Selection, synthesis and evaluation of novel drug-like compounds from a library of virtual compounds designed from natural products with antiplasmodial activities

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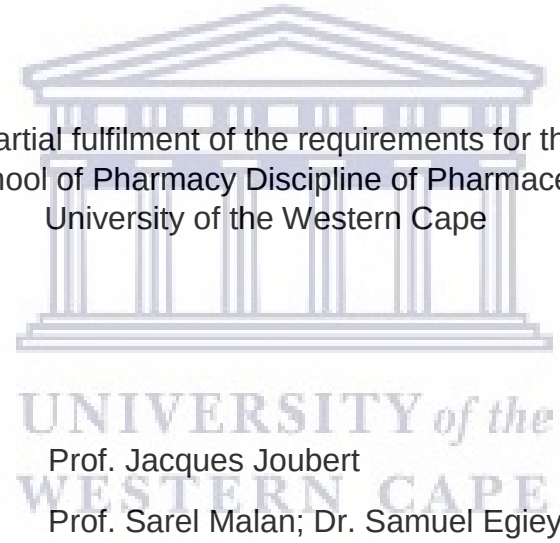

A thesis submitted in partial fulfilment of the requirements for the degree of *Magister Scientiae* in the School of Pharmacy Discipline of Pharmaceutical Chemistry, University of the Western Cape

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KEYWORDS

Malaria

Plasmodium falciparum

Chloroquine resistance

Drug resistance

Cheminformatics

KNIME

Virtual compounds library

Natural products with antiplasmodial activities (NAA)

SDF Format

Lipinski's rule of five

ABSTRACT

Selection, synthesis and evaluation of novel drug-like compounds from a library of virtual compounds designed from natural products with antiplasmodial activities

M.Sc. Thesis, School of Pharmacy, Discipline of Pharmaceutical Chemistry, University of the Western Cape.

Malaria is an infectious disease which continues to kill more than one million people every year and the African continent accounts for most of the malaria death worldwide. New classes of medicine to combat malaria are urgently needed due to the surge in resistance of the *Plasmodium falciparum* (the parasite that causes malaria in humans) to existing antimalarial drugs. One approach to circumvent the problem of *P. falciparum* resistance to antimalarial drugs could be the discovery of novel compounds with unique scaffolds and possibly new mechanisms of action. Natural products (NP) provide a wide diversity of compounds with unique scaffolds, as such, a library of virtual compounds (VC) designed from natural products with antiplasmodial activities (NAA) can be a worthy starting point.

This project aims to develop an automated computerized prioritizing workflow method based on available data (virtual compounds from NAA), to select a set of compounds to be synthesised and evaluated as potential new antimalarial agents. Using the computational tool KNIME, 1805 VC were prioritized from a library of 164532 VC and a final number of 25 diverse VC were selected for further exploration. A series of compounds were selected for synthesis based on the VC results, using multi-step procedures and the structure elucidation was done by using ^{1}H and ^{13}C NMR spectroscopy, IR absorption spectrophotometry and mass spectrometry. Six compounds were successfully synthesised via amination and sulphonamide formation reactions.

The synthesised compounds were evaluated for their cytotoxicity and antimalarial activity against chloroquine (CQ) sensitive (NF54) and resistant (K1) strains of the *P. falciparum* parasite. Although all compounds were found to be non-toxic (CHO IC_{50} > 10000 nM), they were not as active as chloroquine against the CQ-sensitive strain (NF54). However, these compounds displayed statistically significant antimalarial activity against the same strain (NF54). All synthesised compounds maintained their

antimalarial activity against the CQ-resistant strain (K1) compared to chloroquine. Among the synthesised compounds (**1**-**6**), compound **2** showed the best activity against the CQ-sensitive strain (NF54), and the second lowest resistance index (RI = 1.28). This was an indication that it retained its activity against the CQ-resistant strain (K1) compared to chloroquine where a 30-fold reduction in activity was observed. Compound **2** was thus identified as the most promising candidate to overcome the problem of *P. falciparum* CQ resistance among all synthesised compounds. The retention of activity of synthesised compounds in the *P. falciparum* chloroquine resistant strain (K1) could be attributed to the presence of the 6-bromothieno [3.2-*d*] pyrimidin-4-amine moiety. This could be due to a different mechanism of action or that the substances are not substrates of the Chloroquine Resistance Transporter (*Pf*CQRT). Further work should be done to explore and confirm the mechanism(s) of action involved in the parasite-killing.

