Investigating the chemical diversity and biomedicinal potential of South African actinomycetes for tuberculosis drug discovery

by
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Abstract

The chemical diversity and biomedicinal potential of three South African actinomycetes, *Streptomyces speibonae* PK-Blue^T, *Streptomyces africanus* CPJVR-H^T and *Streptomyces pharetrae* CZA14^T, was investigated. The primary aim was the isolation and structure elucidation of anti-tubercular natural products (NPs), including the re-isolation of the compound named PK-B, reported to be produced by *S. speibonae* PK-Blue^T.

Efforts were made to re-isolate the compound named PK-B. However, the results could not be replicated and focus was shifted to the bioassay-guided isolation of antimycobaterial compounds from the three actinobacteria. Culture conditions were optimised for antimycobacterial activity of *S. speibonae* PK-Blue^T extracts by testing against the MTB analogue *Mycobacterium aurum* A+ (a non-pathogenic, fast-growing mycobacterium that has a similar antibiotic susceptibility to *Mycobacterium tuberculosis*). Bioactive crude extracts were prioritised for liquid-liquid partitioning and dereplication by LR LC-MS, followed by prefractionation and purification using a combination of HPLC and benchtop column chromatography. Structure elucidation of isolated compounds was achieved using NMR, LC-MS and GC-MS data. Bioassays against *M. aurum* A+ were implemented at every stage of the isolation process to make sure that the isolated compounds had antimycobacterial activity. This strategy led to the isolation of four known compounds, the alkaloid *N*-phenylpyridin-2-aminium (**3.1**), a 1:1 mixture of the long-chain fatty acids (LCFAs) *n*-hexadecanoic acid (**3.2**) and 14-methylpentadecanoic acid (**3.3**), and the isoflavone 7-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (**3.4**), from *S. speibonae* PK-Blue^T cultures. Of the four isolated compounds, only **3.1** has not previously been reported from a natural source. The approach of using *M. aurum* A+ in searching for anti-tubercular compounds was vindicated as **3.1**, the mixture of **3.2** and **3.3**, and **3.4**, in addition to inhibiting the growth of *M. aurum* A+, exhibited activity against *M. tuberculosis* H37Rv^T with MIC_{90} values of 135 µM, 26 µM and 195 µM, respectively.

Additionally, the effect of different growth media on the chemical diversity of *S. speibonae* PK-Blue^T, *S. africanus* CPJVR-H^T and *S. pharetrae* CZA14^T extracts was demonstrated by bioassay where the extracts were screened against a panel of test organisms. HR LC-MS dereplication was used to identify a list of 11 suggested molecular formulae of potentially

novel, bioactive NPs in liquid-liquid partitioned extracts of theses three filamentous actinobacteria. Unfortunately, these compounds could not be investigated further due to low biomass and time limitations.

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List of abbreviations

1D	one-dimensional
2D	two-dimensional
2YT	2 x yeast extract tryptone medium
ADMET	absorption, distribution, metabolism, excretion and toxicity
AIDS	acquired immune deficiency syndrome
AR	analytical reagent
BCE	before common era
$CaCO_3$	calcium carbonate
$CDCl_3$	deuterated chloroform
DMSO	dimethyl sulfoxide
$CHCl_3$	chloroform
CO_2	carbon dioxide
COSY	correlated spectroscopy
d	day(s)
DAD	diode array detector
DNA	deoxyribonucleic acid
ESI	electrospray ionisation
FA	formic acid
FAME	fatty acid methyl ester
G+C	guanine-cytosine
H_2O	water
HCl	hydrogen chloride
HIPS	Helmholtz Institute for Pharmaceutical Research Saarland
HIV	Human Immunodeficiency Virus
HM	Hacène's medium
HMBC	heteronuclear multiple-bond correlation spectroscopy
HPLC	high-performance liquid chromatography
HR	high resolution
HSQC	heteronuclear single-quantum correlation spectroscopy
HTS	high-throughput screening
Hz	hertz
IDM	Infectious Disease and Molecular Medicine
ISP 2	International *Streptomyces* Project 2
J	coupling constant
LCFA	long-chain fatty acids
LC-MS	liquid chromatography-mass spectrometry
LR	low resolution
M7H9	Middlebrook medium
m/z	*mass-to-charge ratio*
MCP	microchannel plate

MDR	multi-drug resistant
MeOH-d4	deuterated methanol
MeOH	methanol
$MgSO_4$	magnesium sulfate
MIC	minimum inhibitory concentration
MS	mass spectrometry
MTB	*Mycobacterium tuberculosis*
MTT	thiazolyl blue tetrazolium bromide
MHB	Mueller-Hinton broth
Myc	Mycosel medium
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NB	natural botanical
NCE	new chemical entity
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser effect spectroscopy
NP	natural product
OD	optical density
RO5	rule of five
ROESY	rotating frame nuclear Overhauser effect spectroscopy
RP	reversed-phase
SAR	structure-activity relationship
SHG	*Streptomyces hagronensis*
TB	tuberculosis
TFA	trifluoroacetic acid
ToF	time of flight
TSB	tryptic soy broth
UCT	University of Cape Town
UP-LC	ultra-performance liquid chromatography
UV	ultraviolet
WHO	World Health Organisation
WT	wild type
XDR	extensively drug resistant

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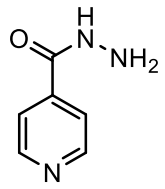
Chapter 1: Introduction

1.1 Tuberculosis

Tuberculosis (TB) is the second most lethal infectious disease globally, behind Human Immunodeficiency Virus infection and the associated acquired immune deficiency syndrome (HIV/AIDS),[1] and is responsible for the deaths of approximately 1.3 million people yearly.[2] This mortality rate is remarkably high given that the disease has been extensively researched and therapeutics are available.[3] *Mycobacterium tuberculosis* (MTB) was identified as the causative agent of TB as far back as 1882[4] but, despite efforts to eradicate the disease, there has been no decline in disease incidence.[5] Instead, recent years have seen greater numbers of TB cases than ever before, with an estimated 1.7 billion people infected with latent TB globally and 10.4 million new cases in 2016,[6] which is a result of the pathogenesis and biology of the bacterium.[5] While this pathophysiology is understood, it does have inherent complications for drug treatment, even in uncomplicated cases.[3] In such cases, activation of the immune system leads to the formation of granulomas, multicellular structures that surround and contain the pathogen, which slows disease progression but also decreases drug susceptibility.[3] This leads to a latent TB infection in most cases, which in 10% of cases results in post-primary reactivation and active and chronic disease.[3]

Another reason for the high rate of TB is treatment cost and duration, which has led to increased TB prevalence in the developing world where public health systems are especially overburdened.[7] The standard chemotherapeutic regimen for uncomplicated TB cases requires a two-month course of combination therapy of the four drugs isoniazid (**1.1**), rifampicin (**1.2**), pyrazinamide (**1.3**) and ethambutol (**1.4**) and a further four-month combination treatment with isoniazid and rifampicin.[8] Failure to implement this regimen successfully has led to drug resistant forms of MTB and this, along with other factors such as coinfection with HIV and/or the patient suffering from diabetes, plays a significant role in the high death rates associated with TB.[9] This has led the World Health Organisation (WHO) to list multi-drug resistant TB (MDR-TB) as a global threat.[10]



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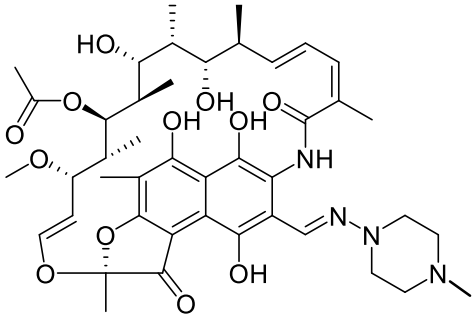

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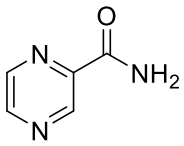

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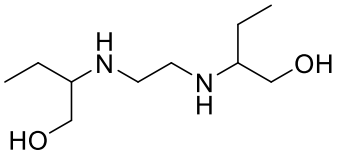

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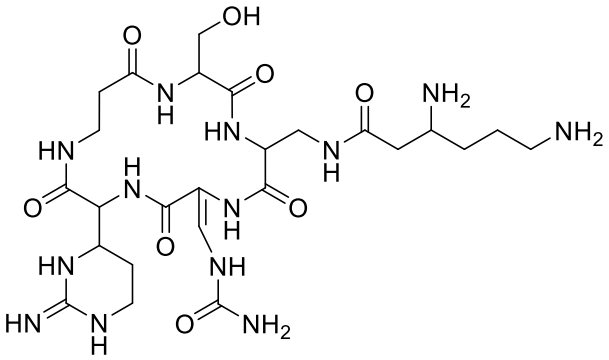
MDR-TB and extensively drug resistant TB (XDR-TB), which accounts for 5.4% of MDR-TB cases, are becoming increasingly common with a worldwide diagnosis rate of almost half a million infections per year and an estimated mortality rate of 33%.[2] This high mortality rate is usually attributed to failure of diagnosis or the lack of appropriate treatment.[2] The treatment regime is longer for MDR-TB cases than for uncomplicated TB cases (at least 8 months), and includes combination treatment with second-line drugs such as capreomycin (**1.5**), cycloserine (**1.6**), ethionamide (**1.7**), kanamycin (**1.8**) and *para*-amino salicylate (**1.9**).[11] Treatment with these second-line drugs is more expensive than regular treatment and is also less effective and more toxic to the patient, which contributes to the spread of the disease.[12] This is especially evident in South Africa where the number of MDR-TB cases is conservatively estimated at 13 000 per year and where XDR-TB which, by definition, has resistance to isoniazid and rifampicin and at least three of the second-line drugs,[13] is a growing epidemic.[14] Additionally, the HIV epidemic has resulted in large numbers of people with increased susceptibility to TB and this has resulted in especially high mortality rates among HIV and TB coinfections.[15] The increased cost and duration of treatment of drug resistant TB and the fact that diagnosis of TB is hampered by HIV coinfection mean that control of both diseases is diminished and highlights the need for new and improved drugs for the treatment of TB.[14]



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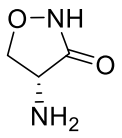

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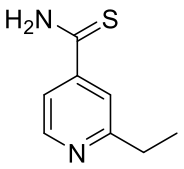

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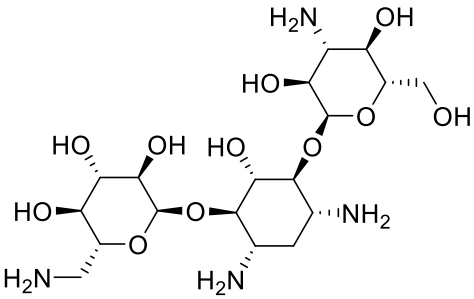

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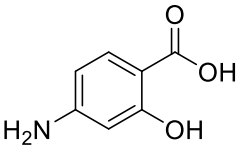

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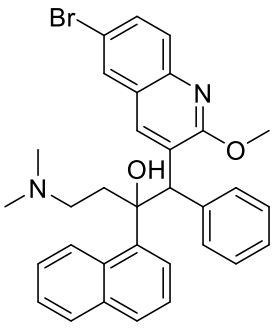
There has been significant investment to this end, but the approval of the anti-MDR TB agent bedaquiline (**1.10**) in 2012 was the first such advance in thirty years, and this drug requires combination therapy with other TB drugs.[16] The problem of complicated drug regimens therefore remains, and consequently the need for the development of new TB drugs that can shorten treatment duration, target drug resistant TB, simplify the pill burden and dose frequency and be co-administered with HIV medications.[17] Discovery of new agents with the required chemical properties for effective TB treatment requires the discovery of novel drugs, a potential source of which are natural products (NPs) which have been and remain an excellent source of novel drugs.[18]



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1.2 Natural Products

NPs are compounds that are naturally produced by living organisms, although the term usually refers more specifically to naturally occurring secondary metabolites.[19] Secondary metabolites are differentiated from primary metabolites as compounds that are not essential to the growth and development of the producing organism, i.e. primary metabolites are required for growth.[19] Secondary metabolites are instead believed to confer a survival advantage to the producing organism as they are responsible for activities such as repelling competitors or predators and they can be classified into five major compound classes: terpenoids and steroids, alkaloids, fatty acids and polyketides, non-ribosomal peptides and enzyme cofactors.[19] Secondary metabolites have had a variety of uses for human-kind and have been of great importance in both traditional and modern medicine, as will be discussed further.[20]

1.2.1 A Brief History of Medicinal Natural Products

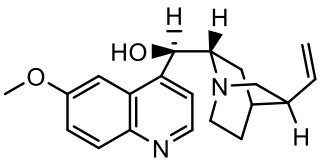
For thousands of years, NPs have been at the centre of traditional medicine systems.[20] The first written records of NPs in medicine are detailed in cuneiform on Mesopotamian clay tablets from *circa* 2600 BCE.[20] These tablets indicate the use of oils from various plants including *Cedrus* species (cedar) and *Cupressus semervirens* (cypress), as well as substances from *Glycyrrhiza glabra* (licorice) and *Papaver somniferum* (poppy juice), all of which are still used today as remedies for a wide array of illnesses.[20] Other instances where NPs are documented to be used in sophisticated traditional medicine systems are the Ebers Papyrus from Egypt (1500 BCE), which detailed 700 primarily plant-derived drugs, the Chinese Materia Medica (52 prescriptions) that was recorded from about 1100 BCE and the Indian Ayervedic from about 1000 BCE.[20] The ancient Greeks were also influential in the development of herbs as drugs, as recorded in Theophastus' *History of Plants* (*ca* 300 BCE), a compendium on the medicinal properties of herbs.[20]

The first privately owned drug stores were introduced in Arabia in the eighth century where Arab resources, Chinese and Indian herbs and Western knowledge was assembled and

recorded.[20] The WHO estimates that plant-based traditional medicine systems such as these continue to provide primary health care for 80% of the global population, mostly in less developed countries, as well as playing an important role in developed countries.[21]

There have also been great advances in NP drug discovery in modern history. The antimalarial drug quinine (**1.11**) was isolated from the then-known antimalarial bark of *Cinchona* species by French pharmacists in 1820.[20] This drug was also used in the synthesis of the antimalarial drugs chloroquine (**1.12**) and mefloquine (**1.13**).[20] As resistance to **1.11** – **1.13** developed, NPs were again a source of antimalarial drugs in the form of artemisinin (**1.14**), which was isolated from *Artemisia annua*, a plant used in traditional Chinese medicine to treat fevers, and its derivatives artemether (**1.15**) and arteether (**1.16**).[20]

Another significant NP discovery in recent history is the 1816 isolation of morphine (**1.17**) from *Papaver somniferum*, which was used in ancient Mesopotamia, as mentioned above.[20] These discoveries underline the importance of NPs as a source of drugs.[22]



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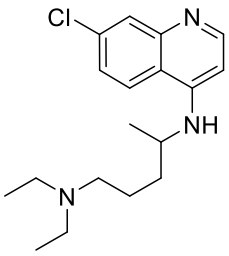

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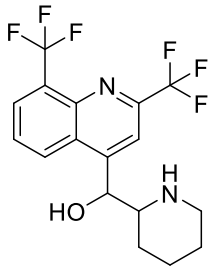

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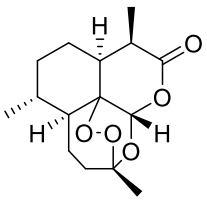

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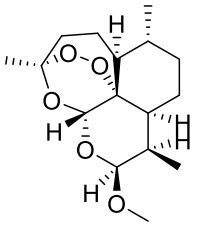

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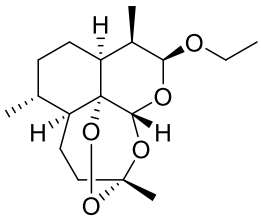

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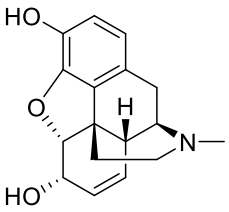

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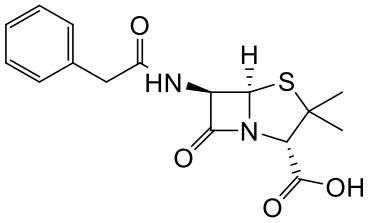
1.2.2 Natural Products and drug discovery

Penicillin G (**1.18**) was discovered in 1929 and has been used therapeutically since 1942.[22] The medical application of **1.18** has led to what is often termed the "golden age of antibiotics".[22] For decades thereafter, most notably the 1950s and '60s, the pharmaceutical industry benefited from consistent drug approvals, including many of the antibiotics and chemotherapies still in use today, and significant drug approval rate increases.[23] The vast majority of these antibiotics were NPs.[24]

However, challenges inherent in NP drug discovery led to a shift away from NP chemistry from the 1980s till the late 2000s.[25] NP chemistry is an expensive undertaking, which is why this research was led by big pharma and other large organizations.[25] Other difficulties include the length of time required to identify and characterise the active components in natural extracts, the risk of re-isolating known compounds and further complications in obtaining sufficient quantities of NP leads.[25,26] This supply issue is due to the NP leads most often being minor components of natural extracts and the need for large-scale acquisition of natural samples can hinder progress to pre-clinical development.[25]

Due to the difficulties in NP drug discovery an alternative method was explored.[25] The advances made in computing in the 1980s allowed the development of compound libraries for high-throughput screening (HTS).[25] Generation of random compound libraries through combinatorial chemistry (the mass synthesis of chemicals from known building blocks)[25] provided large numbers of compounds that could be rapidly screened for activity, and these methods were adopted by pharmaceutical companies at the expense of NP research.[22,23] However, the anticipated success in drug discovery has not been forthcoming and only three *de novo* combinatorial compounds, sorafenib (**1.19**),[27] ataluren (**1.20**)[28] and vemurafenib (**1.21**),[29] have been approved globally since 1981.[18]



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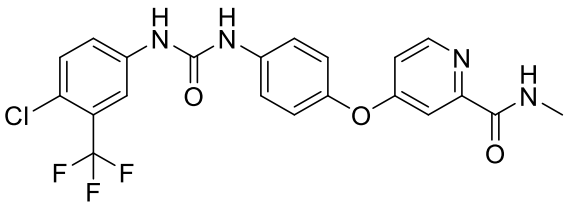

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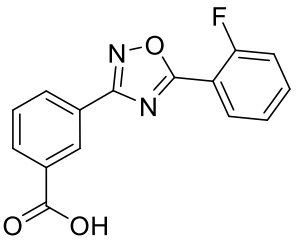

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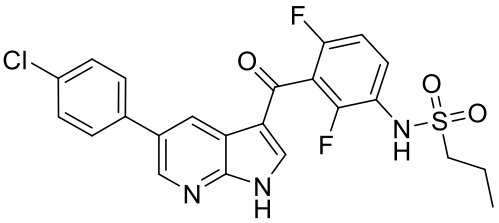

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Between 1981 and 2014, despite the emphasis on HTS and combinatorial chemistry, 26% of total global drug approvals (Figure 1.1) and 33% of small molecule drug approvals were sourced from unaltered NPs, natural botanical compounds or NP derivatives.[18] Of these approved compounds, 24 NP drugs were investigated by Ganesan,[30] consisting mainly of polyketides, peptides and terpenoids and included bestatin (**1.22**), thienamycin (**1.23**), taxol (**1.24**) and rapamycin (**1.25**). This study examined the structural properties of these drugs in terms of Lipinski's Rule of Five (RO5).[30,31] Lipinski's RO5 are a set of guidelines used in medicinal chemistry drug design that allow physico-chemical predictions to be made based on molecular structure.[22,31] These predictions can be used to guide the design of compounds with good oral bioavailability and the desired ADMET (absorption, distribution, metabolism, excretion and toxicity) molecular properties.[22,31] Interestingly, this study demonstrated Lipinski's fifth rule, which states that the first four rules, which outline structural parameters for drugs with effective oral bioavailability, do not apply to NPs.[18,30] NP drugs and drug leads therefore fall outside the parameters considered to be important by medicinal chemists while maintaining successful drug capabilities.[30]

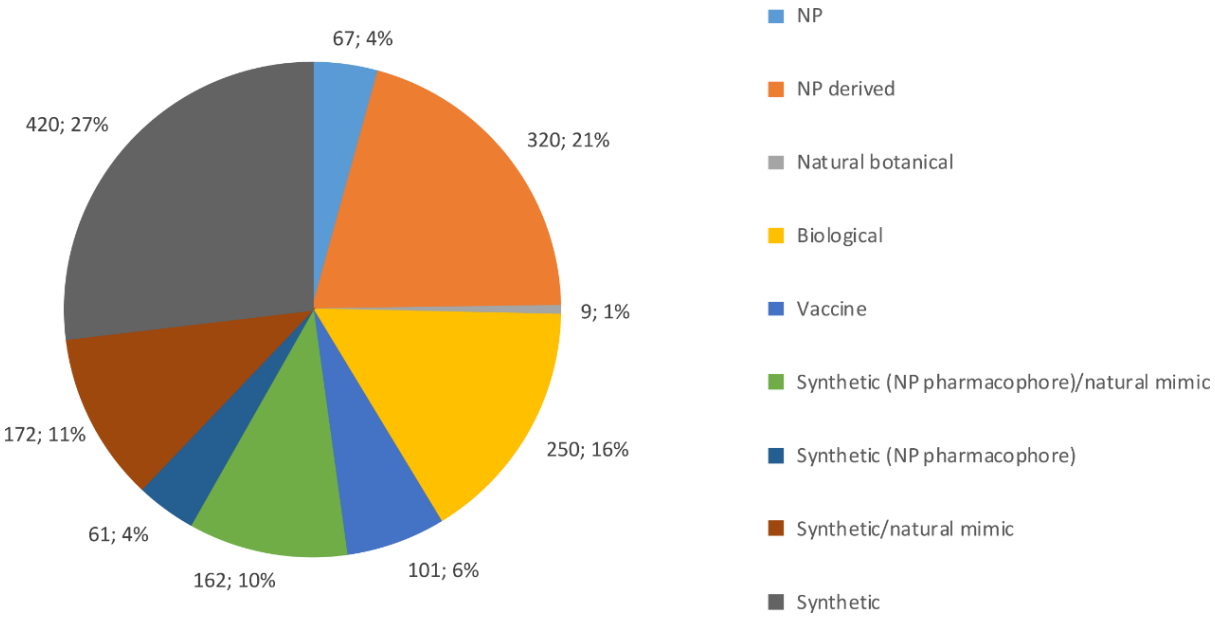
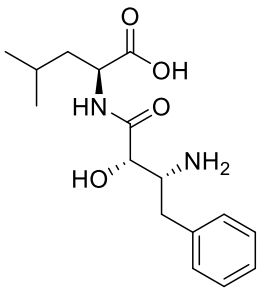


Figure 1.1: Source of all drugs approved globally from 1981 – 2014, adapted from Newman and Cragg.[18]



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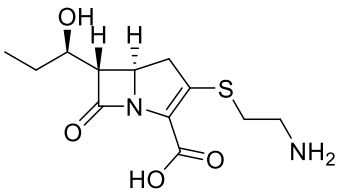

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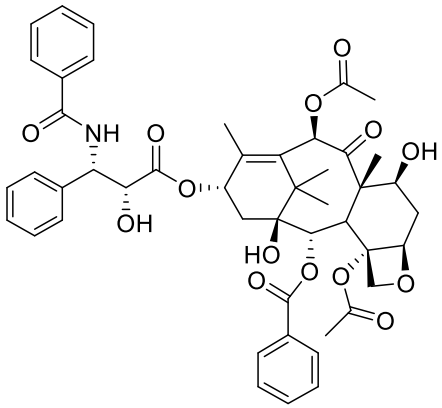

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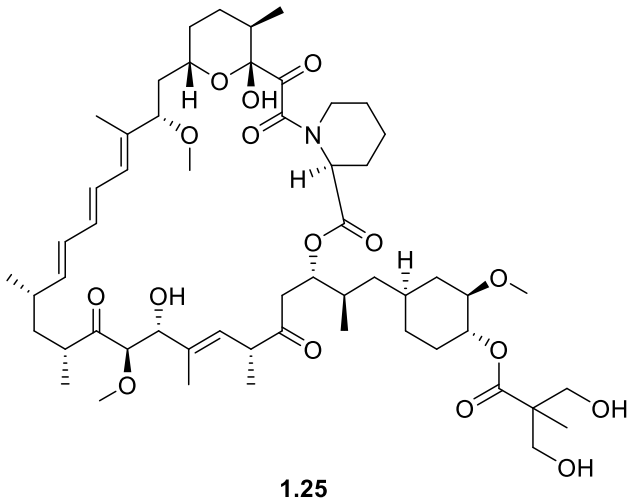

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NPs occupy a larger area of "chemical space" (i.e. have greater structural diversity) than synthetic compounds and hence exhibit greater chemical diversity.[32] This is a result of the differences in the methods employed in the formation of compounds.[30] Biosynthesis makes use of a very limited number of starting materials compared to those available to synthetic chemists, but follows far more complex pathways to achieve greater structural diversity, creating molecules richer in stereochemical features, such as chiral centres.[30] Furthermore, while NPs are not specifically active against human drug targets, they have coevolved over time within their own natural systems to interact with their own host proteins, many of which are structurally similar to those found in human systems.[25,33] NPs are therefore a potential source for novel and bioactive compounds and novel scaffolds that can be readily developed in conjunction with synthetic chemistry for drug discovery.[30] This fact has seen the re-emergence of NP discovery in drug research in recent years.[34]

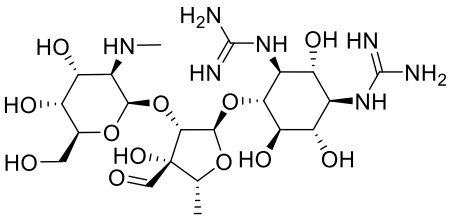
1.2.3 Microbes as a source of Natural Products

Microbes have been shown to be the most promising source for the discovery of novel NP skeletons and microbial NPs have been highly significant in the production of antibiotic drugs.[18] The first major discovery was in 1929 with Alexander Fleming's identification of **1.18** from the fungus *Penicillium notatum*, which went on to be used as an immensely important antibiotic from the 1940s.[24] As described above, this discovery was the catalyst for the "golden age of antibiotics" in the 1950s and '60s, during which time the majority of antibiotic discovery is attributed to actinobacterial sources.[35] In fact, it was during this time that nearly all the important classes of antibacterial antibiotics (tetracyclines, cephalosporins, aminoglycosides, macrolides) were discovered and the actinomycete genus *Streptomyces* accounted for 70 - 80% of all isolated compounds.[35] Actinobacteria are Gram positive bacteria that have a high guanine-cytosine (G+C) content in their deoxyribonucleic acid (DNA).[36] The filamentous actinobacteria are often called actinomycetes.[36]

The discovery rate of new antibiotics was less prolific in the following two decades.[35] Of the newly discovered antibiotics, 65 - 70% were obtained from actinomycetes, making them the major source, but many new chemical entities (NCEs) were found to be analogues of known compounds.[35] At the same time, emergence of MDR pathogens was complicating

chemotherapeutic treatments.[35] In an effort to increase the discovery rate of NCEs, a wider range of microbes was investigated for bioactive NP production.[35] This broadened microbial investigation resulted in an increase in the discovery of "rare actinomycete" products, approximately 30% of new bioactive microbial NPs, but the rate of antibiotic drug discovery was slow.[35] The subsequent shift in focus towards HTS and combinatorial chemistry instead of NP chemistry, while still yielding high numbers of new metabolites, was also not effective in increasing the rate of antibiotic discovery.[35] Three decades after the advent of HTS and combinatorial chemistry, microbial NPs from actinomycetes continue to be responsible for the majority of therapeutic antibiotics.[35]

Actinomycetes, especially soil streptomycetes, are prolific producers of antibiotics[37] and are responsible for many well-known antibiotics, such as the bactericidal aminoglycoside streptomycin (**1.26**), which was the first anti-TB drug, from *Streptomyces griseus*.[38] Other important drugs from streptomycetes include the antibacterial polyketide tetracycline (**1.27**) from *Streptomyces aureofaciens*,[39] the lipopeptide anti-infectant daptomycin (**1.28**) from *Streptomyces roseosporus*[40] and the antimycobacterial agent analogues of griselimycin (**1.29** – **1.31**).[34] Despite producing such a vast array of antibiotics, actinomycetes are still considered a relatively untapped source thereof.[41] Even though 45% of bioactive microbial NPs are reported to be sourced from actinomycetes, there is undoubtedly still huge potential for future work in the actinomycete field,[41] which provides the rationale for this thesis.



