M. (2008). Morphing peptide backbones into heterocycles. *Proceedings of the National Academy of Sciences*, *105*(15), 5655-5656.
63. Milne, B. F., Long, P. F., Starcevic, A., Hranueli, D., & Jaspars, M. (2006). Spontaneity in the patellamide biosynthetic pathway. *Organic & biomolecular chemistry*, *4*(4), 631-638.
64. Morris, L. A., Milne, B. F., Thompson, G. S., & Jaspars, M. (2002). Conformational change in the thiazole and oxazoline containing cyclic octapeptides, the patellamides. Part 1. Cu 2+ and Zn 2+ induced conformational change. *Journal of the Chemical Society, Perkin Transactions 2*, (6), 1072-1075.
65. Magnuson, R., Solomon, J., & Grossman, A. D. (1994). Biochemical and genetic characterization of a competence pheromone from B. subtilis. *Cell*, *77*(2), 207-216.
66. Okada, M., Yamaguchi, H., Sato, I., Tsuji, F., Qi, J., Dubnau, D., & Sakagami, Y. (2007). Acid labile ComX pheromone from Bacillus mojavensis RO-H-1. *Bioscience, biotechnology, and biochemistry*, *71*(7), 1807-1810.
67. Okada, M., Sato, I., Cho, S. J., Iwata, H., Nishio, T., Dubnau, D., & Sakagami, Y. (2005). Structure of the Bacillus subtilis quorum-sensing peptide pheromone ComX. *Nature chemical biology*, *1*(1), 23-24.

68. Wipf, P., & Uto, Y. (2000). Total synthesis and revision of stereochemistry of the marine metabolite trunkamide A. *The Journal of organic chemistry*, *65*(4), 1037-1049.

69. Bulaj, G. (2005). Formation of disulfide bonds in proteins and peptides. *Biotechnology advances*, *23*(1), 87-92.

70. Bulaj, G., & Olivera, B. M. (2008). Folding of conotoxins: formation of the native disulfide bridges during chemical synthesis and biosynthesis of Conus peptides. *Antioxidants & redox signaling*, *10*(1), 141-156.

71. Aymerich, T., Holo, H., Håvarstein, L. S., Hugas, M., Garriga, M., & Nes, I. F. (1996). Biochemical and genetic characterization of enterocin A from Enterococcus faecium, a new antilisterial bacteriocin in the pediocin family of bacteriocins. *Applied and Environmental Microbiology*, *62*(5), 1676-1682.

72. Ireland, C., & Scheuer, P. J. (1980). Ulicyclamide and ulithiacyclamide, two new small peptides from a marine tunicate. *Journal of the American Chemical Society*, *102*(17), 5688-5691.

73. Fimland, G., Johnsen, L., Axelsson, L., Brurberg, M. B., Nes, I. F., Eijsink, V. G., & Nissen-Meyer, J. (2000). A C-terminal disulfide bridge in pediocin-like bacteriocins renders bacteriocin activity less temperature dependent and is a major determinant of the antimicrobial spectrum. *Journal of Bacteriology*, *182*(9), 2643-2648.

74. Castiglione, F., Lazzarini, A., Carrano, L., Corti, E., Ciciliato, I., Gastaldo, L., ... & Parenti, F. (2008). Determining the structure and mode of action of microbisporicin, a potent lantibiotic active against multiresistant pathogens. *Chemistry & biology*, *15*(1), 22-31.

75. Wilson, K. A., Kalkum, M., Ottesen, J., Yuzenkova, J., Chait, B. T., Landick, R., ... & Darst, S. A. (2003). Structure of microcin J25, a peptide inhibitor of bacterial RNA

polymerase, is a lassoed tail. *Journal of the American Chemical Society*, *125*(41), 12475-12483.

76. Colgrave, M. L., & Craik, D. J. (2004). Thermal, chemical, and enzymatic stability of the cyclotide kalata B1: the importance of the cyclic cystine knot. *Biochemistry*, *43*(20), 5965-5975.
77. Qiu, R., Pei, W., Zhang, L., Lin, J., & Ji, G. (2005). Identification of the putative staphylococcal AgrB catalytic residues involving in the proteolytic cleavage of AgrD to generate autoinducing peptide. *Journal of Biological Chemistry*.
78. Malone, C. L., Boles, B. R., & Horswill, A. R. (2007). Biosynthesis of Staphylococcus aureus autoinducing peptides by using the synechocystis DnaB mini-intein. *Applied and environmental microbiology*, *73*(19), 6036-6044.
79. Thomas, X., Destoumieux-Garzón, D., Peduzzi, J., Afonso, C., Blond, A., Birlirakis, N., ... & Rebuffat, S. (2004). Siderophore peptide, a new type of post-translationally modified antibacterial peptide with potent activity. *Journal of Biological Chemistry*, *279*(27), 28233-28242.
80. Kodani, S., Hudson, M. E., Durrant, M. C., Buttner, M. J., Nodwell, J. R., & Willey, J. M. (2004). From The Cover: The SapB morphogen is a lantibiotic-like peptide derived from the product of the developmental gene ramS in Streptomyces coelicolor. Proceedings of the National Academy of Sciences, 101(31), 11448–11453. https://doi.org/10.1073/pnas.0404220101.
81. Machaidze, G., & Seelig, J. (2003). Specific binding of cinnamycin (Ro 09-0198) to phosphatidylethanolamine. Comparison between micellar and membrane environments. *Biochemistry*, *42*(43), 12570-12576.

82. Steiner, I., Errhalt, P., Kubesch, K., Hubner, M., Holy, M. (2008). Pulmonary pharmacokinetics and safety of nebulized duramycin in healthy male volunteers. *Naunyn Schmiedebergs Arch Pharmacol*, 378, 323–333.

83. Paulus, C., Rebets, Y., Tokovenko, B., Nadmid, S., Terekhova, L. P., Myronovskyi, M., Luzhetskyy, A. (2017). New natural products identified by combined genomics-metabolomics profiling of marine Streptomyces sp. MP131-18. Scientific Reports, 7 (February), 1–11. https://doi.org/10.1038/srep42382

84. Mikolajka, A., Liu, H., Chen, Y., Starosta, A. L., Márquez, V., Ivanova, M., ... & Wilson, D. N. (2011). Differential effects of thiopeptide and orthosomycin antibiotics on translational GTPases. *Chemistry & biology*, *18*(5), 589-600.

85. Gonzalez, R. L., Chu, S., & Puglisi, J. D. (2007). Thiostrepton inhibition of tRNA delivery to the ribosome. *Rna*, *13*(12), 2091-2097.

86. Aminake, M. N., Schoof, S., Sologub, L., Leubner, M., Kirschner, M., Arndt, H. D., & Pradel, G. (2011). Thiostrepton derivatives exhibit antimalarial and gametocytocidal activity by dually targeting parasite proteasome and apicoplast. *Antimicrobial agents and chemotherapy*.

87. Hegde, N. S., Sanders, D. A., Rodriguez, R., & Balasubramanian, S. (2011). The transcription factor FOXM1 is a cellular target of the natural product thiostrepton. *Nature chemistry*, *3*(9), 725.

88. Sajid, I., Shaaban, K. A., Frauendorf, H., Hasnain, S., & Laatscha, H. (2008). Val-Geninthiocin: Structure Elucidation and MSn Fragmentation of Thiopeptide Antibiotics Produced by Streptomyces sp. RSF18. *Zeitschrift für Naturforschung B*, *63*(10), 1223-1230.

89. Yamazaki, T. (2016). A monofunctional nonribosomal peptide synthase might be involved in the biosynthesis of marine toxins of the palytoxin family. The FASEB Journal, 30(1_supplement), lb118-lb118. https://doi.org/10.1096/fasebj.30.1_supplement.lb118

90. Nikolouli, K., & Mossialos, D. (2012). Bioactive compounds synthesized by non-ribosomal peptide synthetases and type-I polyketide synthases discovered through genome-mining and metagenomics. Biotechnology Letters, 34(8), 1393–1403. https://doi.org/10.1007/s10529-012-0919-2
91. Reimer, J. M., Haque, A. S., Tarry, M. J., & Schmeing, T. M. (2018). ScienceDirect Piecing together nonribosomal peptide synthesis. Current Opinion in Structural Biology, 49, 104–113. https://doi.org/10.1016/j.sbi.2018.01.011
92. Bloudo, K., & Schmeing, T. M. (2017). BBA - Proteins and Proteomics Structural and functional aspects of the nonribosomal peptide synthetase condensation domain superfamily : discovery , dissection and diversity ☆, 1865(May), 1587–1604. https://doi.org/10.1016/j.bbapap.2017.05.010.
93. Sablé, S. (2014). Novel NonRibosomal Peptide Synthetase (NRPS) genes sequenced from intertidal mudflat bacteria, (July). https://doi.org/10.1111/1574-6968.12532
94. S., Muliandi, A., Katsuyama, Y., Sone, K., Izumikawa, M., … Ohnishi, Y. (2014). Article Biosynthesis of the 4-Methyloxazoline-Containing. Chemistry & Biology, 21(8), 923–934. https://doi.org/10.1016/j.chembiol.2014.06.004
95. http://bioinfo.lifl.fr/norine/
96. Du, L., & Lou, L. (2010). PKS and NRPS release mechanisms. *Natural product reports*, *27*(2), 255-278.
97. Rateb, M. E., Zhai, Y., Ehrner, E., Rath, C. M., Wang, X., Tabudravu, J. Deng, H. (2015).

Legonaridin, a new member of linaridin RiPP from a Ghanaian Streptomyces isolate. *Organic and Biomolecular Chemistry*, 13(37), 9585–9592. https://doi.org/10.1039/c5ob01269d

98. Bingol, K., Bruschweiler-Li, L., Li, D., Zhang, B., Xie, M., & Brüschweiler, R. (2016). Emerging new strategies for successful metabolite identification in metabolomics. *Bioanalysis*, 8(6), 557-73.

99. Chait, B. T. (2011). Mass spectrometry in the postgenomic era. *Annual review of biochemistry*, *80*, 239-246.

100. Kersten, R. D., Yang, Y.-L., Xu, Y., Cimermancic, P., Nam, S.-J., Fenical, W., … Dorrestein, P. C. (2011). A mass spectrometry–guided genome mining approach for natural product peptidogenomics. *Nature Chemical Biology*, 7(11), 794–802. https://doi.org/10.1038/nchembio.684

101. Spencer, R. K., & Nowick, J. S. (2015). A Newcomer′ s Guide to Peptide Crystallography. *Israel journal of chemistry*, *55*(6-7), 698-710.

102. Stubbs, M. T., Taylor J. B., Triggle D. J. (2007). Protein Crystallography. *Comprehensive Medicinal Chemistry II. Elsevier*, 449-472.

103. I Tietz, J., & Mitchell, A. (2016). Using genomics for natural product structure elucidation. *Current topics in medicinal chemistry*, *16*(15), 1645-1694.

104. Christopher, P. R., David, K. V., John, S. M., & Sankarapandian, V. (2009). Antibiotic therapy for Shigella dysentery. *Cochrane Database of Systematic Reviews*, (4).

105. Marler C. (2012). Symptoms Of Shigella Infection. About Shigella.

106. Katzung, B. G., & Lazo de la Vega Sanchez, M. (2007). Basic clinical pharmacology.

107. Baker, K. S., Dallman, T. J., Behar, A., Weill, F. X., Gouali, M., Sobel, J., ... & Nissan, I. (2016). Travel-and community-based transmission of multidrug-resistant Shigella sonnei lineage among international Orthodox Jewish communities. *Emerging infectious diseases*, *22*(9), 1545.

108. Höltzel, A., Schmid, D. G., Nicholson, G. J., Stevanovic, S., Schimana, J., Gebhardt, K., Jung, G. (2002). Arylomycins A and B, new biaryl-bridged lipopeptide antibiotics produced by Streptomyces sp. Tü 6075. II. Structure elucidation. The Journal of Antibiotics, 55(6), 571–577. https://doi.org/10.7164/antibiotics.55.571.

109. Smith, P. A., & Romesberg, F. E. (2012). Mechanism of action of the arylomycin antibiotics and effects of signal peptidase I inhibition. Antimicrobial Agents and Chemotherapy, 56(10), 5054–5060. https://doi.org/10.1128/AAC.00785-12.

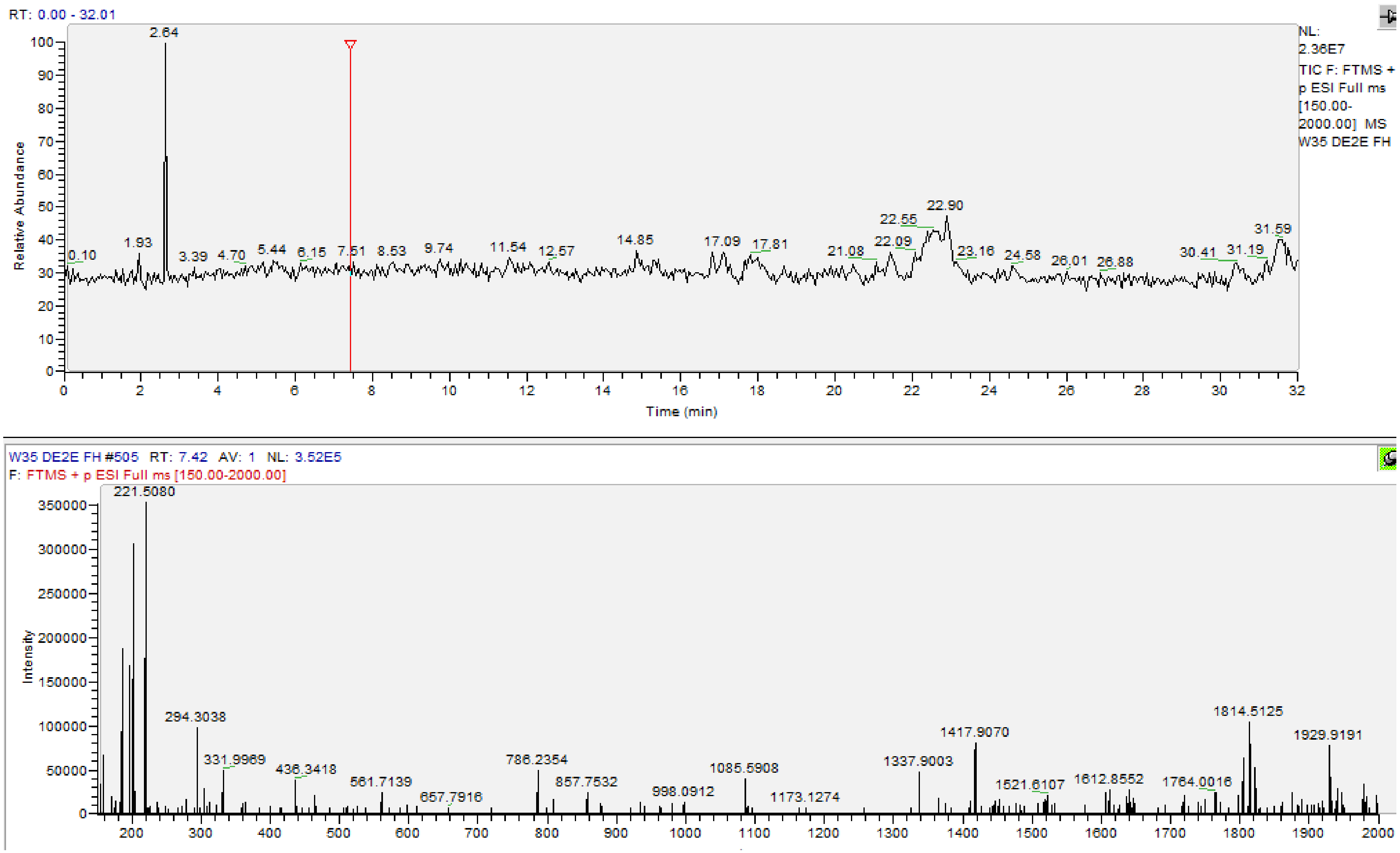
110. Lobanovska, M., & Pilla, G. (2017). Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future? The Yale Journal of Biology and Medicine, 90(1), 135–145.

111. McAuliffe, O., Ross, R. P., & Hill, C. (2001). Lantibiotics: structure, biosynthesis and mode of action. *FEMS microbiology reviews*, *25*(3), 285-308.

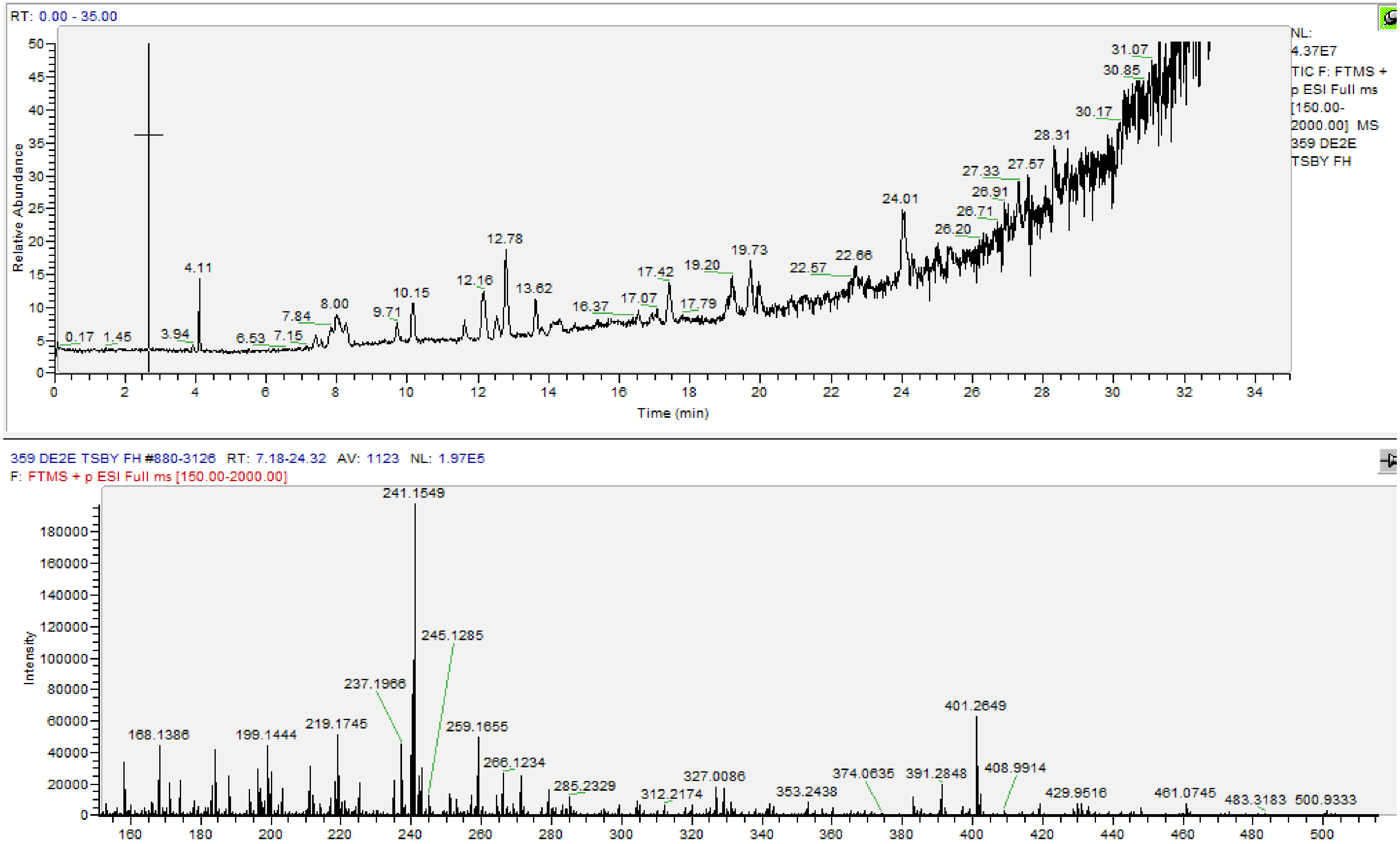
112. Sen, R. (2010). Surfactin: biosynthesis, genetics and potential applications. In *Biosurfactants* (pp. 316-323). Springer, New York, NY.