DECLARATION

I declare that this research title ***"Selection, synthesis and evaluation of novel drug-like compounds from a library of virtual compounds designed from natural products with antiplasmodial activities"*** is my own work, that it has not been submitted for any degree or examination in any other university, and that all sources I have used or quoted have been indicated and acknowledged by complete references.

Rostand Fankam Pokomi Date……17/11/2020…….

Signed……

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SCHEMES

ABBREVIATIONS USED

ACT	Artemisinin-combination therapy
AM	Aminoquinoline
^{13}C-NMR	Carbon 13 nuclear magnetic resonance
CHO	Chinese Hamster Ovarian
CQ	Chloroquine
CQ^R	Chloroquine resistant
CQ^S	Chloroquine sensitive
D	Doublet
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DMSO	Dimethyl sulfoxide
^{1}H-NMR	Proton nuclear magnetic resonance
Hz	Hertz
IR	Infrared radiation
KNIME	Konstanz Information Miner
J	Spin-Spin coupling constant (Hz)
m	Multiplet

MCSS	Most common substructure
m/z	Mass to charge ratio
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazoliumbromide
MW	Microwave
MS	Mass spectrometry
NAA	Natural product with *in vitro* antiplasmodial activities
NP	Natural product
PABA	*p*-aminobenzoic acid
PAINS	Pan assay interference compounds
pLDH	Parasite lactate dehydrogenase
ppm	Part per million
psi	Pounds per square inch
VC	Virtual compound
RDT	Rapid diagnosis test
Ro5	Lipinski's rule of five
rt	Room temperature
s	Singlet
SI	Selectivity index
TLC	Thin-layer chromatography
UV	Ultraviolet
W	Watt
WHO	World Health Organisation
^{o}C	Degrees Celsius

CHAPTER 1

INTRODUCUTION

1.1 Malaria

Malaria is an infectious disease that threatens the life of millions of the people around the world. This infection is caused by transmission of a parasite called *Plasmodium* into human a host by an infected female anopheles mosquito. There are five *Plasmodium* species of which *P. falciparum* is the most prevalent (Greenwood *et al*., 2008; Cox-Singh & Singh, 2008). The Africa continent accounts for about 94% of malaria cases in 2019 and malaria deaths was reduced by 44% in the African region the same year (WHO, 2020).

Chloroquine (CQ) (Figure 1.1) has been used for many years for the treatment of malaria, but due to widespread resistance, this drug has become ineffective in many parts of the world. The World Health Organisation (WHO) now recommends the use of artemisinin-based combination therapy (ACTs) to treat uncomplicated malaria, but unfortunately resistance of *P. falciparum* to ATCs is already emerging in South-East Asia (WHO, 2020). Mutation in a putative ATP-powered multidrug efflux pump known as the *p*-glycoprotein (p-GP) pump and point mutation in the *Plasmodium falciparum* chloroquine resistance transporter (Pf*CRT*) protein are the primary causes of resistance (Sanchez *et al*., 2008; Chinappi *et al*., 2010). The accumulation of CQ in the parasite food vacuole, its primary site of action is reduced by these mutations (Coban, 2020). Over the years different studies have been done to reverse the chloroquine resistance problem. For example the use of agents that potentiate chloroquine activity, known as chemosensitisers (Bray *et al*., 1994; Martiney *et al*., 1995; Bitonti *et al*., 1988; Basco LK *et al*., 1991). However, it has been shown that high concentrations of these chemosensitisers are required to show good reversal ability when co-administered with chloroquine (Basco *et al*., 1994; Van Schalkwyk *et al*., 2006). Also the cost involved with concurrent use of multiple drugs, which are pharmacologically active compounds might be a problem. Hence we need to identify new strategies to counter the increasing and worrying resistance problem.