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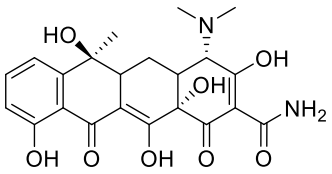

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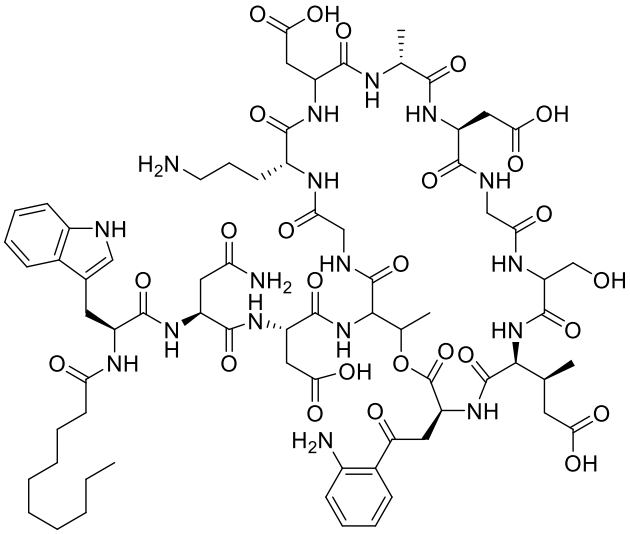

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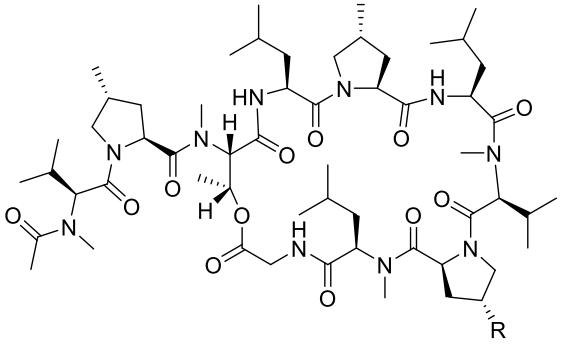
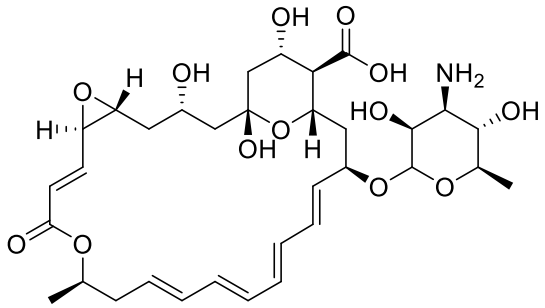


1.29 R = H Griselimycin (GM)
1.30 R = Me Methylgriselimycin (MGM)
1.31 R = Cyclohexyl Cyclohexylgriselimycin (CGM)

1.2.4 South African actinomycetes and NP research

A search of the literature available through the Scifinder database revealed that South African actinomycetes have not been extensively investigated for bioactive compounds.[42] Using the Scifinder database and the terms “South Africa” or “South African” in combination with “bacteria”, “actinobacteria” or “actinomycetes” revealed that few NPs have been reported to be isolated from South African actinomycetes.[42] Two antibiotics were found to be produced by actinomycetes isolated in South Africa, although this work was conducted abroad.[43,44] The first was the polyene macrolide antifungal, pimaricin (**1.32**), which is produced by *Streptomyces natalensis* and is used as a preservative in yoghurt.[43] The second was the broad-spectrum antibiotic against Gram positive bacteria, platensimycin (**1.33**), which is produced by *Streptomyces platensis*.[44] Previous efforts have been made, however, to investigate the microbial biodiversity in South Africa. In a Ph.D. study by Bronwyn Kirby at the University of

Cape Town (UCT), 30 actinomycete strains belonging to six different genera were selected for analysis: 13 *Streptomyces*, eight *Micromonospora*, two *Kribbella*, four *Kineococcus*, two *Gordonia* and one *Nocardia*.[45] These strains were subjected to phylogenetic analysis, phenotypic characterisation and chemotaxonomic classification and then tested for antimycobacterial activity against *M. aurum* A+, *Mycobacterium smegmatis* LR222 and *Mycobacterium bovis* BCG (Tokyo) (Table 1.1).[45]



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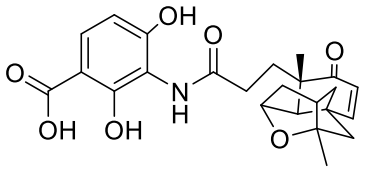


1.33

Of the 30 isolates, 24 inhibited the growth of *M. aurum* A+ through the production of antimycobacterial compounds.[45] As *M. aurum* A+ is a non-pathogenic strain with a similar antibiotic susceptibility profile to MTB, this data can be useful in identifying new strains producing potentially new anti-tubercular compounds.[46] Other South African actinomycetes that have been identified as producers of antimycobacterial compounds are *Streptomyces speibonae* PK-Blue^T,[45] *Streptomyces africanus* CPJVR-H^T,[47] and *Streptomyces pharetrae* CZA14^T.[48]

Table 1.1: Antimycobacterial activity of a collection of South African actinomycete strains.[45]

Genus	Source	Isolate	*M. aurum* A+	*M. bovis* BCG	*M. smegmatis* LR222
Streptomyces	sediment	Berg1C	+	+	+
		Berg2S	+	+	+
		Berg4Y	+	+	-
		Muiz4Y	+	+	+
		NX03U2	+	+	+
		Riet1C	-	+	+
		Zand2Y	+	-	-
		Zand4Y	-	+	-
		Zand8Y	+	+	+
		Zand9Y	+	+	+
	soil	Hel32	+	+	+
		PHelU5	+	-	-
		ShaleUP	-	+	+
Micromonospora	sediment	CGM31	-	-	-
		MuizA5S	-	-	-
		PLU1	-	-	-
		VleiA3C	-	-	-
	plant	DG41	-	-	-
		PBPE	-	-	-
		TVU1	+	-	-
Kribbella	soil	HMC25	-	-	-
		Q41	-	-	-
Gordonia	plant	AC41	+	-	-
Nocardia	soil	M25	-	-	-

+ = antimycobacterial activity; - = no antimycobacterial activity

1.3 An overview of bioassay-guided isolation of NPs

Several approaches can be employed for the isolation of NPs, where the strategy chosen is based on the aim of the research. Examples include bioassay-, chemistry- and metabolomics guided isolation, focusing on bioactive NPs, NPs with novel chemical structures or NPs present in an organism at a specific time and under specific influences, respectively.[49,50] In this thesis, bioassay-guided isolation was employed in an attempt to discover anti-TB NPs.

Bioassay-guided isolation is the incorporation of bioactivity testing throughout the purification process to guide the isolation of bioactive compounds.[51] Accordingly, testing for

bioactivity is performed early in the isolation process in order to identify and prioritise bioactive extracts for further purification.[51] Bioactivity testing is then included at all subsequent fractionation steps, ensuring that isolated NPs are biologically active.[51] Depending on the aims of the research, extracts can be subjected to specific bioassays or screened using a variety of bioassays to get an indication of the bioactivity profile.[52] In this study, the aim was to investigate NPs with anti-TB activity, and hence specific bioassays were used to guide isolation.

In the following sections, an overview of a general NPs isolation process will be described, highlighting the processes of preparing crude extracts from bacterial cultures, bioactivity testing, liquid-liquid partitioning, prefractionation, dereplication, isolation, structure elucidation and chemical synthesis.

1.3.1 Crude extracts from bacterial cultures

Varying the bacterial culture conditions is known to influence the bacterial production, i.e. the yields and variety, of NPs.[53] Numerous culture parameters can be altered that affect the production of NPs including aeration, incubation temperature and the nutrient composition of growth media.[54] Nutrient deficient cultures, cultures lacking in a supply of carbon and nitrogen metabolites, have been shown to induce production of antibiotics.[55] This nutrient deficiency can be due to low nutrient concentration in growth media or by exhaustion of nutrients during culture incubation.[53] In this study, the growth medium, incubation volume and incubation time of actinobacterial cultures were varied. Crude extracts were prepared from bacterial cultures by solvent extraction using ethyl acetate (EtOAc) and methanol (MeOH) or through the use of XAD16 resin (Sigma). The use of XAD16 resin is a form of solid phase extraction in which hydrophobic molecules in a specific size range (up to 40 000 Mw) adsorb to the solid phase. The molecules can later be stripped from the resin by treating it with organic solvents.[56]

1.3.2 Bioactivity testing

Bioassays can be either biochemical or cell-based.[52] Cell-based screening approaches have the advantage of screening for multiple targets in a single assay, while biochemical assays focus on a specific target.[57] Lower activity is often observed in cell-based assays compared to biochemical assays, possibly due to poor membrane permeability, off-target effects and cytotoxicity.[52] However, cell-based assays mimic *in vivo* conditions better than biochemical ones, identifying compounds that can be transported across or permeate through cellular membranes and interact with cellular components.[52]

For the investigation of anti-TB NPs, cell-based assays such as bioautography and *in vitro* minimum inhibitory concentration (MIC) bioassays can be used, as in this study.[45] Bioautography can be performed against the test bacterium *M. aurum* A+. This is a fast, qualitative bioassay and can be used to identify extracts/compounds with potential activity against MTB.[45] Compounds with activity against *M. aurum* A+ can then be rescreened against *M. tuberculosis* H37Rv^T to confirm anti-TB activity.[58] Using these two assays in combination allows rapid screening of extracts/compounds for anti-TB activity.

Bioassays can also be used to obtain a bioactivity profile of extracts/compounds. Although this was not the initial focus of this study, a bioactivity profile was obtained for extracts of a variety of *S. speibonae* PK-Blue^T, *S. africanus* CPJVR-H^T and *S. pharetrae* CZA14^T cultures. *In vitro* antibacterial and antifungal MIC bioassays were used to obtain the bioactivity profiles in a broad, untargeted approach.

1.3.3 Liquid-liquid partitioning

Crude extracts are complex mixtures of biological molecules containing a range of diverse NPs. To reduce the chemical complexity of such extracts, liquid-liquid partitioning can be employed. Liquid-liquid partitioning distributes solutes between two immiscible liquid phases of different polarity. By using a series of solvents with varying polarity, the extract can be separated into fractions containing compounds of similar polarity.[59]

1.3.4 Prefractionation

Prefractionation is performed on crude extracts, separating extract components and yielding fractions each with reduced chemical complexity. Prefractionation can be achieved using several methods including anion exchange chromatography, column chromatography (such as flash chromatography) and high-performance liquid chromatography (HPLC). Anion exchange chromatography is used to separate compounds based on molecular charge, while flash chromatography and HPLC yield fractions containing compounds of different polarities.

Fractions that result from prefractionation can be viewed as semi-purified since they commonly must undergo further purification to yield a pure isolated NP. Semi-purified extracts often display greater bioactivity than crude extracts, as compounds in these fractions are present in higher concentrations.[59,60] However, the biological activity of compounds acting synergistically may be lost.[49]

1.3.5 Dereplication

Dereplication is a multi-faceted tool used to accelerate the drug discovery process by the fast identification of known compounds.[26] Efficient identification of known compounds in complex NP mixtures, i.e. crude and semi-purified extracts, allows researchers to concentrate their efforts on the identification of unknown NPs.[26] Tools used for dereplication include mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, or hyphenated combinations thereof, e.g. liquid chromatography-mass spectrometry (LC-MS), LC-NMR and LC-NMR-MS.[26] The data obtained by dereplication can be compared to literature data in online databases, e.g. the Dictionary of Natural Products.[61]

1.3.6 Isolation of pure compounds

Isolation is performed to yield pure compounds and enable structure elucidation and bioactivity testing. A common chromatographic technique used for the isolation of pure compounds is HPLC. HPLC makes use of a liquid mobile and solid stationary phase.

Compounds are separated based on elution time, with elution time variance arising due to binding affinities to the mobile and stationary phases. The variable binding affinities are attributed to different binding forces between individual compounds and the two phases i.e. hydrogen bonding, Van der Waals forces and dipole-dipole interactions. Stronger binding affinity to the stationary phase results in a longer retention time and vice versa. Reversed phase HPLC (RP-HPLC) is frequently used for the isolation of NPs. In RP-HPLC, non-polar C_{18} (octadecyl) can be used as a stationary phase.[59] Hence, a non-polar mobile phase is required for the elution of non-polar compounds, as these compounds adhere strongly to the stationary phase.[59] HPLC can be time-consuming, involving multiple injections, as each purification sequence is performed on small sample amounts.[59] Isolation of NPs is often performed using semi-preparative HPLC, which employs columns that can separate larger sample masses per run than analytical HPLC.[62]

The elution of NPs by HPLC can be monitored by ultraviolet (UV) light. UV chromatograms generated can be used to guide collection of compounds that absorb UV light, a chemical property that is dependent on the presence of a conjugated system of pi-electrons.[59] Therefore, a disadvantage of this technique is that compounds that do not absorb UV light will not be detected and can contaminate fractions collected by UV-guided HPLC.

1.3.7 Structure elucidation

The structure elucidation of NPs can be achieved using different methods, such as MS (low resolution or high resolution), NMR, UV/visible spectroscopy and infrared absorption spectroscopy. The elucidation of NP structures usually requires the use of two or more of these techniques.

1.3.7.1 MS analysis

MS is an analytical tool used to identify compounds by the molecular or atomic masses of their constituents through the generation of molecular ions. Molecular ions can be separated based on their *mass-to-charge ratio (m/z)* to yield a mass spectrum. The mass spectrometer

consists of an ion source, a mass analyser and a detector.[63] These components function to yield data on molecular mass, relative abundance of ions and isotope patterns of analysed compounds, which are used to calculate a suggested molecular formula and the level of unsaturation of compounds.[63,64] Both LC-MS and gas chromatography MS (GC-MS) hyphenated tools were used for structure elucidation in this study. LC-MS is commonly employed in the analysis of polar compounds and GC-MS used for lipophilic compounds.[65]

In this study, an ultra-performance liquid chromatography time of flight (UP-LC-ToF) system with electrospray ionisation (ESI) and microchannel plate (MCP) detector was used. Through the use of smaller stationary phase particles, higher mobile phase linear velocities and increased pressures, the UP-LC module allows better separation of a complex mixture of compounds than HPLC.[66] After exiting the UP-LC module, the eluting compounds enter the ion source.[67] Compounds entering the ion source are ionised by ESI, a broadly used ionisation technique effective for most samples.[67] This ionisation is facilitated by electrical energy, transferring ions from a solution into the gaseous phase.[67] The gaseous phase ions form in three steps: an electrically charged spray, significant reduction in droplet size and finally the liberation of fully desolvated ions.[67] These gaseous phase ions are then subjected to MS analysis.[67]

Ions with different *m/z*-values travel through the ToF analyser at different, but constant, velocities and therefore reach the detector at different times. This allows separation of ions based on their mass, as ions with less mass travel faster than ions of greater mass and reach the detector first. Ions exiting the quadrupole collide with and are recorded by the MCP detector. The MCP detector is an ion-counting detector that consists of several channel electron multipliers (CEMs) in a cluster. When ions collide with these CEMs, they release a secondary cascade of electrons, thereby amplifying the detected signal.[63] The resulting ions are plotted as *m/z* versus relative abundance (%).[64]

The GC-MS system used in this study had an electron impact (EI) ionisation system and a triple quadrupole mass separator. Unlike LC-MS, samples subjected to GC-MS must be volatile, while stable at the temperatures used for chromatography (up to 250℃ by our method). Volatile samples can be prepared by derivatisation of non-volatile samples e.g. alkylation,

trimethylsilylation and trifluoroacetylation.[68] The choice of derivatising agent depends on the structure of the compound to be analysed. In this study an alkylating agent was used to yield methyl esters from acids of saturated long-chain fatty acids (LCFAs).[69] In GC-MS, EI is an effective and commonly used ionisation technique for a variety of samples.[70] This ionisation uses high-vacuum bombardment of analyte molecules by accelerated electrons and results in extensive fragmentation of most analytes.[70] However, this 'hard' ionisation can render the molecular ion undetectable, posing a challenge for some analyses.[70]

In the MS analyser, ions with different *m/z* are separated as they are transported through the triple quadrupole. Radio frequency (RF) voltage with direct current (DC) offset voltages are applied to the quadrupoles and only ions with a specific *m/z*-value are able to reach the detector for each set of voltage ratios. The voltage ratios can therefore be varied to scan for a range of *m/z*-values. Secondary fragmentation of primary ions, also known as parent ions, occurs in the reaction cell that separates the two quadrupole analysers, generating an MS2 spectrum of selected parent ions.[71] MS2 permits the acquisition of highly detailed ion fragmentation information with a higher level of molecular specificity and selectivity that allows discrimination between very similar compounds (for example fatty acids).[72] GC-MS data are also highly reproducible and linked to comprehensive databases that allow easy identification of analytes.[70]

1.3.7.2 NMR spectroscopy

NMR spectroscopy is a technique used to analyse the chemical composition, structure and bonding of compounds using radio waves.[73] Atomic nuclei that have unpaired valence electrons can be analysed by NMR. These atomic nuclei usually possess spin quantum numbers of 1/2 e.g. ^{1}H, ^{13}C, ^{15}N, ^{19}F and ^{31}P.[64] Nuclei in an NMR sample will have a spin orientation either parallel (+1/2) or antiparallel (-1/2) when placed in a uniform magnetic field. Subjecting the sample to a radio frequency pulse excites the nuclei and rotates the net magnetization away from the Z-axis. Relaxation of the nuclei releases a detectable NMR-signal as it returns to equilibrium.[64,73]

Proton (^{1}H), carbon (^{13}C) and nitrogen (^{15}N) NMR spectra provide information on chemical shifts, integrals, splitting patterns and coupling constants (*J*). Chemical shifts and integrals are used to deduce the nature and number of nuclei present in the sample analysed, while splitting and coupling constants give an indication of the spatial arrangement of nuclei.[74]

^{1}H and ^{13}C NMR are common one-dimensional (1D) techniques. These 1D techniques can be supplemented with two-dimensional (2D) NMR experiments to establish nuclei connectivity in a molecule. Commonly used 2D experiments are correlated spectroscopy (COSY), heteronuclear single-quantum correlation spectroscopy (HSQC) and heteronuclear multiple-bond correlation spectroscopy (HMBC). COSY is used to identify proton spins that are coupled to one another. These proton-proton spin couplings are useful in mapping the proton network of the molecule. HSQC experiments indicate correlations between two different types of nuclei separated by one bond and HMBC detects long range correlations (typically 2 – 3 bonds) between heteronuclear atoms. These heteronuclear experiments feature "through bond" couplings, usually between proton and carbon atoms. HMBC experiments are especially useful in determining the positions of quaternary carbon atoms, e.g. carbonyl carbon atoms. Some NMR experiments identify correlations between nuclei that are spatially near but separated "through space" by 2 – 5 Å. "Through space" NMR experiments include nuclear Overhauser effect spectroscopy (NOESY) and rotating frame nuclear Overhauser effect spectroscopy (ROESY). These two experiments are valuable in assigning the stereochemistry of a molecule.[64]

1.3.7.3 Chemical synthesis

Chemical synthesis has many uses in NP research. One use is the synthesis of a proposed structure to confirm the structure of an isolated compound, as was done in this study. If a compound of interest is produced in trace amounts insufficient for biological analysis, synthesis can also be used to address this supply issue. Additionally, isolated NPs can be used as leads in the creation of a compound library. Bioactivity testing of the synthesised library may reveal structure-activity relationships (SAR), which can facilitate the development of a

molecule exhibiting an optimised biological profile.[75] Synthesis can either be performed *de novo* or by semi-synthesis using an NP as a scaffold. A successful example of antibiotics developed through the semi-synthesis of bacterial NP drug leads is the tetracyclines.[75] The first tetracyclines were broad spectrum antibiotics isolated from actinomycete soil bacteria in 1948, but have since undergone semi-synthesis and SAR profiling to yield two more generations of tetracyclines with more potent bioactivities.[75]

1.4 Aims of this thesis

The aim of this study was to investigate the chemical diversity and biomedicinal potential of South African actinomycetes for TB drug discovery. In this endeavour, we attempted to re-isolate and determine the structure of the compound named PK-B, produced by the novel South African actinomycete, *S. speibonae* PK-Blue^T.[45] In addition, *S. speibonae* PK-Blue^T and two other South African actinomycete strains that have shown activity against *M. aurum* A+, namely, *S. africanus* CPJVR-H^T and *S. pharetrae* CZA14^T, were used in our efforts to isolate, purify and characterise potentially new anti-TB compounds. The objectives are to employ a bioassay guided isolation approach, as described in Section 1.3, incorporating the preparation of crude extracts from bacterial cultures, bioactivity testing, liquid-liquid partitioning, prefractionation, dereplication, isolation, structure elucidation and chemical synthesis.

Chapter 2: Methods

Three different South African actinobacterial strains, *S. speibonae* PK-Blue^T, *S. africanus* CPJVR-H^T and *S. pharetrae* CZA14^T, were examined for bioactive NPs. An effort was also made to re-isolate the compound named PK-B from *S. speibonae* PK-Blue^T.[45] These three bacterial strains were cultivated using various culture conditions, and different solvent extraction methods were investigated to prepare extracts for bioassay-guided isolation and dereplication. Fractions of interest were subjected to purification by liquid-liquid partitioning followed by HPLC and/or benchtop column chromatography. The structures of purified compounds were elucidated using NMR, high resolution (HR) LC-MS and GC-MS. Where required, confirmation of the structure was determined by synthesis of the compound.

2.1 Culture conditions

S. speibonae PK-Blue^T, *S. africanus* CPJVR-H^T and *S. pharetrae* CZA14^T were cultured under various culture conditions where three different parameters i.e. nutrient medium, culture volume and incubation time, were altered. Two different growth media were tested: Hacène's medium[76] (HM) (5 g glucose; 10 g malt extract; 2 g yeast extract; 1 g sodium chloride (NaCl); water (H_2O) to 1 L; pH 7.0) and International *Streptomyces* Project 2 medium (ISP 2) (10 g malt extract; 4 g yeast extract; 4 g glucose; H_2O to 1 L; pH 7.2 ± 0.2).[77] All solid media were prepared by adding 1.5% (w/v) agar to liquid nutrient media.

Three general culture conditions, HM large-scale, ISP 2 small-scale and ISP 2 large-scale, were implemented. The incubation time of each general culture condition was varied from 4 days (d) to 18 d. All cultures were tested for contamination by Gram staining, optical microscopy and by streak-plating for single colonies on agar medium (HM or ISP 2). Cultures were maintained on agar medium from which 20% glycerol spore suspensions were prepared for long term storage at -20˚C.

S. speibonae PK-Blue^T, *S. africanus* CPJVR-H^T and *S. pharetrae* CZA14^T were also cultivated using a method obtained at the Helmholtz Institute for Pharmaceutical Research Saarland (HIPS). Three growth media were tested, namely tryptic soy broth (TSB) (17 g casein peptone; 5 g NaCl; 3 g soy peptone; 2.5 g glucose; 2.5 g dipotassium phosphate; H_2O to 1 L; pH 7.3 ± 0.2),[78] *Streptomyces hagronensis* growth medium (SHG) (20 g corn flour; 20 g glycerol; 10 g

soluble starch; 2 g calcium carbonate ($CaCO_3$); H_2O to 1 L; pH 7.5)[79] and NL2 (30 g starch; 20 g molasses; 15 g soy flour; 10 g $CaCO_3$; 2.5 g yeast extract; H_2O to 1 L) (Sanofi). All inoculations into TSB, SHG and NI2 were performed in a laminar flow fume hood and all cultures were tested for contamination by streak-plating for single colonies on agar medium. Cultures were maintained on agar medium from which 20% glycerol spore suspensions were prepared for long term storage at -20˚C.

See sections 2.1.1 – 2.1.4 for details of the different culture conditions.

2.1.1 HM large-scale culture method

A 1 mL, 20% glycerol stock culture of *S. speibonae* PK-Blue^T was used to inoculate a 100 mL Erlenmeyer flask containing 15 mL HM broth and incubated at 30˚C for 3 d with constant shaking. The resultant ISP 2 broth culture was used to inoculate a 1 L Erlenmeyer flask containing 100 mL HM broth and incubated at 30˚C for 4 d with constant shaking. Finally, the 100 mL HM broth culture was inoculated into a 5 L Erlenmeyer flask containing 1 L HM broth and incubated at 30˚C for 4 d with constant shaking.

2.1.2 ISP 2 small-scale culture method

A 1 mL, 20% glycerol stock culture of *S. speibonae* PK-Blue^T was used to inoculate a 250 mL Erlenmeyer flask containing 15 mL ISP 2 broth and incubated at 30˚C for 4 d with constant shaking. The resultant ISP 2 broth culture was used to inoculate a 1 L Erlenmeyer flask containing 100 mL ISP 2 broth and incubated at 30˚C for 4 d with constant shaking. Finally, 10 mL of the 100 mL ISP 2 broth culture was used to inoculate each of 10 1 L Erlenmeyer flasks containing 100 mL ISP 2 broth and incubated at 30˚C with constant shaking. The incubation duration of the final culture step was 7 d or 14 d.

2.1.3 ISP 2 large-scale culture method

A 1 mL, 20% glycerol stock culture of *S. speibonae* PK-Blue^T was used to inoculate a 250 mL Erlenmeyer flask containing 15 mL ISP 2 broth and incubated at 30˚C for 4 d with constant shaking. The resultant ISP 2 broth culture was used to inoculate a 1 L Erlenmeyer flask containing 100 mL ISP 2 broth and incubated at 30˚C for 4 d with constant shaking. This ISP 2 broth culture was inoculated into a 5 L Erlenmeyer flask containing 1 L ISP 2 broth and incubated at 30˚C with constant shaking. The incubation duration of the final culture step was 14 d or 18 d.

2.1.4 HIPS culture method

A 3 mL, 20% glycerol stock culture was used to inoculate a 50 mL Erlenmeyer flask containing 10 mL culture medium and incubated at 28˚C for 2 d with constant shaking. The resultant broth culture was inoculated into a 300 mL Erlenmeyer flask containing 100 mL culture medium and incubated at 28˚C for 4 d with constant shaking. Finally, the 100 mL broth culture was inoculated into a 5 L Erlenmeyer flask containing 1 L culture medium broth and incubated at 26˚C for 10 d with constant shaking. Sterile XAD16 resin in H_2O (30 % v/v, Sigma) (30 mL) was added to the culture for the last 12 h of incubation.

2.2 Solvent Extraction

Three solvent extraction methods were used: solvent extraction of *S. speibonae* PK-Blue^T HM broth cultures as outlined by Kirby,[45] a general method of solvent extraction for *S. speibonae* PK-Blue^T ISP 2 broth cultures and a HIPS extraction method. See sections 2.2.1 – 2.2.3 for descriptions of the solvent extraction methods.

2.2.1 Solvent extraction method for *S. speibonae* PK-Blue^{T} HM broth cultures

Solvent extraction was performed using the method outlined by Bronwyn Kirby.[45] A 500 mL *S. speibonae* PK-Blue^{T} HM broth culture was filtered through a paper coffee filter and the pH of the culture filtrate was adjusted to pH 2 with 1 M HCl. The acidified culture filtrate was extracted with EtOAc (150 mL), shaken vigorously in a separating funnel and the phases allowed to separate. The acidified, EtOAc extracted culture filtrate fraction was then flash-frozen and lyophilised to yield the culture filtrate extract. 10% of the EtOAc layer was concentrated *in vacuo* and set aside for bioassay (crude EtOAc extract) and the remainder added to 500 mL sterile 10 mM Tris-HCl (pH7), shaken vigorously, and separated using a separating funnel. The organic layer was concentrated *in vacuo* to yield the final EtOAc extract. The aqueous layer was flash-frozen and lyophilised to yield the PK-B extract.[45] The cell mass was extracted with MeOH (3 x 150 mL), shaken for 10 minutes each time, and then filtered through a paper coffee filter. The filtered organic liquid was concentrated *in vacuo* to yield the crude MeOH extract. The cell mass was discarded.

2.2.2 General solvent extraction method for actinomycete broth cultures

Actinomycete ISP 2 broth cultures were filtered through a paper coffee filter. The culture filtrate was extracted with EtOAc (3 x 30% of the culture volume) using a 2 L separating funnel. The organic layers were combined and concentrated *in vacuo* to yield the crude EtOAc extract. The culture filtrate aqueous layer was flash frozen and lyophilised to yield the crude aqueous extract.

MeOH (3 x 20% of the culture volume) was added to the filtered actinobacterial cell mass (scraped off the coffee filter), shaken for 10 minutes each time and then filtered through a paper coffee filter. The filtered organic liquid was concentrated *in vacuo* to yield the crude MeOH extract. The cell mass was discarded.

2.2.3 HIPS Solvent Extraction

The 1 L culture from the 5 L Erlenmeyer flask containing 30 mL sterile XAD16 resin in H_2O (30 % v/v, Sigma) was centrifuged and the supernatant discarded. MeOH (2 x 250 mL) was added to the combined cell mass and XAD16 resin, shaken for 2 h, filtered through glass wool (Sigma), and the two organic extracts combined and concentrated *in vacuo*. Dry extracts were immediately subjected to liquid-liquid partitioning as described in section 2.5.1.

2.3 Biological assays

Three different bioassays were performed i.e. bioautography against *M. aurum* A+, *in vitro* MIC_{90} anti-TB bioassay against *M. tuberculosis* $H37Rv^T$ and *in vitro* MIC_{50} bioassay against a panel of test organisms at HIPS. Bioautography and MIC_{50} bioassays at HIPS were performed by the author, while MIC_{90} bioassays against *M. tuberculosis* $H37Rv^T$ were done on the author's behalf at the Institute of Infectious Disease and Molecular Medicine (IDM) at UCT. See sections 2.3.1 – 2.3.3 for specifics of the three bioassays performed. See Table 2.1 for a summary of bioactivities tested for, the test organisms and types of assays used in this study.

Table 2.1: Full bioassay screening panel used in this study. Crude extracts, partitioned fractions and pure compounds were tested for antimycobacterial, antibacterial and antifungal activity against a range of test organisms, employing three different bioassay techniques.

Bioactivity	Test microorganism	Bioassay
Antimycobacterial	*M. aurum* A+	Cellular (Bioautography)
	M. tuberculosis H37Rv^{T}	Cellular (MIC_{90})
	M. smegmatis mc^{2}155	Cellular (MIC_{50})
Antibacterial	*Escherichia coli* DSM-1116 (WT)	Cellular (MIC_{50})
	E. coli DSM-26863 (*tolC3*)	Cellular (MIC_{50})
	Staphylococcus aureus ATCC-29213	Cellular (MIC_{50})
	Pseudomonas aeruginosa DSM-24600	Cellular (MIC_{50})
	Bacillus subtilis DSM-10^{T}	Cellular (MIC_{50})
Antifungal	*Candida albicans* DSM-1665	Cellular (MIC_{50})
	Mucor hiemalis	Cellular (MIC_{50})

2.3.1 Bioautography against *M. aurum* A+

EtOAc, MeOH and aqueous extracts of *S. speibonae* PK-Blue^{T} were tested for antimycobacterial activity against *M. aurum* A+ through the use of bioautography.

Bioautography was performed using the method described by Kirby.[45] *M. aurum* A+ was streak-plated onto 2 x yeast extract tryptone agar (2YT)(16 g tryptone; 10 g yeast extract; 5 g NaCl; H_2O to 1 L; pH 7)[80] from a 20% glycerol stock culture and incubated at 37˚C for 3 d. One loopful of *M. aurum* A+ from this 2YT agar culture was used to inoculate a 20 mL universal container containing 5 mL 2YT broth, which was then incubated at 37˚C for 2 d with constant shaking.