APPENDICES

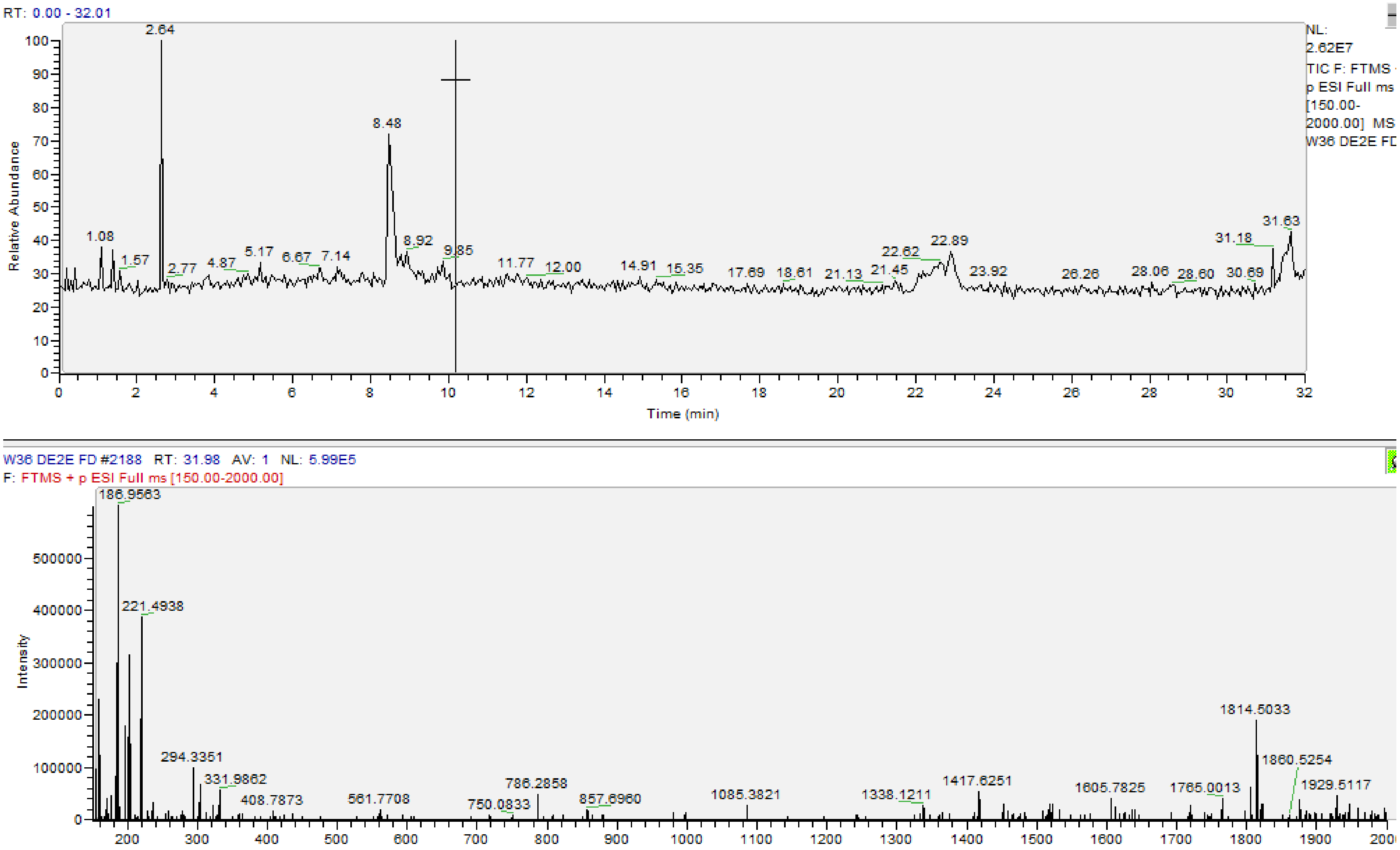
Appendix 1a: HRESI-MS spectrum of DE2E-FH



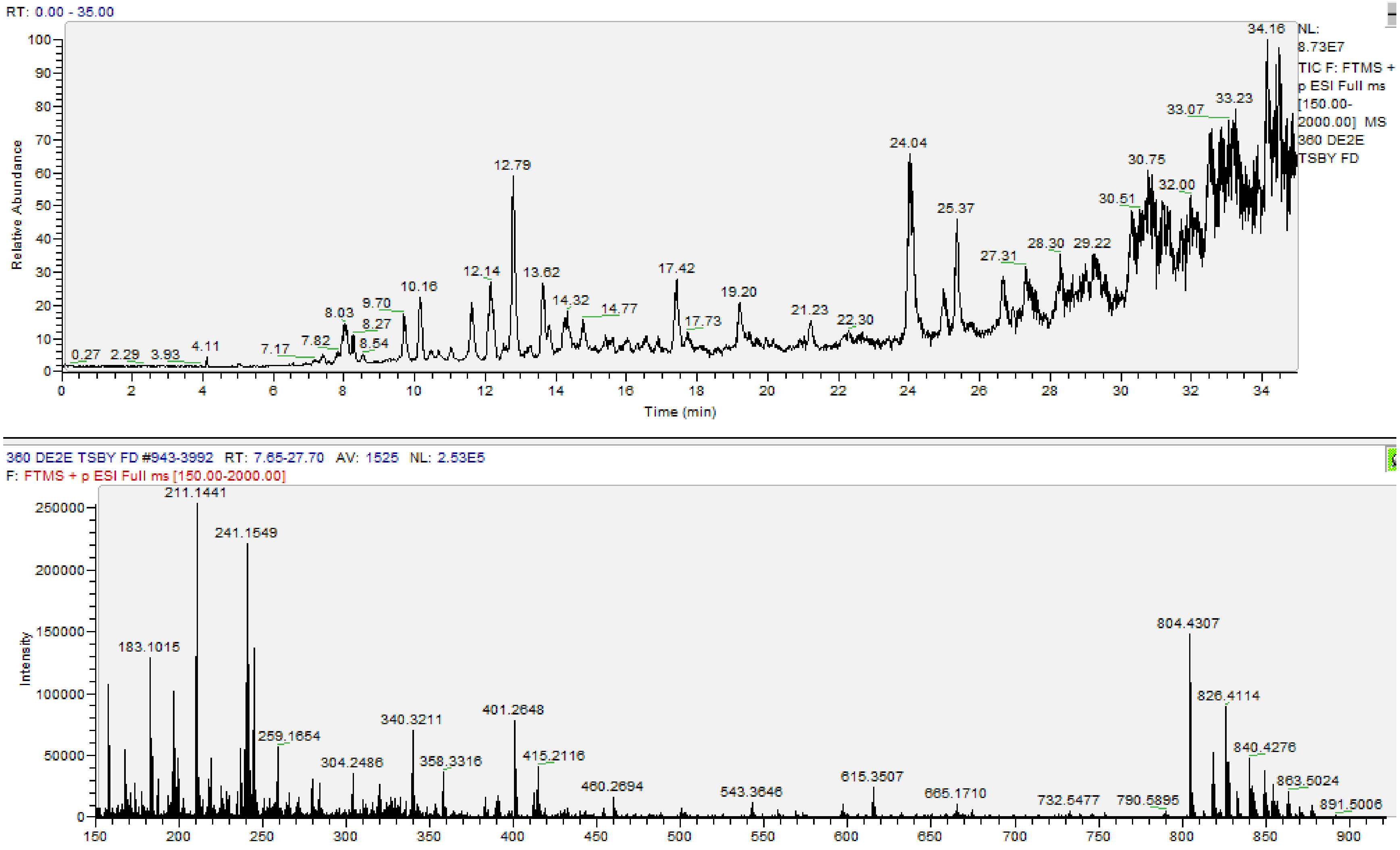
Appendix 1b: HRESI-MS spectrum of DE2E-TSBY-FH



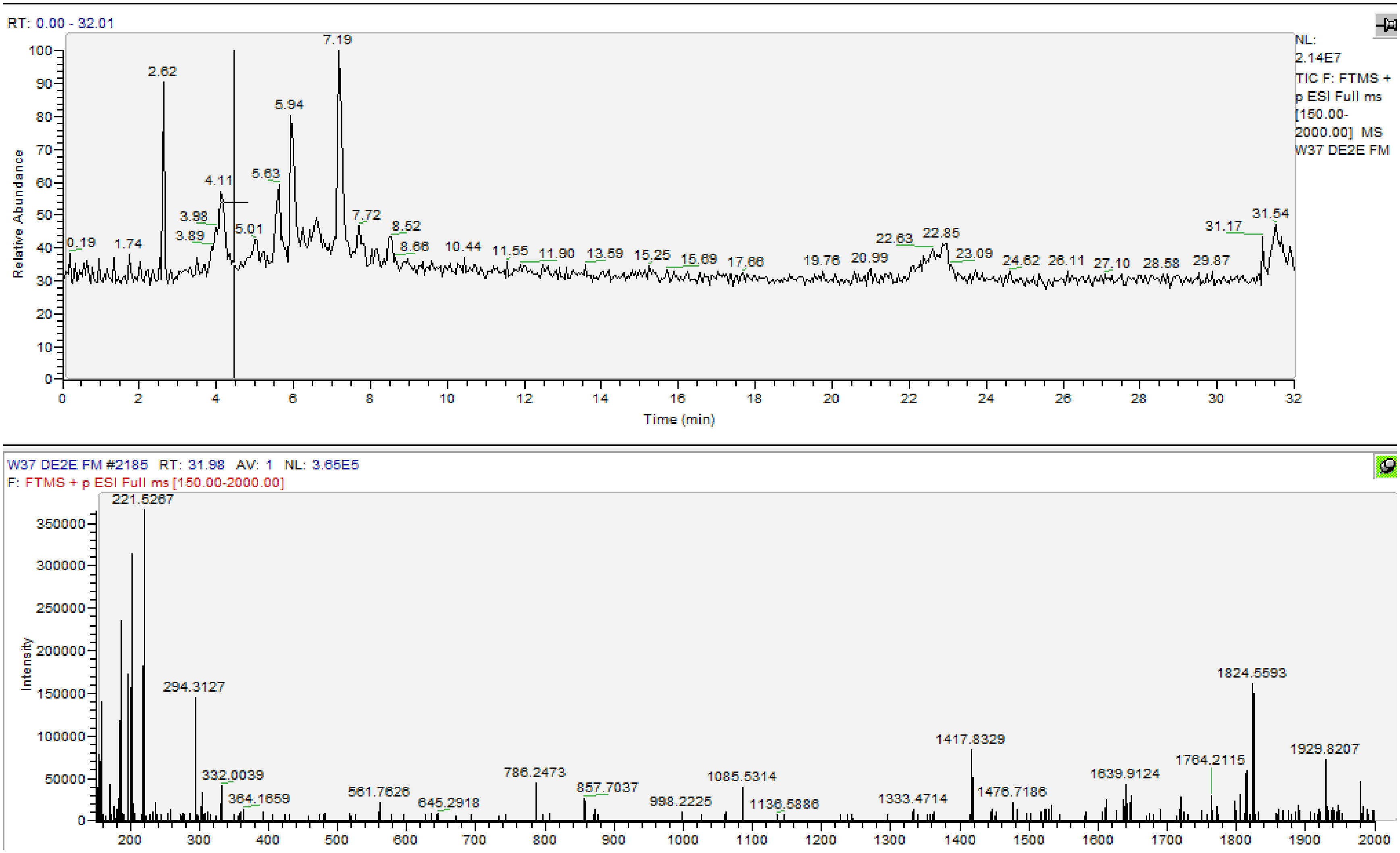
Appendix 2a: HRESI-MS spectrum of DE2E-FD



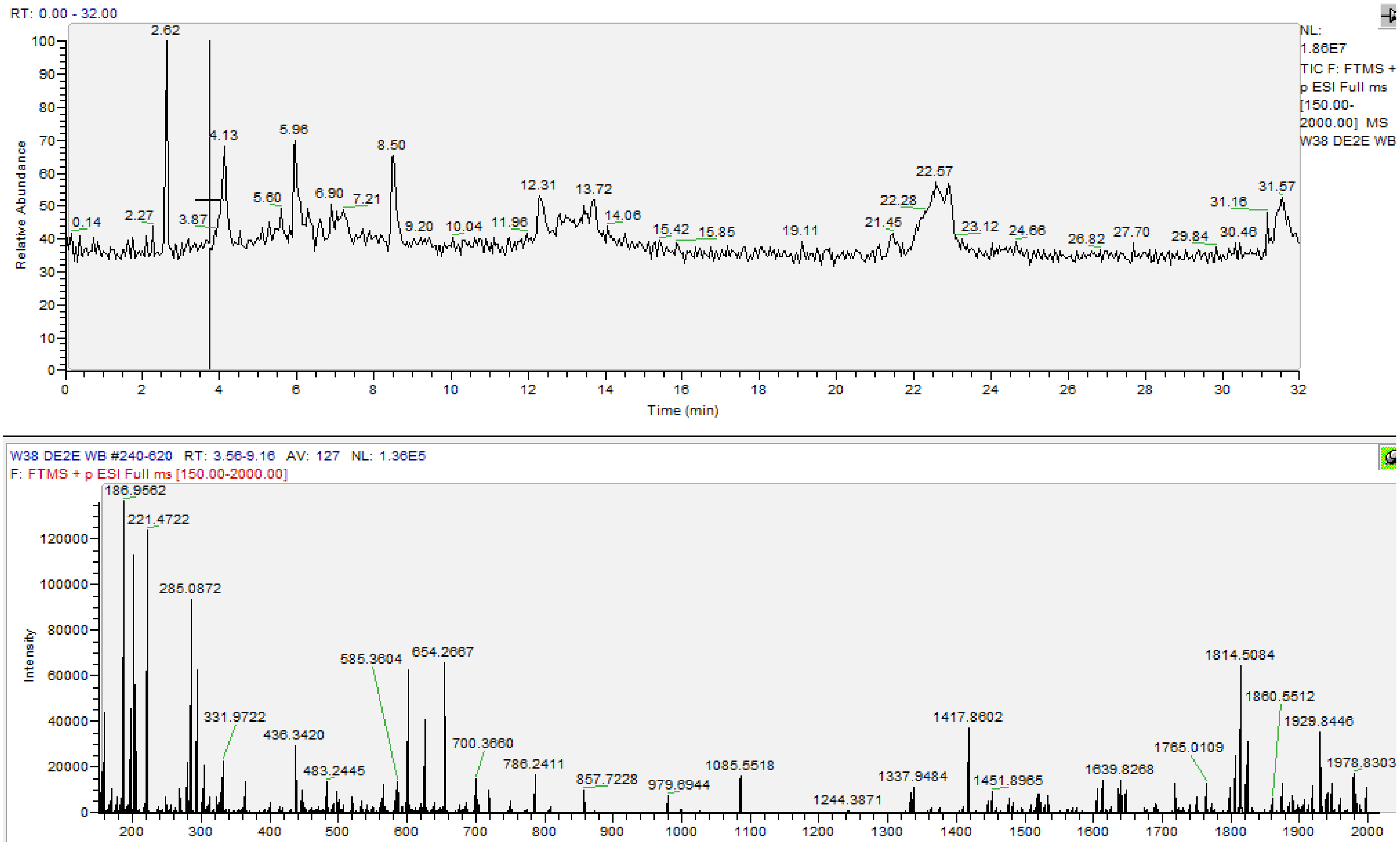
Appendix 2b: HRESI-MS spectrum of DE2E-TSBY-FD



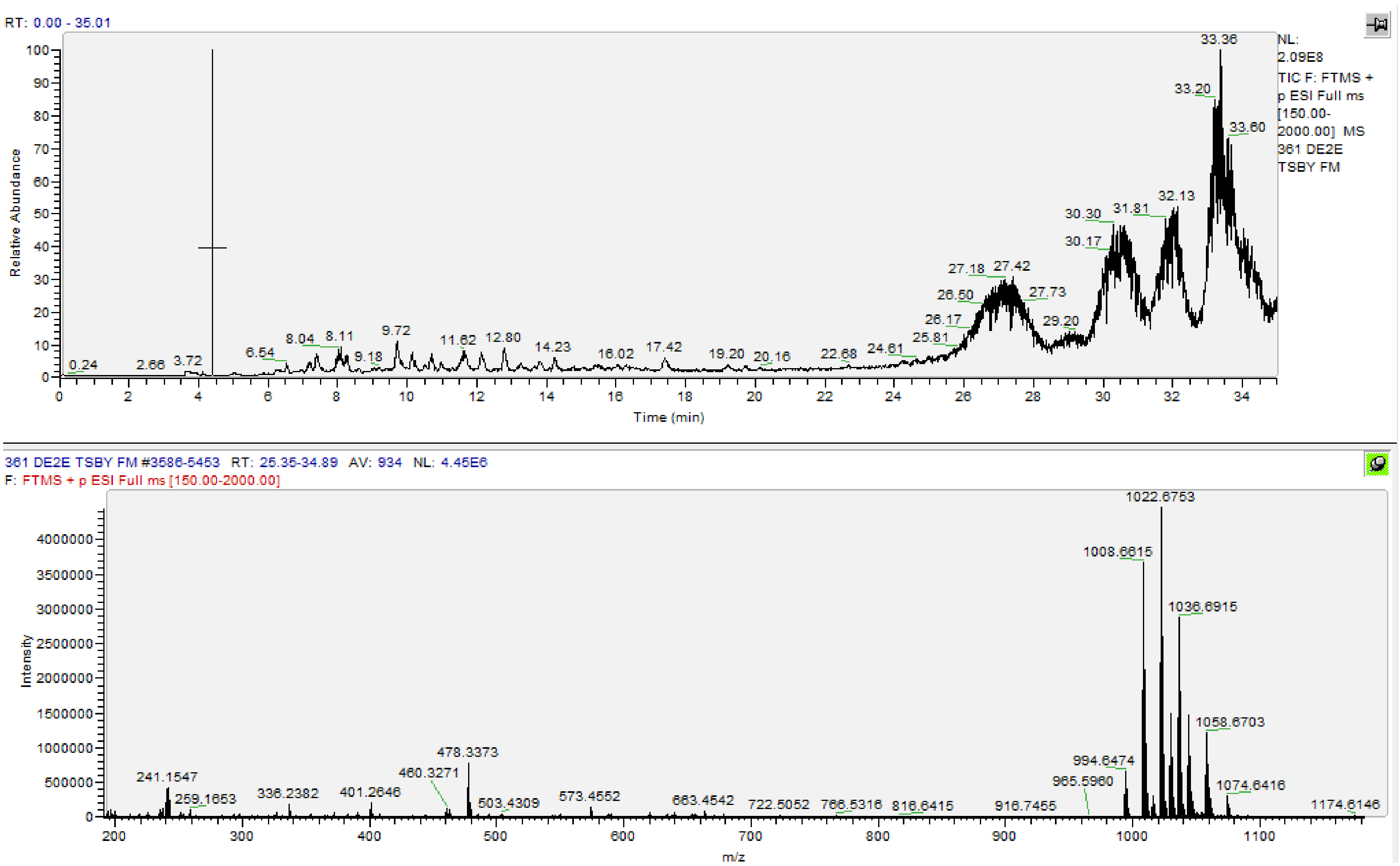
Appendix 3a: HRESI-MS spectrum of DE2E-FM