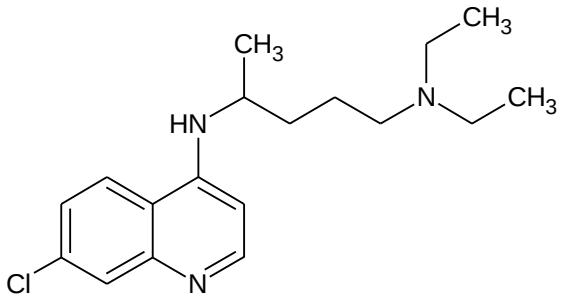


Figure1.1: Chloroquine (CQ), a blood-stage antimalarial agent

One approach to circumvent the problem of *P. falciparum* resistance to anti-malarial drugs could be the discovery of novel compounds with unique chemical scaffolds with the potential to act through new mechanisms. In the past, natural products (NP) and their derivatives have played a powerful role in the fight against malaria and NP scaffolds have been the basis of the majority of current anti-malarial drugs (Wells *et al*., 2011; Soh *et al*., 2007). Thus NP have the potential to provide new scaffolds for drug discovery. Natural products possess highly complex structures and their synthesis on an industrial scale is quite problematic (Yun *et al*., 2012). So the design of a library of virtual compounds (VC) with pharmacological attributes of natural products with antiplasmodial activities (NAA) but with a simpler structure that can be synthesised is a good starting point. The design of a VC library generates a larger number of compounds and because it is not economically viable to synthesise and test all of the generated compounds, a strategy need to be develop to select the most promising compounds (Cramer *et al*., 1998).

1.2 Cheminformatics

One interdisciplinary flied which can aid in the discovery of novel chemical entities and ultimately in the design of new molecules is cheminformatics. Cheminformatics is an important instrument for collection (information acquisition), storing (information management) and analysing (information use) enormous amounts of chemical data to be transformed into information and information into knowledge with the intended goal of making better decisions in the area of drug discovery. Cheminformatics modules comprise computer-assisted synthesis design, structure representation and chemometrics (Begam *et al*., 2012). Different tools and software have been developed over the years for computer-assisted organic synthesis. Some of the software and

database include ISIS-Draw, ChemDraw, ChemWindow, ChemReader, PubChem, ChemMine, LogCHEM, KNIME, Wendi, ChemSketch and so on. The explosion of information generated by chemists requires an effective collection and analysis of chemical information for the rapid development of compounds in drug discovery.

In the current study a library of virtual compounds, which could be easy to synthesize in theory, will be designed from NAA with the aid of computerized approaches (Figure 1.2). The virtual compounds that will be generated will have structural features of natural products but with simplified structures (Egieyeh *et al*., 2016).

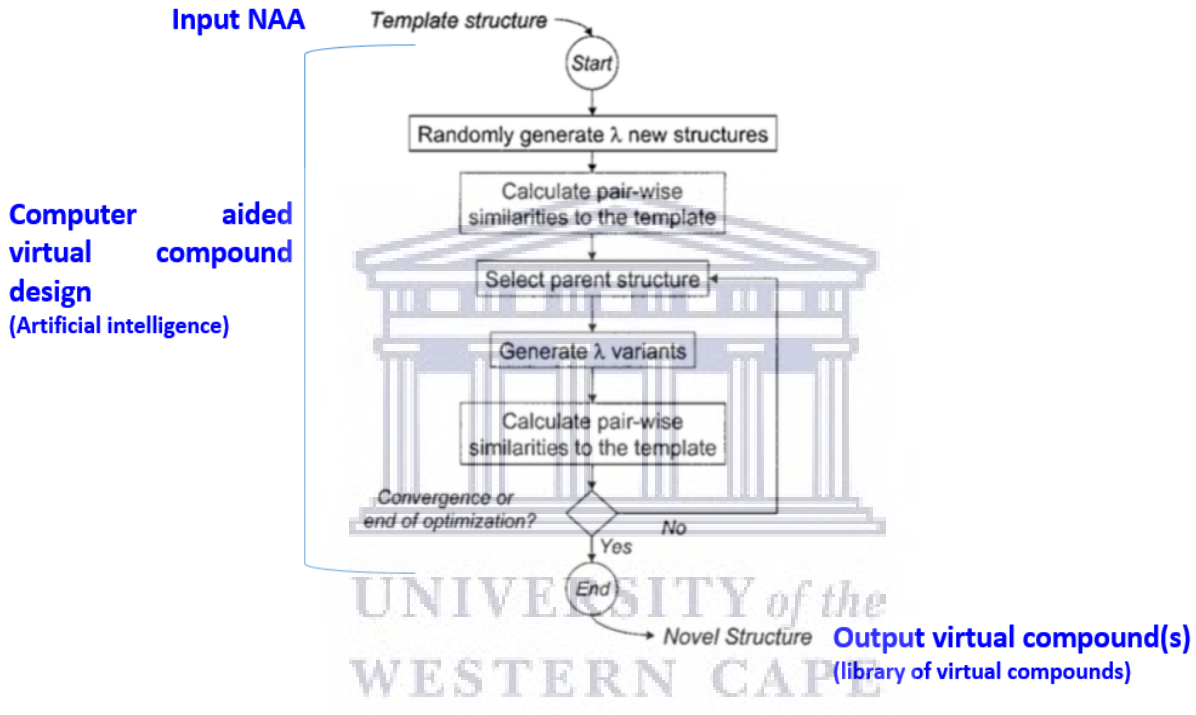


Figure 1.2: Generation of virtual compounds library (Egieyeh *et al*., 2016).