Dry extracts, see sections 2.2.1 and 2.2.2, were dissolved in MeOH (0.5 mL) and 10 µL spotted on thin layer chromatography (TLC) plates (aluminium-backed pre-coated silica gel 60 F_{254} sheets, Merck). Rifampicin (0.6 µL, 8.4 mg/mL) was used as a positive control. The TLC plates were dabbed with an *M. aurum* A+ 2YT broth culture, with an optical density at 600 nm (OD_{600}) adjusted to 0.5, using sterile Dove cotton wool pads. The loaded TLC plates were placed on water-dampened tissue paper in a sealed plastic container and incubated at 37˚C for 24 h. After incubation, the TLC plates were dabbed with 0.25% thiazolyl blue tetrazolium bromide (MTT) (Sigma) in phosphate buffered saline (1.78 g Na_2HPO_4; 8.50 g NaCl; 1 L distilled H_2O; pH 7.3), resealed and incubated at 37˚C for a further 1 h.

2.3.2 *In vitro* MIC_{90} *M. tuberculosis* $H37Rv^T$ bioassay

M. tuberculosis $H37Rv^T$ MIC_{90} bioassays were performed on my behalf at the UCT IDM unit in a biosafety level 3 laboratory using the broth microdilution method described by Jorgensen *et al*.[81] with a modified plate method.[82] A 10 mL of *M. tuberculosis* $H37Rv^T$ was grown to an OD_{600} of 0.6 – 0.7 in Middlebrook 7H9 medium (M7H9) (4 mL glycerol; 2 vials M7H9 ADC growth supplement (Sigma); H_2O to 1 l; pH 6.6 ± 0.2)[83] supplemented with 0.03 % casitone, 0.4 % glucose and 0.05 % tyloxpol.[84] The culture was diluted 1:500. Dry samples were reconstituted in dimethyl sulfoxide (DMSO) (1 mg/mL) then added to a 96-well microtitre plate (50 µL). The activity of a blank with only DMSO was not reported. The test material was diluted in a duplicated two-fold serial dilution with the *M. tuberculosis* $H37Rv^T$ culture across 10 wells. The microtitre plate was sealed in a secondary container and incubated at 37˚C with 5% carbon dioxide (CO_2) and humidification for 6 d after which Alamar Blue reagent was added to each well and the microtitre plate re-incubated for 24 h.

The assay was then scored visually with the lowest concentration of material displaying no visible growth scored as the MIC_{90}. A minimum growth control (Rifampicin at 2 x MIC: 0.150 µM), a maximum growth control (DMSO), and a rifampicin dose response (range 0.15 – 0.0002 µM) were used. Relative fluorescence (excitation 540 nm; emission 590 nm) was measured using a SpecraMax i3x Plate reader at day 7 and analysed using Softmax Pro 6 software.

2.3.3 HIPS Bioassay

Crude extracts and liquid-liquid partitioned fractions, i.e. the hexane, chloroform ($CHCl_3$), H_2O, and EtOAc fractions, were tested against a panel of bacteria and fungi. All bioassay procedures were conducted in a Biological Safety Cabinet in a biosafety level 2 laboratory. The test organisms were the bacteria *Escherichia coli* wild type (WT) (DSM-1116), *E. coli tolC3* (DSM-26863), *Staphylococcus aureus* ATCC-29213, *Pseudomonas aeruginosa* DSM-24600*, Bacillus subtilis* DSM-10^T and *M. smegmatis* mc^2155, and the fungi *Candida albicans* DSM-1665 and *Mucor hiemalis*. M7H9 medium was used as the screening medium for *M. smegmatis*.[83] Mueller-Hinton broth (MHB) (17.5 g Casein hydrolysate; 2 g beef extract; 1.5 g starch; H_2O to 1 L; pH 7)[85] was used for the remaining bacterial strains and Mycosel medium (Myc) (10 g Papaic Digest of Soybean Meal; 10 g glucose; 0.4 g cycloheximide; 0.05 g chloramphenicol; H_2O to 1 L; pH 5.6 ± 0.2)[86] was used for fungal strains.

The test organisms were cultured at 30˚C overnight in 50 mL Falcon tubes containing 20 mL screening medium. The OD_{600} of each overnight culture was measured. These cultures were inoculated into 50 mL Falcon tubes containing 20 mL of the respective screening medium to an OD_{600} of 0.01 (bacteria), 0.05 (*M. smegmatis*) and 0.02 (fungi). Cultures were transferred to sterile 96-well microtitre plates (150 µL per well, 1 plate per strain) using a multichannel pipette. Extracts were dissolved in MeOH and 20 µL added to row A of each plate to yield a concentration of 640 µg/mL. A multichannel pipette was used to perform an extract dilution series to obtain extract concentrations of 640 µg/mL, 320 µg/mL, 160 µg/mL, 80 µg/mL, 40 µg/mL, 20 µg/mL, 10 µg/mL and 5 µg/mL across the plate. Pure MeOH was used as a control. All plates were wrapped with Parafilm and placed on a Heidolph Titramax 1000 microplate shaker. Plates were incubated at 30˚C and 800 rpm. All plates were incubated for 16 h except

M. smegmatis which was incubated for 24 h., while *M. hiemalis* was incubated for a further 24 h at room temperature, without shaking. MIC_{50} values were assigned by visually estimating a 50% zone of inhibition i.e. plate wells were visualised under a microscope and the concentration threshold determined at which 50% of the plate surface was cleared of microbial growth. Semi-purified extracts and pure compounds were rescreened for activity using the same method.

2.4 Dereplication

Dereplication was performed using low resolution (LR) LC-MS at UCT and HR LC-MS at HIPS. Details of dereplication methods using these two instruments are given in sections 2.4.1 – 2.4.2.

2.4.1 LR LC-MS dereplication

Dereplication of culture extracts was performed by LC-MS. LC-MS samples were prepared by dissolving culture extracts in MeOH (10 mg/mL). Samples were run on an LR Agilent Technologies HPLC system. This system consisted of an Agilent Technologies 1260 Infinity Binary Pump, Agilent Technologies 1260 Infinity diode array detector (DAD), Agilent Technologies 1290 Infinity Column Compartment, Agilent Technologies 1260 Infinity Standard Autosampler and Agilent Technologies 6120 Quadrupole LC/MS. All dereplication was performed using a Zorbax, 300SB-C18 column (4.6 x 150 mm, 5 µm). All solvents used for HPLC were of HPLC grade (Merck or Sigma). Data was analysed using Agilent Technologies OpenLAB CDS ChemStation software.

Samples were analysed using both positive-ion and negative-ion ESI MS. Extracts containing molecular masses abundant in active extracts, while absent in inactive extracts and uninoculated growth media, were investigated further. These molecular masses were

compared with the Dictionary of Natural Products and StreptomeDB databases to establish if they could be known compounds.[61,87]

2.4.2 HR LC-MS dereplication

Crude extracts and liquid-liquid partitioned fractions, i.e. hexane, $CHCl_3$, H_2O and EtOAc, were dissolved in MeOH. HR LC-MS mass spectra were obtained on an Agilent 1200 series HPLC-UV system combined with an ESI-ToF-MS (Maxis, Bruker) using a Waters Acquity UP-LC BEH column (2.1 x 50 mm, 1.7 µm). Data was analysed using Thermo Scientific Chromeleon Chromatography Data System software. HR LC-MS data were compared with growth media HR LC-MS data and with the Dictionary of Natural Products database.[61]

2.5 Purification of extracts

Crude EtOAc extracts were subjected to an initial purification process of liquid-liquid partitioning, which is described in section 2.5.1. Fractions obtained from liquid-liquid partitioning were then further purified by semi-preparative HPLC and/or benchtop column chromatography as required to obtain pure compounds. Here, semi-preparative HPLC was used for the purification of chemically complex extracts and benchtop column chromatography, as required, was used for the purification of extracts with few components and a significant amount of material. A third chromatographic technique, anion exchange chromatography, was used for the separation of charged molecules. For details of the chromatographic techniques used see sections 2.5.2 – 2.5.4.

2.5.1 Liquid-liquid partitioning

Crude EtOAc extracts were dissolved in 200 mL MeOH and partitioned with 3 x 200 mL hexane using a separating funnel. The hexane fraction was concentrated under vacuum to yield the

hexane extract. The MeOH fraction was concentrated under vacuum, reconstituted in 200 mL H_2O and extracted with 3 x 200 mL $CHCl_3$, followed by 3 x 200 mL EtOAc. The H_2O, $CHCl_3$ and EtOAc partitions were individually concentrated under vacuum to yield the H_2O, $CHCl_3$ and EtOAc extracts, respectively.

2.5.2 Semi-preparative HPLC

For semi-preparative HPLC, the acetonitrile used was of HPLC grade (Merck or Sigma), while H_2O was filtered (0.22 µm membrane filter). Both the H_2O and acetonitrile were supplemented with 0.1% trifluoroacetic acid (TFA) or 0.1% formic acid (FA) (Sigma). Semi-preparative HPLC at UCT was performed using an Agilent Technologies 1200 series HPLC system equipped with an Agilent Technologies 1200 series quad pump and Agilent Technologies 1200 series DAD. Samples were analysed using a Phenomenex Luna C18(2) (10 µm, 250 x 10 mm) column or a Develosil Diol-100 column (5 µm, 4.6 x 150 mm).

At HIPS, HPLC was performed on liquid-liquid partitioned extracts using a Gilson HPLC system (Gilson 322 pump, Gilson UV/Vis-151 detector and Gilson 402 syringe 3 pump). HPLC gradients were developed using a Phenomenex Jupiter Proteo LC column (250 x 4.60 mm, 4 µm) then samples separated using a Phenomenex Jupiter Proteo LC column (250 x 10 mm, 4 µm).

2.5.3 Benchtop column chromatography

Solvents used for benchtop column chromatography were analytical reagent (AR) grade that had been further purified by distillation. Benchtop columns were performed using silica gel 60 (Fluka 70 – 230 mesh, 63 – 200 µm) (Sigma) and monitored by TLC using aluminium-backed pre-coated silica gel 60 F_{254} sheets (Merck). TLC plates were viewed under UV light (254 nm and 330 nm) and developed using either ceric ammonium sulphate or 1:1 concentrated H_2SO_4 and MeOH stain, both followed by heating.

2.5.4 Anion Exchange Chromatography

Whatman DE52 Matrix was prepared in 100 mM Tris-HCl (pH7) according to the manufacturer's instruction and packed into a 10 mL glass pipette. The packed column was washed with 30 mL 10 mM Tris-HCl (pH7).

The lyophilised *S. speibonae* PK-Blue^{T} HM broth culture extract was dissolved in 2 mL sterile H_2O, diluted with 10 mL 10mM Tris-HCl (pH7), the pH adjusted to pH7 and applied to the column. A step gradient of 20 mL of each of 20, 50, 100, 250, 500 and 1000 mM NaCl in 10 mM Tris-HCl (pH7) was used sequentially and then the column was flushed with 2.5 M NaCl in 10 mM Tris-HCl (pH7). 22 x 5 mL fractions were collected and individually concentrated *in vacuo*.

2.6 Structure elucidation

The structures of purified compounds were determined by HR LC-MS and GC-MS, NMR analysis, UV spectroscopy, and, where required, confirmed by synthesis of the compound. These techniques are detailed in sections 2.6.1 – 2.6.5.

2.6.1 HR LC-MS

For HR LC-MS analysis, pure compounds were dissolved in HPLC grade solvent. HR LC-MS data for UCT samples was obtained using a Waters Synapt G2 LC-ToF-MS instrument at the Stellenbosch University Central Analytical Facility (CAF) or a Thermo Instruments MS system (LTQ XL/LTQ Orbitrap Discovery) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler and Accela pump) at the University of Aberdeen Marine Biodiscovery Centre. HR LC-MS data for HIPS samples was obtained using a Bruker maXis 4G UHR-ToF MS system.

2.6.2 GC-MS

Lipid compounds were derivatised for GC-MS according to the method described by Ichibara and Fukubayashi.[69] Toluene (0.2 mL), MeOH (1.5 mL), and an 8% (w/v) solution of HCl in MeOH/ H_2O (85:15, v/v) (0.3 mL) were added sequentially to the lipid compound(s). The resulting solution was vortexed then incubated overnight at 45°C. After cooling to room temperature, H_2O (1.0 mL) and hexane (1.0 mL) were added, followed by vortexing. The hexane layer was collected and analysed by GC-MS using an Agilent Technologies J&W column (30 m x 250 µm x 0.25 µm) on an Agilent Technologies 7890A GC system equipped with an Agilent Technologies 7693 Autosampler and Agilent Technologies 7000A GC/MS Triple Quadrupole. GC-MS data was analysed using Agilent MassHunter Workstation software.

2.6.3 NMR

Pure compounds were dissolved in 160 µL deuterated methanol (MeOH-d4) or chloroform ($CDCl_3$) NMR solvent for NMR analysis (Sigma). NMR data were collected using a Bruker 600-MHZ spectrometer equipped with a 5-mm Prodigy cryoprobe. Chemical shifts are reported in ppm and spectra referenced to residual solvent resonances ($CDCl_3$ δ_H 7.26, δ_C 77.0, MeOH-d4 δ_H 3.30, δ_C 49.0).[88] Coupling constants (*J*) are reported in Hertz (Hz).

2.6.4 UV Spectroscopy

UV absorption data was obtained using an Agilent Technologies Cary 60 UV-Vis spectrometer. Samples were dissolved in MeOH (0.5 mg/mL) for analysis and molar extinction coefficients calculated using the Beer-Lambert law.[89]

2.6.5 Chemical Synthesis

All reagents used in synthesis were purchased from Sigma. Reaction mixtures were heated using an oil bath and the reaction progress monitored by TLC. Upon completion of the

reaction, reaction mixtures were dissolved in 1 M sodium hydroxide (NaOH), extracted into EtOAc, and the organic extract washed with saturated sodium bicarbonate ($NaHCO_3$) before drying over magnesium sulfate ($MgSO_4$). Products were concentrated *in vacuo*. See section 4.2 for a detailed outline of synthetic methods.

Chapter 3: Results and discussion

The primary objective of this study was the re-isolation and structure elucidation of the antimycobacterial compound PK-B from the South African actinomycete, *S. speibonae* PK-Blue^T, following the method outlined by Kirby.[45] This chapter summarises the efforts made to achieve this objective and discusses potential reasons for the fact that it ultimately proved unsuccessful. Owing to difficulties in reproducing the previously reported results, culture conditions suitable for the production of other antimycobacterial compounds by *S. speibonae* PK-Blue^T were explored, with success. These culture conditions were implemented in the bioassay-guided isolation of compounds from *S. speibonae* PK-Blue^T extracts. Purification of *S. speibonae* PK-Blue^T extracts resulted in the isolation of the alkaloid, *N*-phenylpyridin-2-aminium (**3.1**); the fatty acids, *n*-hexadecanoic acid (**3.2**) and 14-methylpentadecanoic acid (**3.3**); and the isoflavone, 7-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (**3.4**). The biological activity of these compounds against *M. tuberculosis* H37Rv^T was also determined.

In addition to *S. speibonae* PK-Blue^T, two other bacterial strains, namely *S. africanus* CPJVR-H^T and *S. pharetrae* CZA14^T, were investigated for the production of bioactive NPs. All three strains were analysed at UCT, and also during a six-week internship at the Helmholtz Institute for Pharmaceutical Research Saarland (HIPS). Section 3.1 describes the strategies implemented in the bioactivity-guided isolation of compounds at UCT, while the investigation of the same strains at HIPS is covered in section 3.2.

3.1 *Streptomyces speibonae* PK-Blue^T, *S. africanus* CPJVR-H^T and *S. pharetrae* CZA14^T at UCT

This section provides details of the work performed on the three South African actinomycetes *S. speibonae* PK-Blue^T, *S. africanus* CPJVR-H^T and *S. pharetrae* CZA14^T at UCT.

3.1.1 Attempted re-isolation of compound PK-B

The initial attempt to re-isolate compound PK-B from *S. speibonae* PK-Blue^T was performed by replicating the method described by Kirby.[45] *Streptomyces speibonae* PK-Blue^T was cultivated in 1L HM broth, as described in section 2.1.1. After incubation, the culture was

subjected to the prescribed method of solvent extraction using EtOAc and MeOH to yield five crude culture extracts; i.e., the extracted culture broth, crude EtOAc extract, final EtOAc extract, MeOH extract and PK-B extract. The PK-B extract was reported by Kirby to contain compound PK-B; however, as a precaution, all five extracts were tested for bioactivity. *Mycobacterium aurum* A+ was used as the bioautography test organism (see section 2.3.1), as compound PK-B was reported to have activity against this bacterium.[45] Unfortunately, none of the extracts displayed activity against *M. aurum* A+. The process was repeated three times to exclude any potential errors that could result in a change in the NPs produced, such as inadvertent changes in the culture conditions i.e. incubation temperature and constant shaking for aeration; however, repeated attempts yielded no extracts with activity against *M. aurum* A+.

As it was possible that the negative bioassay result was due to compound PK-B being a very minor component of the extracts, the PK-B extract was purified by anion exchange chromatography (see section 2.5.4), as reported by Kirby.[45] This technique is used to separate compounds based on their charge and was performed at pH7, a pH at which PK-B is reported to be negatively charged.[45] All 22 fractions resulting from the anion exchange chromatography were screened for activity against *M. aurum* A+; again, no activity was found.

Since the extracts were not active against *M. aurum* A+, it was concluded that PK-B was not being produced under the implemented culture conditions. It was hypothesised that, at the end of the incubation period, the *S. speibonae* PK-Blue^T culture had yet to reach stationary phase. If the *S. speibonae* PK-Blue^T culture had not yet reached stationary phase, secondary metabolism and the production of antibiotic NPs might not have been activated. There is some evidence to support this hypothesis since the production of aerial mycelium in streptomycetes is linked to secondary metabolism and antibiotic production.[90] *Streptomyces speibonae* PK-Blue^T has also been shown to produce grey aerial mycelium on agar medium.[91] The production of this grey aerial mycelium on HM agar was consistently observed to coincide with the appearance of the blue colour in the substrate mycelium that gave rise to the species name, PK-Blue (see Figure 3.1). *Streptomyces speibonae* PK-Blue^T is known to produce blue substrate mycelium,[91] but failed to do so under the implemented culture conditions, a possible indication that the culture had not matured to the point of inducing secondary metabolism. It was further hypothesised that *S. speibonae* PK-Blue^T cultures had stopped

producing PK-B due to the bacterium becoming accustomed to living on rich laboratory growth media. This adaptation to the rich laboratory growth medium could have resulted in *S. speibonae* PK-Blue^{T} no longer expressing all the genes it expressed as a "wild" bacterium, as it is known that growth medium nutrient composition affects gene expression of bacteria and therefore antibiotic production.[54] It was therefore decided that the culture conditions should be optimised for activity against *M. aurum* A+ by the induction of secondary metabolism through the use of an alternative growth medium using the appearance of blue mycelium as an indicator.

Figure 3.1: *Streptomyces speibonae* PK-Blue^{T} HM agar plate showing blue substrate mycelium after incubation at 30°C for 14 days.

3.1.2 Optimisation of *Streptomyces* culture conditions for activity against *M. aurum* A+

ISP 2 medium was used as the growth medium for the optimisation of activity against *M. aurum* A+ in *S. speibonae* PK-Blue^{T} cultures, as ISP 2 medium is commonly used in the culturing of actinobacteria. All cultures were incubated at 30°C with constant shaking while the final incubation culture volume and time were varied based on the time of appearance of the blue pigment in the mycelium. After incubation, a solvent extraction was performed (see section 2.2.2) and the extracts tested for activity against *M. aurum* A+ by bioautography, as described in section 2.3.1. As a control, uninoculated, sterile ISP 2 broth medium was subjected to solvent extraction using the same method adopted for the cultured extracts and tested against *M. aurum* A+.

Both small- and large-scale *S. speibonae* PK-Blue^T cultures were prepared. Small-scale cultures using a 1 L Erlenmeyer flask containing 100 mL ISP 2 broth culture incubated for a minimum of 7 d exhibited blue mycelia, with solvent extracts showing activity against *M. aurum* A+ (for full culture conditions see section 2.1.2). Large-scale *S. speibonae* PK-Blue^T cultures using a 5 L Erlenmeyer flask containing 1 L ISP 2 broth culture exhibited the same blue mycelium and activity against *M. aurum* A+ when incubated for a minimum of 14 d (culture conditions are detailed in section 2.1.3). Good activity against *M. aurum* A+, indicated by a significant zone of inhibition by bioautography, was consistently found in crude EtOAc extracts. However, weak activity against *M. aurum* A+ was observed for MeOH extracts and no activity observed in the extracted culture broth. This suggested that the bioactive compounds produced by *S. speibonae* PK-Blue^T were secreted by the cells into the growth medium and are readily soluble in EtOAc. Active EtOAc extracts were then prioritised for purification as part of our strategy of bioassay-guided isolation of antimycobacterial compounds.

The optimised large-scale culture conditions developed for *S. speibonae* PK-Blue^T were used in the cultivation of *S. africanus* CPJVR-H^T and *S. pharetrae* CZA14^T. Cultures of these two actinobacteria were subjected to solvent extraction and the resulting crude EtOAc extracts were revealed to be active against *M. aurum* A+ by bioautography.

3.1.3 Dereplication and liquid-liquid partitioning of bacterial extracts

All bacterial extracts and an uninoculated, sterile ISP 2 broth medium EtOAc extract were analysed by LR LC-MS for dereplication (see section 2.4.1). The purpose of dereplication was to facilitate efficient identification of known compounds by comparing extract molecular masses against the Dictionary of Natural Products and StreptomeDB online databases.[61,87] LR LC-MS was used in the dereplication process, as the instrument was readily available. Using such low-resolution MS data, which measures molecular ion masses to only one decimal place, is not ideal for dereplication of NPs. These data are not accurate enough to confidently assign molecular formulae to NPs, although they could be used to give an indication of the masses of the molecular ions present in the samples. Ideally HR LC-MS data with an the

accuracy of four decimal places should be recorded as this can resolve differences between compounds with very similar *m/z*-values.[92,93]

The LR LC-MS profiles of crude culture extracts, liquid-liquid partitioned fractions and pure compounds were compared with that of the uncultured, sterile ISP 2 broth medium EtOAc extract. An EtOAc extract of the sterile, uncultured ISP 2 broth medium (prepared using the method described in section 2.2.2) was used as a blank sample. For dereplication of the bioactive crude extracts and semi-purified extracts, the LC-MS ion molecular masses and retention times of major peaks were compared with that of the ISP 2 medium blank. This was done to confidently identify ions present in extract samples, but absent from the ISP 2 medium blank. The knowledge gained by this comparison allowed purification efforts to be concentrated towards isolation of compounds of bacterial origin.

The first purification step of bioactive crude EtOAc extracts was liquid-liquid partitioning with hexane, $CHCl_3$, H_2O and EtOAc (see section 2.5.1). Compounds in the extracts were partitioned into the four different solvents based on their polarity and therefore their solubility in the respective solvents. The hexane fraction, hexane being the least polar solvent, accumulated the least polar compounds. The H_2O fraction, the most polar fraction, accumulated the most polar compounds. This is consistent with the principle that "like dissolves like".[62] Similarly, intermediate polarity compounds were partitioned between the two medium polarity solvent fractions of EtOAc and $CHCl_3$. The liquid-liquid partitioning of crude extracts resulted in four fractions that were each less chemically complex than the parent fraction; i.e., containing a smaller variety of compounds. This can be seen by comparing LC-MS chromatogram profiles of a fraction resulting from liquid-liquid partitioning with its parent extract, and is illustrated in Figure 3.2. This figure displays an overlay of the positive-ion ESI LC-MS chromatograms (5 – 95% acetonitrile) of an *S. speibonae* PK-Blue^T crude EtOAc extract and liquid-liquid partitioned $CHCl_3$ fraction, showing the decrease in chemical complexity after solvent partitioning. Figure 3.2 also illustrates that the $CHCl_3$ fraction, as a medium polarity fraction, accumulated the compounds of intermediate polarity; i.e., compounds that eluted in the middle of this gradient on a RP-HPLC system.

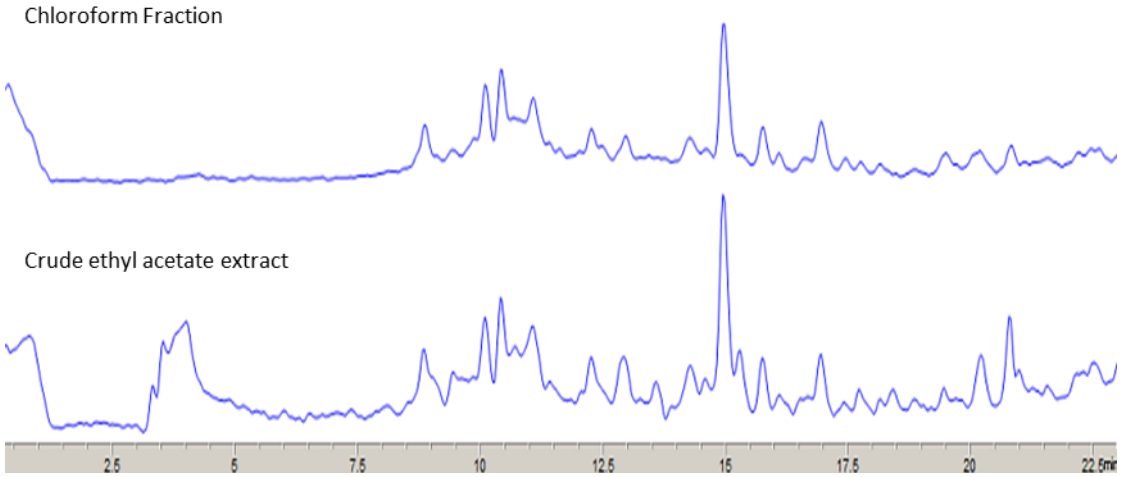


Figure 3.2: Overlay of the positive-ion ESI LC-MS chromatograms of an *S. speibonae* PK-Blue^T crude EtOAc extract and liquid-liquid partitioned $CHCl_3$ fraction (5 – 95% acetonitrile).

All fractions from liquid-liquid partitioning were screened for activity against *M. aurum* A+ by bioautography as part of the strategy of bioassay-guided isolation of antimycobacterial compounds. Active fractions were selected for further purification and the individual purification strategies of compounds isolated from *S. speibonae* PK-Blue^T are discussed in the following section.

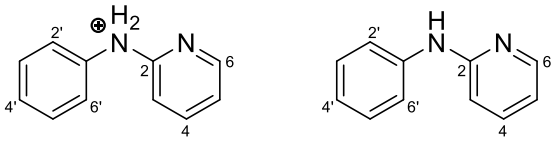
3.1.4 Compounds isolated from *S. speibonae* PK-Blue

Four compounds were isolated from *S. speibonae* PK-Blue^T using the culture conditions optimised for activity against *M. aurum* A+. The isolation and structure elucidation of these compounds, **3.1** – **3.4**, are detailed below.

3.1.4.1 Isolation and structure elucidation of N-phenylpyridin-2-aminium

The antimycobacterial alkaloid, *N*-phenylpyridin-2-aminium (**3.1**, 2.5 mg), was isolated from a 14 d, ISP 2 small-scale culture of *S. speibonae* PK-Blue^T (see section 2.1.2 for ISP 2 small-scale culture method). An extensive review of the Scifinder database[42] has shown that the

deprotonated form of this compound, *N*-phenylpyridin-2-amine (**3.5**),[94] has previously been synthesised, however this is the first instance of isolation of either **3.1** or **3.5** from a natural source.



3.1 **3.5**

An EtOAc extract (145 mg) with bioactivity against *M. aurum* A+ (see Figure 3.3) was obtained from a 14 d *S. speibonae* PK-Blue^T culture and was subsequently subjected to liquid-liquid partitioning. The $CHCl_3$ fraction from liquid-liquid partitioning had activity against *M. aurum* A+ and hence this fraction was further purified by RP-HPLC (40 - 100% acetonitrile, 0.1% TFA in H_2O). All fractions collected from RP-phase HPLC purification were tested for activity against *M. aurum* A+ and the bioactive compound **3.1** was shown to elute at 100% acetonitrile in the gradient (see Scheme 3.1 for isolation protocol of **3.1** from *S. speibonae* PK-Blue^T).

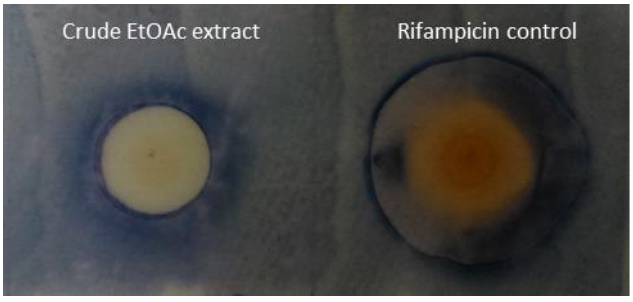
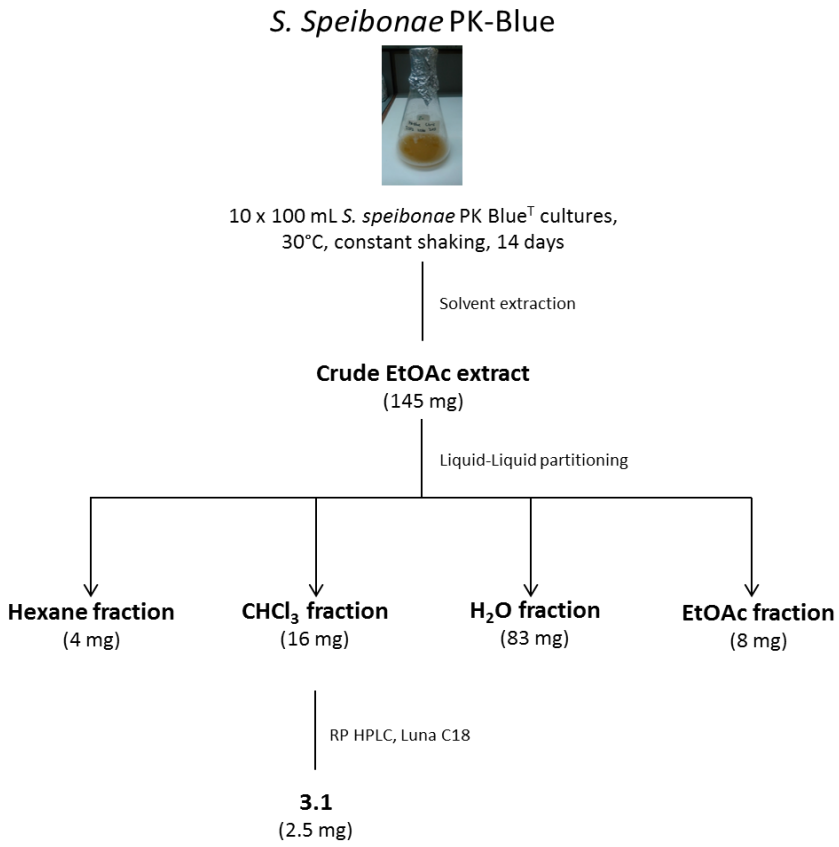


Figure 3.3: Bioautography against *M. aurum* A+ of an *S. speibonae* PK-Blue^T crude EtOAc extract. Bioactivity is indicated by a clear yellowish zone of inhibition. Rifampicin was used as a positive control.