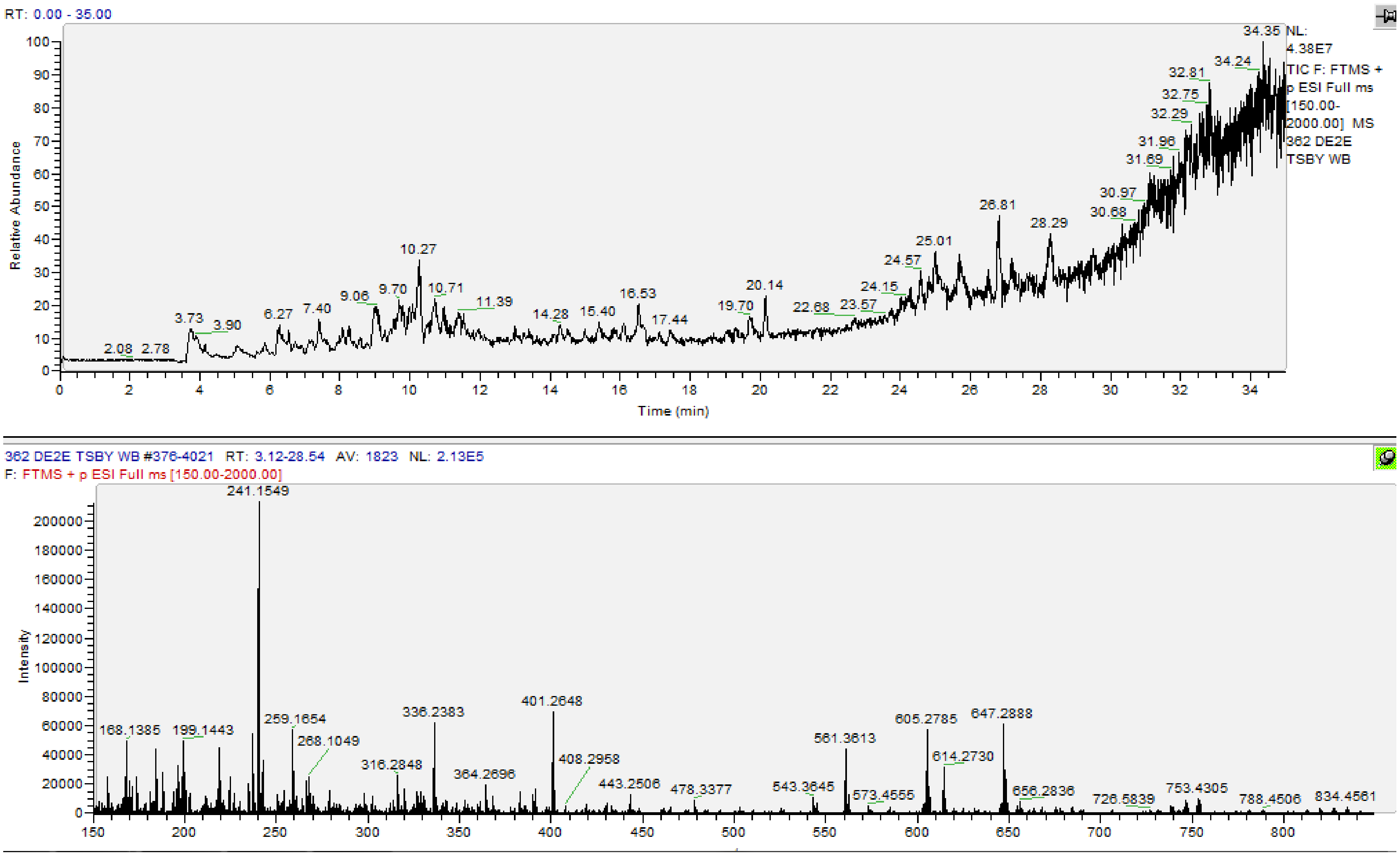
Appendix 3b: HRESI-MS spectrum of DE2E-TSBY-FM



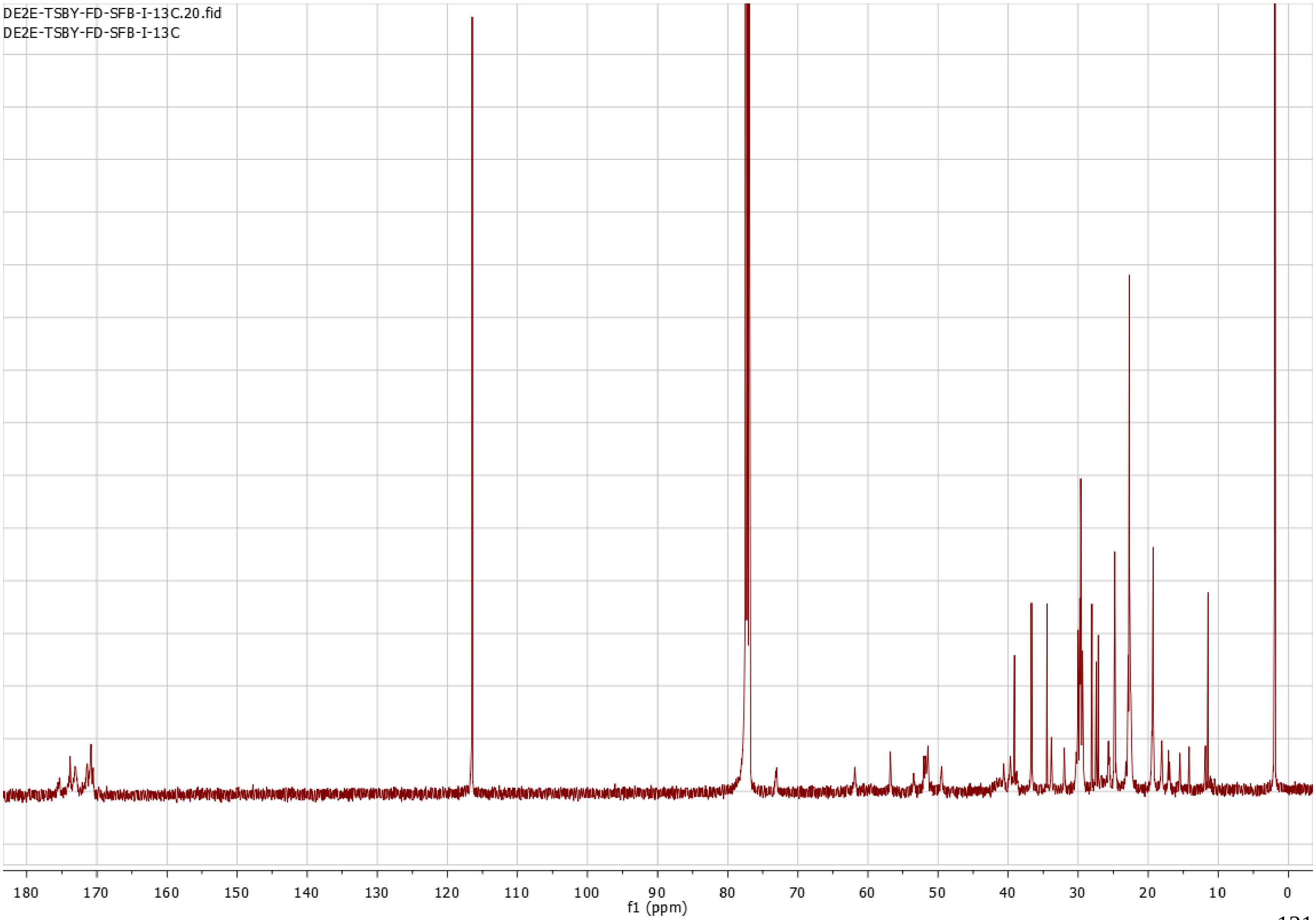
Appendix 4a: HRESI-MS spectrum of DE2E-WB



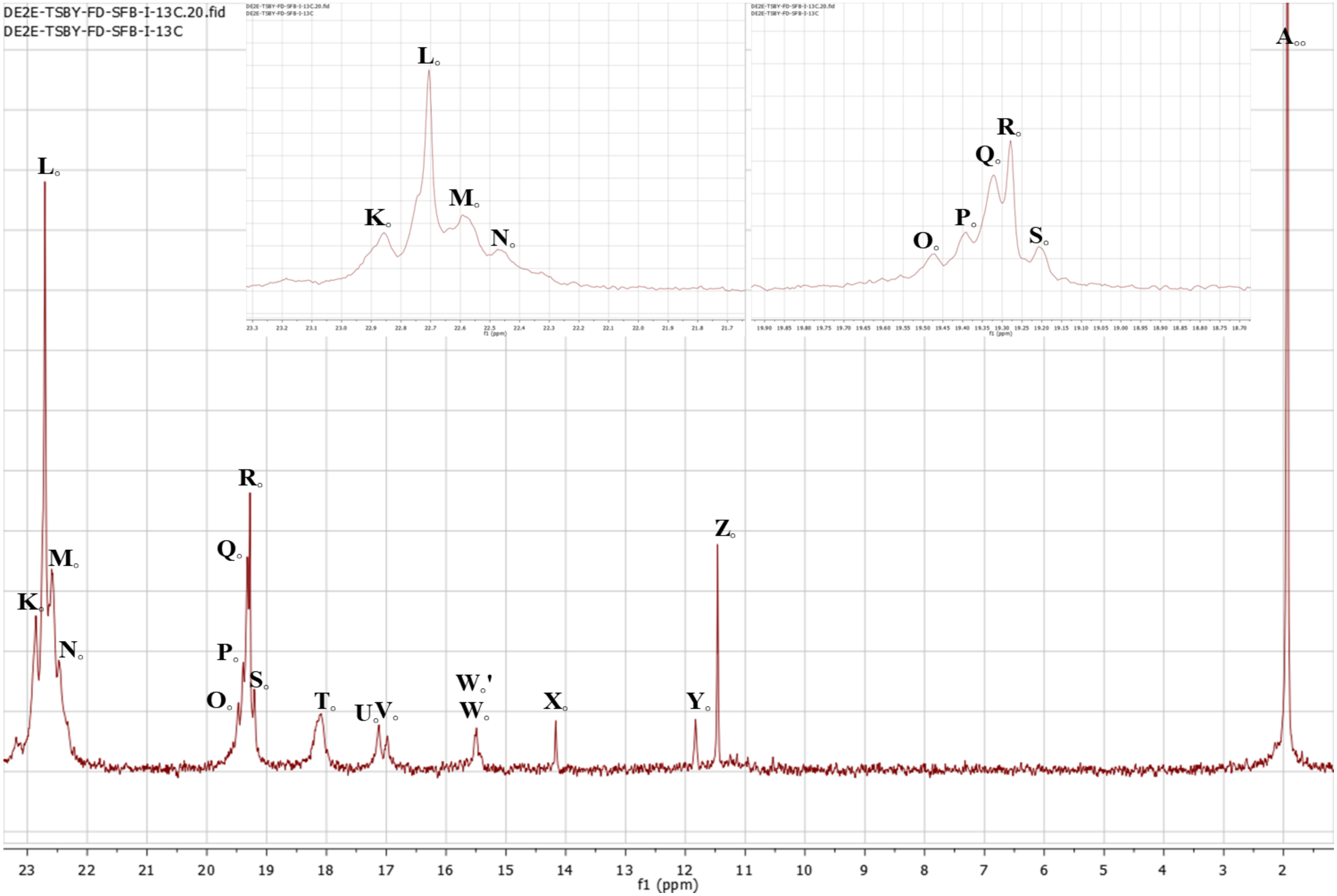
Appendix 4b: HRESI-MS spectrum of DE2E-TSBY-WB



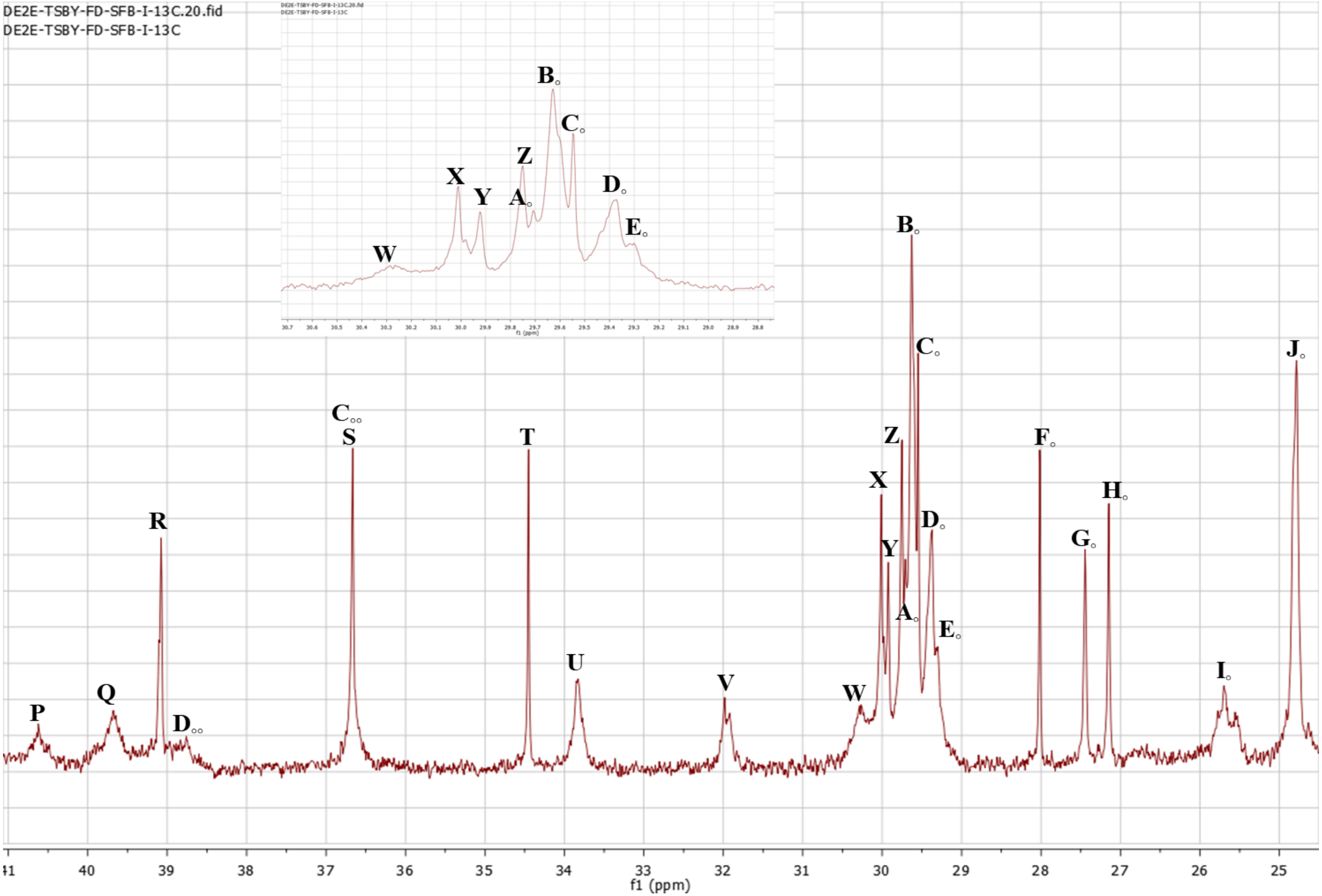
Appendix 5a: ^{13}C-NMR spectrum of compound DE2E-TSBY-FD-SFB-I



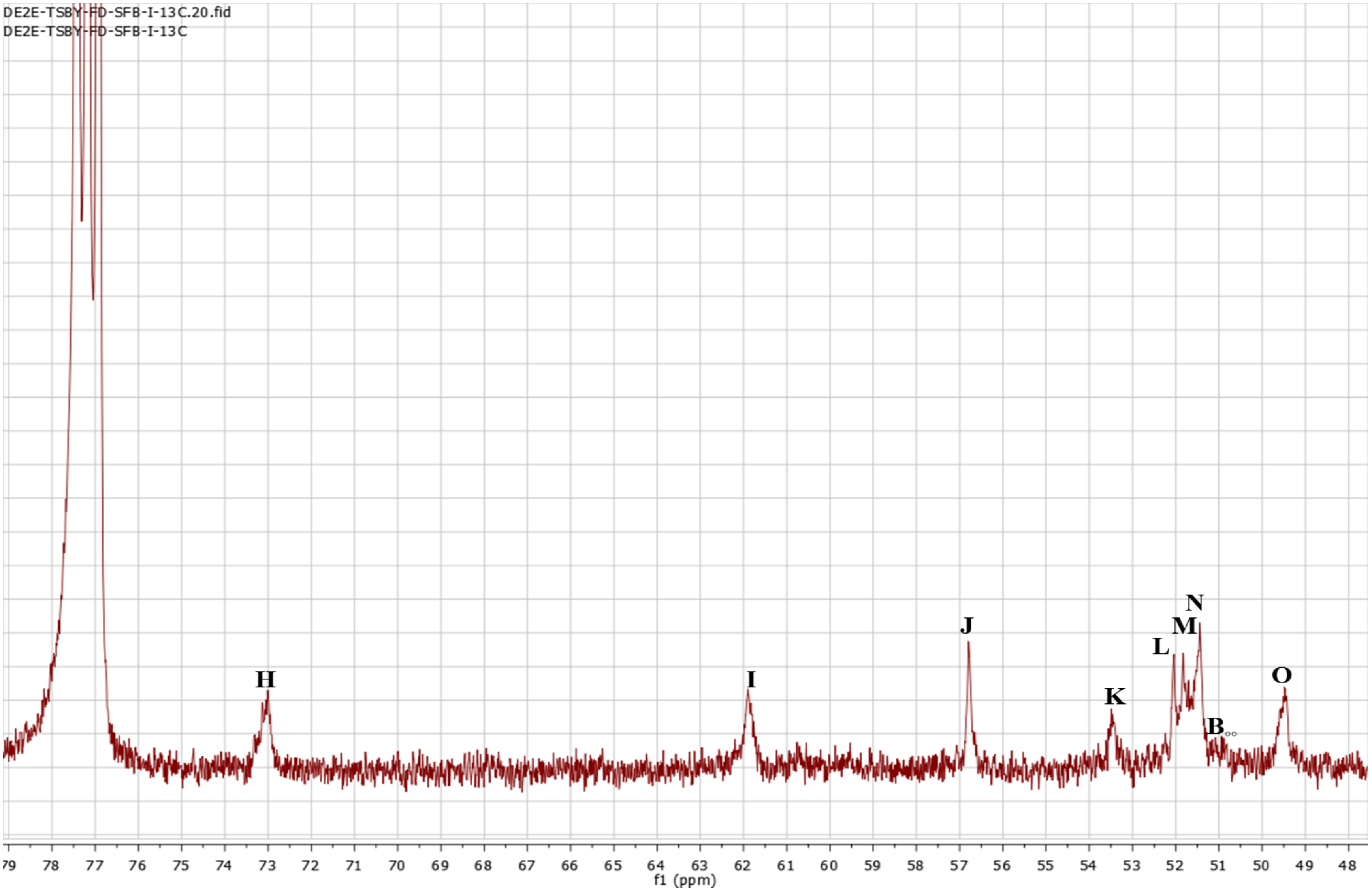
Appendix 5b: Expanded ^{13}C-NMR spectrum of compound DE2E-TSBY-FD-SFB-I