The main problem with the design of a library of virtual compounds is that a significantly large number of compounds are generated and could be up to millions of compounds (Cramer *et al*., 1998). Since it is not possible to synthesize and test all the compounds generated, a library of virtual compounds is only useful if individual molecules can be practicably selected using predefined parameters (Cramer *et al.,* 1998). The aim of the current study is to synthesise a series of novel compounds from a virtual compound library with potentially new mechanisms of action against *P. falciparum*. Therefore, the prioritization and selection of virtual compounds with the most promising anti-malarial potential is paramount.

1.3 Objectives for this study

In pursuit to identify novel antimalarial drugs with new mechanisms of action and to potentially circumvent the growing and alarming resistance of the *P. falciparum* to antimalarial drugs, the following objectives were set:

- Prioritization and selection of compounds from a virtual compound library designed from natural product with antimalarial activities.
- Synthesis and evaluation of the most promising compounds

1.3.1 Prioritization and selection

The prioritization and selection of compounds from a library of virtual compounds designed from natural products with antiplasmodial activities will be done using computational tools with predefined parameters. KNIME (Konstanz Information Miner) is a computational tool that can be used to develop an automated computerized prioritizing workflow method based on available data (i.e. virtual compounds from NAA) for selection of new sets of compounds to be synthesised and evaluated as potential new antimalarial agents with unique chemical scaffolds (Fig 1.3.1) (Mc Guire *et al*., 2017; Berthold *et al*., 2009).

Using a well-designed KNIME workflow which consists of 6 steps (Figure 1.3), this study will commence using a large number of virtual compounds (VC) in SDF format. The Lipinski's rule of five (Ro5) will then be used as molecular descriptor, simply because it characterises the potential bioavailability profile of the VC. There is a high probability that a compound will encounter oral bioavailability problems if it fails the Ro5 (Lipinski *et al.*, 2004). Each physicochemical parameter which define the Ro5 will be used as a filtering and selection criteria with cut-off values. Any VC that fail the Ro5 will not be retained. A first pairwise comparison of molecules based on the most common substructure (MCSS) will be done amongst the retained VC to assess their similarity. Furthermore, a selection of a diverse set of VC will be done based on their fingerprints. A second pairwise comparison of previously retained VC will be performed to assess their similarity based on their MCSS. The selected VC will be used to perform similarity searches against molecules found in ChEMBL, which is a chemical database of bioactive molecules with drug-like properties. Finally the selected VC which could have a specific or a nonspecific interaction with multiple

biological targets will be identified using PAINS (pan assay interference compounds) (Dahlin *et al*., 2015).