Scheme 3.1: Isolation protocol for **3.1** from *S. speibonae* PK-Blue^T ISP 2 cultures.

Data obtained by ^{13}C and ^{1}H NMR (see Figure 3.4) and HR LC-MS (molecular ion *m/z* = 171.0918, ESI+) were used to deduce a molecular formula for **3.1** of $C_{11}H_{10}N_2$ (for NMR and HR LC-MS methods see sections 2.6.1 and 2.6.3). 1D NMR spectroscopy (^{13}C and ^{1}H) showed all carbon and proton signals to be significantly deshielded and in the aromatic range, and 2D NMR experiments (COSY, HSQC, HMBC and NOESY) indicated that the aromaticity of the compound was due to two independent conjugated pi-electron systems. This information was used to initially assign the structure as (**3.5**). UV analysis indicated the presence of two conjugated aromatic systems absorbing at 265 nm and 310 nm, which was supported by NMR data. COSY and HMBC (Figures 3.5 and 3.6) correlations indicated the presence of two isolated aromatic ring systems; one phenyl group and one heteroaromatic six-membered ring. As the molecule was shown to be purely aromatic and all $^{13}C/^{1}H$ signals appeared in the downfield region of the NMR spectra, it followed that the two aromatic ring systems could not be bridged by a carbon atom. This was consistent with the deduced molecular formula and a nitrogen atom as the bridging atom (N-1) accounted for the observed molecular ion mass.

The heteroaromatic substituent was assigned as 2-pyridine based on COSY and HMBC correlations and the C-2 quaternary carbon signal (δ_c 153.8), which is characteristic for this type of system.[95]

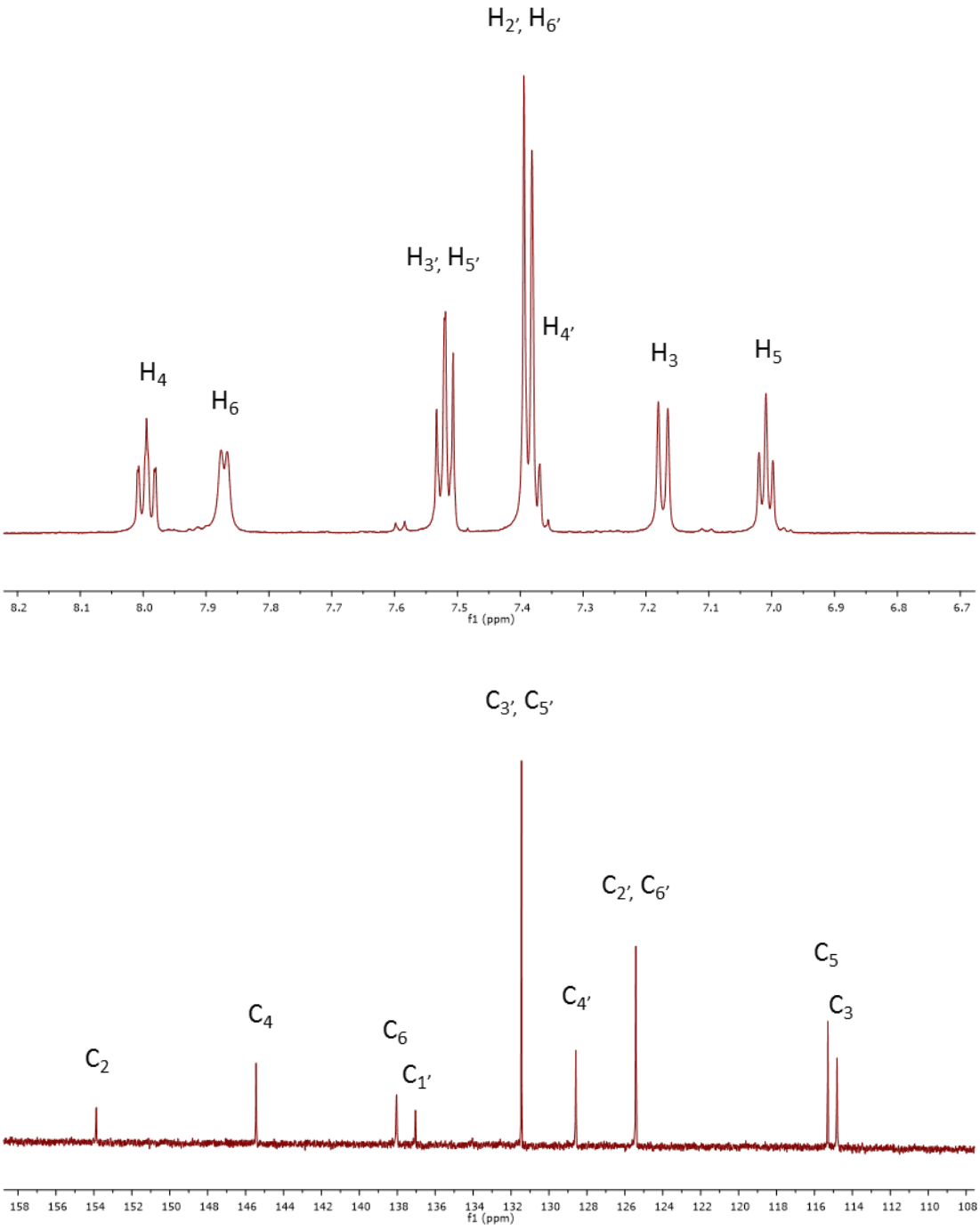


Figure 3.4: ^{1}H (MeOH-d4, 600 MHz) and ^{13}C (MeOH-d4, 150 MHz) NMR spectra obtained for **3.1**.

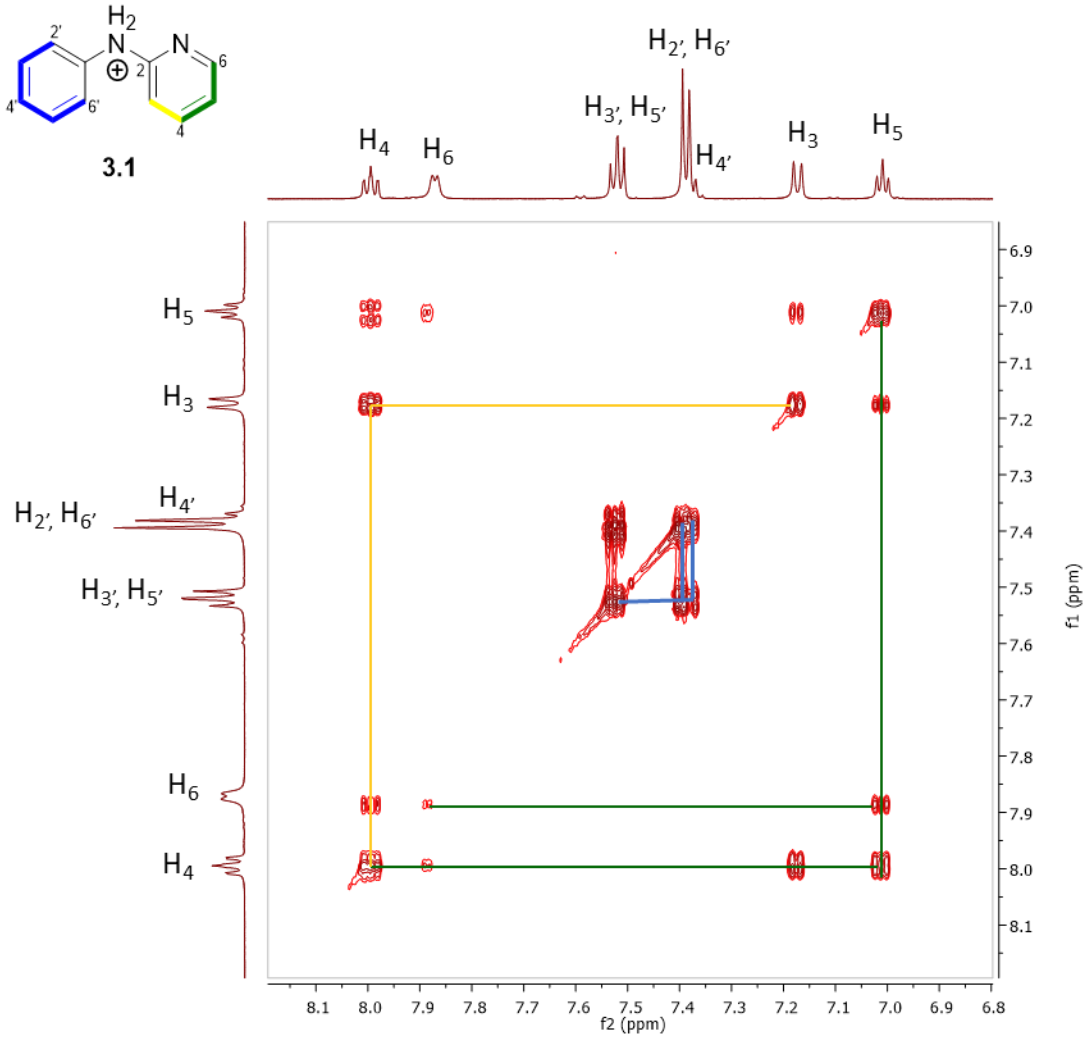


Figure 3.5: COSY spectrum (MeOH-d4, 600 MHz) of **3.1**. The accompanying structure shows the key COSY correlations used to deduce the structure of **3.1**.

However, experimental 1D NMR data did not correlate with literature 1D NMR data of **3.5**.[94] As the 2D NMR data strongly supported the assignment of **3.5**, this compound and its protonated form **3.1** were synthesised (see section 4.2). By comparing NMR data of the isolated compound with that of both synthesised compounds, the isolated compound was assigned as compound **3.1** (see Figure 3.7). NMR data of the isolated and synthesised **3.1** correlated well, but imperfectly. However, the assignment of the isolated **3.1** was further supported by LC-MS data. It was observed that the synthesised **3.1** and **3.5** both yielded

m/z=171.0 (ESI+) by LR LC-MS but with different retention times, and the retention time of the isolated molecule matched that of the synthesised **3.1**.

NMR prediction software (both ACD/Labs NMR Spectroscopy Software and Chemdraw Professional) was used to guide the approach of using chemical synthesis. As we were confident that the backbone of the structure was correctly identified (a phenyl ring and pyridine ring bridged by a nitrogen atom), it was hypothesised that the differences in chemical shifts between **3.1** and **3.5** was due to a protonation/deprotonation effect. This was supported by the NMR prediction software which showed that protonation of the bridging nitrogen of **3.5** would yield a compound with an NMR profile similar to **3.1**, while protonation of the pyridyl nitrogen or both of the nitrogen atoms in the compound were predicted to create greater differences in the NMR profiles. This effect was observed experimentally after synthesis of **3.1**, as literature NMR data on **3.1** were not available. As the experimental NMR data for the synthesised and isolated **3.1** were very similar but not perfectly so, it was considered that the slight discrepancy could be due to the degree of protonation, but titrating **3.5** against acid and analysing by NMR did not show protonation of N-2 and this was not confirmed experimentally. Also, as there was insufficient material of the isolated **3.1**, it was not possible to manipulate it with acid/base treatments. Another option that was considered for the observed differences between the synthesised and isolated **3.1** were a counter-ion effect. To investigate this, **3.5** was protonated using HBr and HCl, but these yielded the same result by NMR.

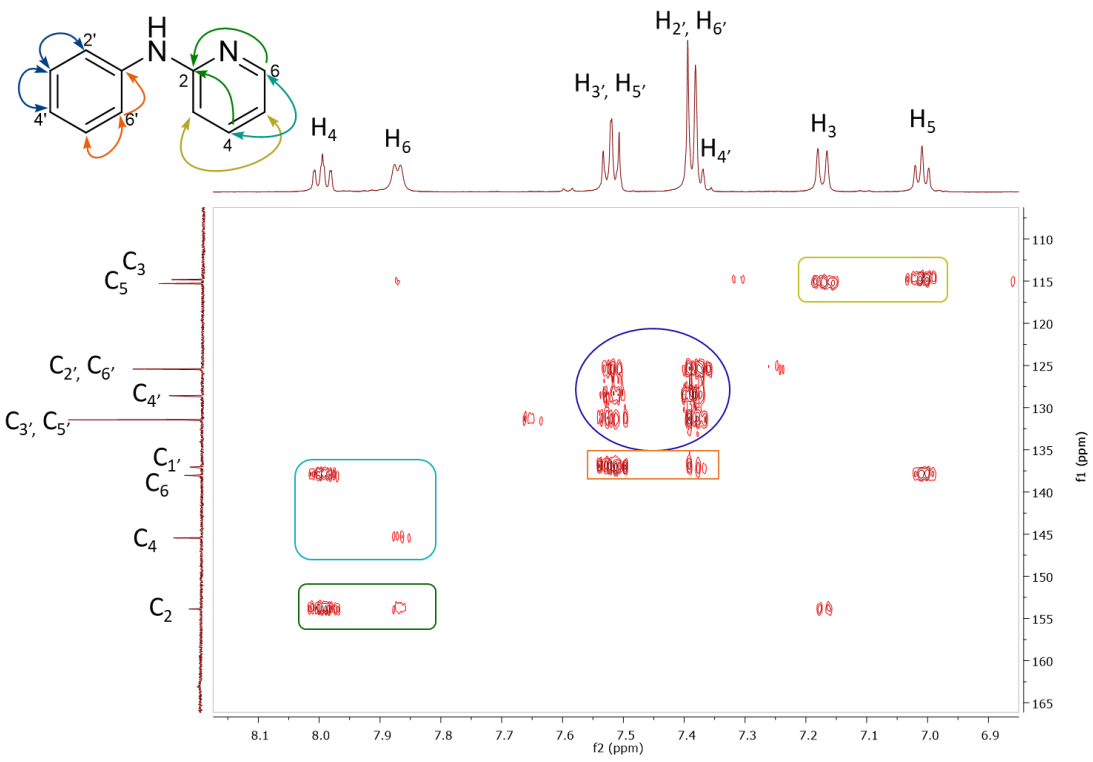


Figure 3.6: HMBC spectrum (MeOH-d4, 600 MHz) of **3.1**. The accompanying structure shows the key HMBC correlations used to deduce the structure of **3.1**.

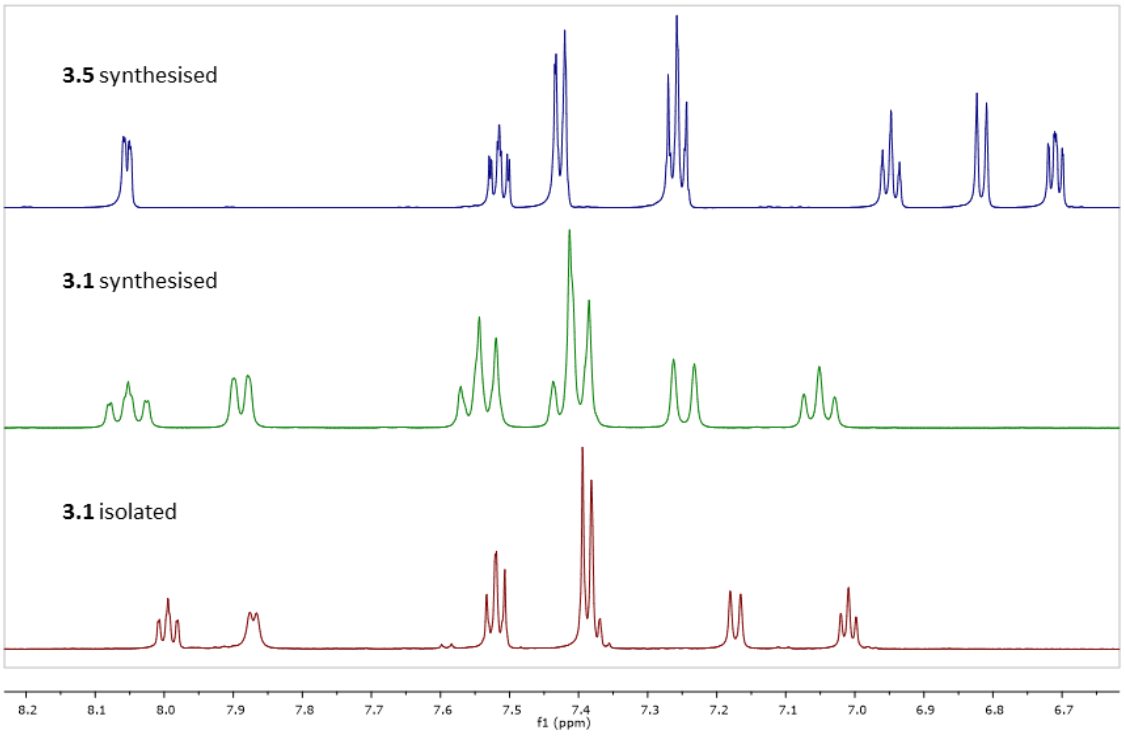


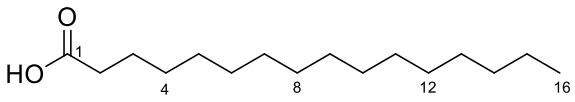
Figure 3.7: Overlay of ^{1}H NMR spectra (MeOH-d4, 600 MHz) of the isolated **3.1**, synthesised **3.1** and synthesised **3.5**.

During the structure elucidation of **3.1**, LR LC-MS data of the parent fractions of the isolated **3.1** were reviewed, but the molecular ion *m/z* = 171.0 was not found prior to HPLC purification. Hence there can be some debate as to whether **3.1** is produced by *S. speibonae* PK-Blue^T or if it has another origin. Efforts were made to ascertain if the origin of this compound could be other than the *S. speibonae* PK-Blue^T culture, but no plausible other source was identified. It was confirmed that compound **3.1** was not owned by any of the research groups in the buildings where work was performed. Additionally, the author was the only HPLC operator for two months prior to and after the isolation of this compound and the only samples purified were *S. speibonae* PK-Blue^T extracts, making it unlikely that the compound arose from contamination during purification. It can therefore not be concluded that **3.1** is produced by *S. speibonae* PK-Blue^T, however it also cannot be concluded that the bacterium is not the producer of the compound. A possible reason for not observing **3.1** in the parent fractions is that it could be the break-down product of another molecule produced by *S. speibonae* PK-Blue^T and present in the crude EtOAc extract, although possible precursors were not identified in the parent fraction.

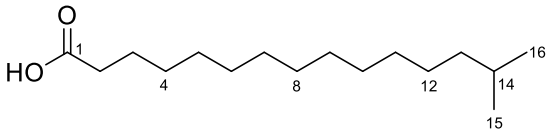
3.1.4.2 Isolation and structure elucidation of n-Hexadecanoic acid and 14-methylpentadecanoic acid

The known saturated LCFAs, *n*-hexadecanoic acid (**3.2**) and 14-methylpentadecanoic acid (**3.3**), were isolated as a mixture (2.2 mg) from six culture broths of *S. speibonae* PK-Blue^T, both small- and large-scale.[96,97] The crude EtOAc extracts of these six cultures displayed activity against *M. aurum* A+ by bioautography and were subjected to liquid-liquid partitioning. The resulting liquid-liquid partitions – i.e., hexane, $CHCl_3$, H_2O and EtOAc – were screened for activity against *M. aurum* A+ by bioautography and the $CHCl_3$ fractions were found to be active. The $CHCl_3$ fractions were then individually semi-purified by RP-HPLC (40 - 100% acetonitrile, 0.1% TFA in H_2O). In each case, the RP-HPLC 100% acetonitrile wash showed activity against *M. aurum* A+. The six semi-purified wash fractions were then combined based on the similarity of their ^{1}H NMR profiles and purified further by semi-preparative RP-HPLC (20 - 100% acetonitrile, see section 2.5.2) to yield **3.2** and **3.3** as a

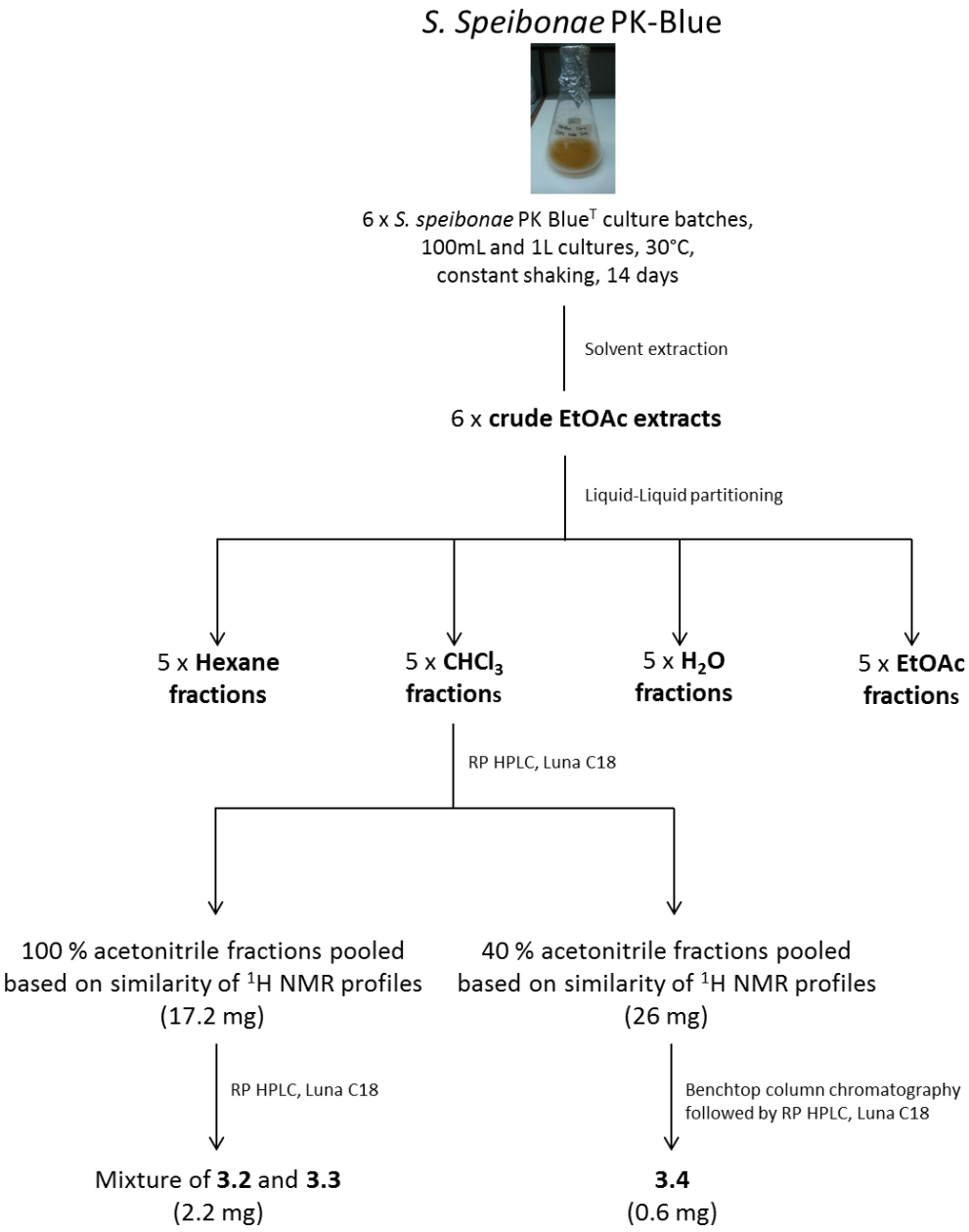
mixture (2.2 mg). The HPLC fraction containing the mixture of **3.2** and **3.3** exhibited activity against *M. aurum* A+ by bioautography. See Scheme 3.2 for the isolation protocol for **3.2**, **3.3** and **3.4** from *S. speibonae* PK-Blue^{T}.



3.2



3.3



Scheme 3.2: Isolation protocol for **3.2**, **3.3** and **3.4** from *S. speibonae* PK-Blue^T ISP 2 cultures.

1D NMR of the mixture of **3.2** and **3.3** showed the presence of a long-chain hydrocarbon, with a characteristic broad signal at δ_H 1.29 – 1.30 ppm and multiple signals at δ_C 30 – 31 ppm in addition to a characteristic carboxyl signal at δ_C 177.7, as seen in Figure 3.8.[95] The mixture of **3.2** and **3.3** was therefore expected to be a fatty acid. Fatty acids are commonly analysed by GC-MS, as this technique is highly sensitive and reproducible, and also allows the characterisation of complex mixtures of geometric isomers.[98] Hence the sample was

derivatised to form the fatty acid methyl esters (FAMEs) before GC-MS was used to determine the length of the hydrocarbon chain (see section 2.6.2 for GC-MS derivatisation methods and protocols). GC-MS analysis showed hydrocarbon chain lengths corresponding to those of **3.2** and **3.3**, where they were present as methyl esters. The structures of **3.2** and **3.3** were supported by 2D NMR data (see Figure 3.9) and confirmed by comparison of experimental NMR data (1D and 2D) and GC-MS data with literature NMR and GC-MS data.[96,97]

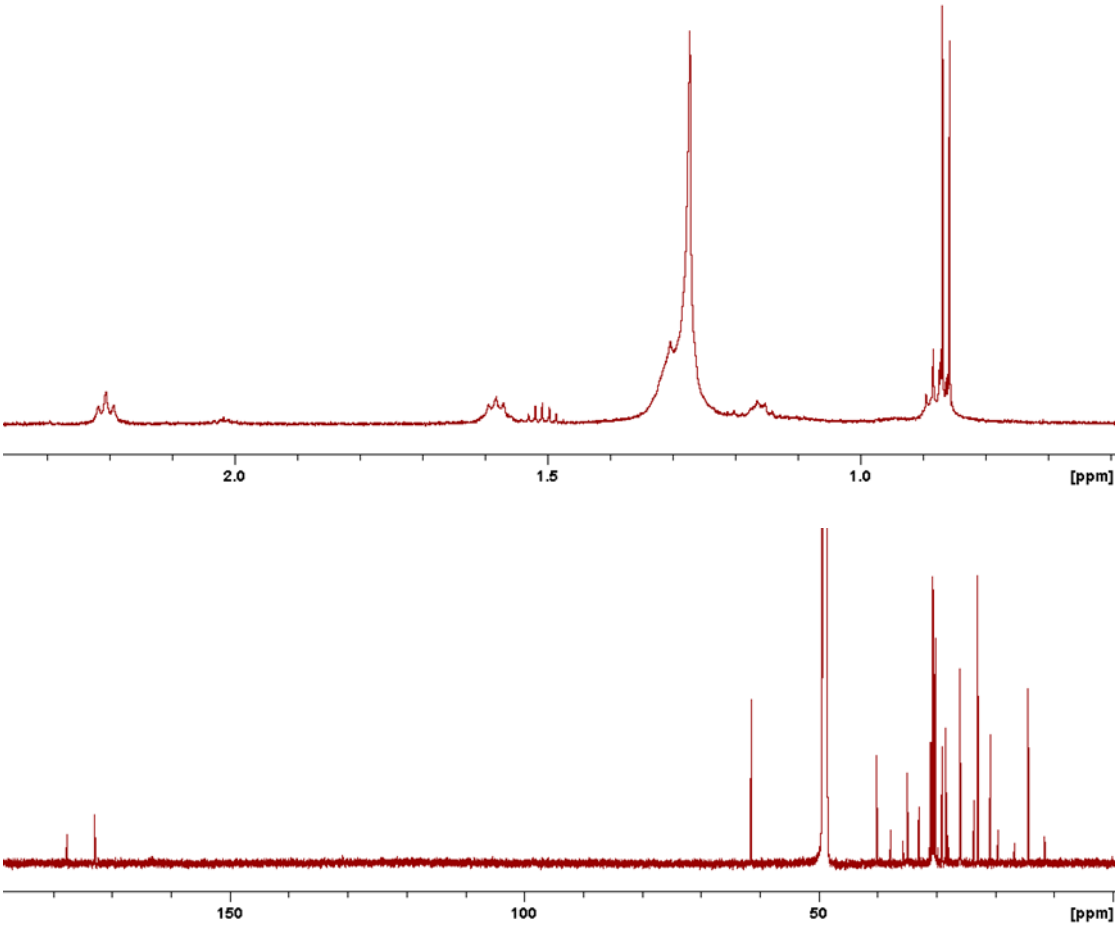


Figure 3.8: ^{1}H (MeOH-d4, 600 MHz) and ^{13}C (MeOH-d4, 150 MHz) NMR spectra obtained for the mixture of **3.2** and **3.3**. EtOAc is present in the ^{13}C NMR sample and accounts for the carboxyl signal observed at δ_C 172.99.