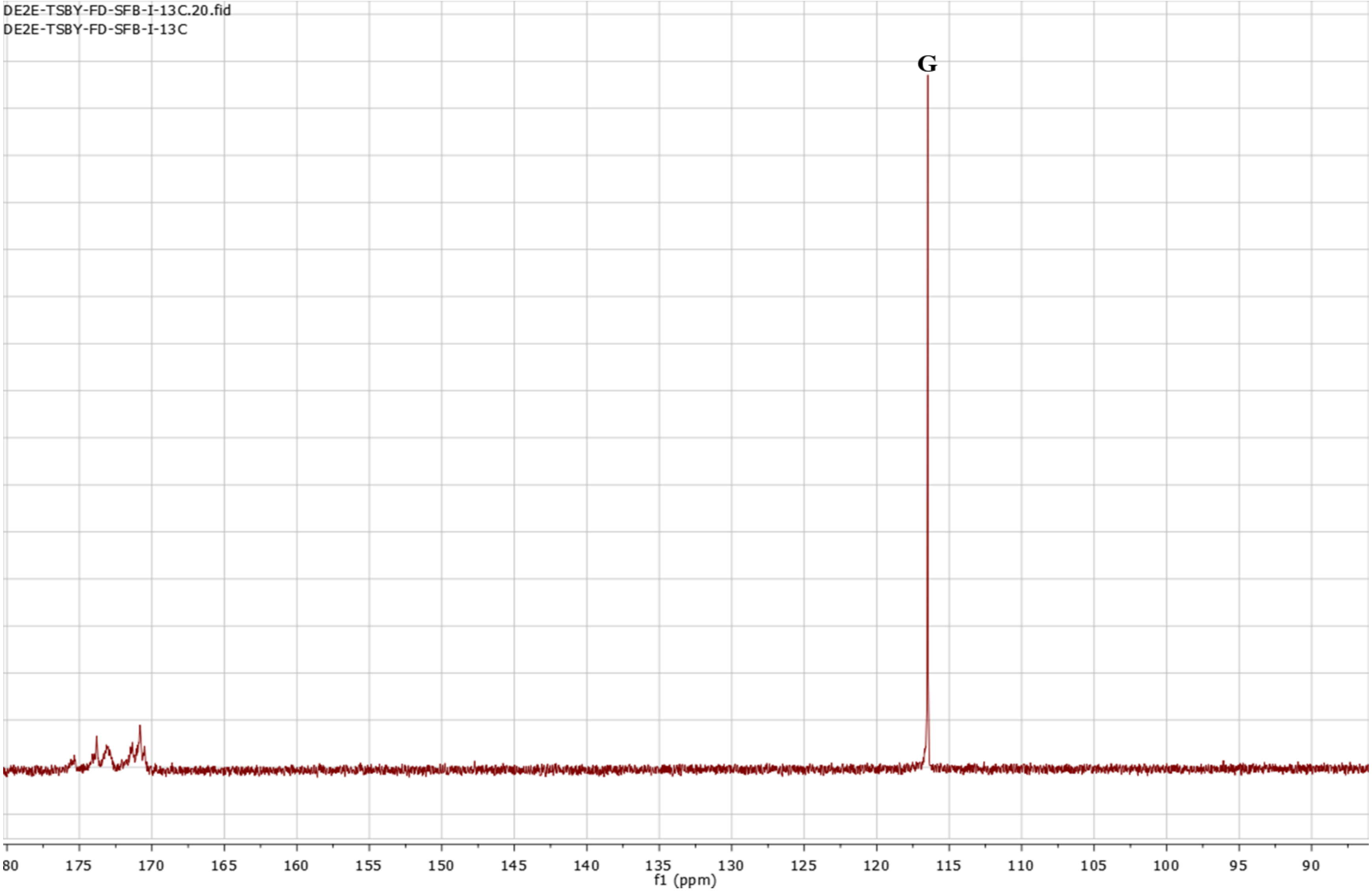
Appendix 5c: Expanded ^{13}C-NMR spectrum of compound DE2E-TSBY-FD-SFB-I



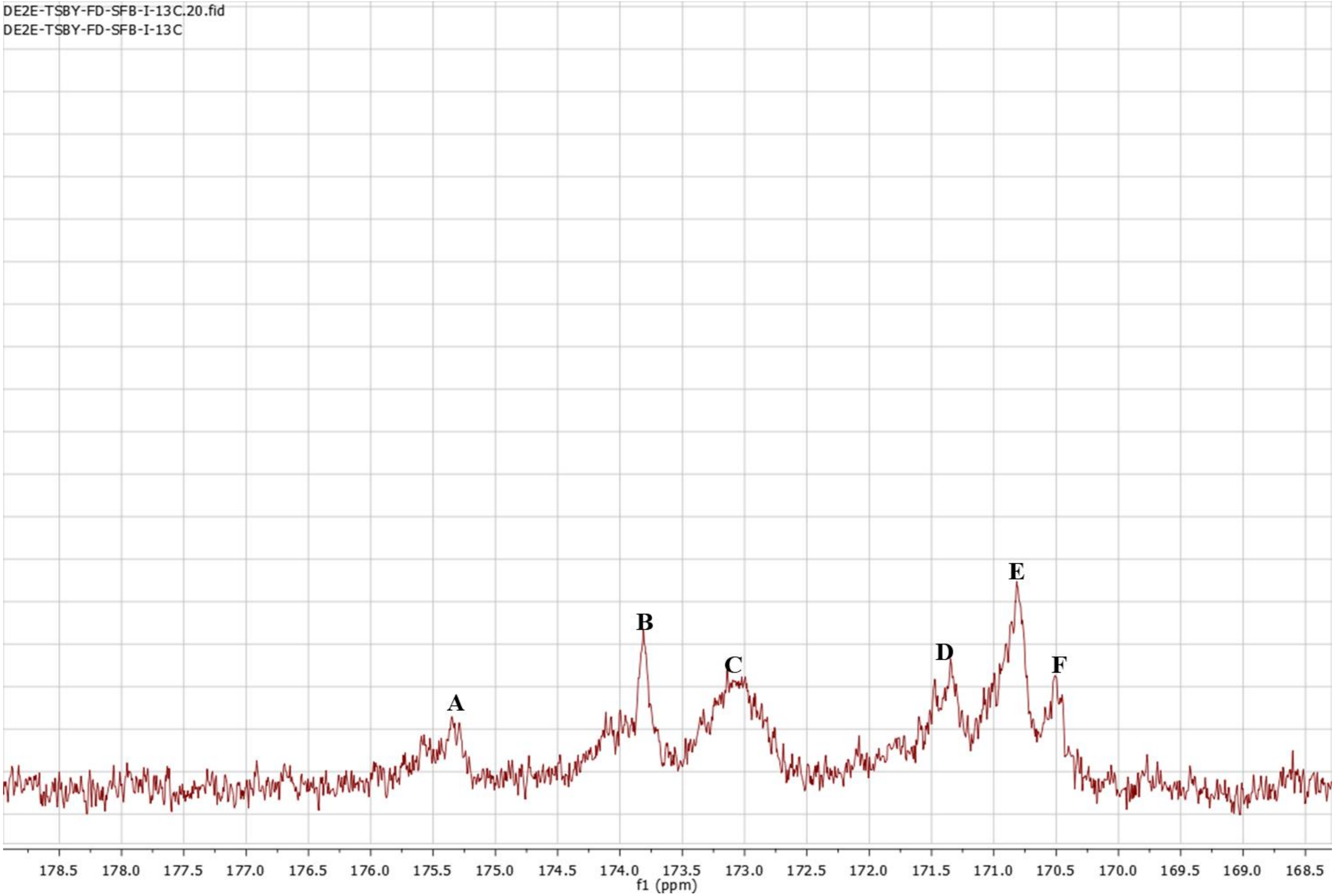
Appendix 5d: Expanded ^{13}C-NMR spectrum of compound DE2E-TSBY-FD-SFB-I



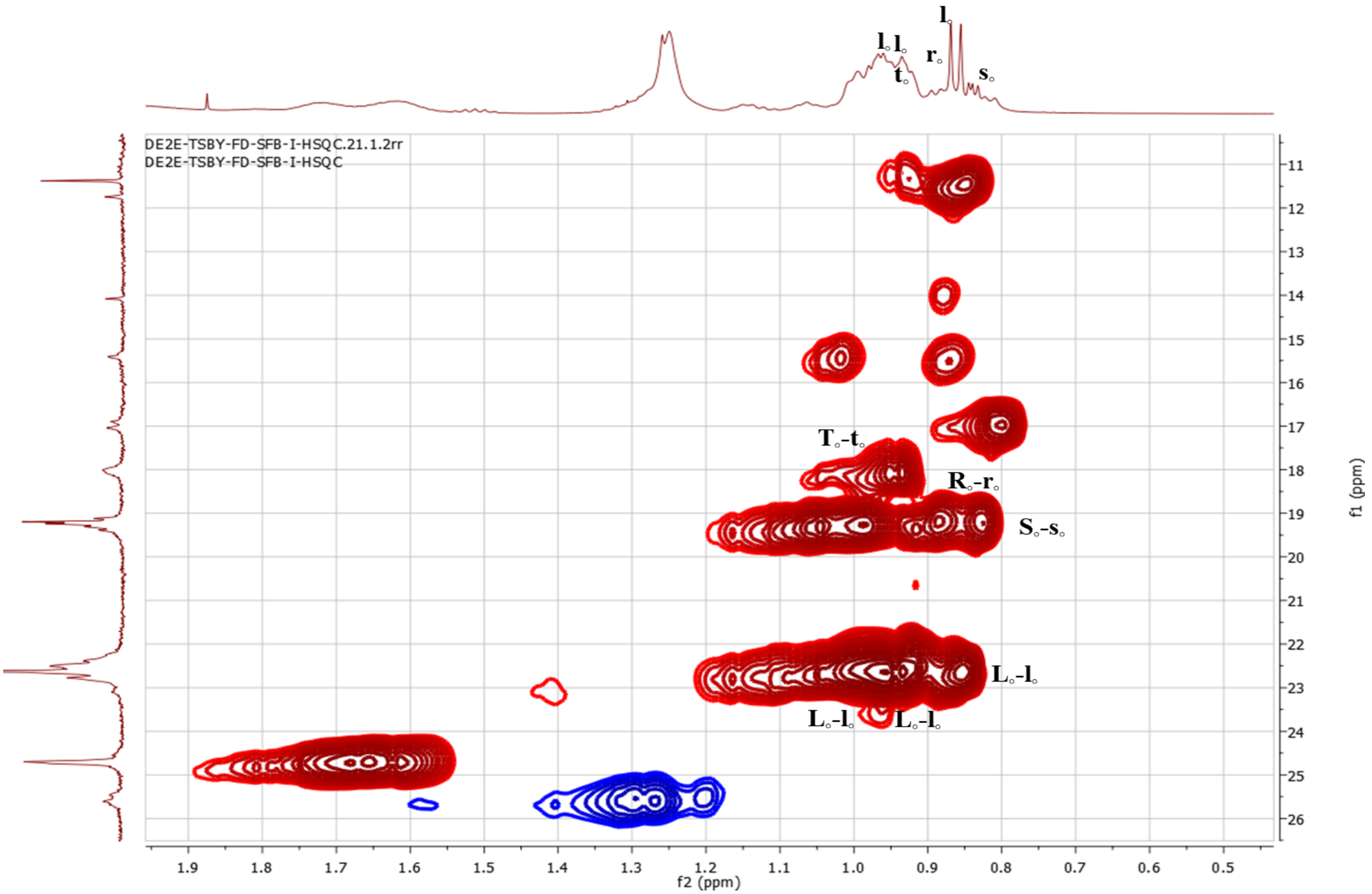
Appendix 5e: Expanded ^{13}C-NMR spectrum of compound DE2E-TSBY-FD-SFB-I



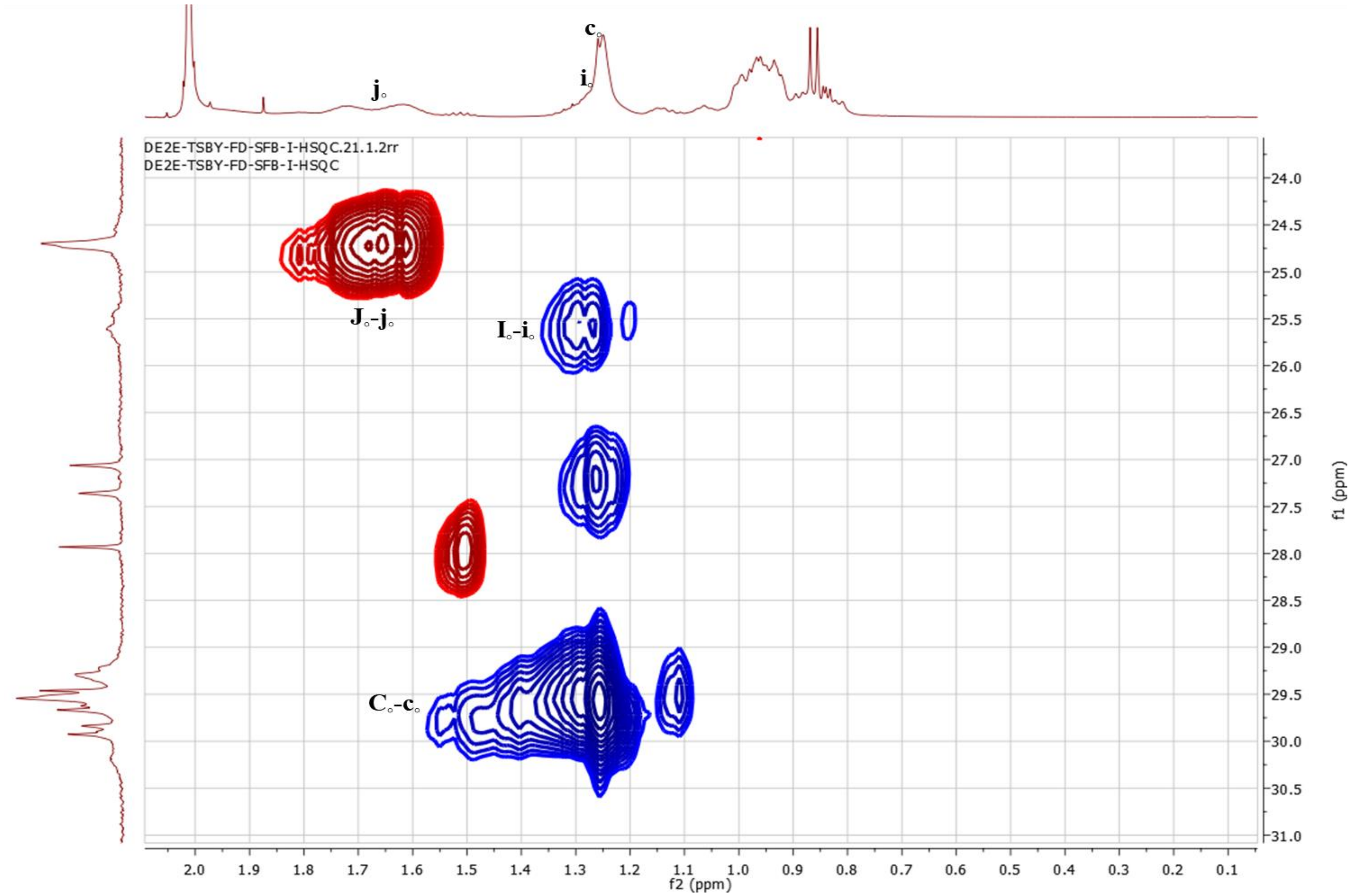
Appendix 5f: Expanded ^{13}C-NMR spectrum of compound DE2E-TSBY-FD-SFB-I



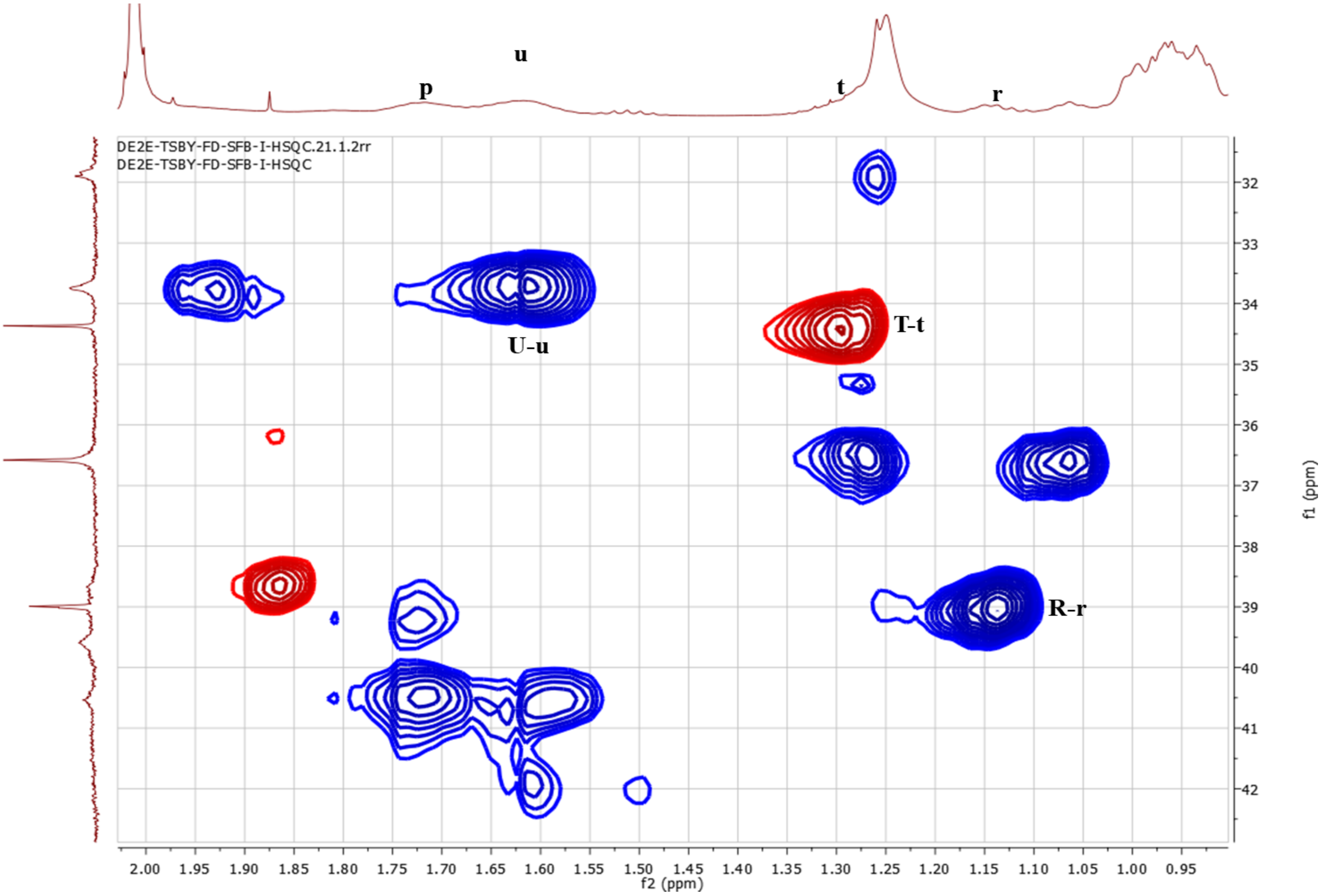
Appendix 6a: HSQC spectrum of compound DE2E-TSBY-FD-SFB-I