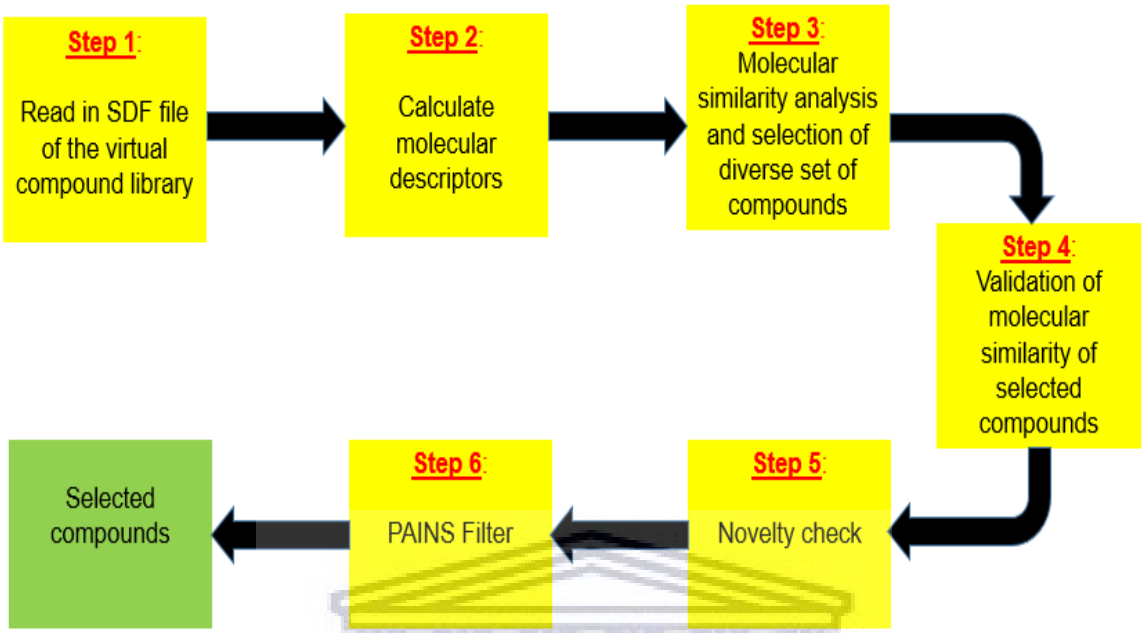


Figure 1.3: Cheminformatics workflow for virtual compounds prioritization and selection.

1.3.2 Synthesis and evaluation

The ease of synthesis of selected VC will be estimated by determining the synthetic accessibility score using SWISSADME (http://www.swissadme.ch/). As proof of concept, a series of compounds will be selected based on the VC results and synthesised using multi-step procedures. The elucidation of the novel synthesised compounds will be done using ^{1}H and ^{13}C NMR, IR and MS. Biological evaluation of the synthesised compounds will be conducted on sensitive and resistant strains of *P. falciparum* using a cell-based assay.

1.4 Proposed series of test compounds

The aim of the current study is to synthesise a series of novel compounds from a virtual compound library with potentially new mechanisms of action against *P. falciparum*. This is done using the new computational approach, as described above, in order to discover novel chemical entities. As proof of concept, the automated KNIME workflow was used for filtering and selection of new compounds with promising antimalarial

activities to be synthesised and evaluated (Begam *et al.,* 2012). The compounds planned for synthesis and biological evaluation, based on the cheminformatics results, include:

(1.1) 6-bromothieno[3,2-d]pyrimidin-4-amine

(1.2) *N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)- 4-bromo-benzenesulfonamide

(1.3) *N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)- 4-methylbenzenesulfonamide

(1.4) *N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)- 4-methoxybenzenesulfonamide

(1.5) *N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)benzenesulfonamide

(1.6) *N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)- 4-chlorobenzenesulfonamide