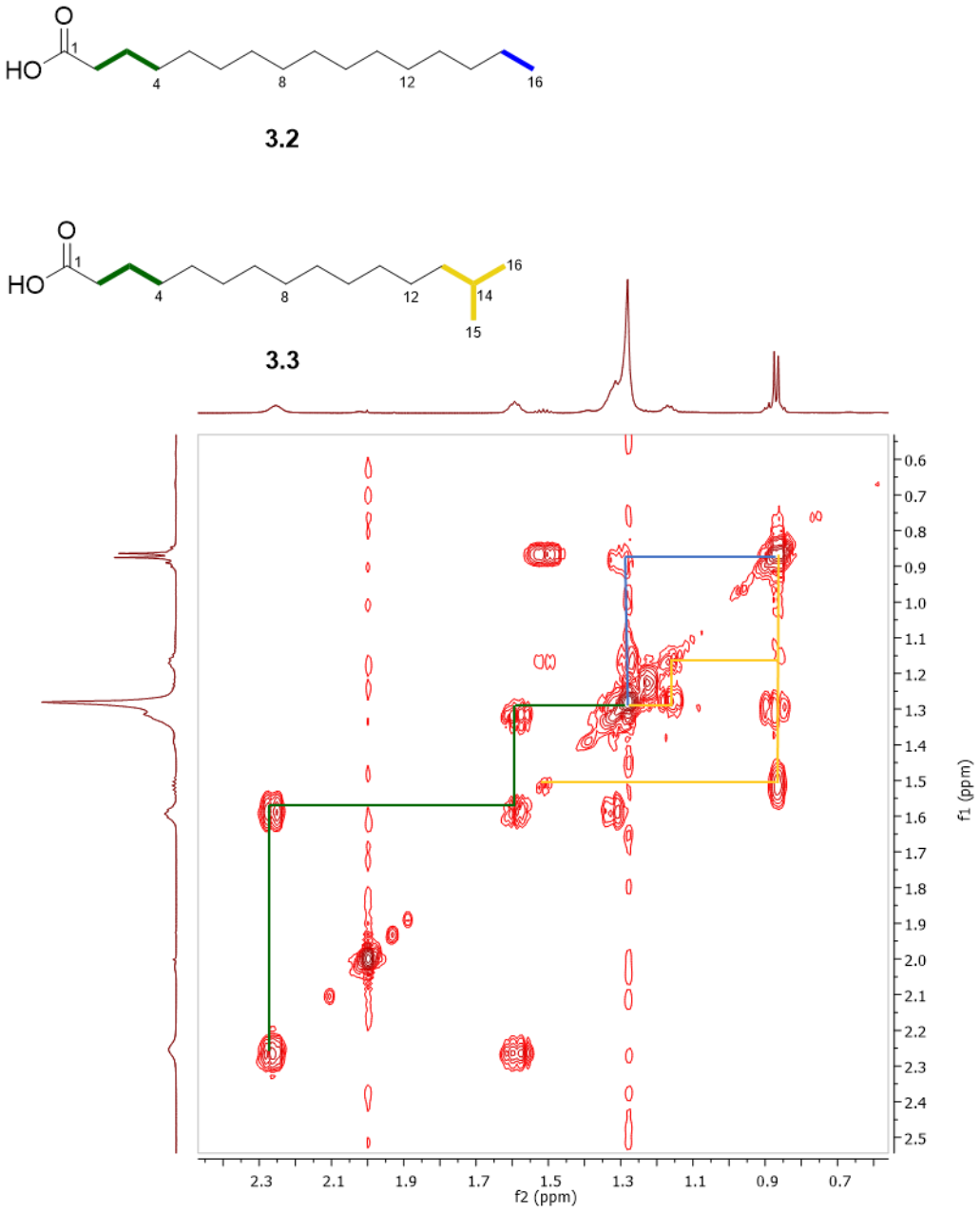


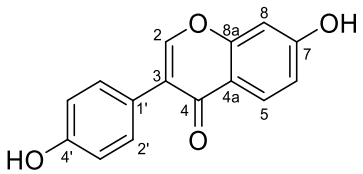
Figure 3.9: COSY spectrum (MeOH-d4, 600 MHz) of **3.2** and **3.3**. The accompanying structures show the key COSY correlations used to deduce the structures of **3.2** and **3.3**.

3.1.4.3 Isolation and structure elucidation of 7-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one

The known isoflavone, 7-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (**3.4**, 0.6 mg),[99] was isolated from the same six *S. speibonae* PK-Blue^{T} cultures (small- and large-scale) as **3.2** and **3.3**. The crude EtOAc extracts of these six cultures displayed activity against *M. aurum* A+ by bioautography. Subsequent liquid-liquid partitioning of the crude EtOAc extracts yielded

$CHCl_3$ fractions with bioactivity against *M. aurum* A+. Semi-preparative RP-HPLC (40 - 100% acetonitrile, 0.1% TFA in H_2O) was performed on the $CHCl_3$ fractions. Activity against *M. aurum* A+ was found in the first fraction collected (40% acetonitrile, 0.1% TFA in H_2O) and the HPLC UV chromatogram showed this to be a complex mixture of compounds. This first HPLC fraction from the six cultures was combined and analysed by TLC (see section 2.5.3). As the TLC profile was simple, the combined fraction was purified further by benchtop column chromatography (isocratic 50% hexane/EtOAc). ^{1}H NMR showed that **3.4** was present, with minor peaks indicating the presence of impurities, in the second of seven fractions from benchtop column chromatography. This second fraction was further purified by semi-preparative RP-HPLC (2 - 100% acetonitrile, 0.1% FA, see section 2.5.2) to yield **3.4** (0.6 mg). The isolation procedure of **3.4** is outlined in Scheme 3.2.

The structure of **3.4** was determined using the LR LC-MS molecular ion mass of *m/z* = 255.0 (ESI+) in conjunction with 1D and 2D NMR data (^{1}H, ^{13}C, COSY, HSQC and HMBC). ^{1}H and ^{13}C NMR data showed **3.4** to be purely aromatic due to the absence of upfield signals as seen in Figure 3.10. Correlations observed in a 2D COSY NMR experiment showed three aromatic spin systems which were pieced together based on the HMBC correlations to C-3, C-4, C-1', and C-8a (see Figure 3.11 and Figure 3.12). The chemical shift of C-4 (δ_c 178.2) was characteristic of a carbonyl group and the substituents at C-4' and C-7 were based on their characteristic chemical shift values and to account for the molecular ion mass obtained by LR LC-MS.[95] The structure of **3.4** was confirmed by comparison of experimental NMR data with literature NMR data for this compound.[100]



3.4

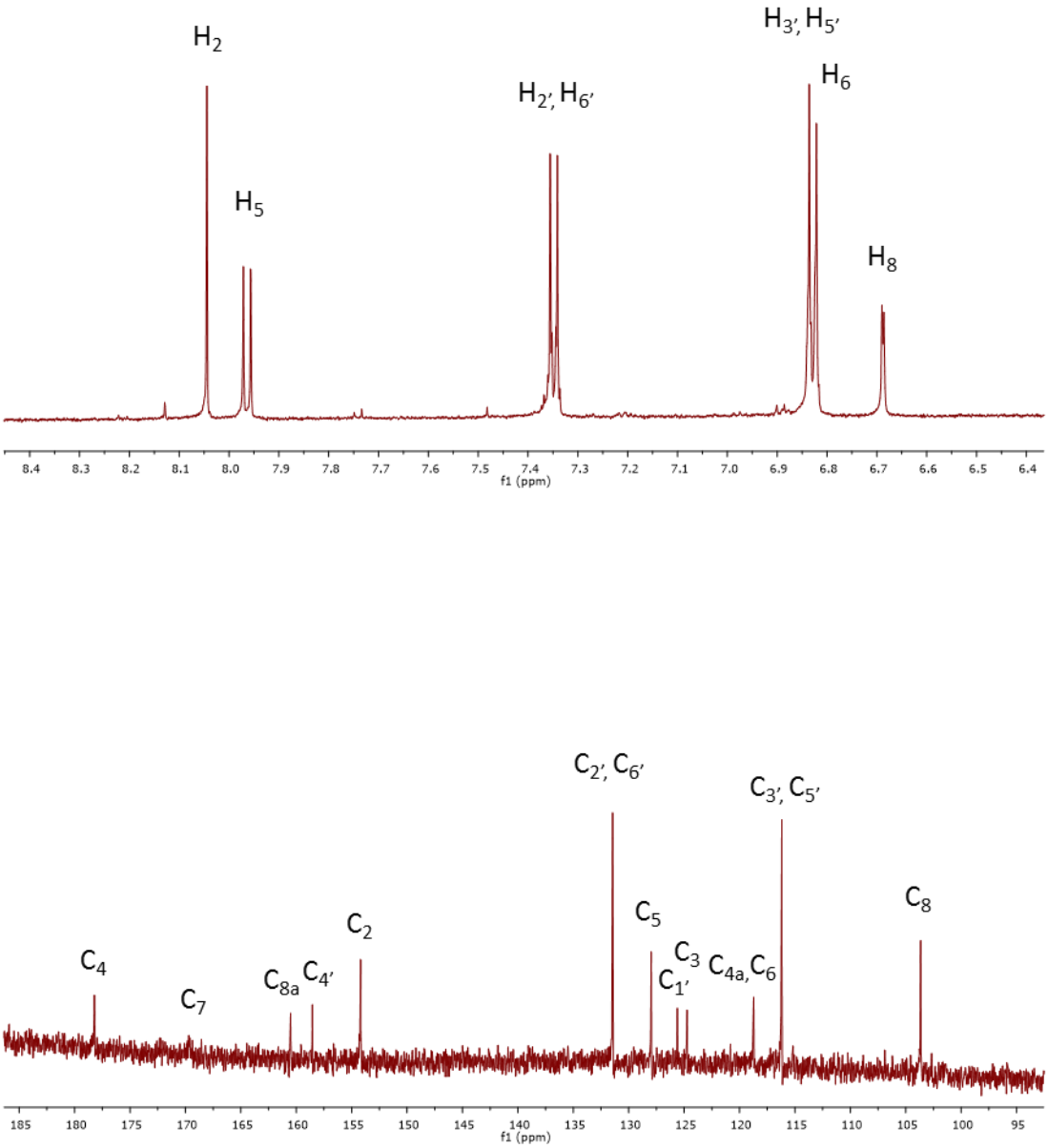


Figure 3.10: ^{1}H (MeOH-d4, 600 MHz) and ^{13}C (MeOH-d4, 150 MHz) NMR spectra obtained for **3.4**.

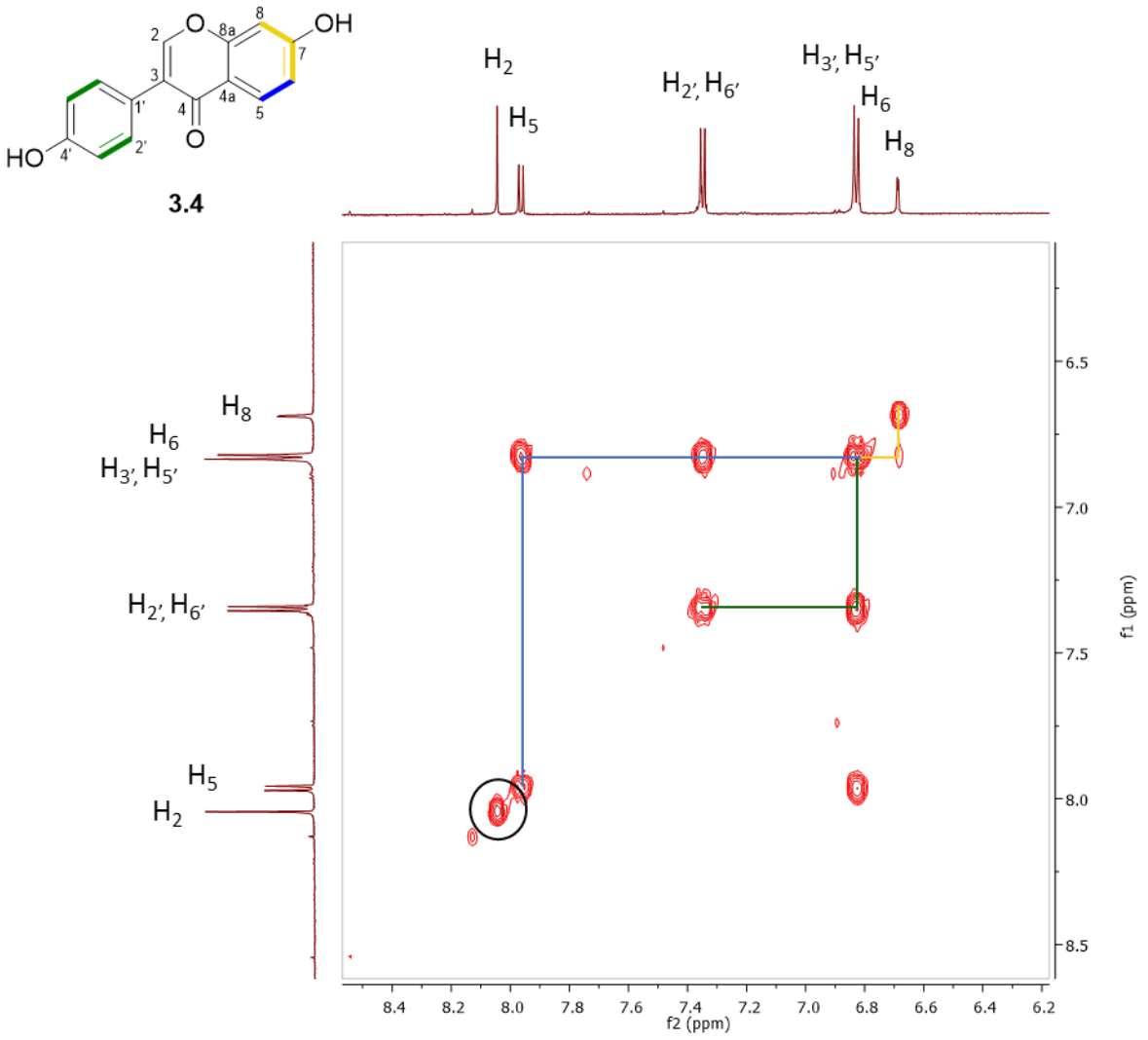
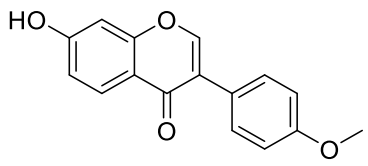


Figure 3.11: COSY spectrum (MeOH-d4, 600 MHz) of **3.4**. The accompanying structure shows the key COSY correlations used to deduce the structure of **3.4**.

After the structure elucidation of **3.4**, a literature search identified **3.4** as a demethylated conjugate of formononetin (**3.6**), a compound common to legumes such as soy beans and red clover.[99] Therefore it was speculated that **3.5** was produced through the metabolism of **3.6**, which was present as a component of the ISP 2 growth medium, rather than produced *de novo* by *S. speibonae* PK-Blue^T. The most likely source of **3.6** is the 10 g/L of malt extract used in preparing ISP2 broth. Malt extract is prepared from malted barley.

LR LC-MS data of an uninoculated, sterile ISP 2 broth medium EtOAc extract showed a molecular ion mass of *m/z* = 269.3 (ESI+), indicative of the presence of **3.6**. The ability to

demethylate of **3.6** has been reported to be common among lactic acid bacteria and bifidobacteria.[99] Bifidobacteria are actinobacteria, but are distantly related to *Streptomyces*.[101]



3.6

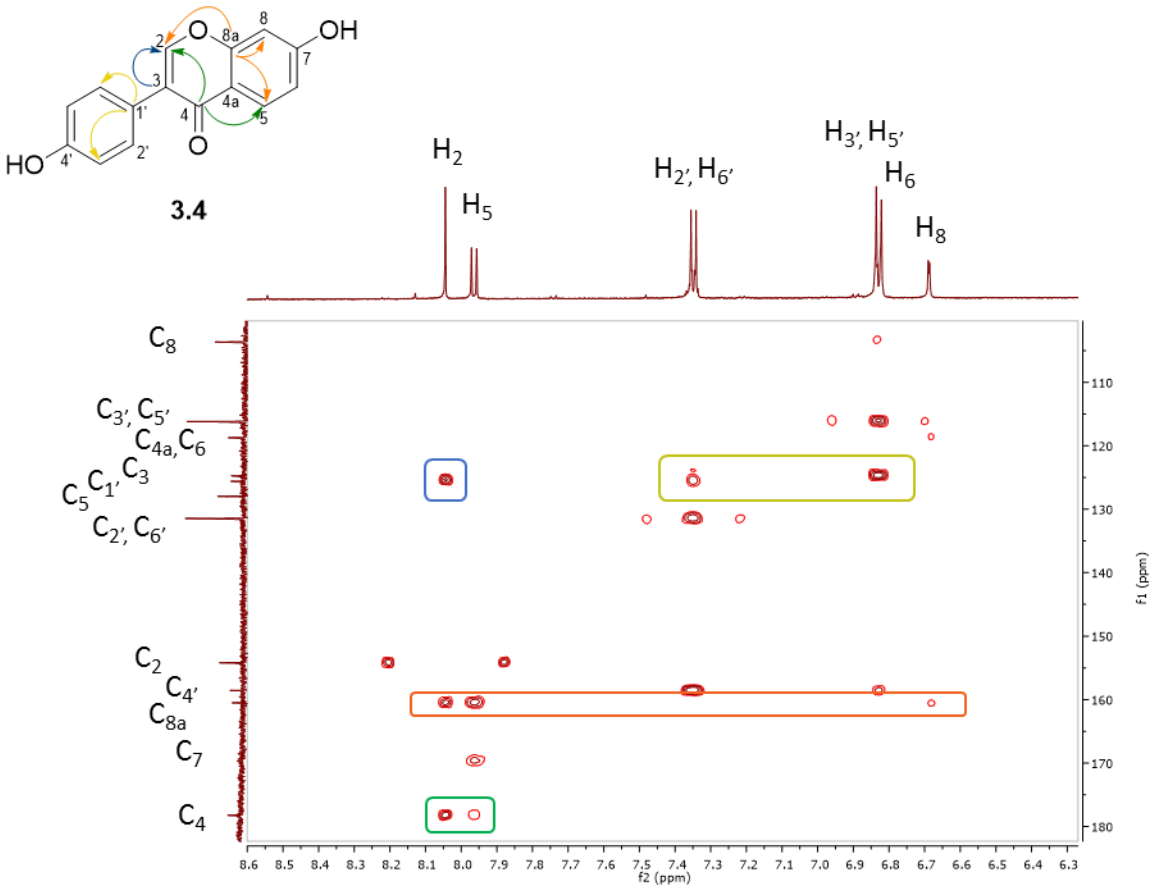


Figure 3.12: HMBC spectrum (MeOH-d4, 600 MHz) of **3.4**. The accompanying structure shows the key HMBC correlations used to deduce the structure of **3.4**.

3.2 *S. speibonae* PK-Blue^T, *S. africanus* CPJVR-H^T and *S. pharetrae* CZA14^T at HIPS

During a six-week internship at HIPS, the three South African actinomycetes, *S. speibonae* PK-Blue^T, *S. africanus* CPJVR-H^T and *S. pharetrae* CZA14^T, were cultured and subjected to bioassay-guided isolation for the discovery of novel NPs. This internship was an excellent opportunity, as it allowed access to and training on different instruments and bioassays which were incorporated in the study, including first-hand experience of running HR LC-MS experiments and MIC_{50} bioassays.

3.2.1 Culture conditions, solvent extraction and liquid-liquid partitioning

Cultures of *S. speibonae* PK-Blue^T, *S. africanus* CPJVR-H^T and *S. pharetrae* CZA14^T were prepared using three different growth media: TSB, SHG and NL2 (see section 2.1.4). These three growth media were selected as they have very of different nutrient compositions. The three different growth media were used to test the effect of the growth medium on the production of NPs by the three actinobacteria, as discussed in the following section. On the final day of incubation, cultures were treated with XAD16 resin for 12 h, after which the cells and resin were harvested by centrifugation. XAD16 resin adsorbs organic molecules from the culture broth and releases the adsorbed compounds by treatment with organic solvent. The XAD16 resin and cell mass were extracted with MeOH and an organic extract was obtained (see section 2.2.3). The components of organic extracts were separated based on polarity by liquid-liquid partitioning with hexane, $CHCl_3$, H_2O and EtOAc (see section 2.5.1) as an initial purification.

3.2.2 Bioassay, dereplication and purification

Liquid-liquid partitions were screened for activity against a test panel that included the bacteria *Escherichia coli* WT (DSM-1116), *E. coli tolC3* (DSM-26863), *S. aureus* ATCC-29213, *P. aeruginosa* DSM-24600, *B. subtilis* DSM-10^T and *M. smegmatis* mc^{2}155 (another mycobacterium used as a proxy for MTB),[45] and the fungi *C. albicans* DSM-1665 and *M.*

hiemalis (see section 2.3.3). The results for this initial screening of semi-purified extracts provided a clear illustration of the effect of the growth medium on the production of NPs (see Table 3.1). *S. speibonae* PK-Blue^T cultured using TSB showed weak activity against *M. smegmatis* mc^{2}155 (EtOAc fraction, MIC_{50} = 160 µg/mL) while the SHG culture of the same bacterium exhibited no activity against *M. smegmatis* mc^{2}155. Similarly, *S. africanus* CPJVR-H^T cultured using NL2 broth had strong activity against *B. subtilis* DSM-10^T ($CHCl_3$ fraction, MIC_{50} = 40 µg/mL) and *S. pharetrae* CZA14^T cultured using SHG broth also showed strong activity against *B. subtilis* DSM-10^T (EtOAc fraction, MIC_{50} = 5 µg/mL) while neither was active against *B. subtilis* DSM-10^T in TSB cultures. Hence, it was demonstrated that the growth medium used in bacterial cultivation of these three South African actinobacteria affected the specificity and degree of biological activities. This variation in biological activity was due to differences in the nutrient composition of the growth media. Different media components can activate or repress different biochemical pathways, and therefore affect the production of bioactive NPs.[53] The fractions with the strongest biological activities were selected for dereplication by HR LC-MS (see section 2.4.2).

Table 3.1: Selected bioassay results for liquid-liquid partitioned fractions of *S. speibonae* PK-Blue^T, *S. africanus* CPJVR-H^T and *S. pharetrae* CZA14^T culture extracts.

	***S. speibonae* PK-Blue^T**			***S. africanus* CPJVR-H^T**			***S. pharetrae* CZA14^T**		
Medium	TSB	TSB	SHG	SHG	NL2	TSB	SHG	SHG	TSB
Partition	$CHCl_3$	EtOAc	EtOAc	EtOAc	$CHCl_3$	$CHCl_3$	$CHCl_3$	EtOAc	EtOAc
E. coli WT (DSM-1116)	-	-	-	-	-	-	-	-	-
M. hiemalis	C	B	B	-	D	B	-	-	-
S. aureus ATCC-29213	-	A	-	A	C	-	-	-	-
E. coli tolC3 (DSM-26863)	B	C	-	-	B	-	B	B	A
B. subtilis DSM-10^T	C	B	D	D	E	-	G	H	-
C. albicans DSM-1665	-	-	A	C	A	-	-	-	-
M. smegmatis mc^{2}155	-	C	-	-	B	A	-	D	-

*MIC_{50} value key (µg/mL): A = 640; B = 320; C = 160; D = 80; E = 40; F = 20; G = 10; H = 5.

Active fractions were run on a HR LC-MS system and molecular formulae generated by the Chromeleon MS software were compared to molecular formulae found in the uninoculated broth media. Compounds that were present in the active fractions and absent from the uninoculated broth media were then compared to the Dictionary of Natural Products database.[61] This was done to identify potentially novel NPs and to prioritise which fractions to select for further purification. Table 3.2 lists the molecular formulae obtained that are potentially novel, as they elicited no hits on the Dictionary of Natural Products database and did not match any compounds from the growth media.[61] As time was limited, only the *S. africanus* CPJVR-H^T NL2 culture $CHCl_3$ extract, containing the suggested molecular formulae of $C_{10}H_{14}O_{14}$, $C_{10}H_{12}O_{13}$ and $C_{12}H_8N_6O_2$, and the *S. pharetrae* CZA14^T SHG culture EtOAc extract, containing the suggested molecular formula of $C_{11}H_{16}O_3$, were further purified by semi-preparative HPLC. The HPLC purification of the *S. africanus* CPJVR-H^T NL2 culture $CHCl_3$ extract yielded 15 fractions, none of which exhibited biological activity when rescreened against the test panel. Hence, it was hypothesised that the activity previously observed for the $CHCl_3$ extract was due to the combined effect of the compounds present in the crude mixture. The HPLC purification of the *S. pharetrae* CZA14^T SHG culture EtOAc extract yielded 14 fractions, only one fraction of which showed biological activity when rescreened against the test panel (against *B. subtilis*, MIC_{50} = 16 µg/mL). This active fraction was investigated by HR LC-MS and yielded an *m/z* = 1245.3178 (ESI+) and a suggested molecular formula of $C_{58}H_{52}N_8O_{24}$. This molecule is also potentially novel. Unfortunately, no fractions purified by HPLC contained sufficient material to allow structure elucidation by NMR.

Table 3.2: Suggested molecular formulae of potentially novel bioactive compounds from extracts of *S. speibonae* PK-Blue^T, *S. africanus* CPJVR-H^T and *S. pharetrae* CZA14^T.

Source	Growth medium	Solvent fraction	Suggested molecular formula
S. speibonae PK-Blue^T	NL2	$CHCl_3$	$C_{18}H_{14}N_2O_4$
		EtOAc	$C_{11}H_6N_6O_3$
S. africanus CPJVR-H^T	NL2	$CHCl_3$	$C_{10}H_{14}O_{14}$
		$CHCl_3$	$C_{10}H_{12}O_{13}$
		$CHCl_3$	$C_{12}H_8N_6O_2$
S. pharetrae CZA14^T	SHG	$CHCl_3$	$C_{14}H_{24}O_3$
		$CHCl_3$	$C_{14}H_{26}O_4$
		$CHCl_3$	$C_{18}H_{34}O_3$
		$CHCl_3$	$C_{18}H_{36}O_4$
		EtOAc	$C_{11}H_{16}O_3$
		EtOAc	$C_{58}H_{52}N_8O_{24}$

3.3 Biological activity against *M. tuberculosis* H37Rv^T

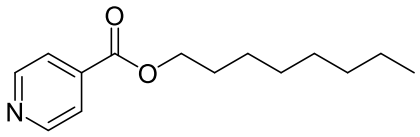
All *S. speibonae* PK-Blue^T, *S. africanus* CPJVR-H^T and *S. pharetrae* CZA14^T crude extracts, semi-purified extracts and purified compounds with activity against *M. aurum* A+ were also tested for activity against *M. tuberculosis* H37Rv^T using the protocol outlined in section 2.3.2. The crude EtOAc extract of ISP 2 growth medium cultures of *S. speibonae* PK-Blue^T exhibited good activity against *M. tuberculosis* H37Rv^T with an MIC_{90} value of 2.0 µg/mL (see Table 3.3). Liquid-liquid partitioning of the *S. speibonae* PK-Blue^T crude EtOAc extract yielded a $CHCl_3$

fraction with an increased biological activity against *M. tuberculosis* H37Rv^T of 1.3 µg/mL. All compounds isolated from *S. speibonae* PK-Blue^T extracts were obtained from $CHCl_3$ fractions.

Table 3.3: *In vitro* activity against *M. tuberculosis* H37Rv^T (MIC_{90}) of crude extracts, semi-purified extracts and pure compounds from *S. speibonae* PK-Blue^T, *S. africanus* CPJVR-H^T and *S. pharetrae* CZA14^T ISP 2 cultures.

Source	Sample	Activity against *M. tuberculosis* H37Rv^T (MIC_{90}) (µg/mL)
S. speibonae PK-Blue^T	Crude EtOAc extract	2.0
	$CHCl_3$ fraction	1.3
	3.1	22.9 (135 µM)
	3.2 and **3.3** mixture	6.7 (26 µM)
	3.4	50
S. africanus CPJVR-H^T	Crude EtOAc extract	>50
	$CHCl_3$ fraction	13.1
S. pharetrae	Crude EtOAc extract	13.0
	$CHCl_3$ fraction	22.0

The alkaloid **3.1** was moderately active against *M. tuberculosis* H37Rv^T with an MIC_{90} value of 135 µM. This was not unexpected, as similar alkaloids have displayed good anti-TB activity.[8] A good example is isoniazid (**1.1**), which is also a pyridine alkaloid and one of the frontline TB chemotherapeutic drugs.[8] Another pyridine alkaloid with good anti-TB activity against *M. tuberculosis* H37Rv^T is octyl isonicotinate (**3.7**) (MIC_{100} = 8 µg/mL).[102]



3.7

The mixture of **3.2** and **3.3** showed strong activity against *M. tuberculosis* H37Rv^T (26 µM). This strong activity was expected, as **3.2** is known to interfere with the hydroxyl group of lipopolysaccharides present in the cell wall of Gram negative bacteria.[96] This disrupts the balance in the membrane lipid structure, damaging cytoplasm membrane flexibility, leading to cell swelling and lysis.[96] Compound **3.3** is also known to possess highly selective antifungal properties with an MIC_{50} value of 5 µg/mL against *C. albicans*.[97] However, **3.3** displayed negligible antibiotic activity against the Gram negative bacterial pathogens *E. coli* and *P. aeruginosa*.[97] Unfortunately, no assumption can be made about which of **3.2** or **3.3** is responsible for the observed anti-TB activity, as this activity could be either one or a combination effect of the two. Compounds **3.2** and **3.3** should ideally be separated and tested individually for antimycobacterial activity.

The isoflavone **3.4** showed weak activity against *M. tuberculosis* H37Rv^T (MIC_{90} = 195 µM). However, the oestrogenic potential of **3.4** in stimulating the growth of oestrogen-dependent breast cancer (MCF-7) has been investigated and was found to stimulate MCF-7 cell growth at concentrations of 0.001 µM *in vitro* and 50 µM *in vivo* in ovariectomised athymic mice.[103] This stimulation of MCF-7 cells by dietary **3.4** resulted in a slight, but significant, stimulation of tumour growth.[103] Therefore, although **3.4** is produced naturally in the human gut through the metabolism of legumes by intestinal bacteria, the effect of high doses of **3.4** on human cancer cell lines requires investigation.[99] This possible side-effect makes further research on **3.4** as a potential anti-tubercular drug less attractive.

Therefore, all the compounds isolated from *S. speibonae* PK-Blue^T extracts displayed activity against *M. tuberculosis* H37Rv^T. However, these isolated compounds were all significantly less potent than the first-line drug, rifampicin (**1.2**). Rifampicin was used as a control in the *M. tuberculosis* H37Rv^T bioassay and yielded an MIC_{90} value of 0.043 µM.