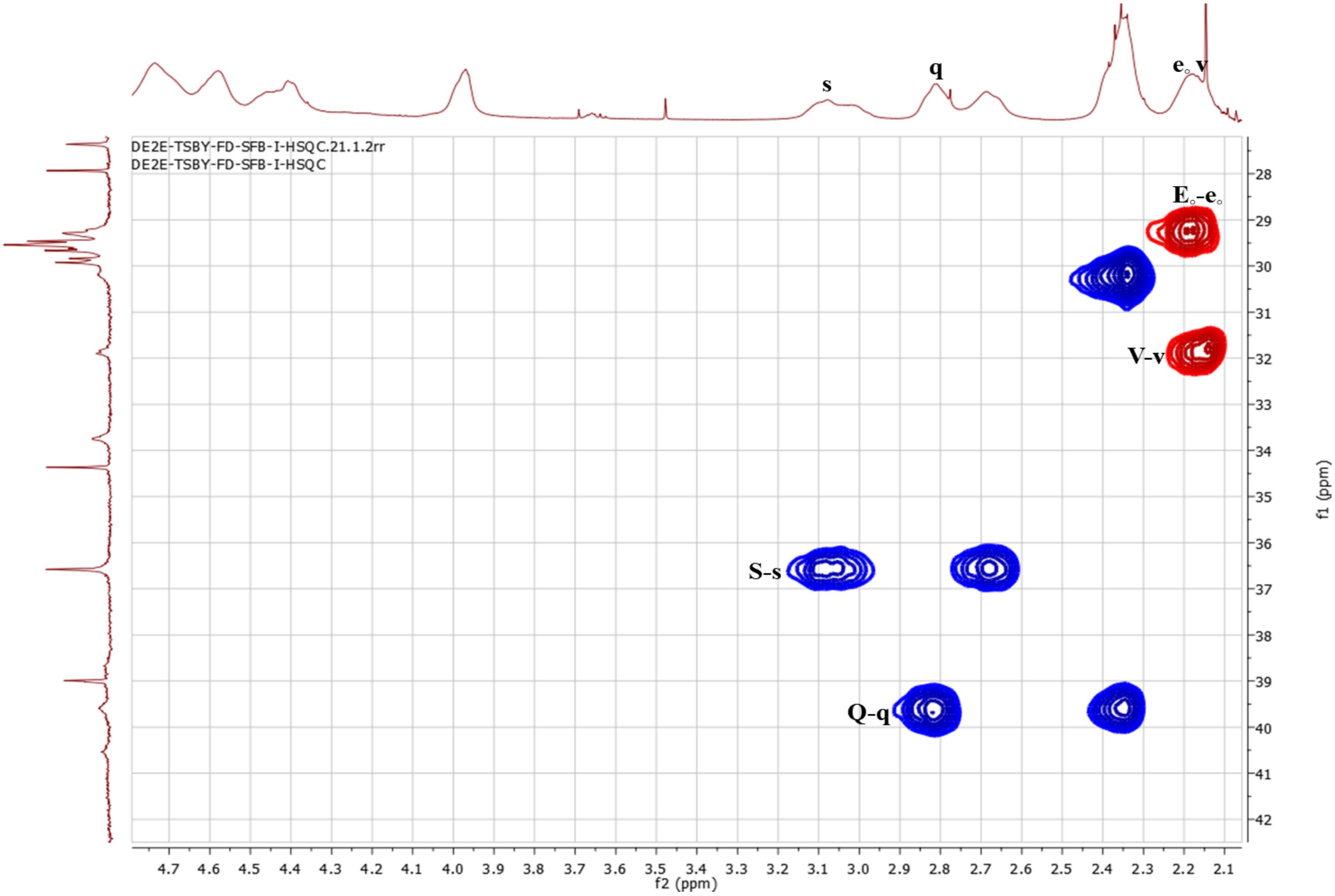
Appendix 6b: HSQC spectrum of compound DE2E-TSBY-FD-SFB-I



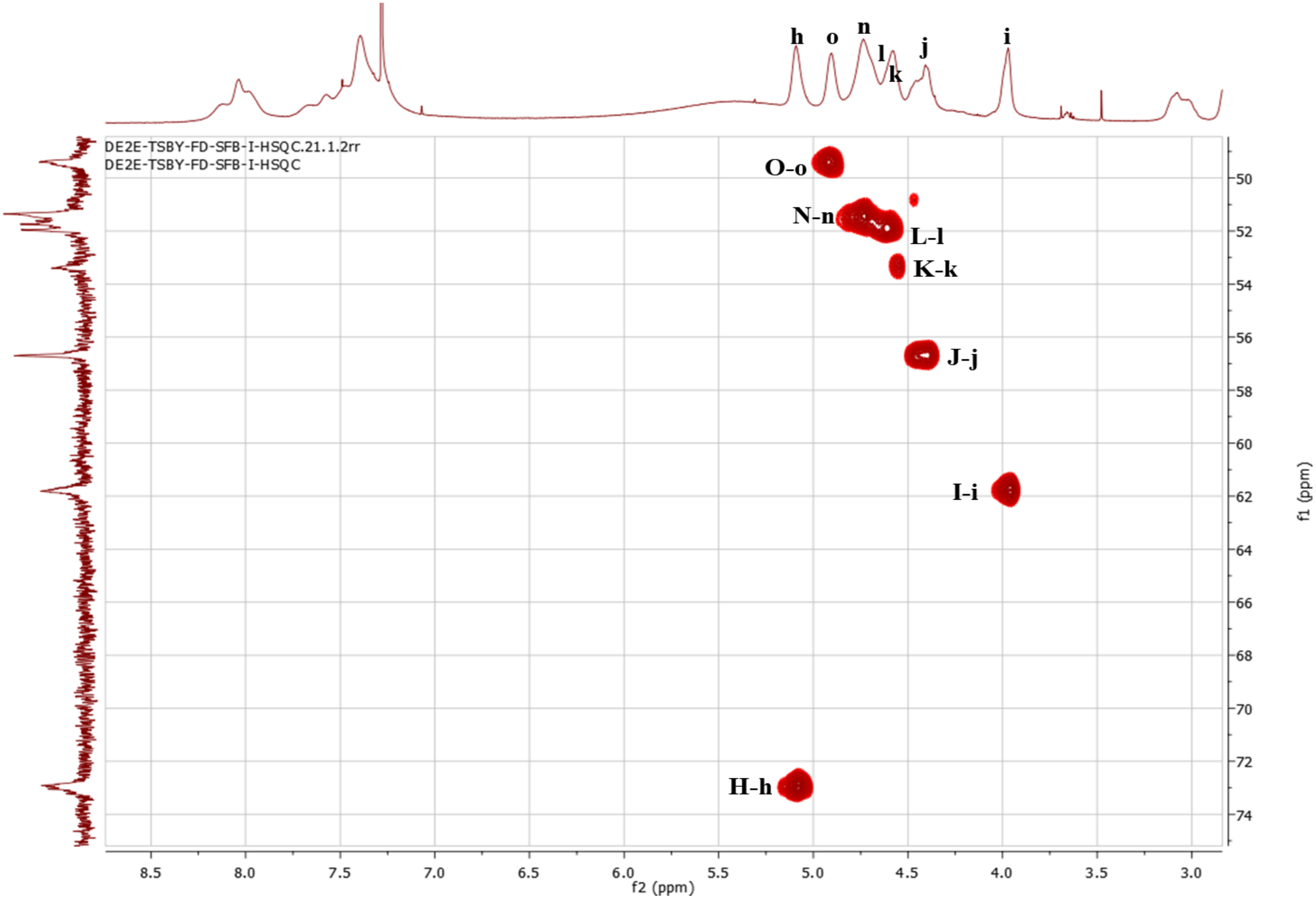
Appendix 6c: HSQC spectrum of compound DE2E-TSBY-FD-SFB-I



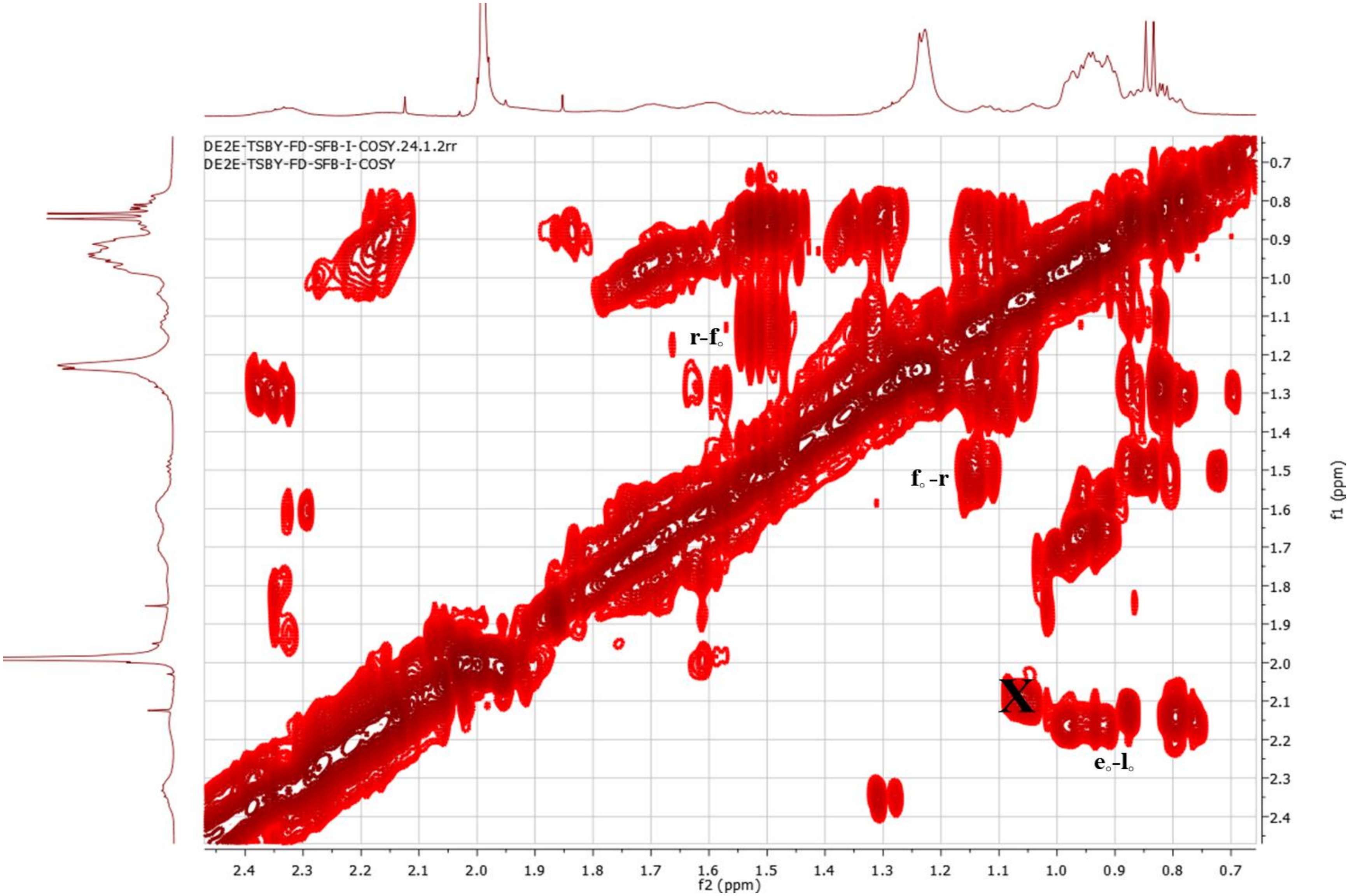
Appendix 6d: HSQC spectrum of compound DE2E-TSBY-FD-SFB-I



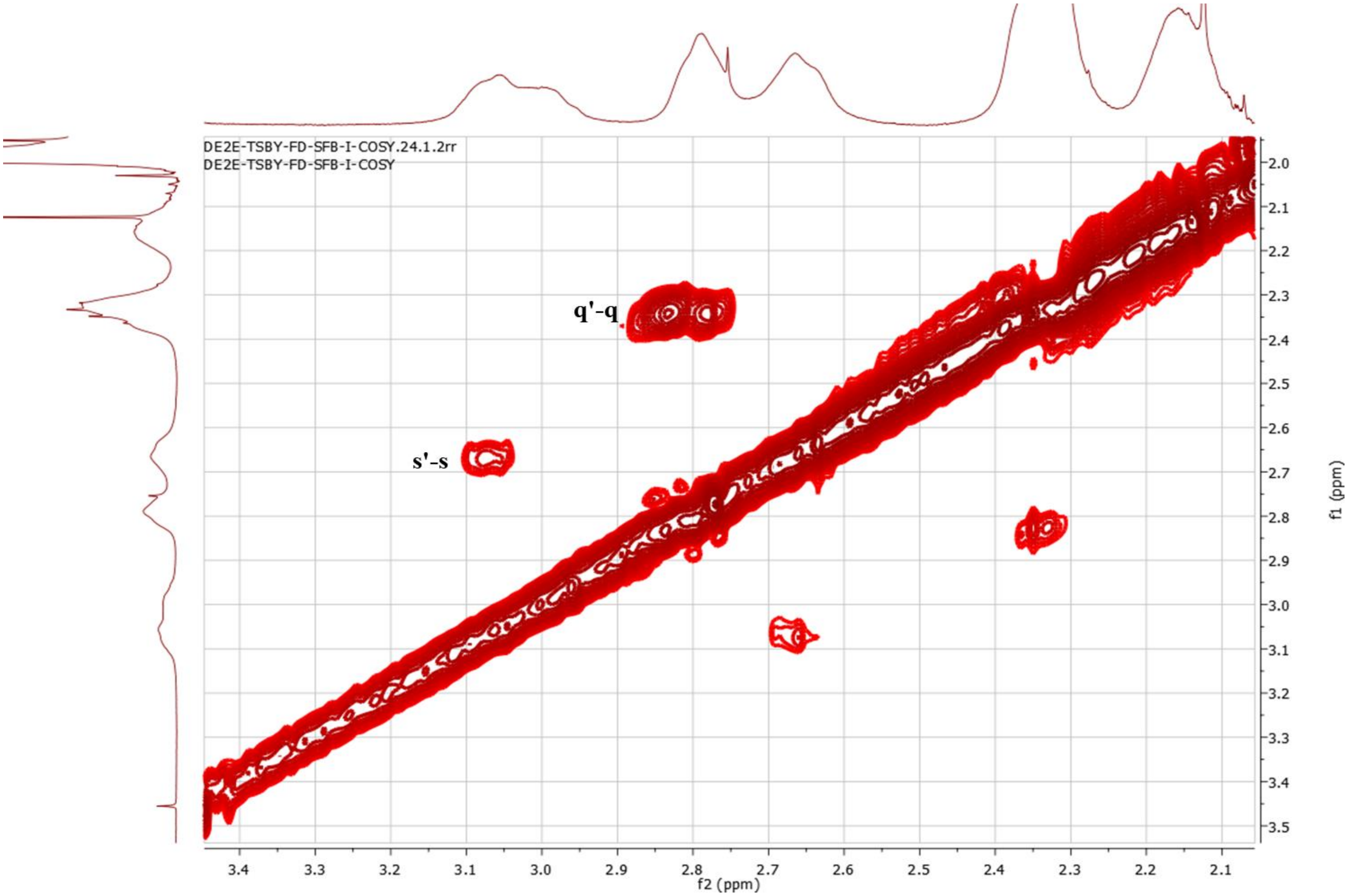
Appendix 6d: HSQC spectrum of compound DE2E-TSBY-FD-SFB-I



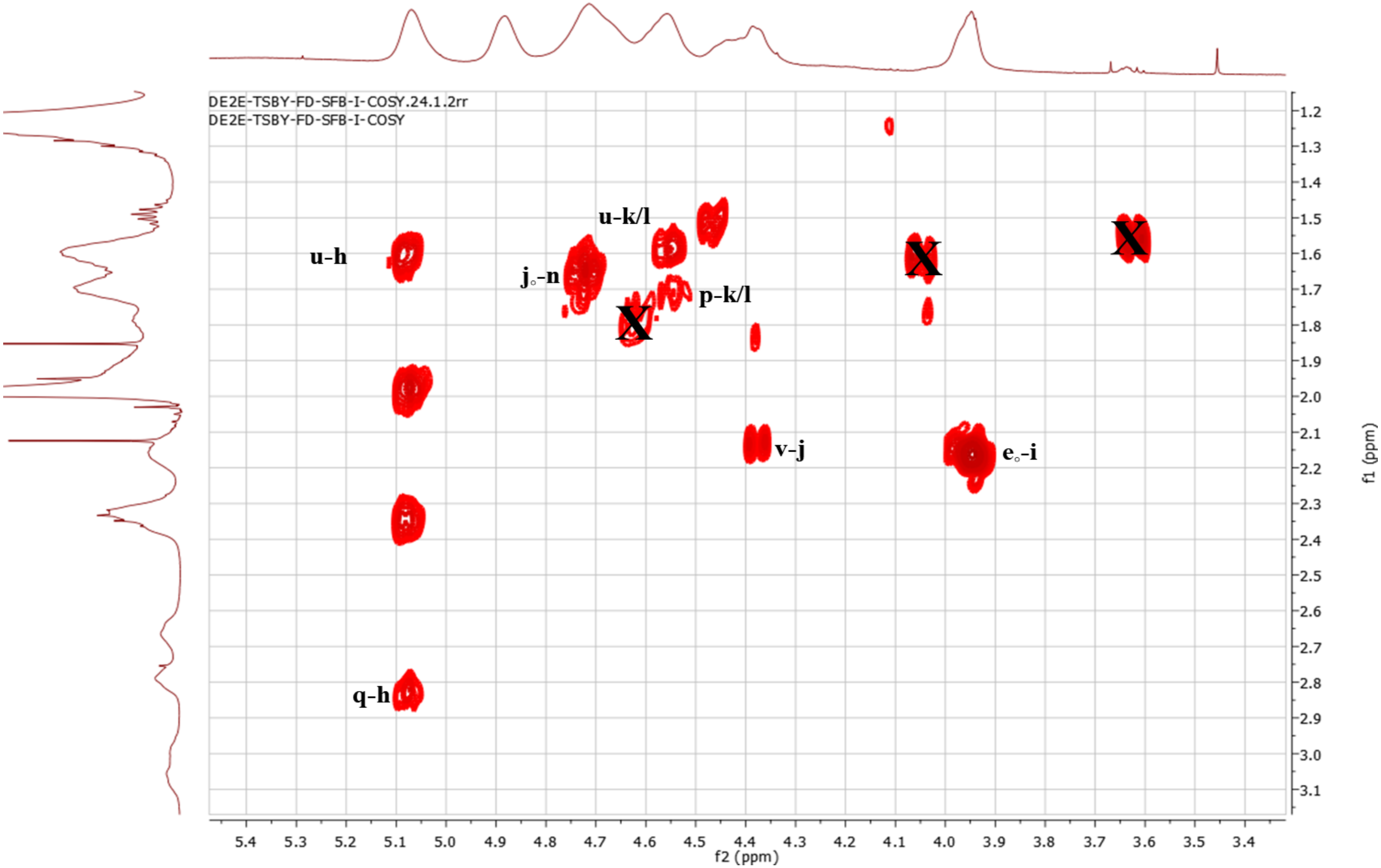
Appendix 7a: COSY spectrum of compound DE2E-TSBY-FD-SFB-I