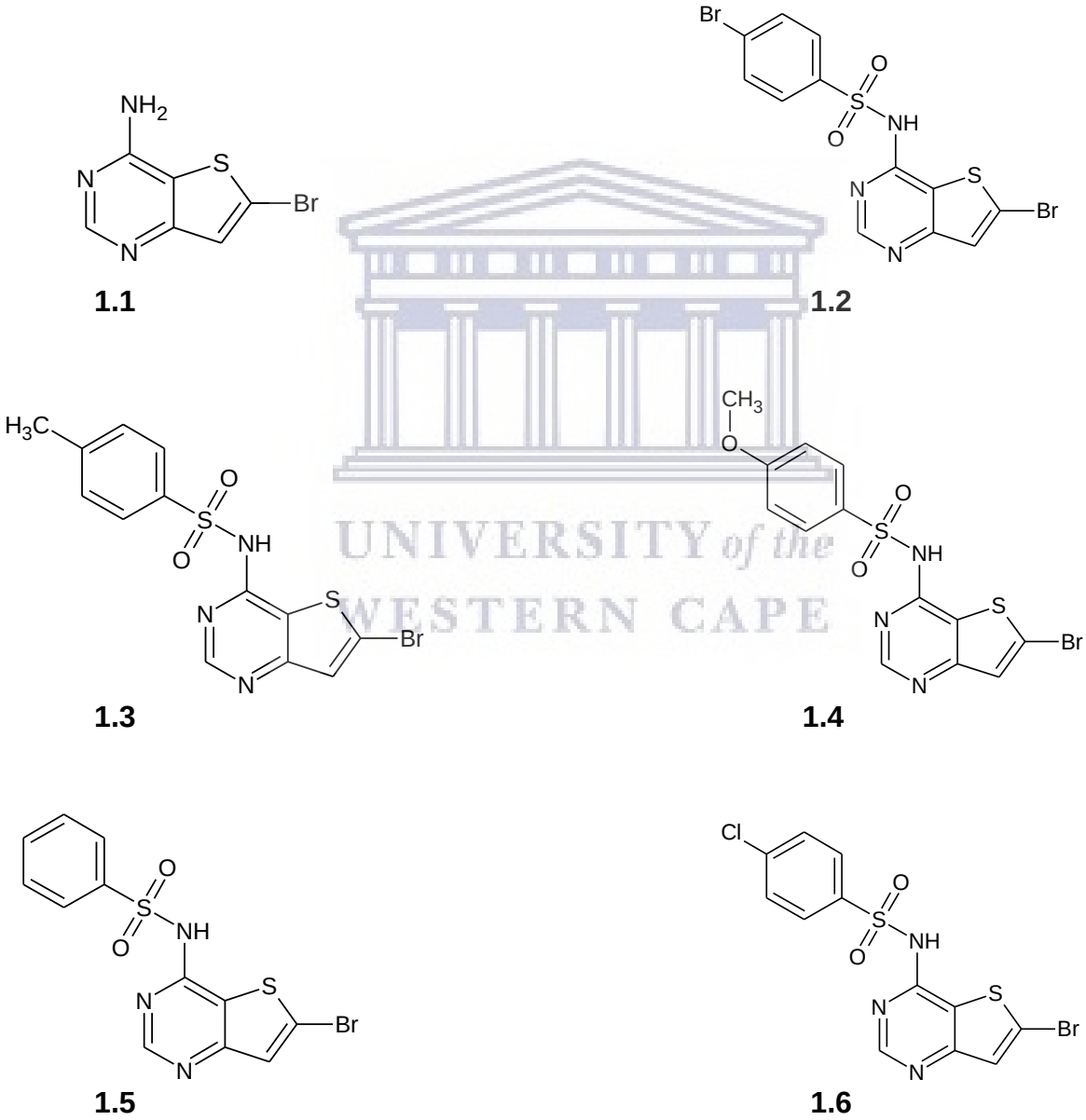


Figure 1.4: Compounds synthesised in this study

It is hypothesized that these novel agents with unique chemical scaffolds will exhibit activity against both sensitive and resistance strains of *P. falciparum* using cell-based assays.

CHAPTER 2

LITERATURE REVIEW

The aim of this chapter is to describe the life cycle of the plasmodium parasite, clinical features of malaria, the chemotherapeutic agents used for the prevention and treatment of the disease and finally briefly describe a new approach using artificial intelligence in the quest to identify novel antimalarial drugs with potentially new mechanisms of action in order to circumvent the growing and alarming resistance of the *P. falciparum* to antimalarial drugs.

2.1 Malaria

Malaria is an infectious disease caused by infection of the red blood cells with protozoan parasites of the genus plasmodium transmitted into a human host by a feeding and infected female anopheles mosquito (Greenwood *et al*., 2008; Cox-Singh & Singh, 2008). There are four *Plasmodium* species that cause malaria in humans, namely *P. falciparum, P. ovale, P. malariae, P. vivax* and the more recent *P. knowlesi* (Greenwood *et al*., 2008; Cox-Singh & Singh, 2008).

Nearly half of the world's population is threatened by this disease and it is estimated that more than one million people are killed every year due to malaria. Among the different *Plasmodium* species that cause malaria, *P. falciparum* is the more virulent and it accounts for 99% of estimated malaria case in sub-Saharan Africa. The African continent accounts for 94% of all malaria cases (WHO, 2020). The high prevalence on the African continent is merely due to the humid and warm climate of the sub-Saharan region of the continent which provides a favourable breeding ground for the malaria vector.

It has been shown that the lower rate of economic growth and poverty of the malaria-endemic countries are also a burden in the fight against malaria. For instance the population cannot afford to buy insecticides or treated mosquito nets and this make it almost impossible to control the malaria vector (mosquito). The unaffordability of antimalarial drugs is another problem that are encountered by these impoverish population (Sachs *et al*., 2002).

The main concern in the fight against malaria in Africa is the *P.falciparum* resistance to commercially available antimalarial drugs. The WHO recommend the use of artemisinin-combination therapy (ACTs), which remain efficacious in all endemic settings in Africa. Unfortunately, resistance to ACTs has been reported in *P. falciparum* in five countries of South-East Asia (WHO, 2020; Winstanley, 2000). Therefore a need to find novel compounds with potential new mechanisms of action to circumvent the *P. falciparum* resistance problem is urgently required.