The crude EtOAc extract of ISP 2 growth medium cultures of *S. africanus* CPJVR-H^T showed poor activity against *M. tuberculosis* H37Rv^T (MIC_{90} >50 µg/mL) while improved activity was observed in the $CHCl_3$ fraction of this extract following liquid-liquid partitioning (MIC_{90} =13.1

µg/mL). A reason for the observed increase in bioactivity of the $CHCl_3$ fraction is that the bioactive compound(s) is/are present in greater concentration(s) than in the parent fraction. Conversely, moderate activity against *M. tuberculosis* H37Rv^T was observed in the crude EtOAc extract of *S. pharetrae* CZA14^T ISP 2 growth medium cultures (MIC_{90} = 13.0 µg/mL), with slightly poorer activity in the $CHCl_3$ fraction (MIC_{90} = 22.0 µg/mL). This decrease in activity could be due to the separation of compounds that displayed bioactivity through a combination effect in the parent fraction.

3.4 Conclusions and future work

Three South African actinobacteria, *S. speibonae* PK-Blue^T, *S. africanus* CPJVR-H^T and *S. pharetrae* CZA14^T were found to produce compounds with antimycobacterial activity. Crude extracts and semi-purified extracts from cultures of all three bacteria showed activity against the MTB analogue *M. aurum* A+ and against *M. tuberculosis* H37Rv^T. *In vitro* bioassays for anti-TB activity against *M. tuberculosis* H37Rv^T for crude EtOAc extracts and semi-purified extracts of the three actinobacteria showed *S. speibonae* PK-Blue^T to have the strongest activity. These bioassay results suggested that *S. speibonae* PK-Blue^T is the most promising of the three actinobacteria for further anti-TB research.

While the re-isolation of the antimycobacterial compound PK-B was not successful, it has been shown that *S. speibonae* PK-Blue^T extracts contain other compounds with antimycobacterial activity. Compound **3.1**, a 1:1 mixture of **3.2** and **3.3**, and **3.4** all showed antimycobacterial activity against *M. aurum* A+ by bioautography, and therefore provide evidence that the bioassay-guided isolation of compounds from *S. speibonae* PK-Blue^T extracts was successful. More promisingly, and supporting the strategy of screening against *M. aurum* A+ as an analogue of MTB, the compounds isolated – i.e., **3.1**, a 1:1 mixture of **3.2** and **3.3**, and **3.4** – all showed activity against *M. tuberculosis* H37Rv^T with MIC_{90} values of 135 µM, 26 µM and 195 µM, respectively.

Streptomyces speibonae PK-Blue^T was therefore successfully investigated in the context of NP anti-TB research. However, there were two main aspects of the isolation process that could be improved. The masses of crude extracts obtained from growth cultures were low and

crude extracts proved to be complex mixtures of compounds. This paucity of biomass hampered the isolation of novel NPs. Access to large-scale fermenters could potentially have resolved this issue, although there is also the possibility that the same compounds would not be produced by *S. speibonae* PK-Blue^T under the conditions of larger-scale cultivation. This is certainly a possibility, as this study has shown that compound production by all three actinobacteria was dependent on the growth culture conditions. It was demonstrated that antimycobacterial activity of *S. speibonae* PK-Blue^T extracts was dependent on culture volume, culture duration and growth medium, with best activity against *M. aurum* A+ observed in ISP 2 cultures with prolonged incubation times. MIC_{50} bioassay results against a panel of test organisms also established that the growth medium used in bacterial cultivation of all three actinobacteria did affect the degree and specificity of biological activity, although the cause of this phenomenon is unknown. Therefore, it could be worthwhile to experiment further with culture condition parameters – i.e., culture volume, culture duration and growth medium – in the cultivation of *S. speibonae* PK-Blue^T, as this could prompt production of compound PK-B and allow the re-isolation of this compound.

The second aspect of the implemented isolation process that could be improved is easy access to HR LC-MS. Monitoring of all stages of the isolation process by HR LC-MS would enable a more thorough dereplication of extracts. HR LC-MS, as a more sensitive technique than LR LC-MS, could be used to more reliably identify known compounds and compounds originating from culture growth media. This information would save time and allow concentration of efforts to where they will be most constructive in achieving the aims of the research. This method of dereplication was successfully implemented during a research visit to HIPS and resulted in the identification of culture conditions and suggested molecular formulae of potentially novel, bioactive NPs from *S. speibonae* PK-Blue^T, *S. africanus* CPJVR-H^T and *S. pharetrae* CZA14^T. In future, these compounds identified through HR LC-MS can be investigated further by re-isolation from upscaled growth cultures. Upscaling of these growth cultures could facilitate the isolation of pure, novel, bioactive compounds with sufficient biomass for NMR analysis.

Chapter 4: Experimental

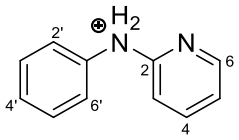
4.1 Extraction and isolation of compounds from *S. speibonae* PK-Blue^T cultures

The South African actinomycete *S. speibonae* PK-Blue^T was cultured using ISP 2 growth medium on a small scale (10 x 100 mL) and large scale (1 L in a single Erlenmeyer flask). Culture broths were extracted with EtOAc (3 x 30% of the culture volume) and extracts subjected to liquid-liquid partitioning and dereplication by LR LC-MS. Fractions with activity against *M. aurum* A+ by bioautography were further purified by benchtop column chromatography and HPLC and purified fractions rescreened against *M. aurum* A+. The structures of pure compounds with activity against *M. aurum* A+ were determined using LC-MS, NMR and GC-MS analysis.

A 14 d small-scale *S. speibonae* PK-Blue^T culture yielded a bioactive liquid-liquid partitioned chloroform fraction. The bioactive compound *N*-phenylpyridin-2-aminium (**3.1**, 2.5 mg) was isolated by RP-HPLC (40 - 100% acetonitrile, 0.1% TFA in water) from this fraction.

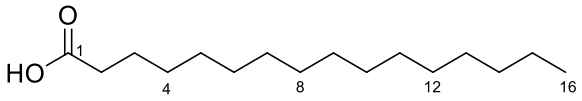
Six 14 d *S. speibonae* PK-Blue^T cultures (small- and large-scale) yielded a bioactive liquid-liquid partitioned chloroform fraction. This fraction was semi-purified by RP-HPLC (40 - 100% acetonitrile, 0.1% TFA in water) and the 100% acetonitrile fractions combined and purified further by RP-HPLC (20 - 100% acetonitrile). A fraction with activity against *M. aurum* A+ by bioautography contained a 1:1 mixture of the fatty acids *n*-hexadecanoic acid (**3.2**) and 14-methylpentadecanoic acid (**3.3**) (mixture total 2.2 mg). These carboxylic acids were derivatised to FAMEs for GC-MS analysis.

Six 14 d *S. speibonae* PK-Blue^T cultures (small- and large-scale) yielded a bioactive liquid-liquid partitioned chloroform fraction. This fraction was semi-purified by RP-HPLC (40 - 100% acetonitrile, 0.1% TFA in water) and the 40% acetonitrile fractions combined and purified further by benchtop column chromatography (isocratic 50% hexane/ethyl acetate and fractions rescreened for activity against *M. aurum* A+. The second of seven fractions obtained from benchtop column chromatography showed activity against *M. aurum* A+ was further purified by RP-HPLC (2 - 100% acetonitrile, 0.1% FA) to yield 7-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (**3.4**, 0.6 mg).



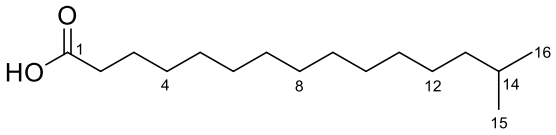
3.1

***N*-Phenylpyridin-2-aminium (3.1)** : yellow oil; UV (MeOH) ε_{265} = 600.44, ε_{310} = 437.58; ^{1}H NMR (MeOH-d4, 600 MHz) δ 7.99 (1H, dd, *J* = 7.9 Hz, H-4), 7.87 (1H, d, *J* = 6.1 Hz, H-6), 7.52 (2H, dd, *J* = 7.5 Hz, H-3', H-5'), 7.39 (2H, d, *J* = 7.7 Hz, H-2', H-6'), 7.37 (1H, m, H-4'), 7.17 (1H, d, *J* = 9.1 Hz, H-3), 7.01 (1H, dd, *J* = 6.6 Hz, H-5); ^{13}C NMR (MeOH-d4, 150 MHz) δ 153.8 (q_c, C-2), 145.4 (CH, C-4), 138.0 (CH, C-6), 137.0 (q_c, C-1'), 131.5 (2 x CH, C-3', C-5'), 128.5 (CH, C-4'), 125.4 (2 x CH, C-2', C-6'), 115.2 (CH, C-5), 114.8 (CH, C-3); HR-MS (ESI+) *m/z* 171.0918 (calcd for $C_{11}H_{11}N_2$ [M], 171.0922).



3.2

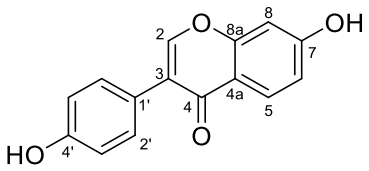
***N*-Hexadecanoic acid (3.2)[96]** : yellow oil; ^{1}H NMR (MeOH-d4, 600 MHz) δ 2.22 (2H, t, *J* = 7.6 Hz, H_2-2), 1.58 (2H, m, H_2-3), 1.30 (2H, m, H_2-4), 1.29 (2H, m, H_2-15), 1.27 (20H, m, H_2-5 – H_2-14), 0.86 (3H, m, H_3-16); ^{13}C NMR (MeOH-d4, 150 MHz) δ 177.7 (q_c, C-1), 35.0 (CH_2, C-2), 26.1 (CH_2, C-3), 30.2 – 31.1 (11 x CH_2, C-4 – C-14), 23.6 (CH_2, C-15), 14.4 (CH_3, C-16); Hexadecanoic acid methyl ester: GC-EIMS: m/z (rel. int.) 270 [M^+] (15), 239 (12), 227 (16), 143 (25), 83 (74), 77 (100), 55 (32), 43 (33).



3.3

14-Methylpentadecanoic acid (3.3)[97] : yellow oil; ^{1}H NMR (MeOH-d4, 600 MHz) δ 2.22 (2H, t, *J* = 7.6 Hz, H_2-2), 1.58 (2H, m, H_2-3), 1.51 (1H, m, H-14), 1.30 (2H, m, H_2-4), 1.29 (2H, m, H_2-

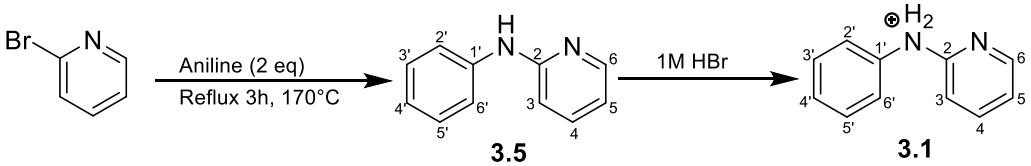
12),1.27 (14H, m, H_2-5 – H_2-11), 1.15 (2H, m, H_2-13), 0.88 (6H, m, H_3-15, H_3-16); ^{13}C NMR (MeOH-d4, 150 MHz) δ 177.7 (q_c, C-1), 40.2 (CH_2, C-13) 35.0 (CH_2, C-2), 26.1 (CH_2, C-3), 30.2 – 31.1 (8 x CH_2, C-4 – C-11), 30.2 (CH_2, C-14), 28.5 (CH_2, C-12), 23.0 (2 x CH_3, C-15, C-16); 14-Methylpentadecanoic acid methyl ester: GC-EIMS: m/z (rel. int.) 270 [M^+] (15), 239 (12), 227 (16), 143 (25), 83 (74), 77 (100), 55 (32), 43 (33).



3.4

7-Hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (3.4)[100] : white solid; ^{1}H NMR (MeOH-d4, 600 MHz) δ 8.05 (1H, s, H-2), 7.97 (1H, d, *J* = 9.0 Hz, H-5), 7.35 (2H, d, *J* = 8.9 Hz, H-2', H-6'), 6.83 (2H, d, *J* = 8.7 Hz, H-3', H-5'), 6.83 (1H, d, *J* = 8.7 Hz, H-6), 6.69 (1H, d, *J* = 2.3 Hz, H-8);); ^{13}C NMR (MeOH-d4, 150 MHz) δ 178.2 (q_c, C-4), 169.7 (q_c, C-7), 160.5 (q_c, C-8a), 158.6 (q_c, C-4'), 154.2 (CH, C-2), 131.5 (2 x CH, C-2', C-6'), 128.0 (CH, C-5), 125.6 (q_c, C-1'), 124.7 (q_c, C-3), 118.8 (q_c, C-4a), 118.7 (CH, C-6), 116.2 (2 x CH, C-3', C-5'), 103.7 (CH, C-8); LR-MS (ESI+) *m/z* 255.0 (calcd for $C_{15}H_{11}O_4$ [M^+], 255.0657).

4.2 Synthesis of 3.1 and *N*-phenylpyridin-2-amine (3.5)



3.5 **3.1**

Aniline (0.44 mL, 4.8 mmol, 2 eq) was added to 2-bromopyridine (0.22 mL, 2.4 mmol) and refluxed at 170°C for 3 h with constant stirring and then cooled. The reaction mixture was dissolved in 1M NaOH (30 mL) and partitioned with ethyl acetate (3 x 30 mL). The ethyl acetate layer was washed with sat. $NaHCO_3$ (30 mL) and brine (30 mL), dried over $MgSO_4$ and

concentrated *in vacuo* to yield a grey powder.[104] The crude grey powder was purified by benchtop column chromatography (gradient 2:1 hexane/dichloromethane to 100% dichloromethane) to yield *N*-phenylpyridin-2-amine (**3.5**, 67 mg) as a purple/grey solid.

Compound **3.5** (20 mg) was dissolved in chloroform (10 mL), treated with 1M HCl (2 x 10 mL), shaken, partitioned with H_2O (20 mL) and the aqueous layer concentrated *in vacuo* to yield **3.1** (22 mg) as a yellow oil. No further purification was necessary.

***N*-phenylpyridin-2-amine (3.5)**[94] : purple/grey solid; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.05 (1H, d, *J* = 5 Hz, H-6), 7.51 (1H, dd, *J* = 7.9 Hz, H-4), 7.43 (2H, d, H-2', H-6'), 7.26 (2H, dd, H-3', H-5'), 6.95 (1H, dd, H-4'), 6.82 (1H, d, *J* = 8.4 Hz, H-3), 6.7 (1H, dd, *J* = 6.2 Hz, H-5); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 157.8 (q_c, C-2), 148.2 (CH, C-6), 142.5 (q_c, C-1'), 138.9 (CH, C-4), 129.8 (2 x CH, C-3', C-5'), 122.9 (CH, C-4'), 120.7 (2 x CH, C-2', C-6'), 115.5 (CH, C-5), 111.4 (CH, C-3); LR-MS (ESI+) *m/z* 171.1 (calcd for $C_{11}H_{11}N_2$ [M^+], 171.0922).

***N*-phenylpyridin-2-aminium (3.1**, synthesised**)**[94] : yellow oil; ^{1}H NMR (MeOH-d4, 600 MHz) δ 8.05 (1H, dd, *J* = 8.2 Hz, H-4), 7.89 (1H, d, *J* = 6.2 Hz, H-6), 7.54 (2H, dd, *J* = 7.3 Hz, H-3', H-5'), 7.41 (1H, m, H-4'), 7.40 (2H, d, *J* = 8.1 Hz, H-2', H-6'), 7.25 (1H, d, *J* = 9.2 Hz, H-3), 7.05 (1H, dd, *J* = 6.9 Hz, H-5); ^{13}C NMR (MeOH-d4, 150 MHz) δ 153.3 (q_c, C-2), 146.1 (CH, C-4), 137.1 (CH, C-6), 136.4 (q_c, C-1'), 131.6 (2 x CH, C-3', C-5'), 129.0 (CH, C-4'), 125.8 (2 x CH, C-2', C-6'), 115.3 (CH, C-5), 115.1 (CH, C-3); LR-MS (ESI+) *m/z* 171.1 (calcd for $C_{11}H_{11}N_2$ [M], 171.0922).

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Appendix 1: MS spectra

Contents

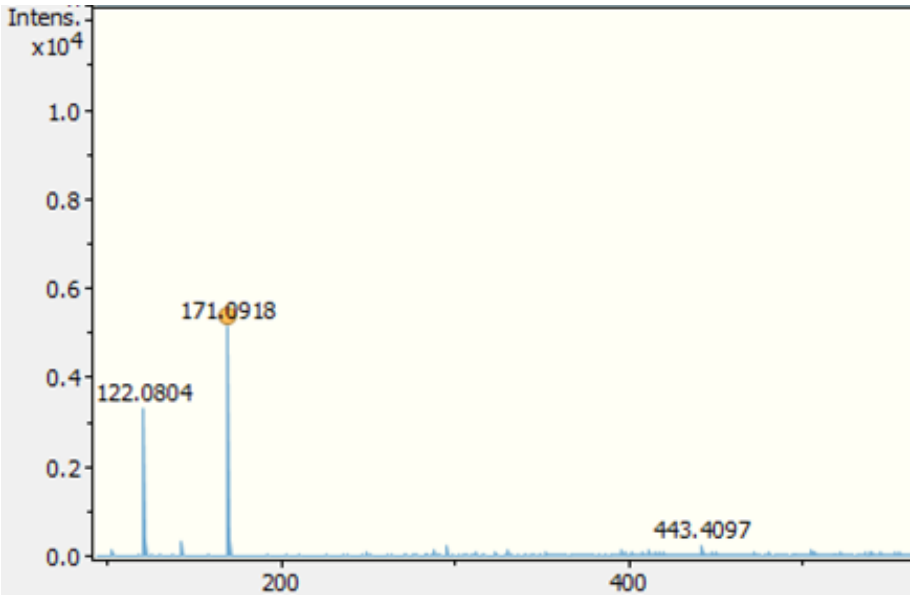

Figure A1.1: HR-MS spectrum (ESI+) of **3.1**.

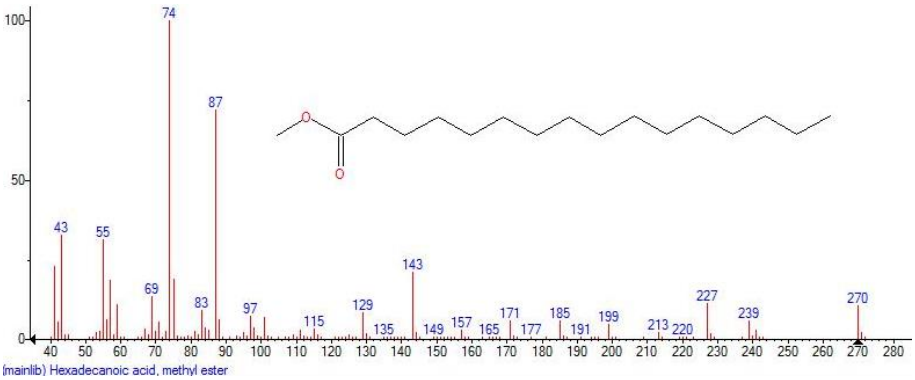

Figure A1.2: GC-MS spectrum (EI+) of **3.2**.

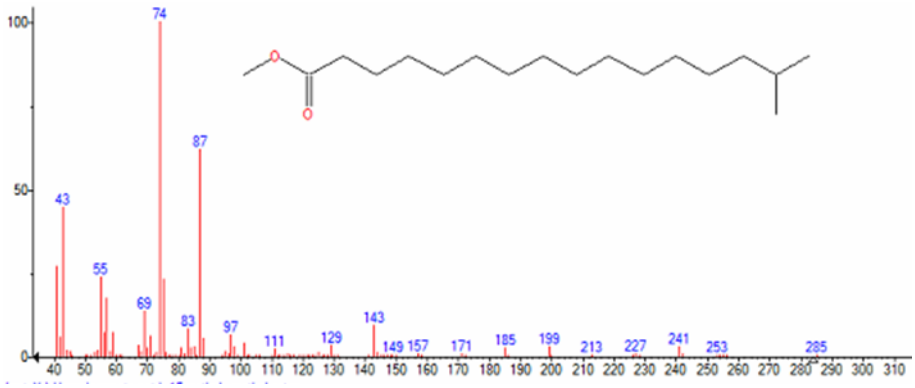

Figure A1.3: GC-MS spectrum (EI+) of **3.3**.

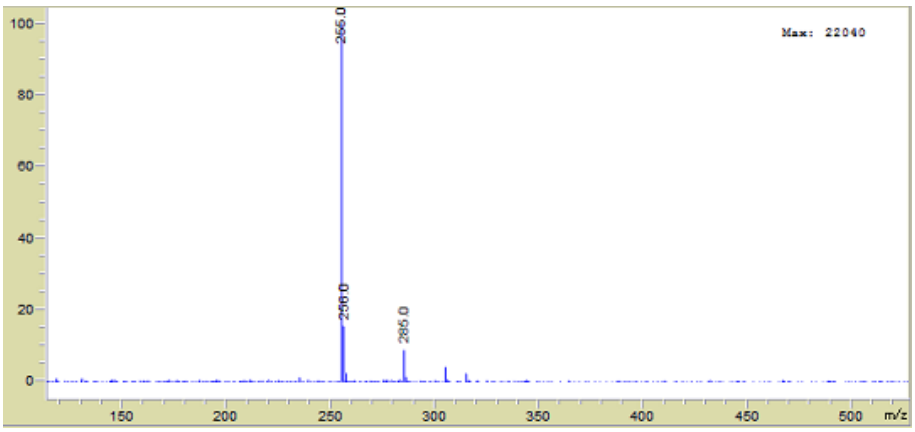

Figure A1.4: LR-MS spectrum (ESI+) of **3.4**.

Appendix 2: NMR spectra

Contents

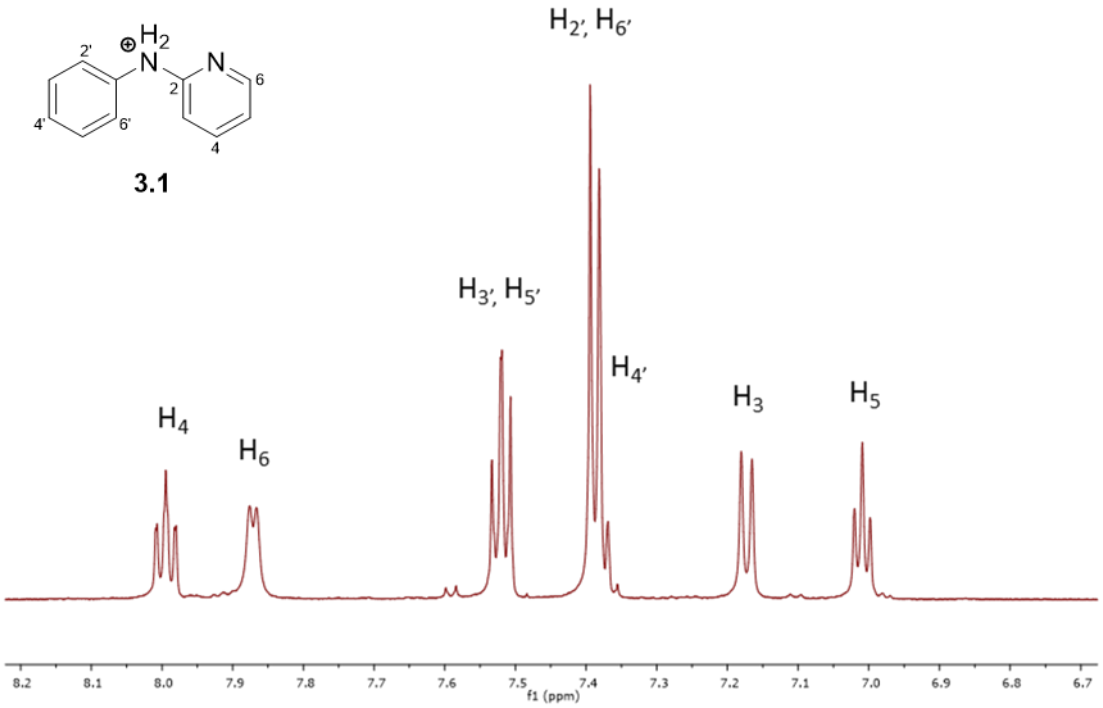

Figure A2.1: ^{1}H NMR spectrum (MeOH-d4, 600 MHz) obtained for **3.1**.

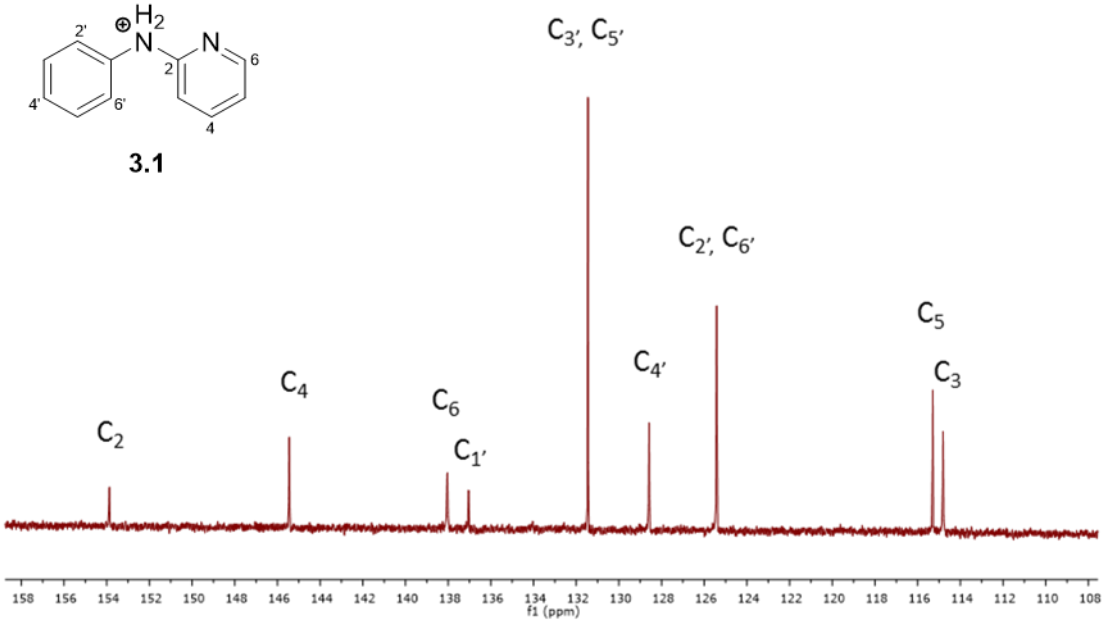

Figure A2.2: ^{13}C NMR spectrum (MeOH-d4, 150 MHz) obtained for **3.1**.

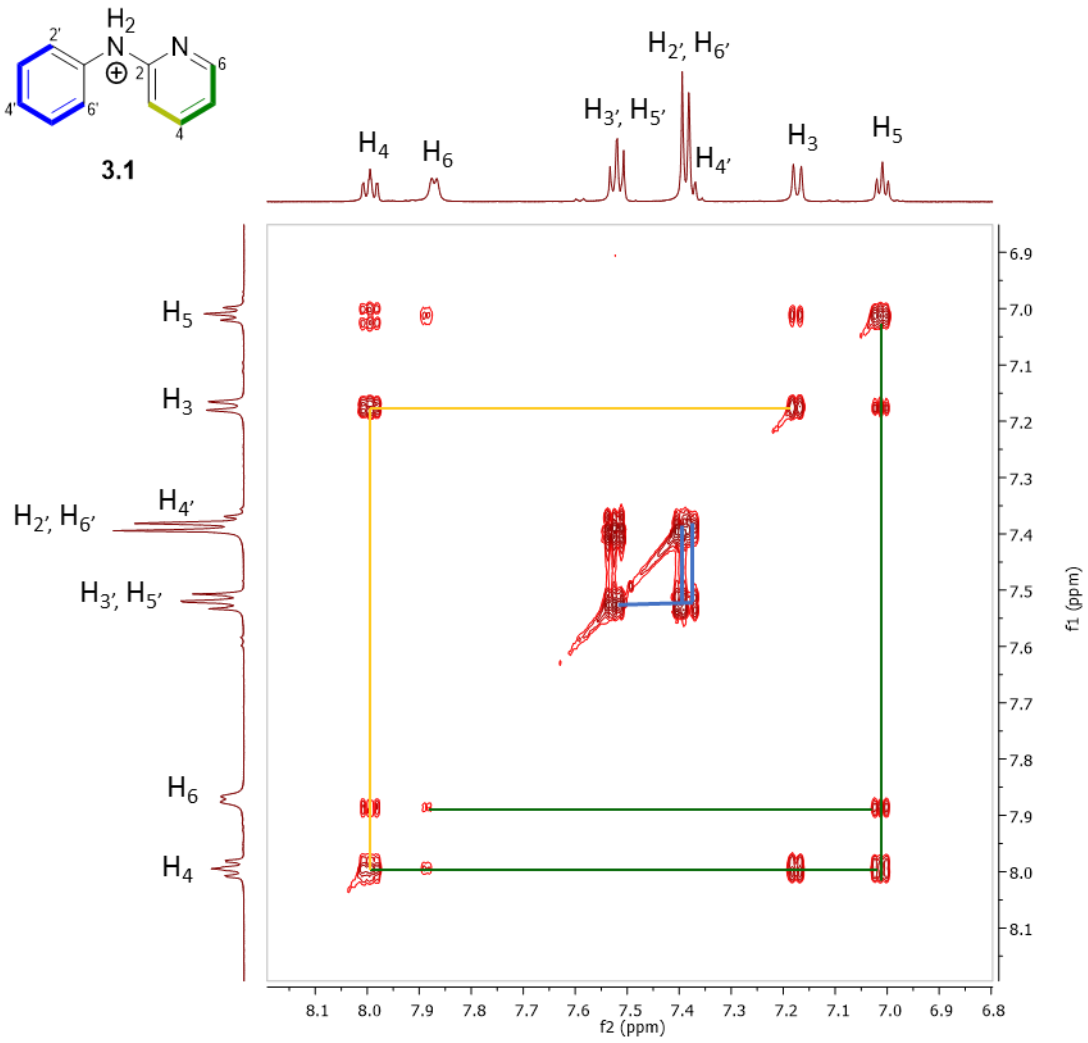

Figure A2.3: COSY NMR spectrum (MeOH-d4, 600 MHz) obtained for **3.1**.