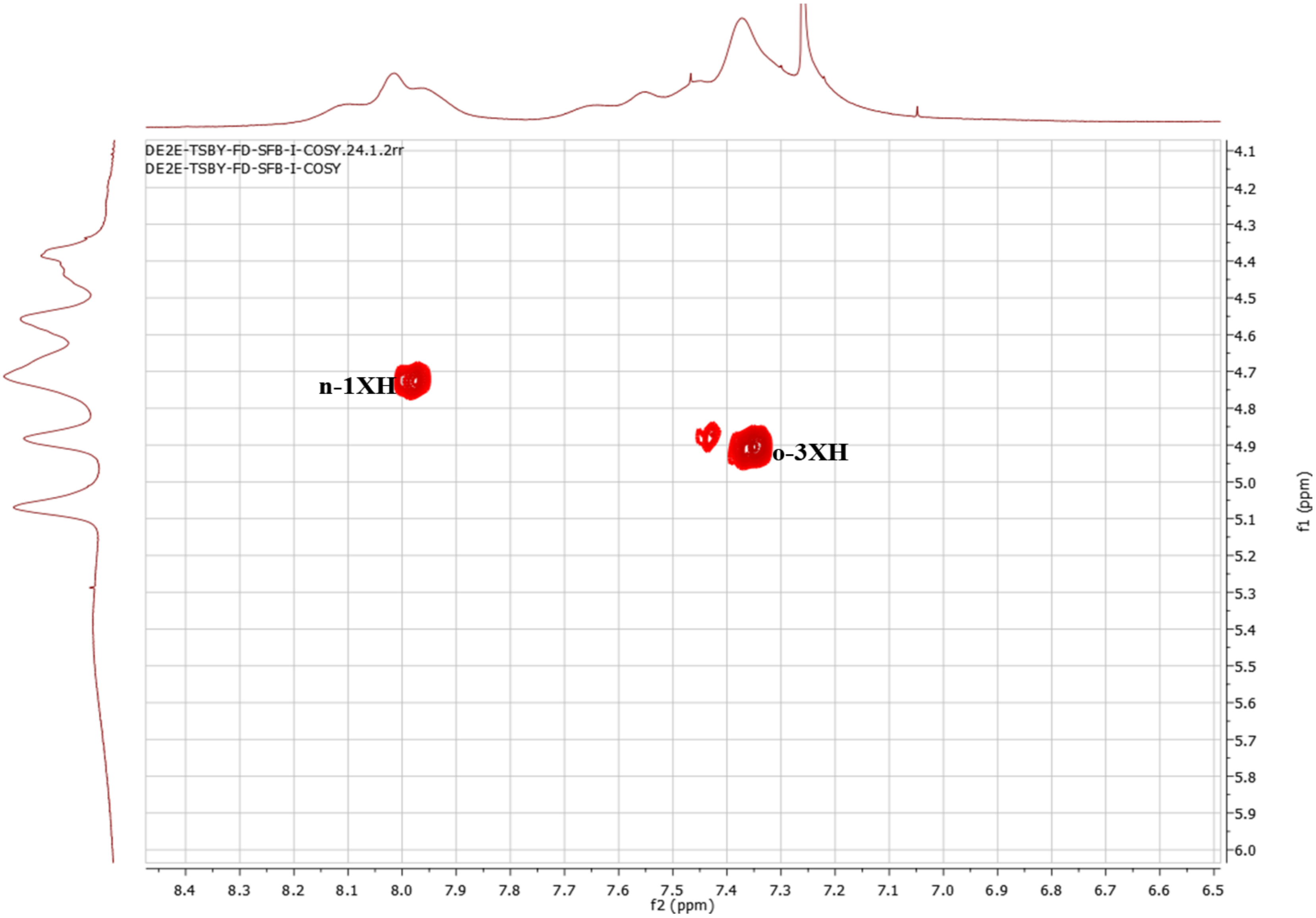
Appendix 7b: COSY spectrum of compound DE2E-TSBY-FD-SFB-I



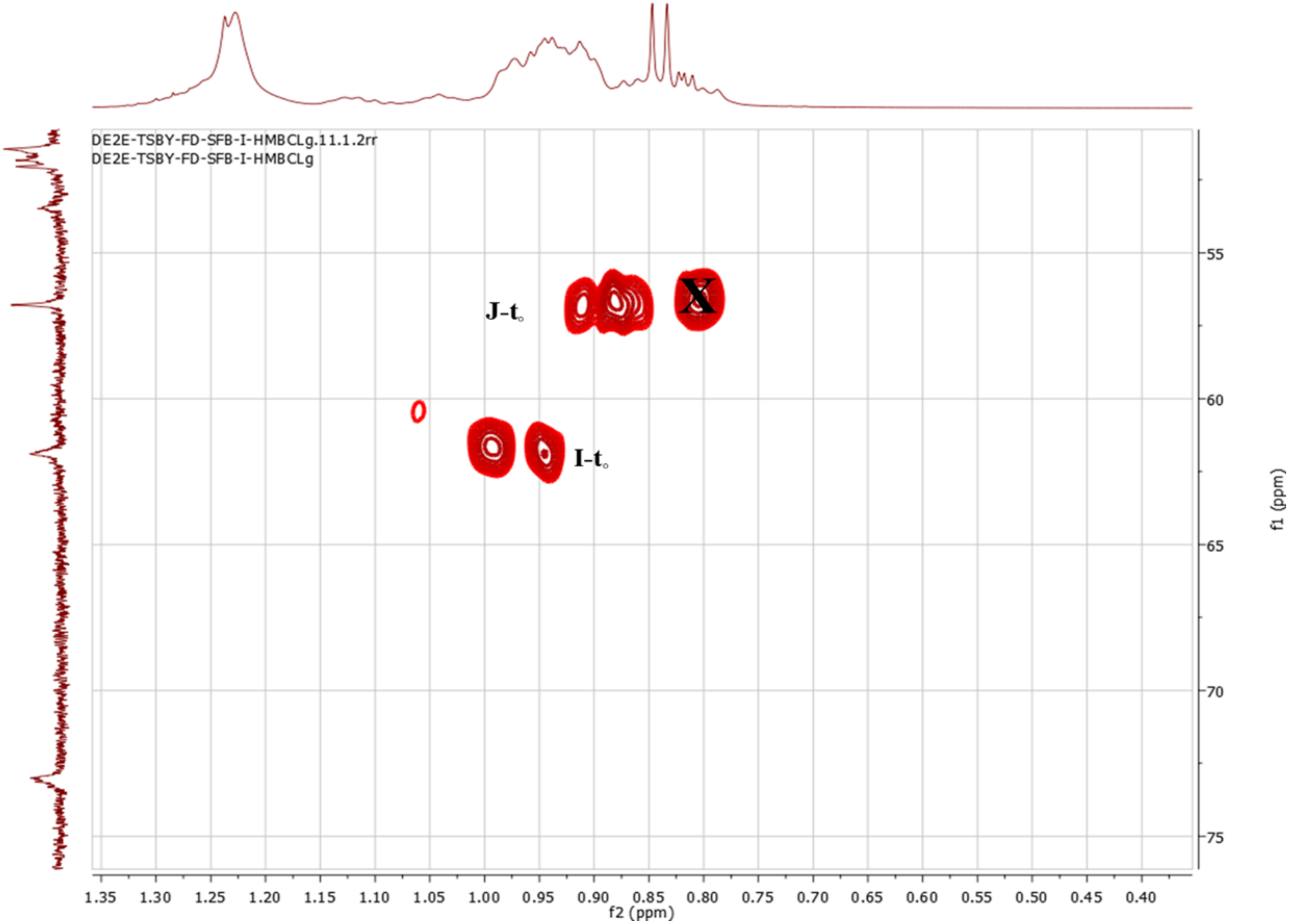
Appendix 7c: COSY spectrum of compound DE2E-TSBY-FD-SFB-I



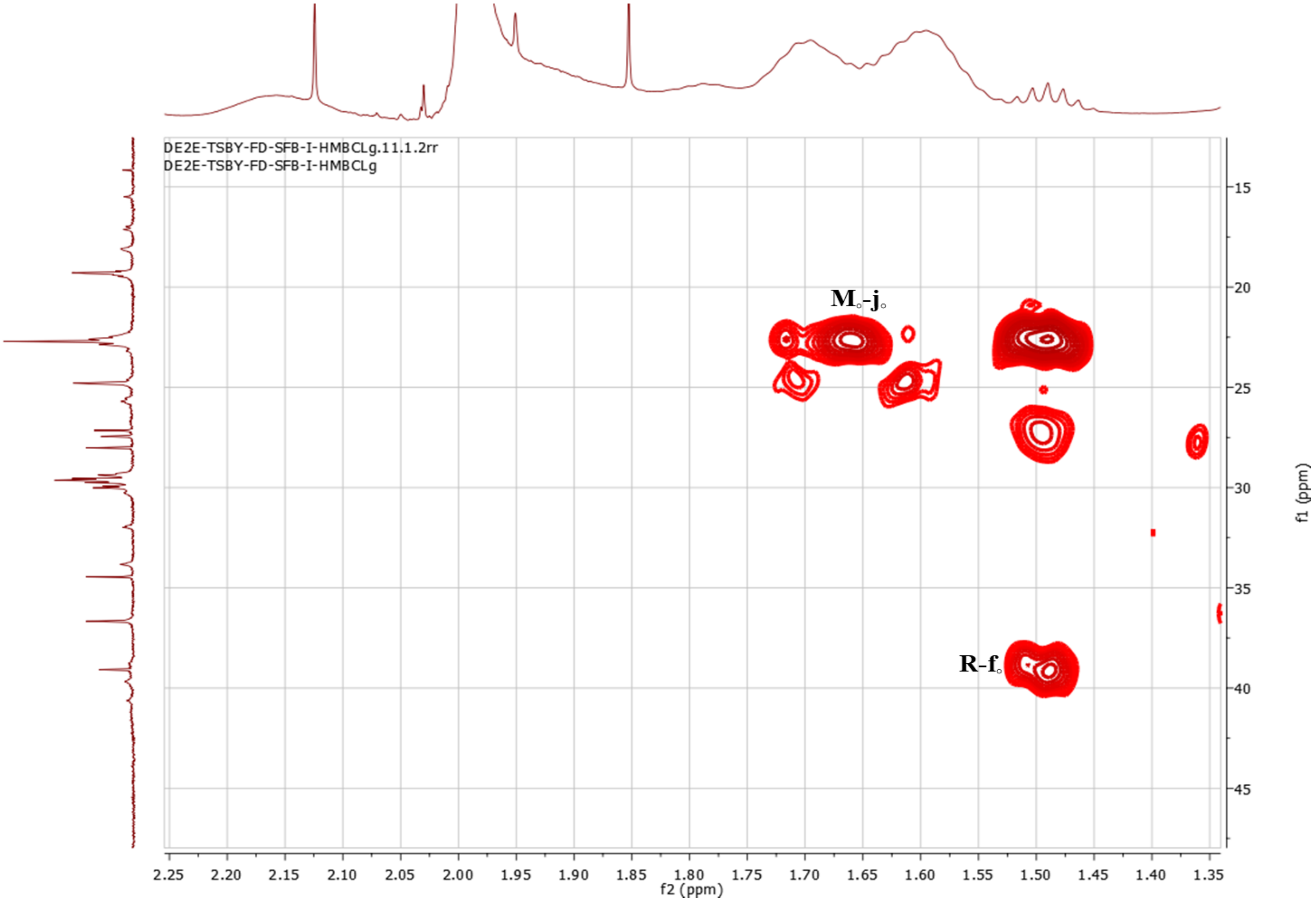
Appendix 7d: COSY spectrum of compound DE2E-TSBY-FD-SFB-I



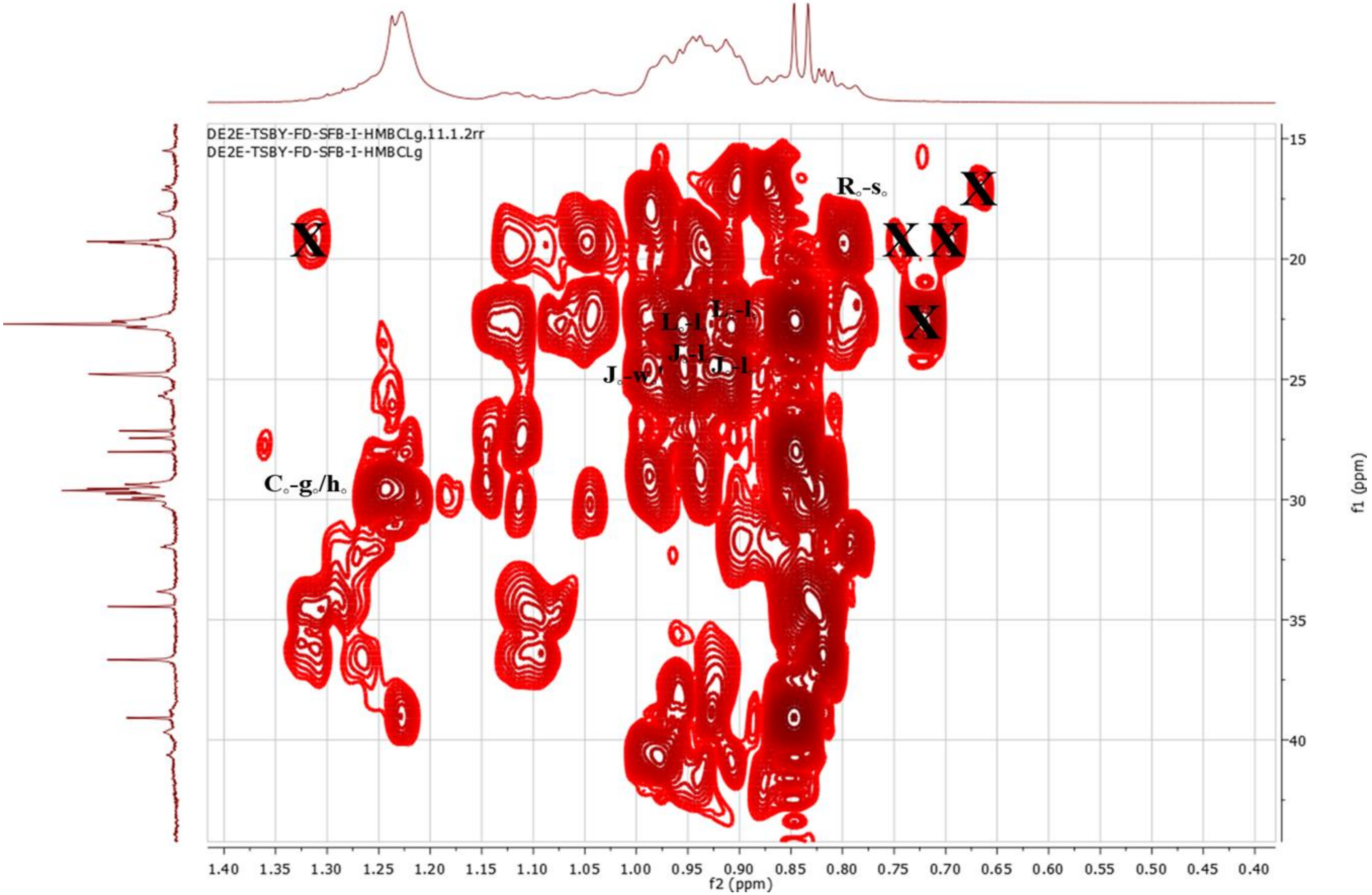
Appendix 8a: HMBC spectrum of compound DE2E-TSBY-FD-SFB-I



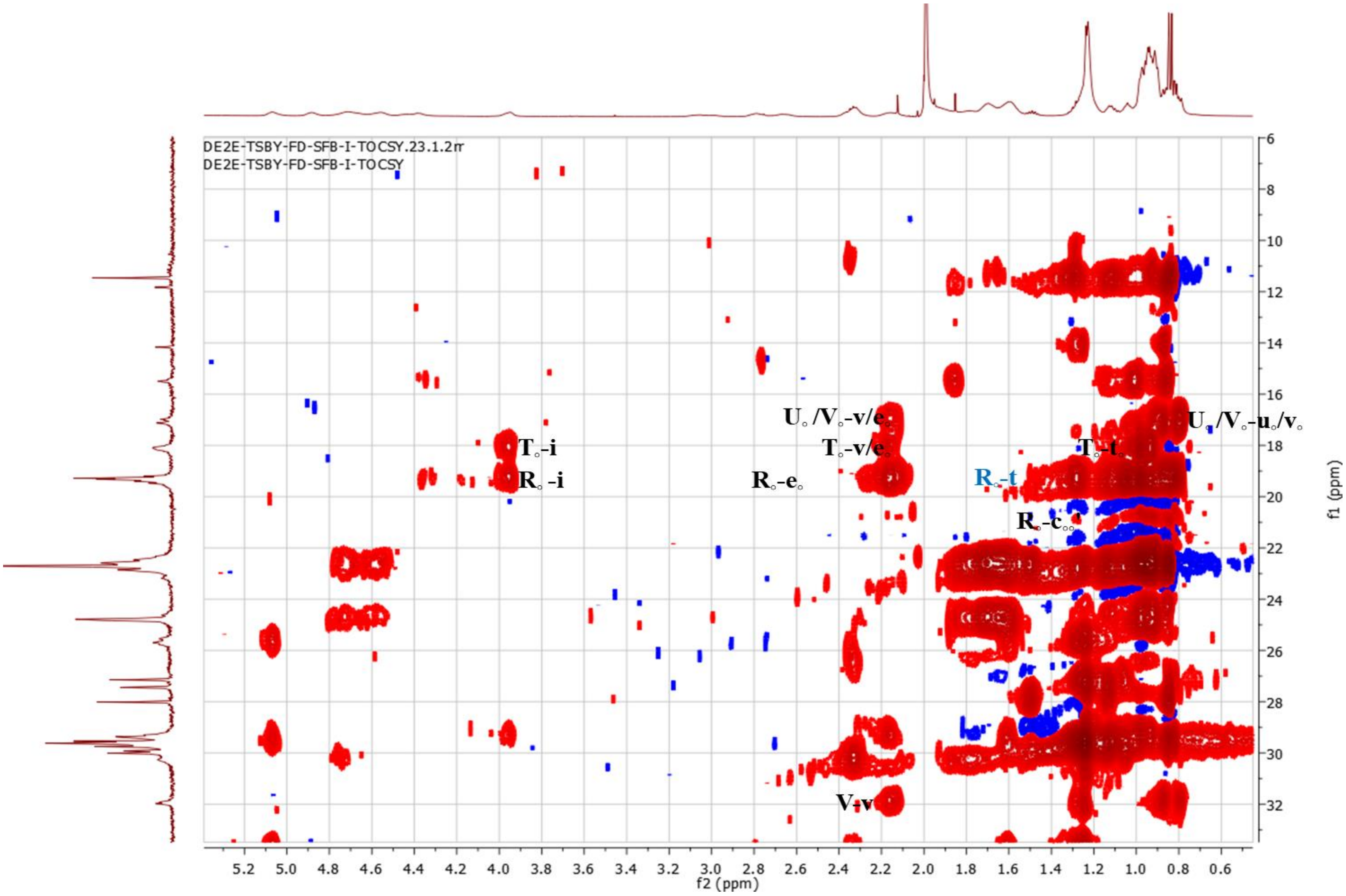
Appendix 8b: HMBC spectrum of compound DE2E-TSBY-FD-SFB-I