2.1.1 The life cycle of plasmodium parasite

The malaria parasite life cycle (figure 2.1) is complex and requires two hosts which are the human body and the female anopheles mosquito. Upon a blood meal feeding by the malaria mosquito, sporozoites are injected into the blood stream of the human host. The sporozoites go to the liver where it infects the liver cells. This is the human liver stage (exo-erythrocyclic cycle). At this stage there is no symptoms experienced by the human host. The sporozoites then mature in the liver into schizonts after an incubation of about 10 days. The number of schizonts increase and differentiate to merozoites in the liver hepatocytes and after rupture of the liver cells the merozoites are released into the blood stream where they will infect the red blood cells. This is the human blood stage (erythrocytic cycle) and the human host starts to experience symptoms. The blood stage is complete when merozoites differentiate into sexual forms known as male and female gametocytes that are taken up in a mosquito's blood meal. The mosquito stage is the last stage of the parasite life cycle. During a blood meal feeding, the female anopheles mosquito ingests the gametocytes into its mid-gut which causes fusion into a zygote which will eventually develop new sporozoites that invades the mosquito salivary glands. This then completes the life cycle of the malaria parasite in the mosquito host (Mota *et al.,* 2004; Greenwood *et al.,* 2008; Cowman *et al.,* 2012).