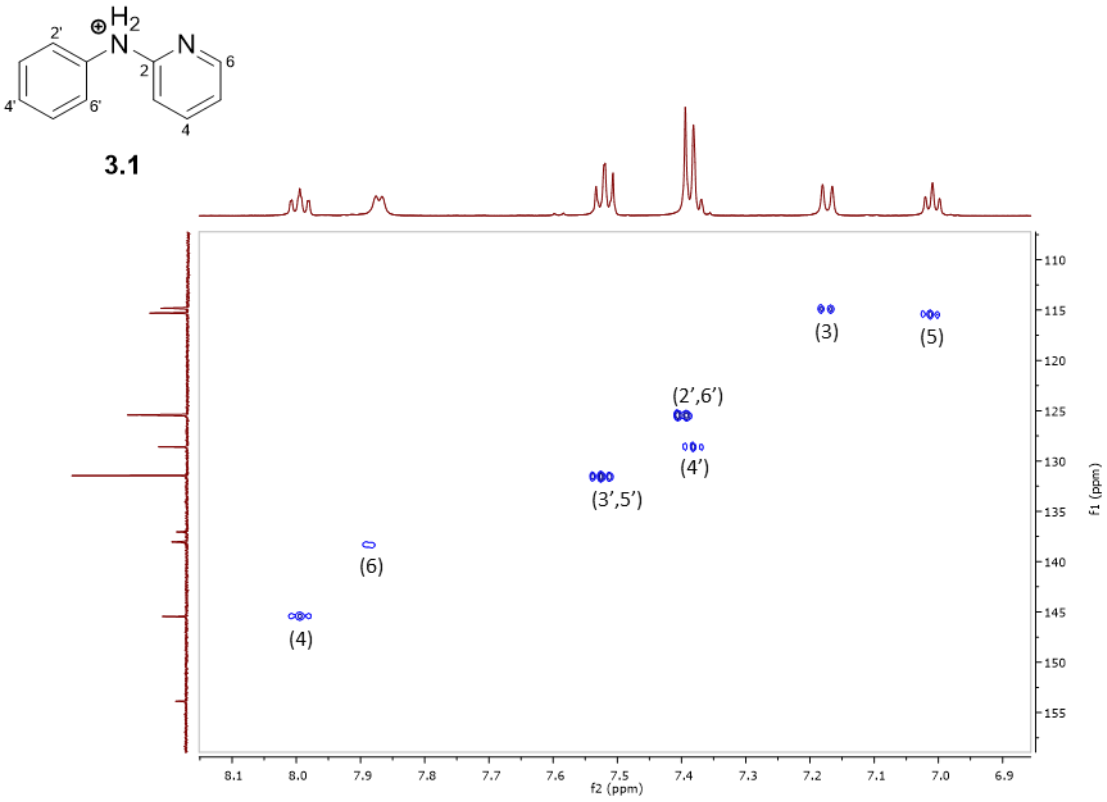

Figure A2.4: HSQC NMR spectrum (MeOH-d4, 600 MHz) obtained for **3.1**.

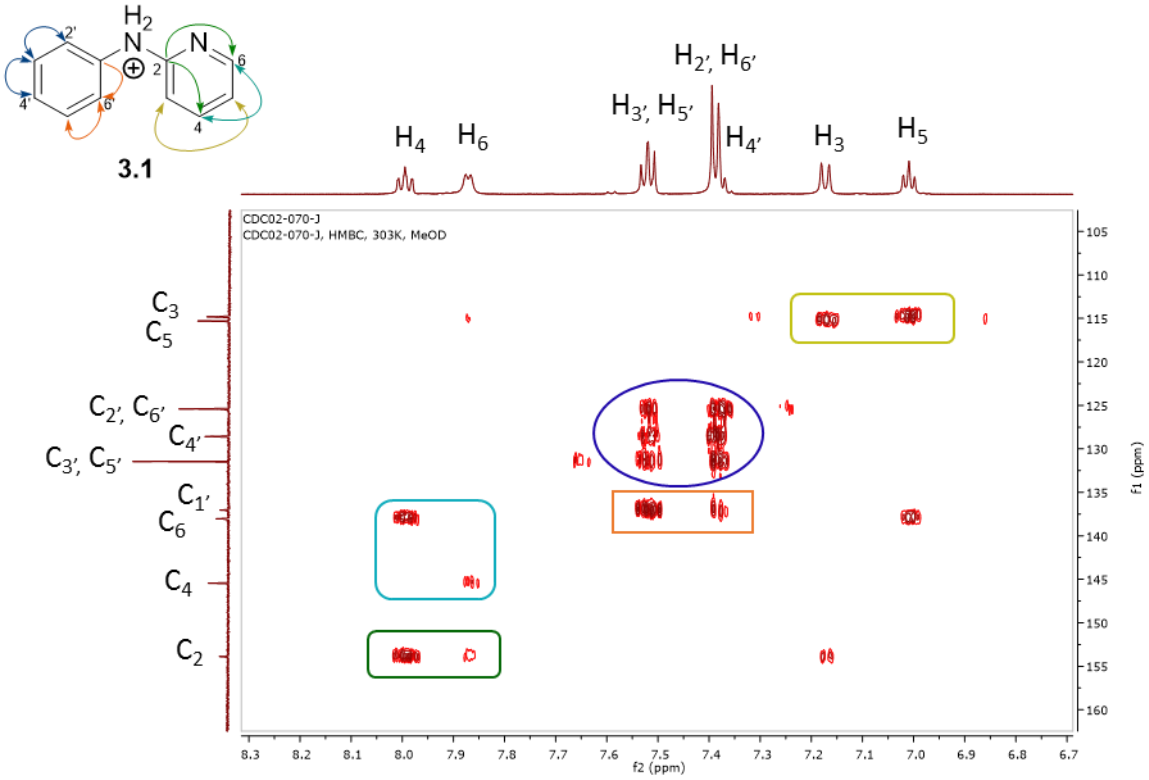

Figure A2.5: HMBC NMR spectrum (MeOH-d4, 600 MHz) obtained for **3.1**.

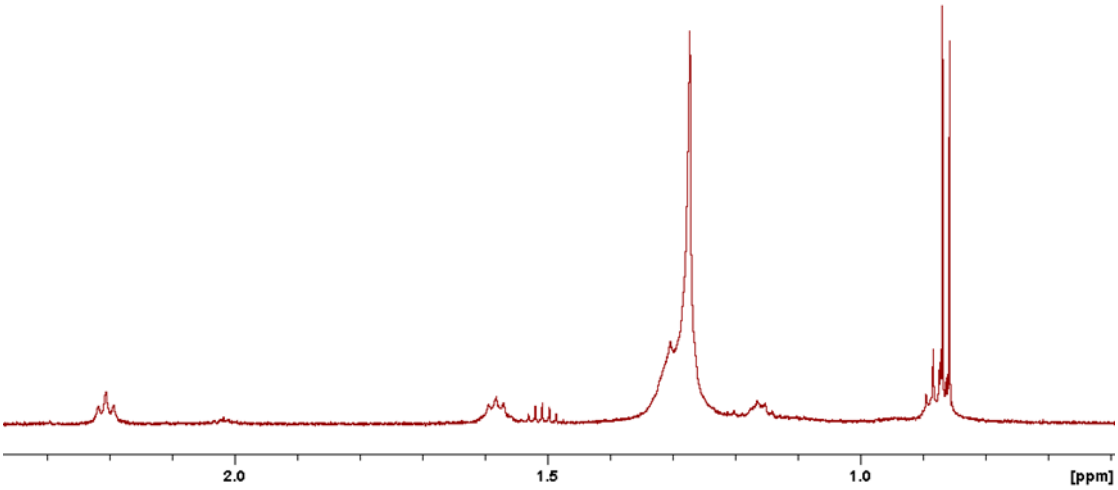

Figure A2.6: ^{1}H NMR spectrum (MeOH-d4, 600 MHz) obtained for a mixture of **3.2** and **3.3**.

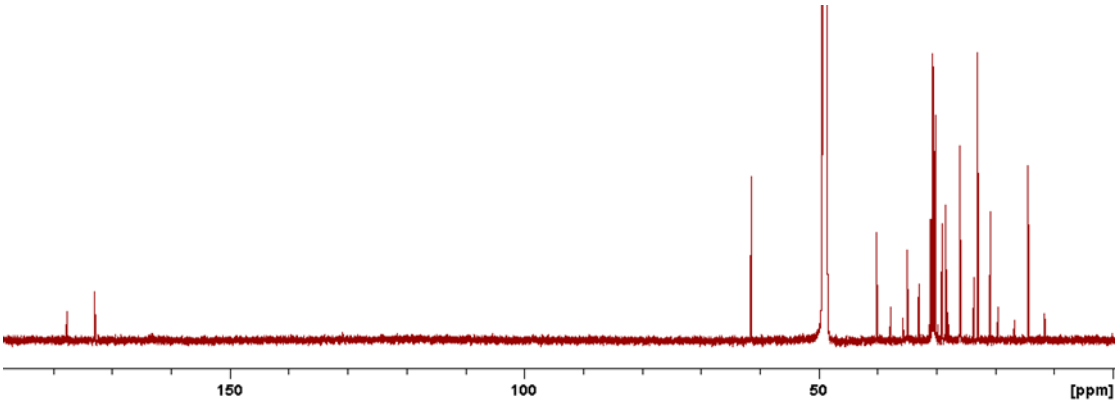

Figure A2.7: ^{13}C NMR spectrum (MeOH-d4, 150 MHz) obtained for a mixture of **3.2** and **3.3**.

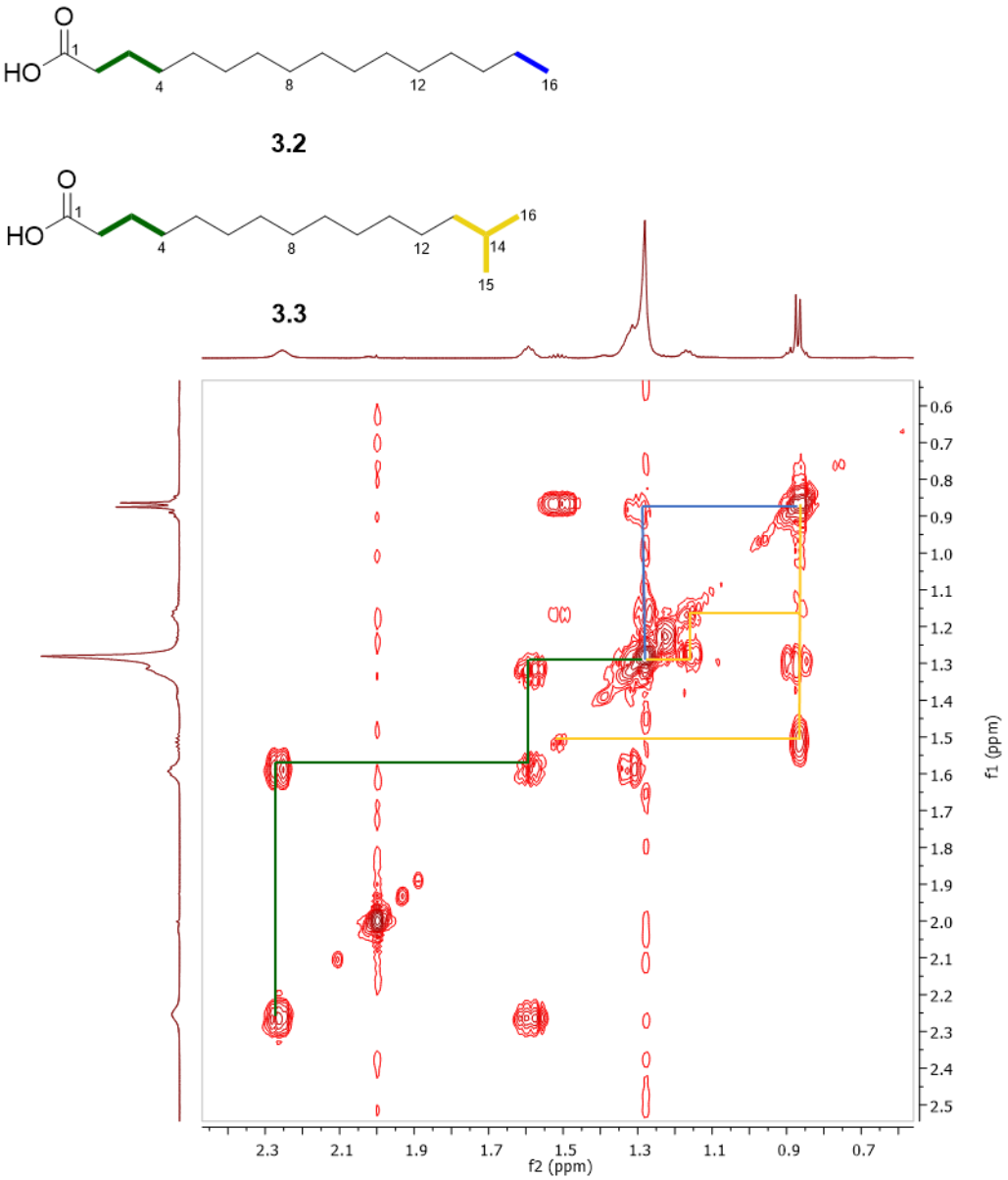

Figure A2.8: COSY NMR spectrum (MeOH-d4, 600 MHz) obtained for a mixture of **3.2** and **3.3**.

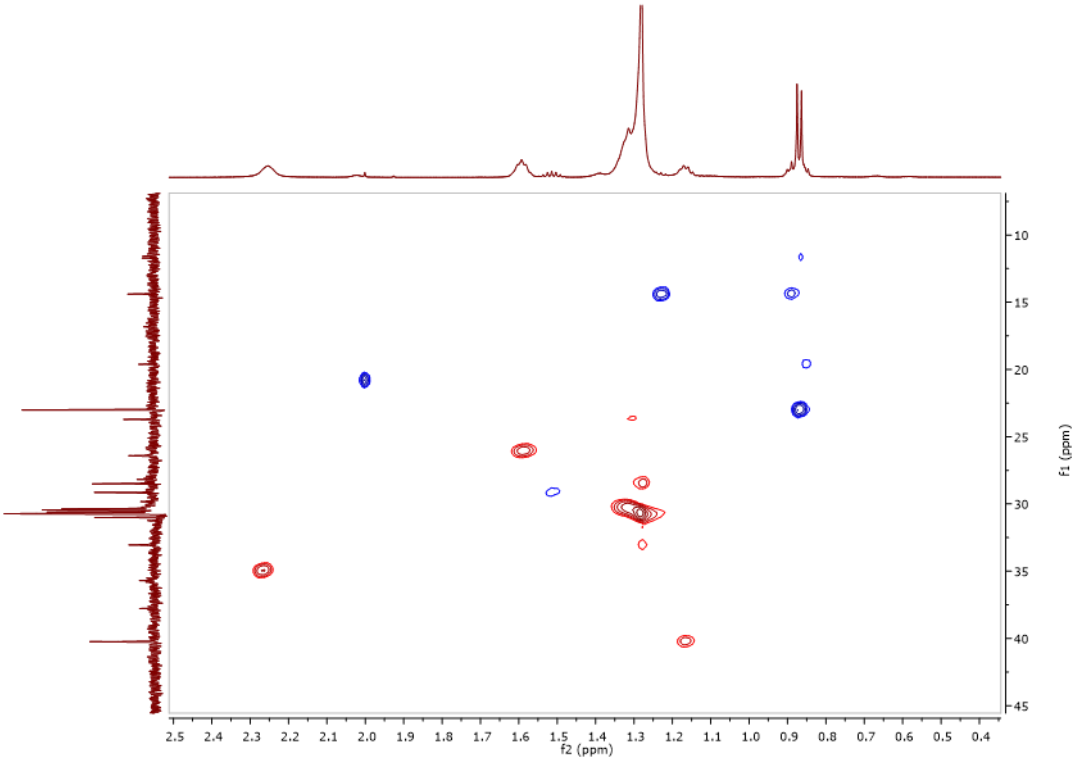

Figure A2.9: HSQC NMR spectrum (MeOH-d4, 600 MHz) obtained for a mixture of **3.2** and **3.3**.

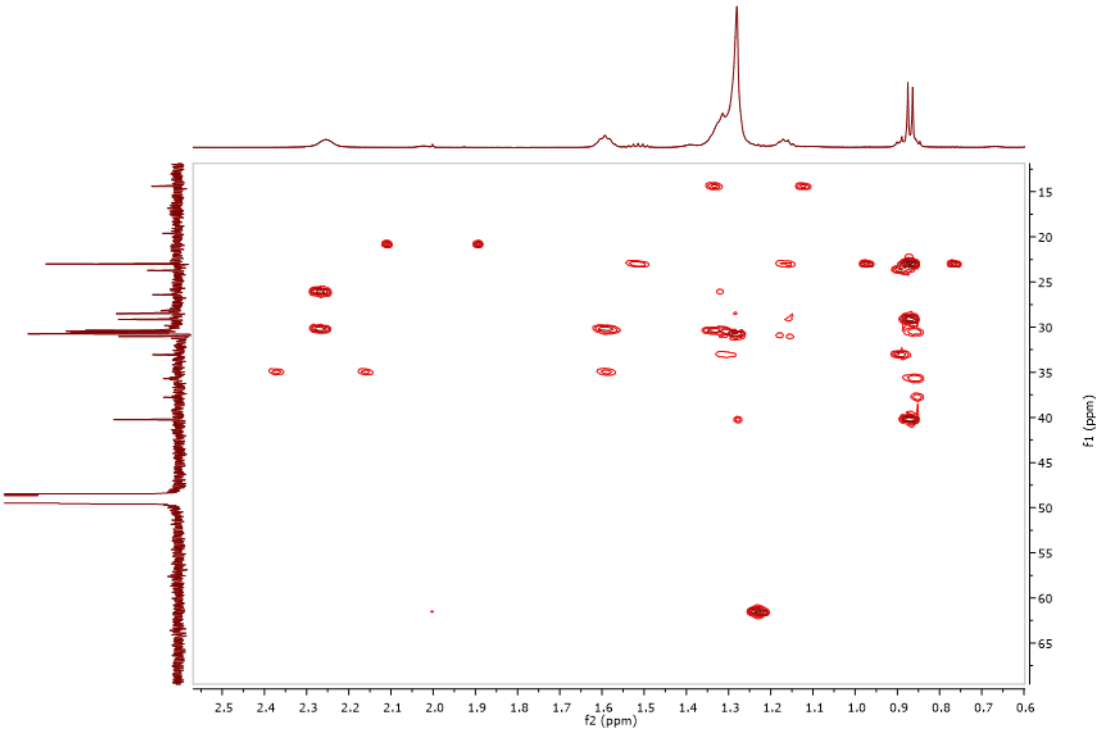

Figure A2.10: HMBC NMR spectrum (MeOH-d4, 600 MHz) obtained for a mixture of **3.2** and **3.3**.

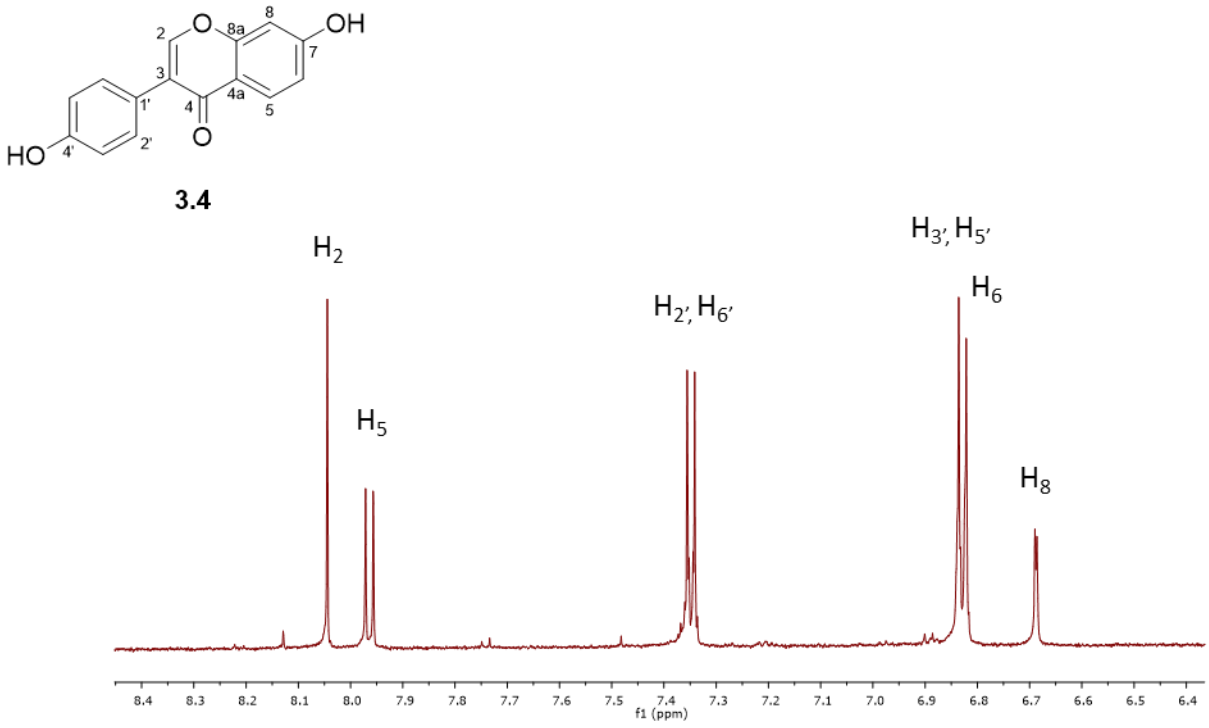

Figure A2.11: ^{1}H NMR spectrum (MeOH-d4, 600 MHz) obtained for **3.4**.

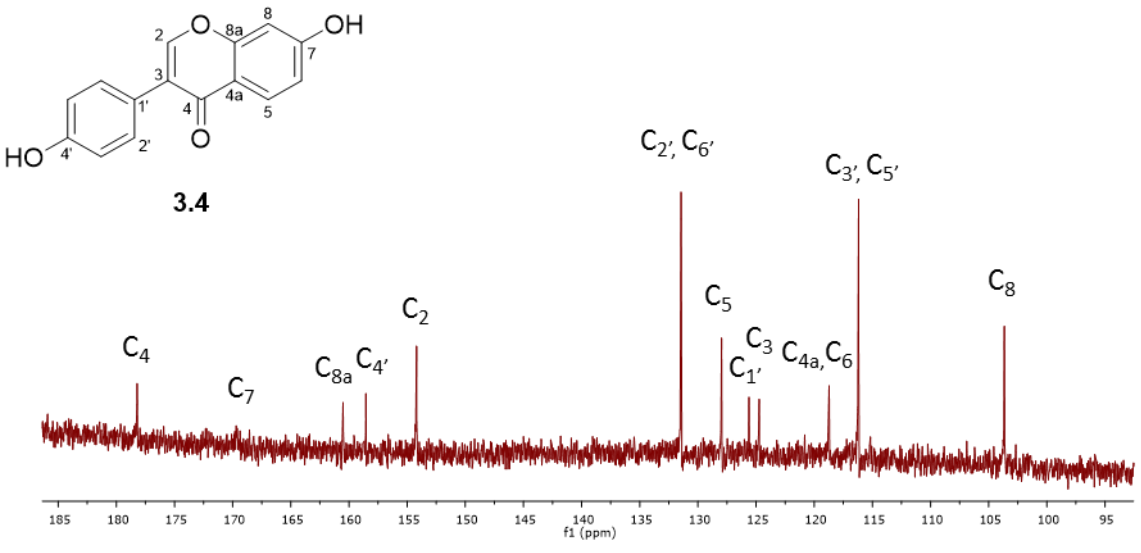

Figure A2.12: ^{13}C NMR spectrum (MeOH-d4, 150 MHz) obtained for **3.4**.

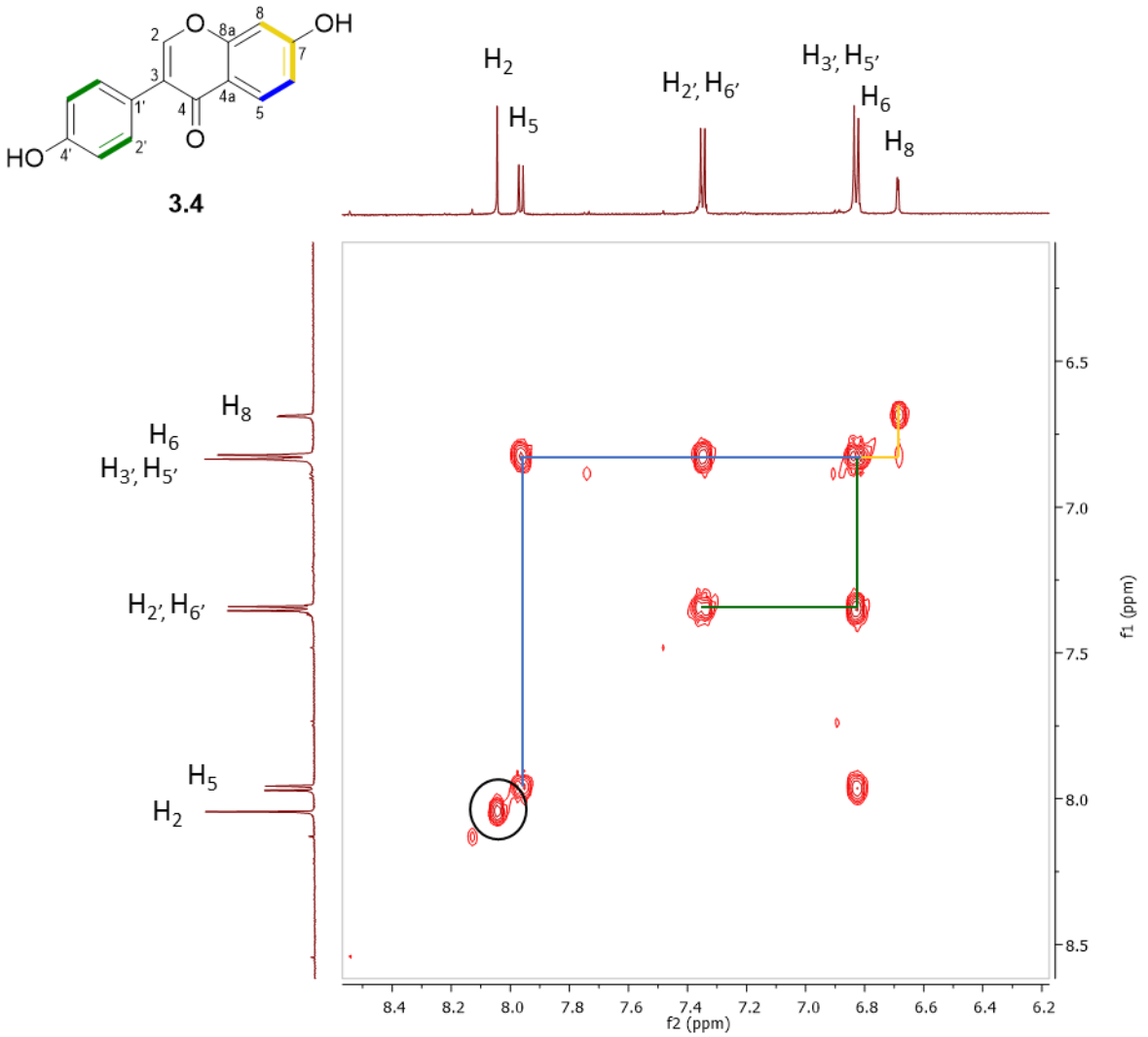

Figure A2.13: COSY NMR spectrum (MeOH-d4, 600 MHz) obtained for **3.4**.

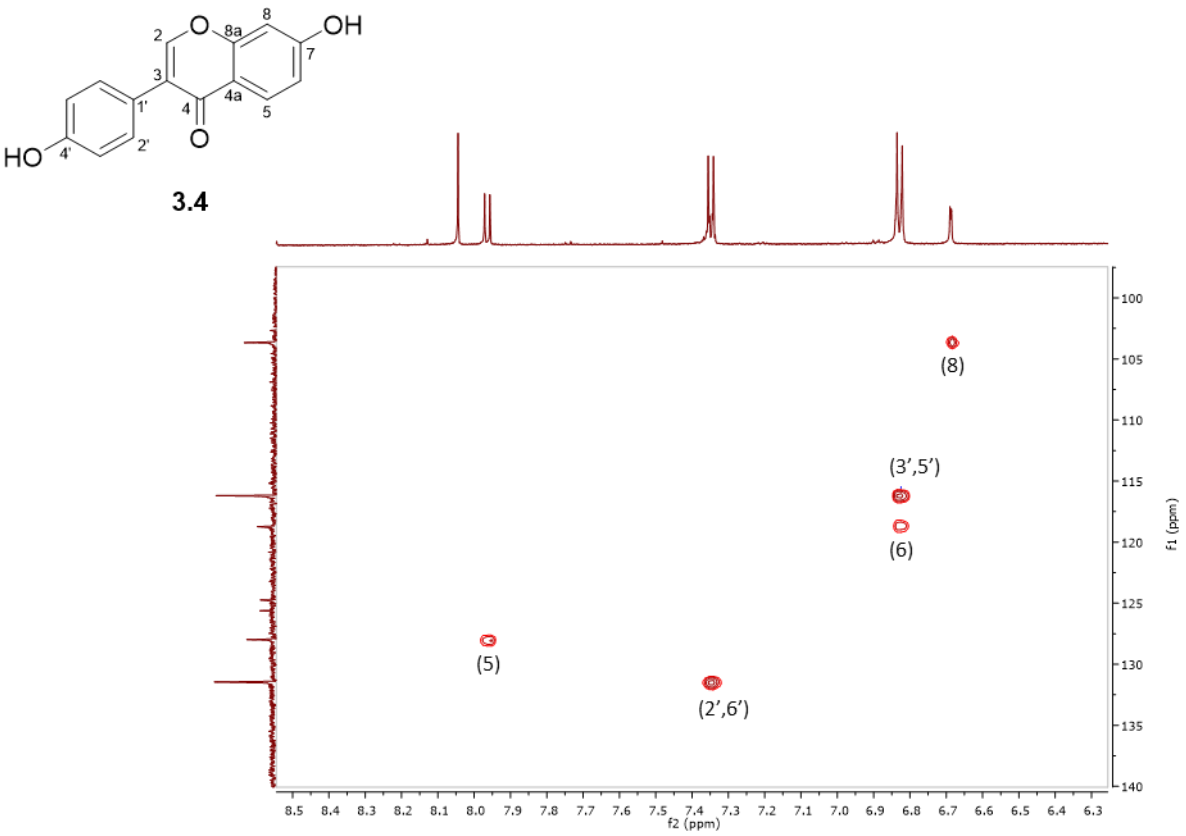

Figure A2.14: HSQC NMR spectrum (MeOH-d4, 600 MHz) obtained for **3.4**.