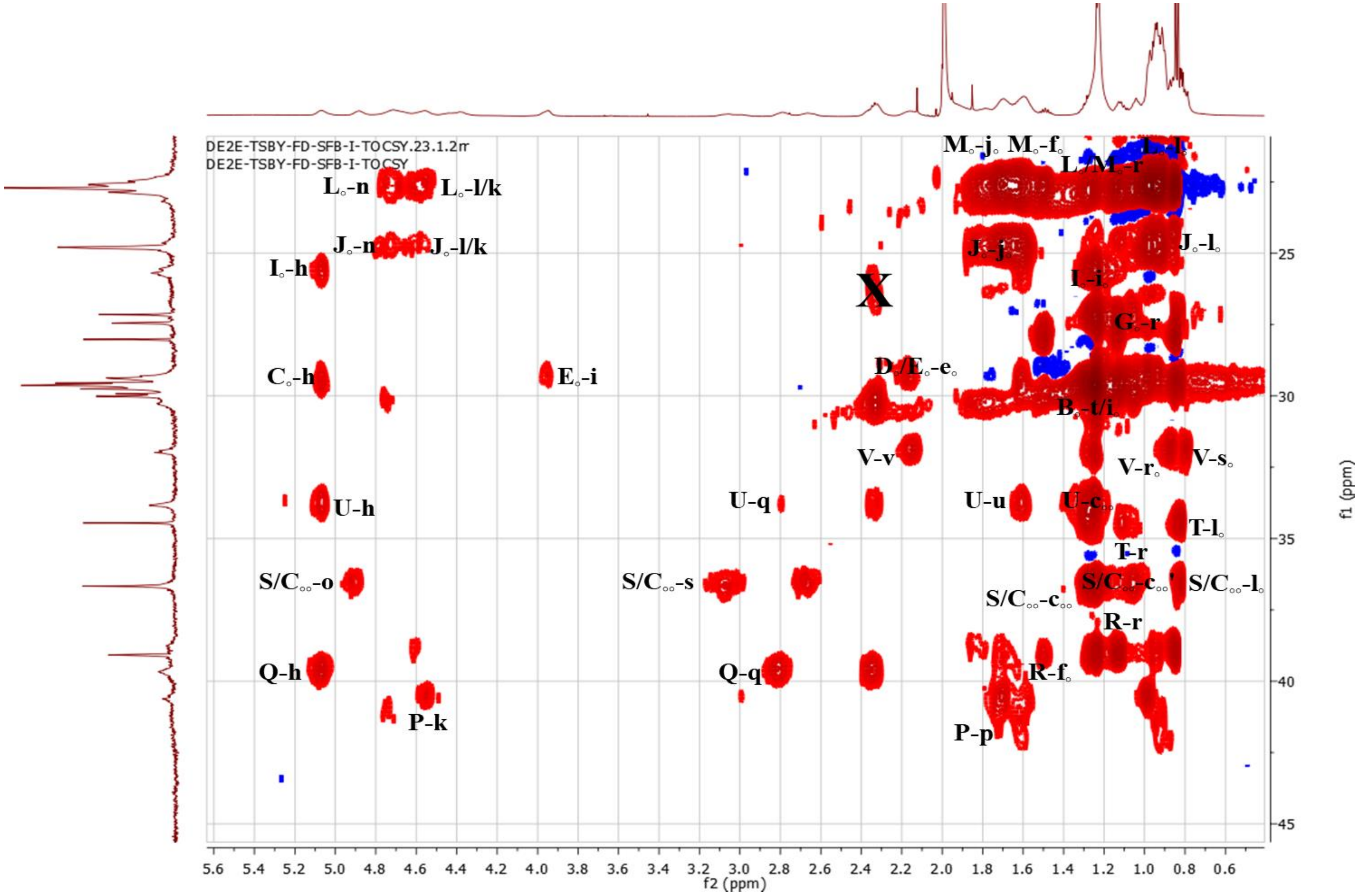
Appendix 8c: HMBC spectrum of compound DE2E-TSBY-FD-SFB-I



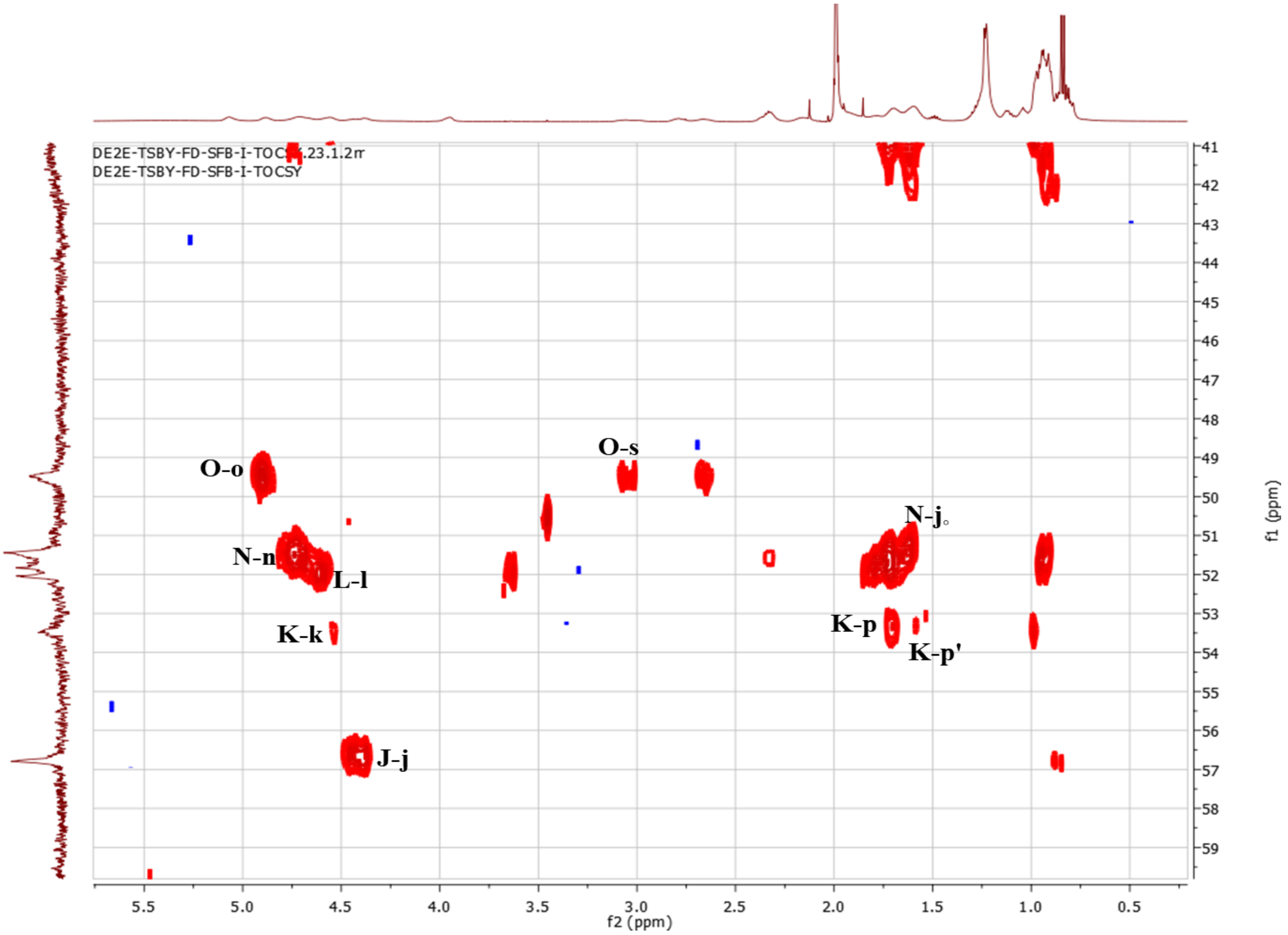
Appendix 9a: TOCSY spectrum of compound DE2E-TSBY-FD-SFB-I



Appendix 9b: TOCSY spectrum of compound DE2E-TSBY-FD-SFB-I

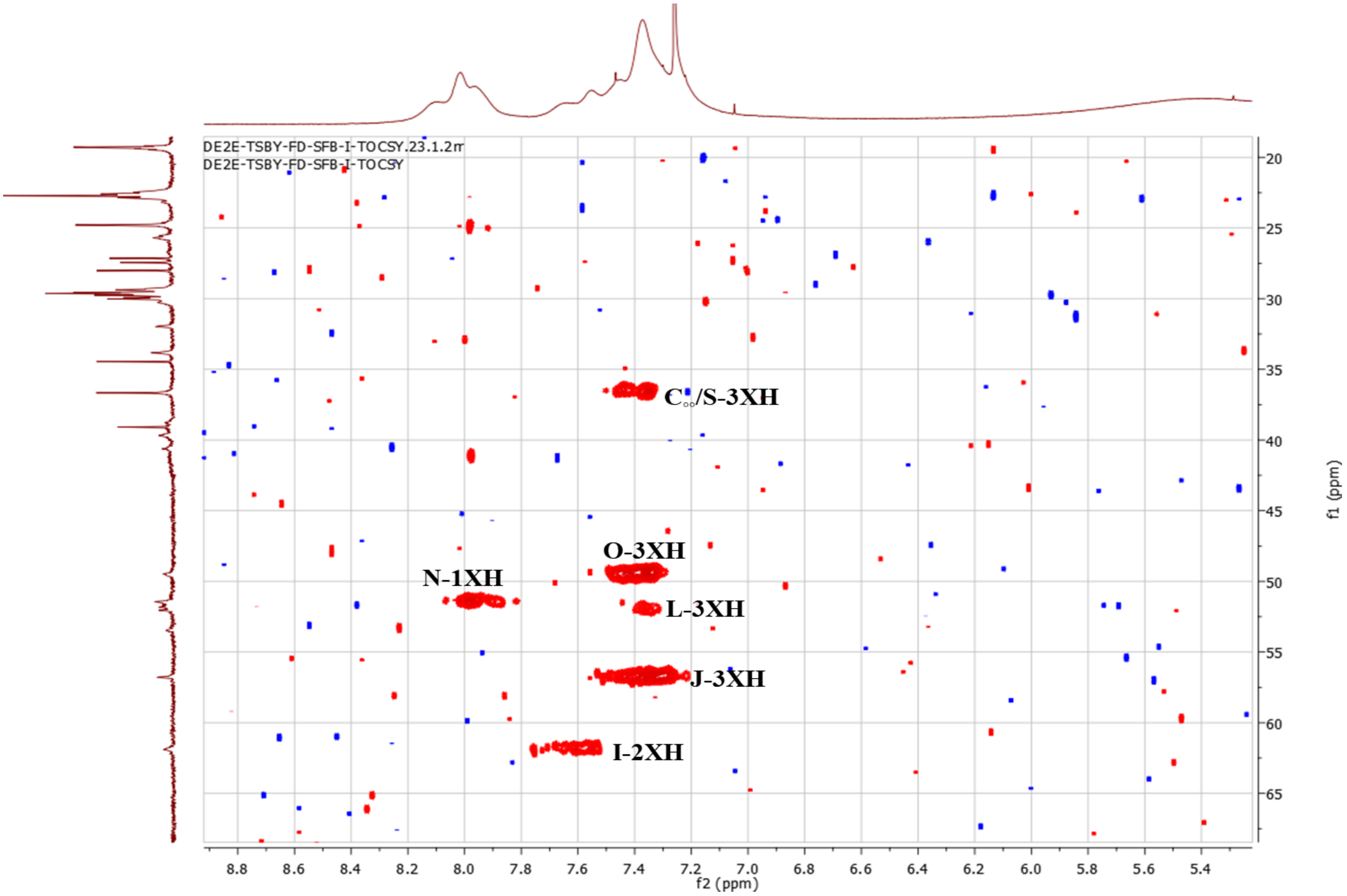


Appendix 9c: TOCSY spectrum of compound DE2E-TSBY-FD-SFB-I

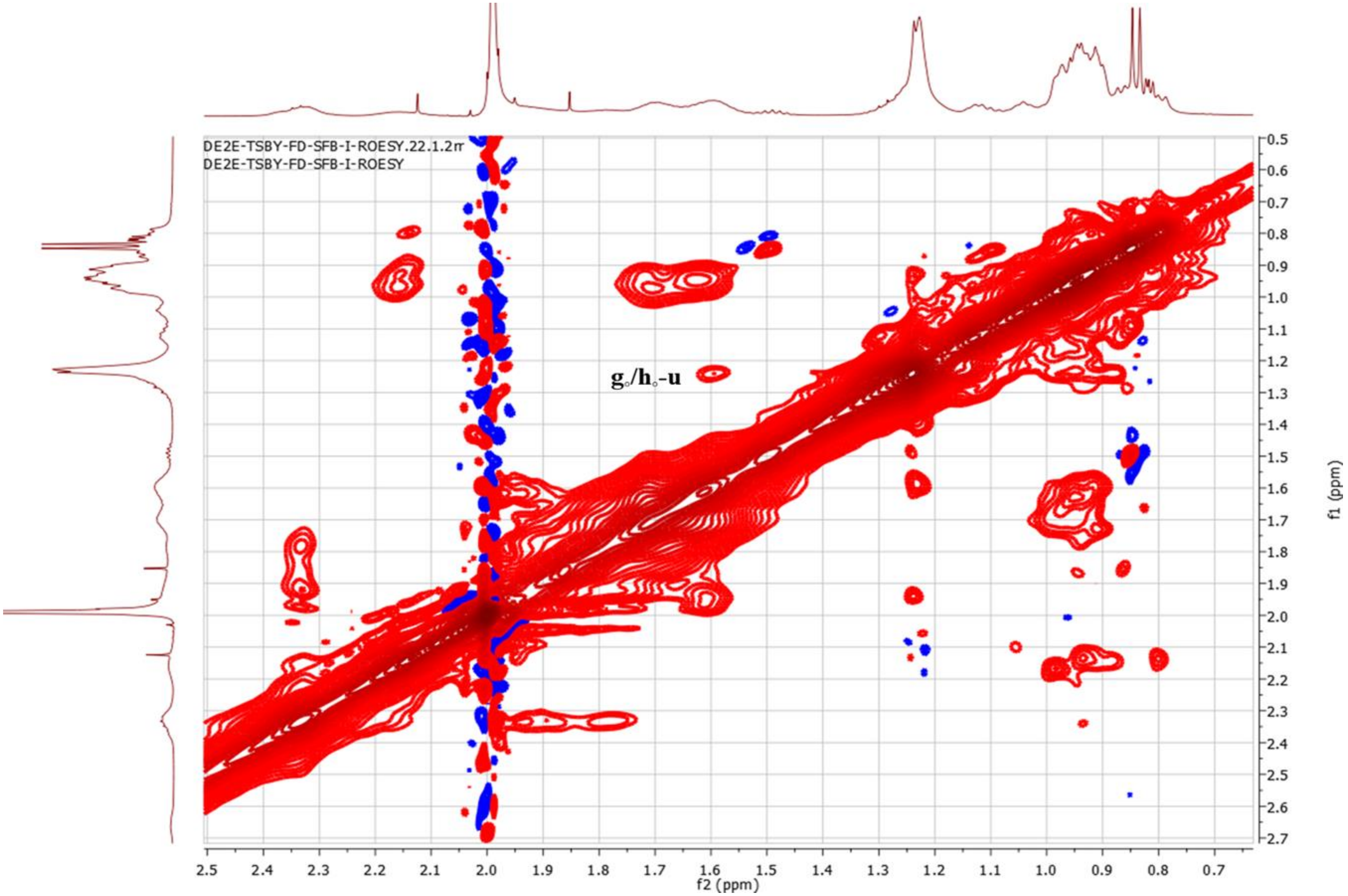


Appendix 9d: TOCSY spectrum of compound DE2E-TSBY-FD-SFB-I

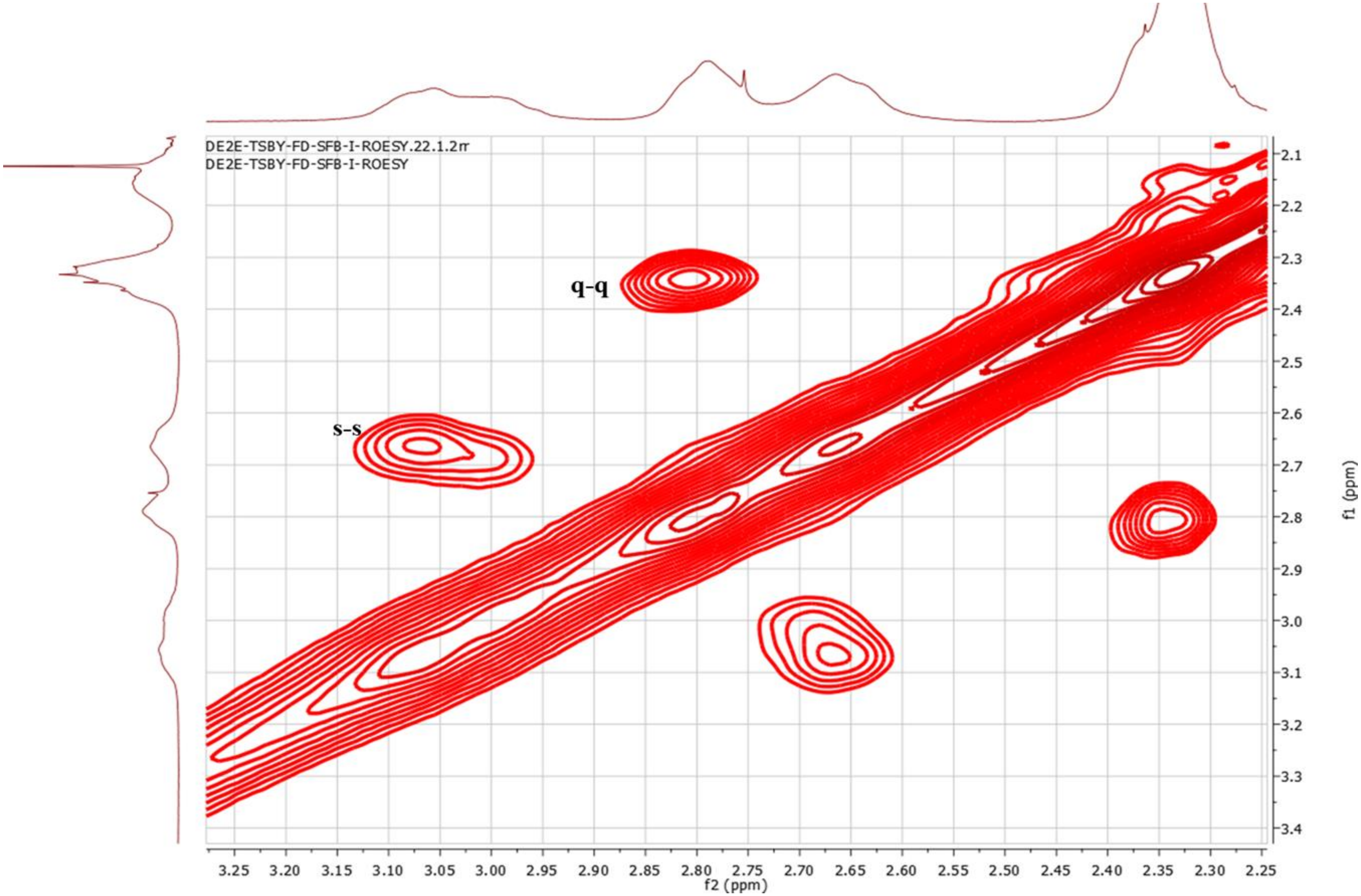
Appendix 9d: TOCSY spectrum of compound DE2E-TSBY-FD-SFB-I



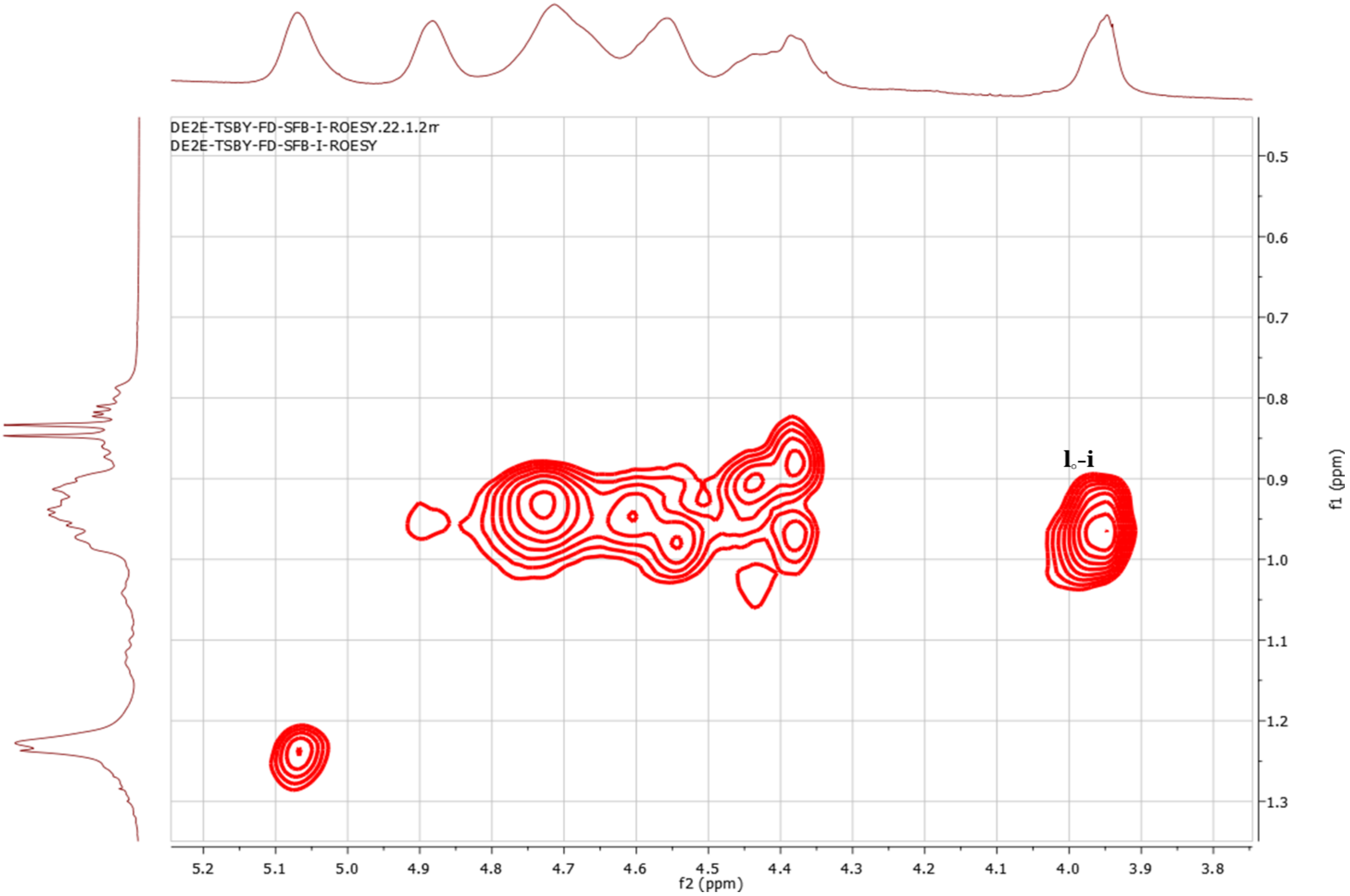
Appendix 10a: ROESY spectrum of compound DE2E-TSBY-FD-SFB-I



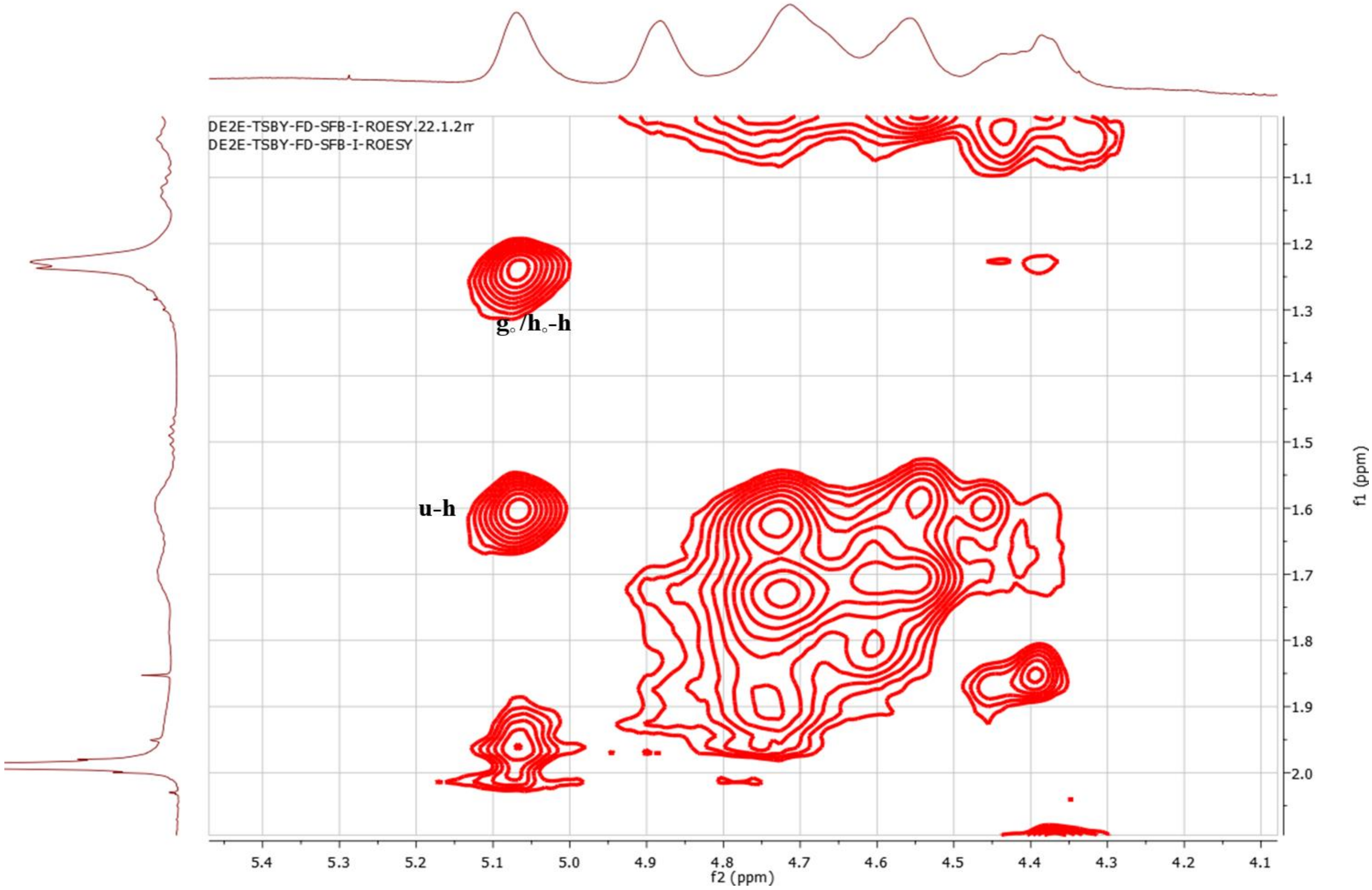
Appendix 10b: ROESY spectrum of compound DE2E-TSBY-FD-SFB-I



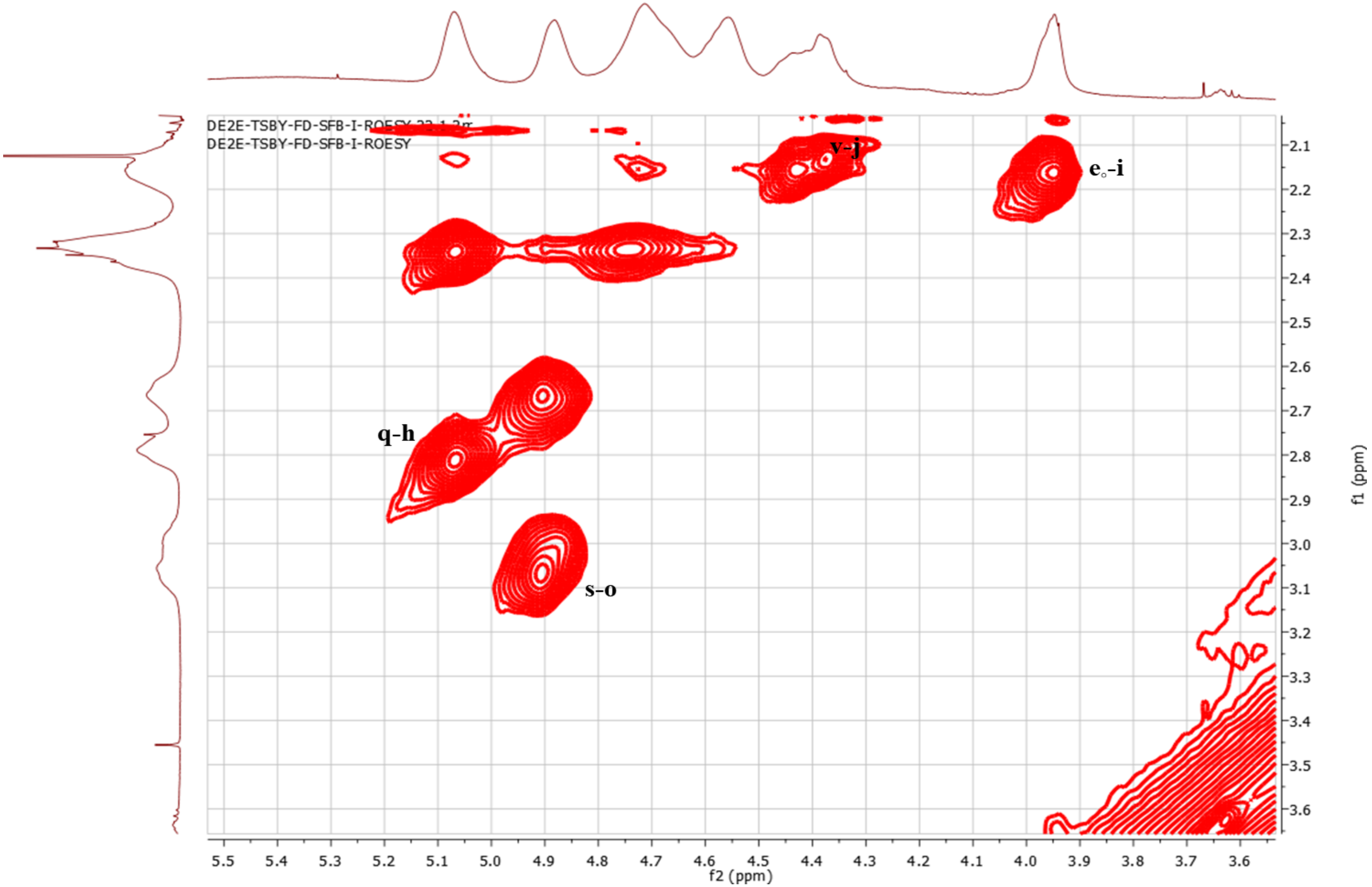
Appendix 10c: ROESY spectrum of compound DE2E-TSBY-FD-SFB-I



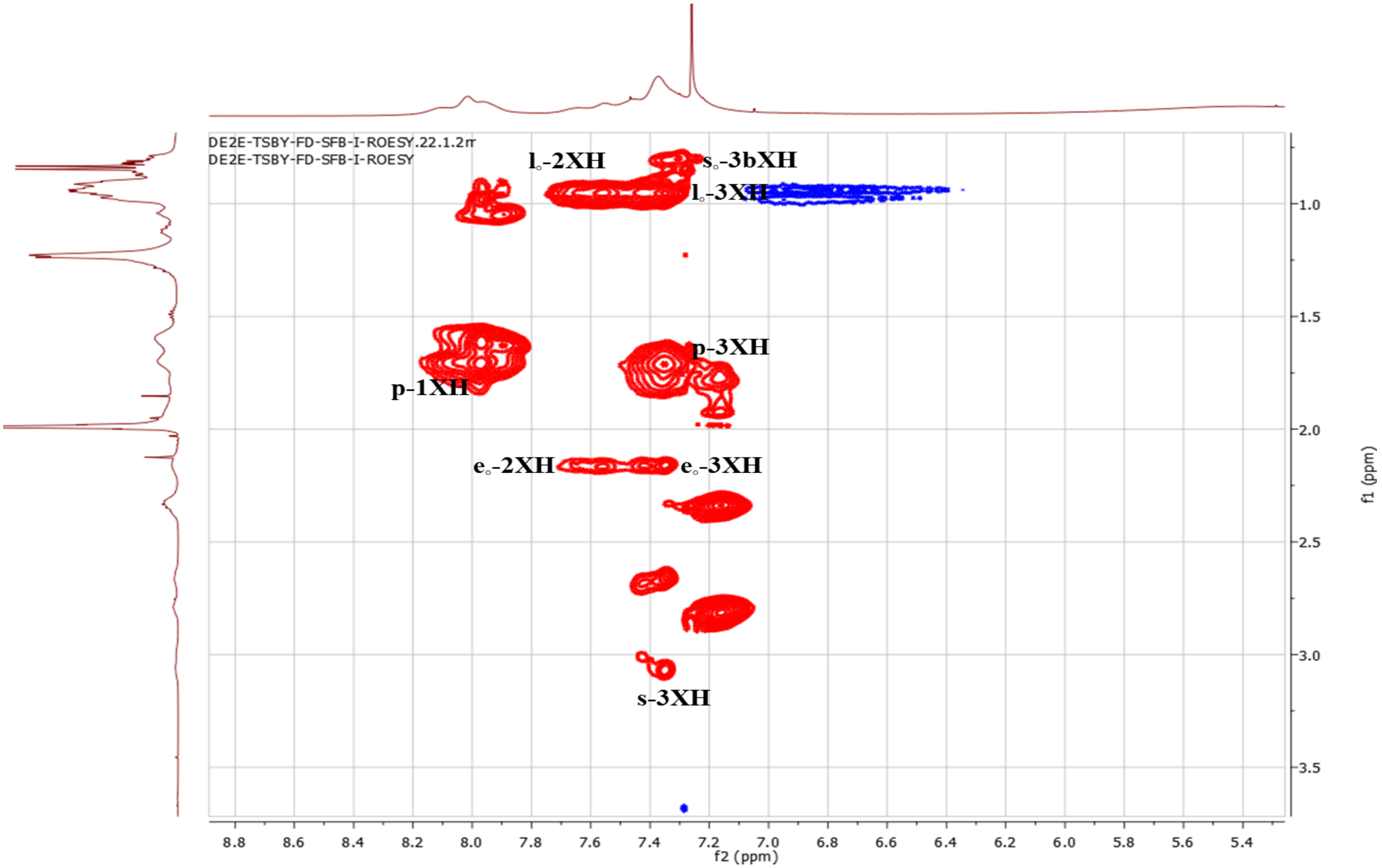
Appendix 10d: ROESY spectrum of compound DE2E-TSBY-FD-SFB-I



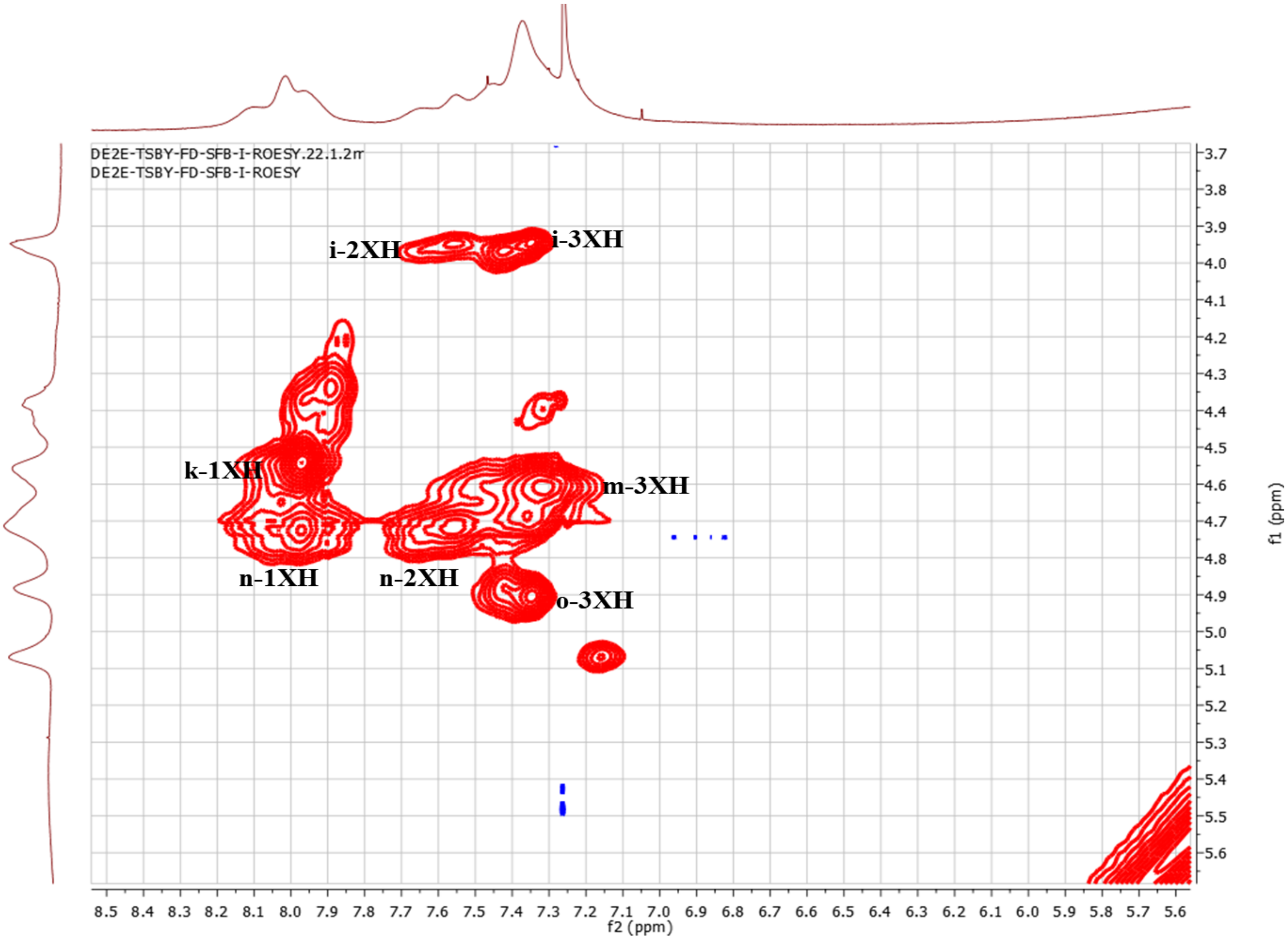
Appendix 10e: ROESY spectrum of compound DE2E-TSBY-FD-SFB-I



Appendix 10f: ROESY spectrum of compound DE2E-TSBY-FD-SFB-I



Appendix 10g: ROESY spectrum of compound DE2E-TSBY-FD-SFB-I



Appendix 11a: Biological activity results for the isolated peptides E, F and G

SAMPLE	Recorded IC_{50} values of compounds against respective pathogen (µM)					
	T. brucei	*L. donovani*	*E.coli*	*S. aureus*	*B. cereus*	*Sh. flexneri*
DE2E-TSBY-FD-SFB-E	24.79	4.848	88.65	>100	>100	74.09
DE2E-TSBY-FD-SFB-F	14.37	8.175	>100	93.15	16.39	55.67
DE2E-TSBY-FD-SFB-G	15.51	98.2	>100	13.07	>100	>100
Positive controls with respective their IC_{50} values (µM)						
Ampicillin			10.44	0.18	1.70	1.76
Amphotericin B		0.96				
Suramin	4.78					

Appendix 11b: Biological activity results for the isolated peptides E, F and G

SAMPLE	**Recorded IC$_{50}$ values of compounds against respective pathogen (µM)**				
	Sa. para B	*S. sonnei*	*Sh. dysentriae*	*Sa. typhomorium*	*Sa. enteritidis*
DE2E-TSBY-FD-SFB-E	73.50	58.43	19.35	>100	73.66
DE2E-TSBY-FD-SFB-F	58.86	20.03	93.01	43.69	30.09
DE2E-TSBY-FD-SFB-G	59.52	37.84	45.11	92.10	>100
Positive controls with respective their IC$_{50}$ values (µM)					
Ampicillin		1.53	1.07	1.27	0.76