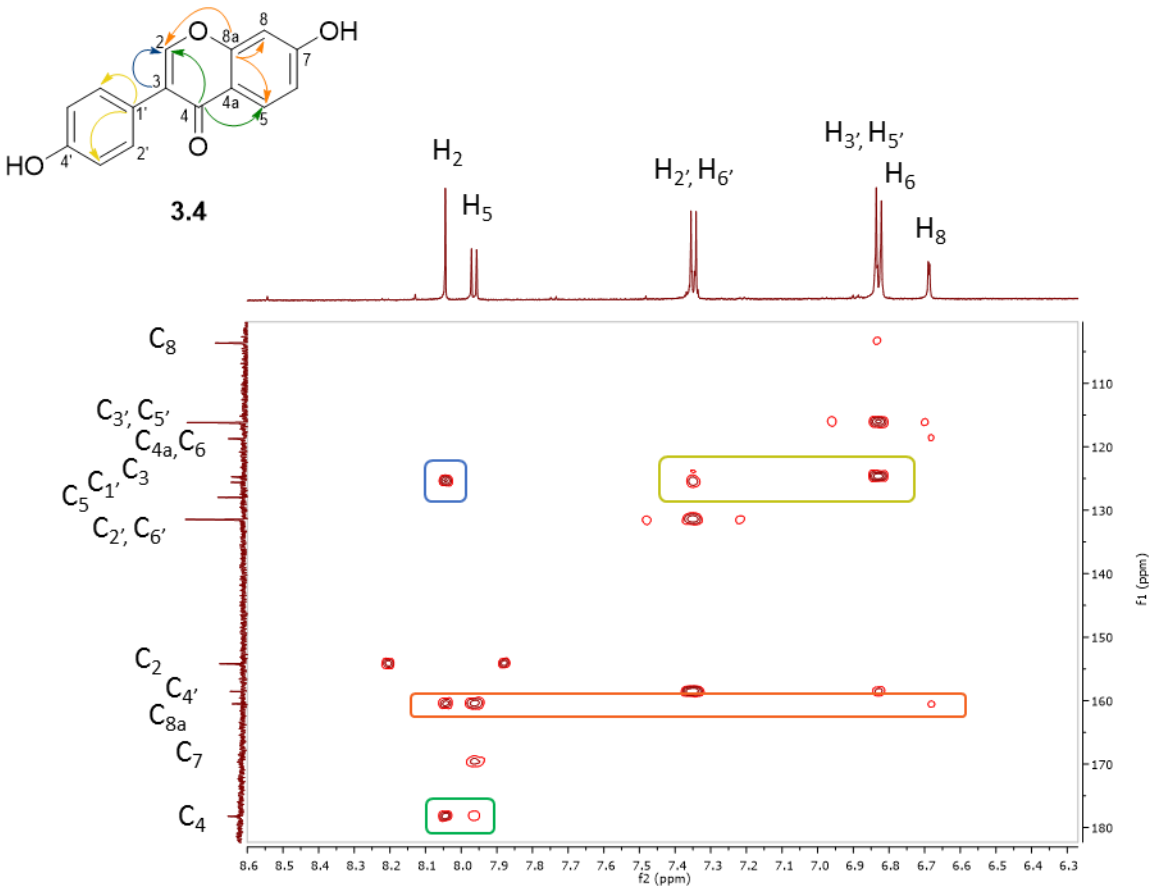

Figure A2.15: HMBC NMR spectrum (MeOH-d4, 600 MHz) obtained for **3.4**.

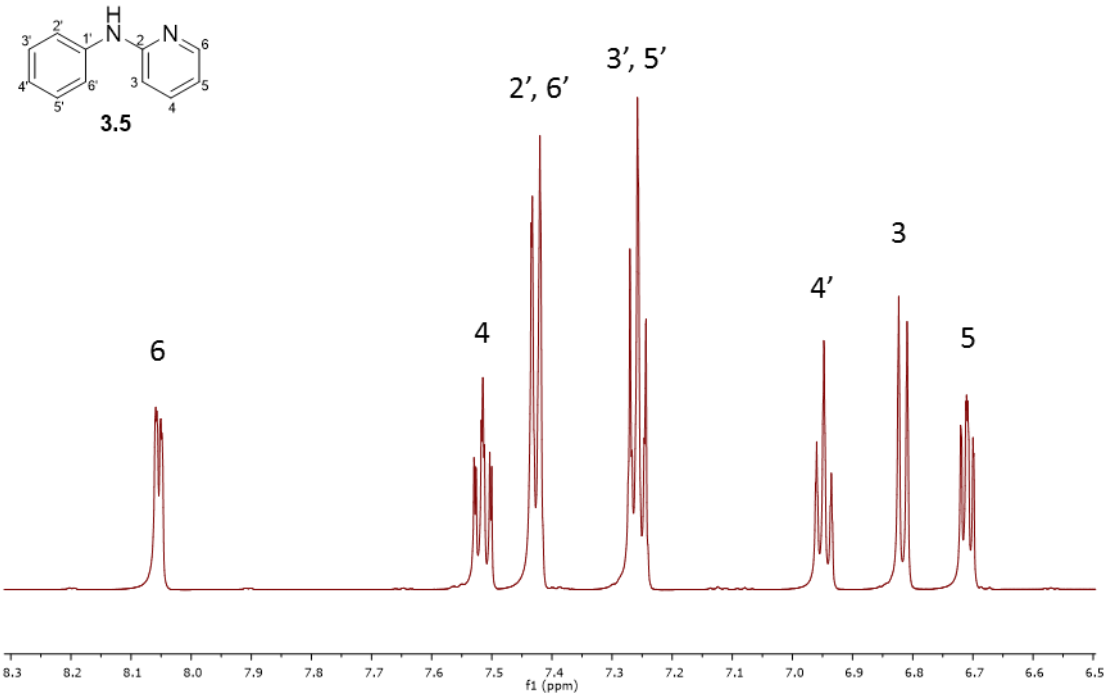

Figure A2.16: ^{1}H NMR spectrum (MeOH-d4, 600 MHz) obtained for **3.5**.

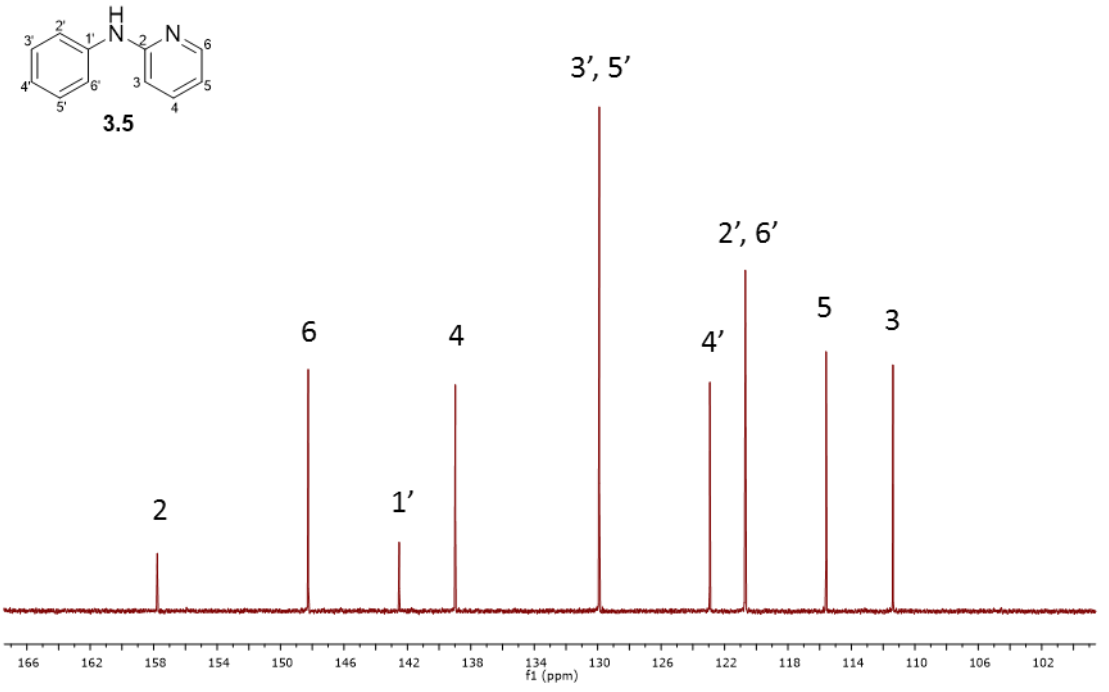

Figure A2.17: ^{13}C NMR spectrum (MeOH-d4, 150 MHz) obtained for **3.5**.

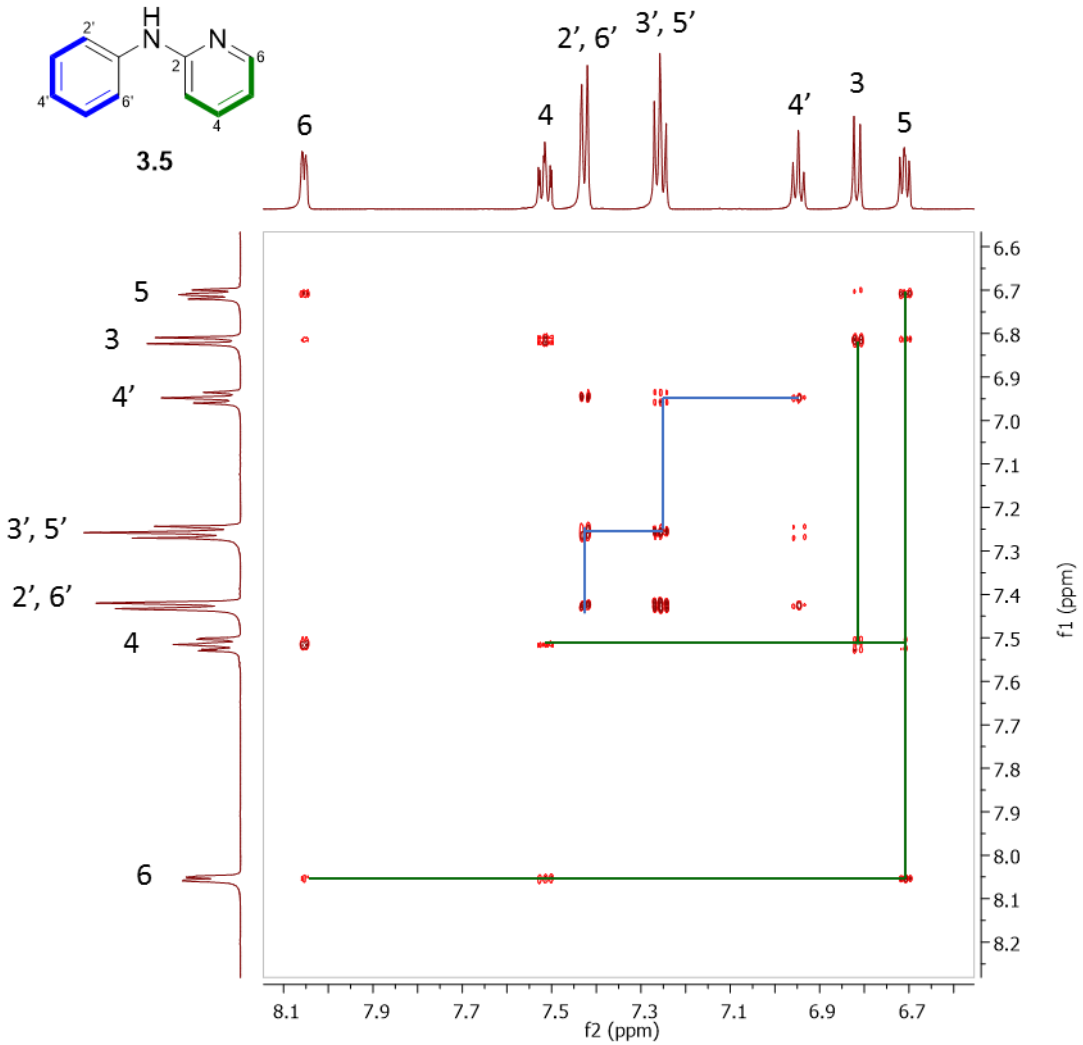

Figure A2.18: COSY NMR spectrum (MeOH-d4, 600 MHz) obtained for **3.5**.

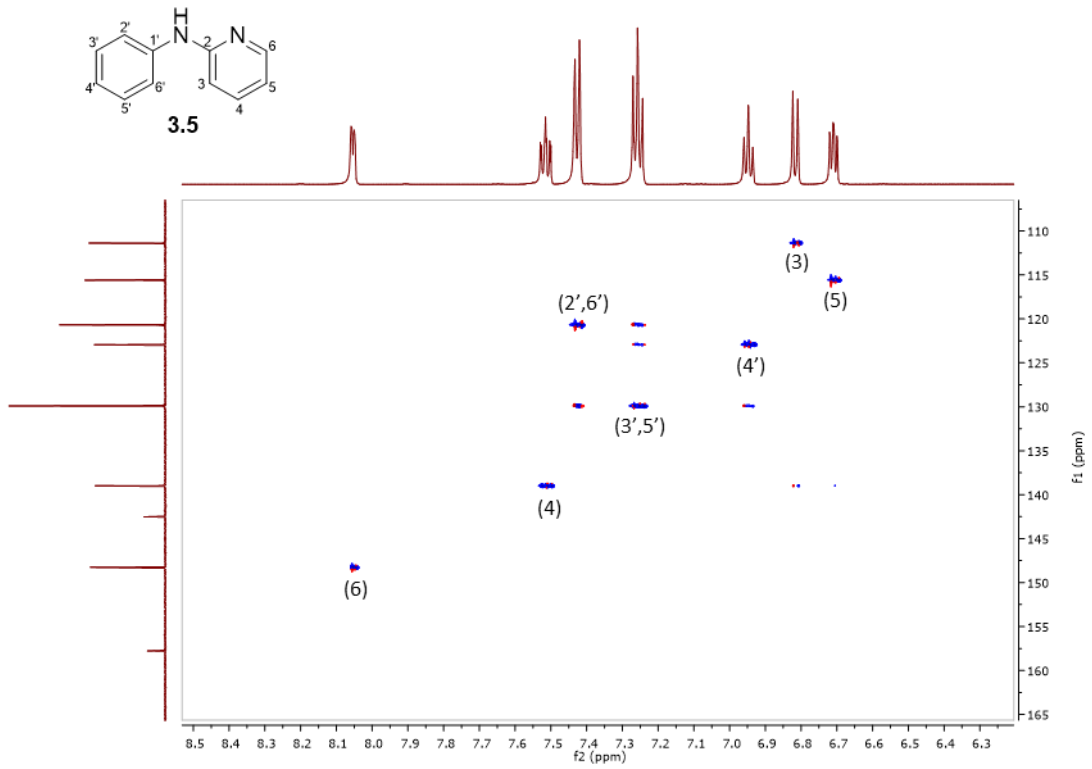

Figure A2.19: HSQC NMR spectrum (MeOH-d4, 600 MHz) obtained for **3.5**.

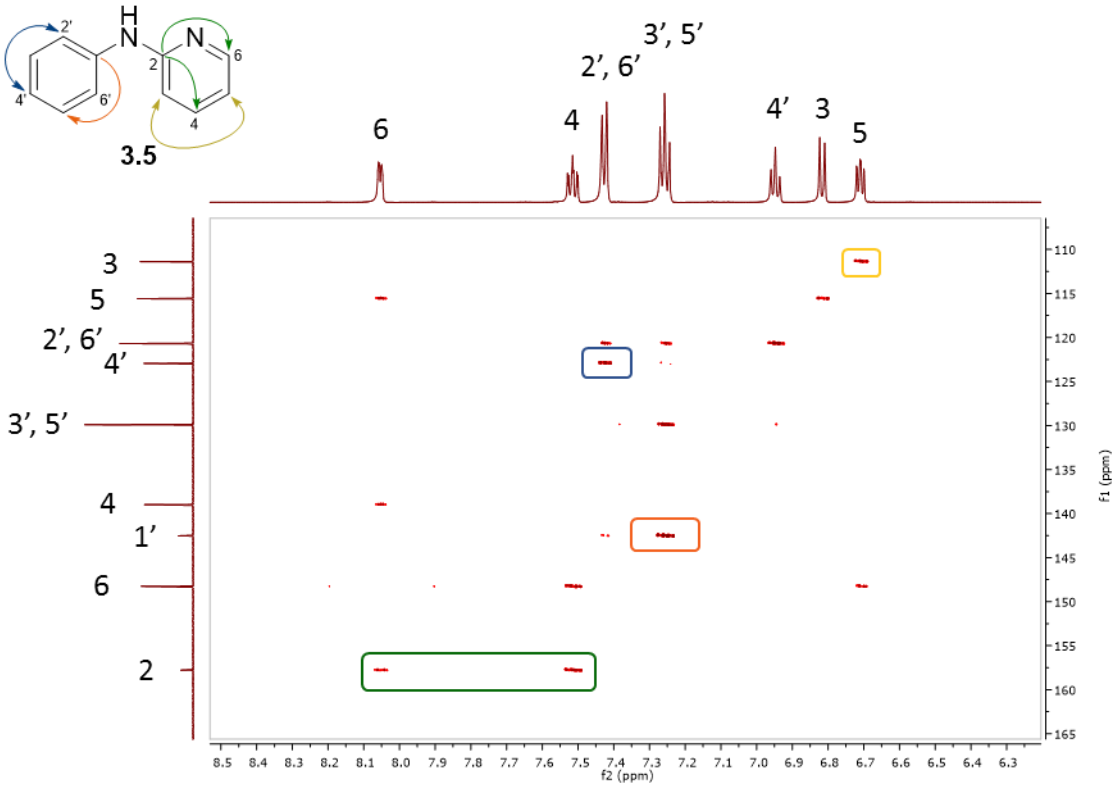

Figure A2.20: HMBC NMR spectrum (MeOH-d4, 600 MHz) obtained for **3.5**.

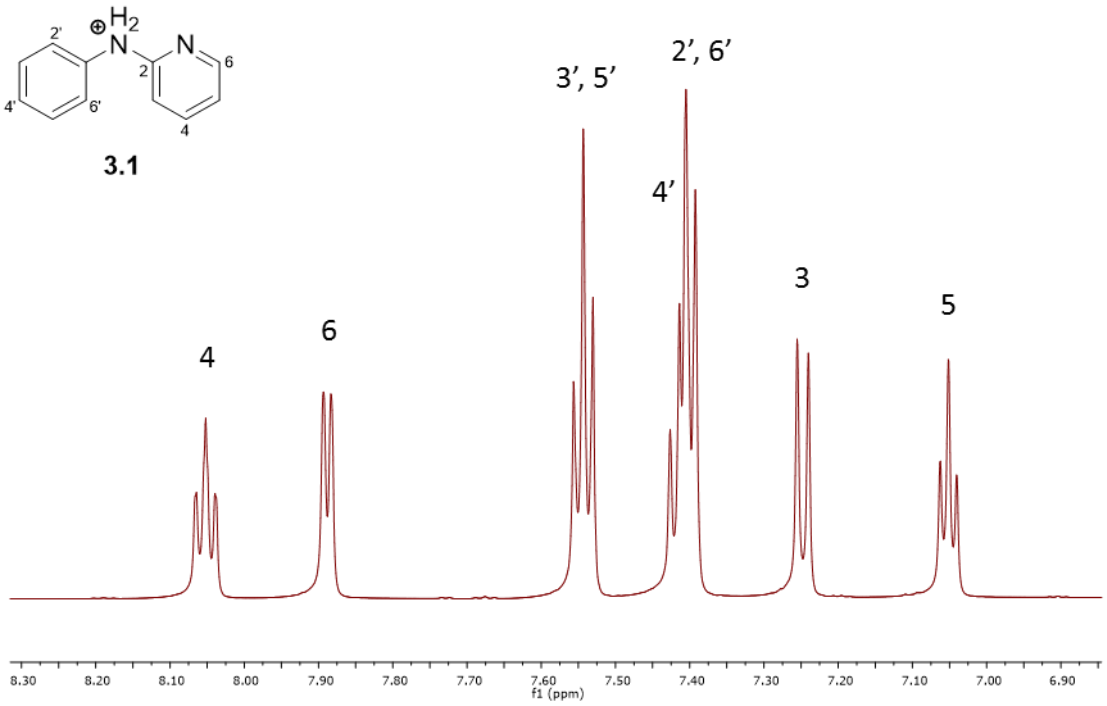

Figure A2.21: ^{1}H NMR spectrum (MeOH-d4, 600 MHz) obtained for **3.1** (synthesised).

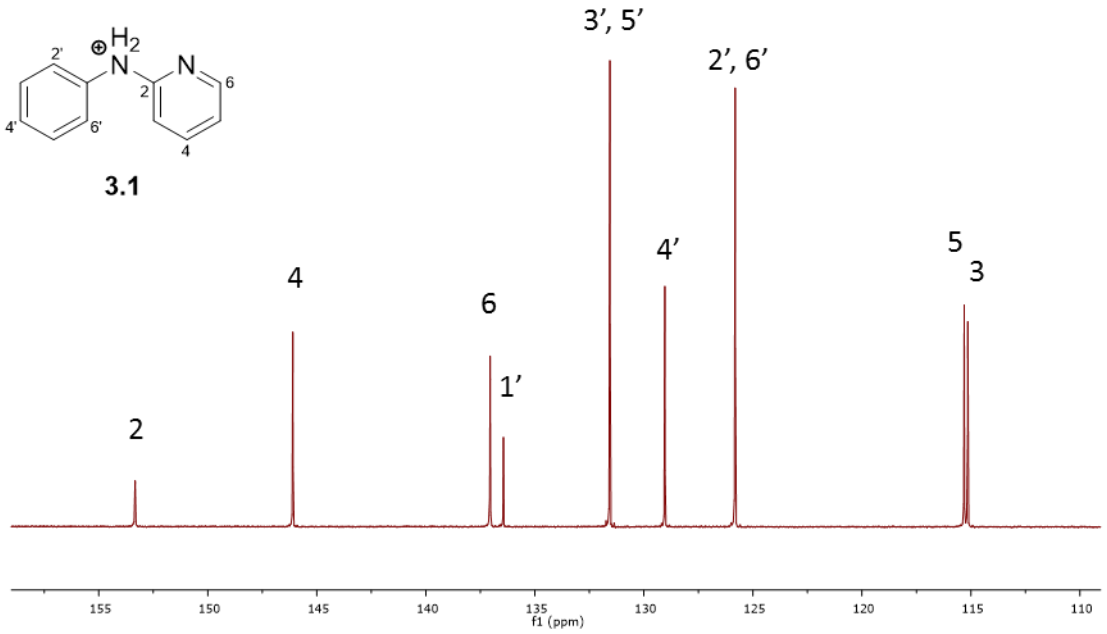

Figure A2.22: ^{13}C NMR spectrum (MeOH-d4, 150 MHz) obtained for **3.1** (synthesised).

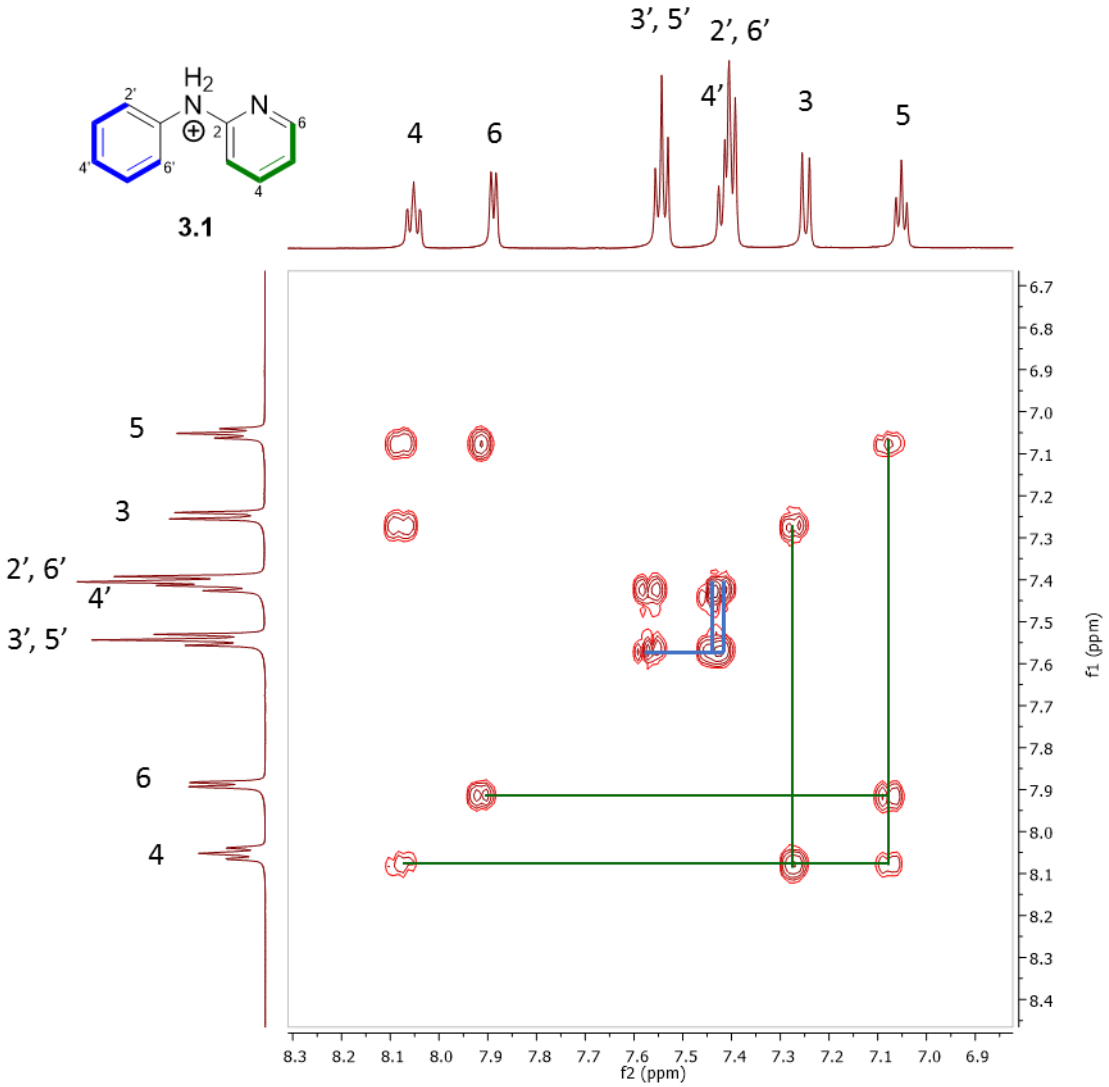

Figure A2.23: COSY NMR spectrum (MeOH-d4, 600 MHz) obtained for **3.1** (synthesised).

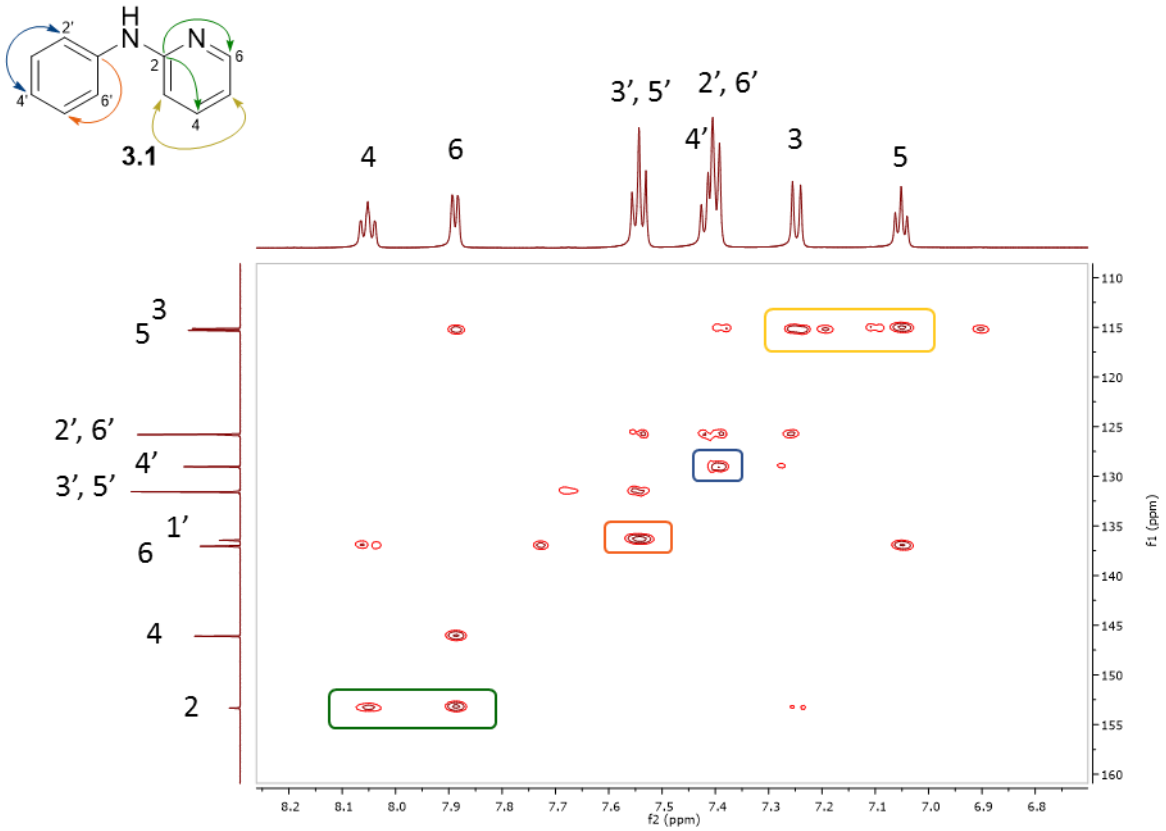

Figure A2.24: HMBC NMR spectrum (MeOH-d4, 600 MHz) obtained for **3.1** (synthesised).