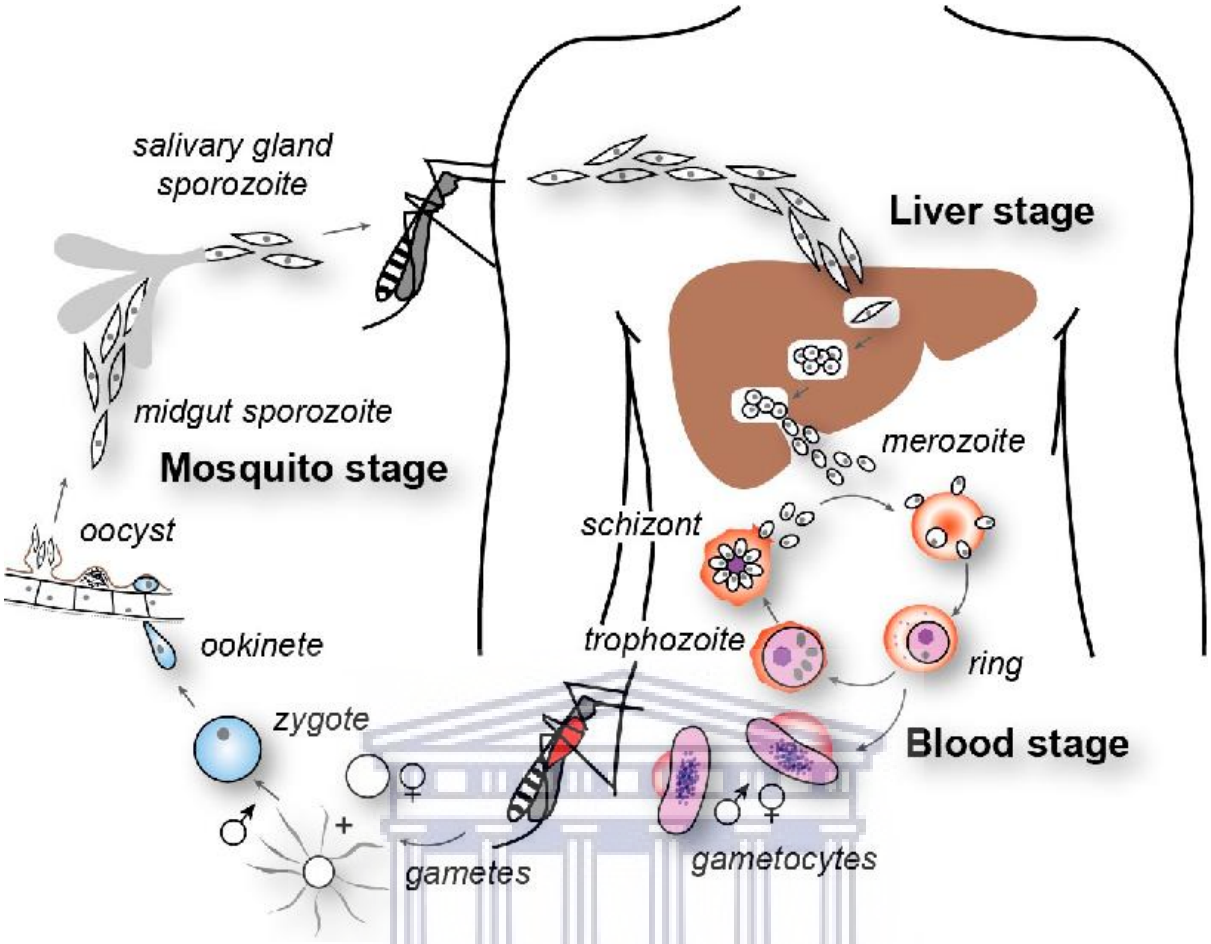


Figure 2.1: Malaria parasite life cycle (Cowman *et al.,* 2012)

2.1.2 Signs and symptoms of malaria

Malaria is an infectious disease caused by protozoan parasites of the genus *Plasmodium* which is transmitted from person to person (human host) via the anopheles female mosquito (malaria vector). The symptoms associated with malaria is experienced during the blood stage of the parasite's life cycle in the human body. The severity of disease for the four species of *Plasmodium* that causes malaria varies but the symptoms remain the same. Prompt and efficacious treatment of uncomplicated malaria is required to avoid its progression to severe malaria and death. The symptoms characterizing uncomplicated malaria comprise malaise, sweating, headache, fatigue, fever, chills, anorexia, vomiting, muscle and joint aches (also known as malaria paroxysm). Symptoms such as coma (cerebral malaria), hypoglycaemia, renal failure and severe anaemia are associated with severe malaria which may lead to death if not treated appropriately (WHO, 2015; Karunaweera *et al*., 2007).

Along with the symptoms presented by a patient for uncomplicated and/or severe malaria, the World Health Organisation recommend the use of a microscopy examination or RDT (rapid diagnosis test) testing of a blood sample for the diagnosis of malaria (WHO, 2015).

2.1.3 Antimalarial agents used for prevention and treatment of malaria

Malaria is a significant problem worldwide, especially on the African continent where hundreds of thousands of people are killed every year due to the disease. There is a limited arsenal of chemotherapeutic agents used to combat malaria and these are classified into three main categories, namely:

- Aminoquinolines
- Antifolates
- Artemisinin and related derivatives

2.1.3.1 Aminoquinolines

Quinine is a naturally occurring organic compound isolated from the cinchona bark with a relative low potency and modest therapeutic potential discovered during the 17th century (figure 2.2). Quinine has shown good gametocytocidal activity against the *P*. *falciparum* gametocytes and good growth inhibition of *falciparum* cultures *in vitro* (Chotivanich *et al*., 2006; Dorn *et al*., 1998). Methylene blue is the first synthetic antimalarial drug ever used in humans and to demonstrate antiparasitic activity. It has shown to disturb the redox homoeostasis of the parasite by selective inhibition of the glutathione reductase enzyme (Schirmer *et al.,* 2003). Pamaquine was synthesized in 1925 by structural modification of methylene blue. This 8-aminoquinoline demonstrated good gametocytocidal activity when used in combination with quinine and was the first drug capable to prevent relapse from *P. vivax* malaria. The toxicity of pamaquine was a concern and led to the development of another 8-aminoquinoline called primaquine (Peters, 1999). To obtaine primaquine, the diethylamino group (side chain) of pamaquine was replaced with a primary amine and it is the only exoerythrocytic drug available for the eradication of *P. vivax* and *P. ovale* infections

in the liver stages. Primaquine has shown insignificant activity against erythrocytic stages, but possess gametocytocidal activity against all *plasmodium* strains. The mechanism of action of primaquine is not well-known, but literature suggests that it generates reactive oxygen species (ROS) resulting in the formation of superoxides and hydroxyl radicals which are responsible for the parasite killing (Kouznetsov *et al*., 2009; Vennerstorm *et al*., 1999; Fletcher *et al*., 1988).

Although primaquine is the only drug available for the eradication of pre-erythrocytic stages of *P. vivax* and *P. ovale*, a congener named tafenoquine (WR238605) is being developed. Preliminary clinical studies has shown that tafenoquine has a longer half-life (about 14 days) than primaquine and was a safer drug in the prevention of relapse of *P. vivax* malaria (Brueckner *et al*., 1998; Walsh *et al*., 1999).

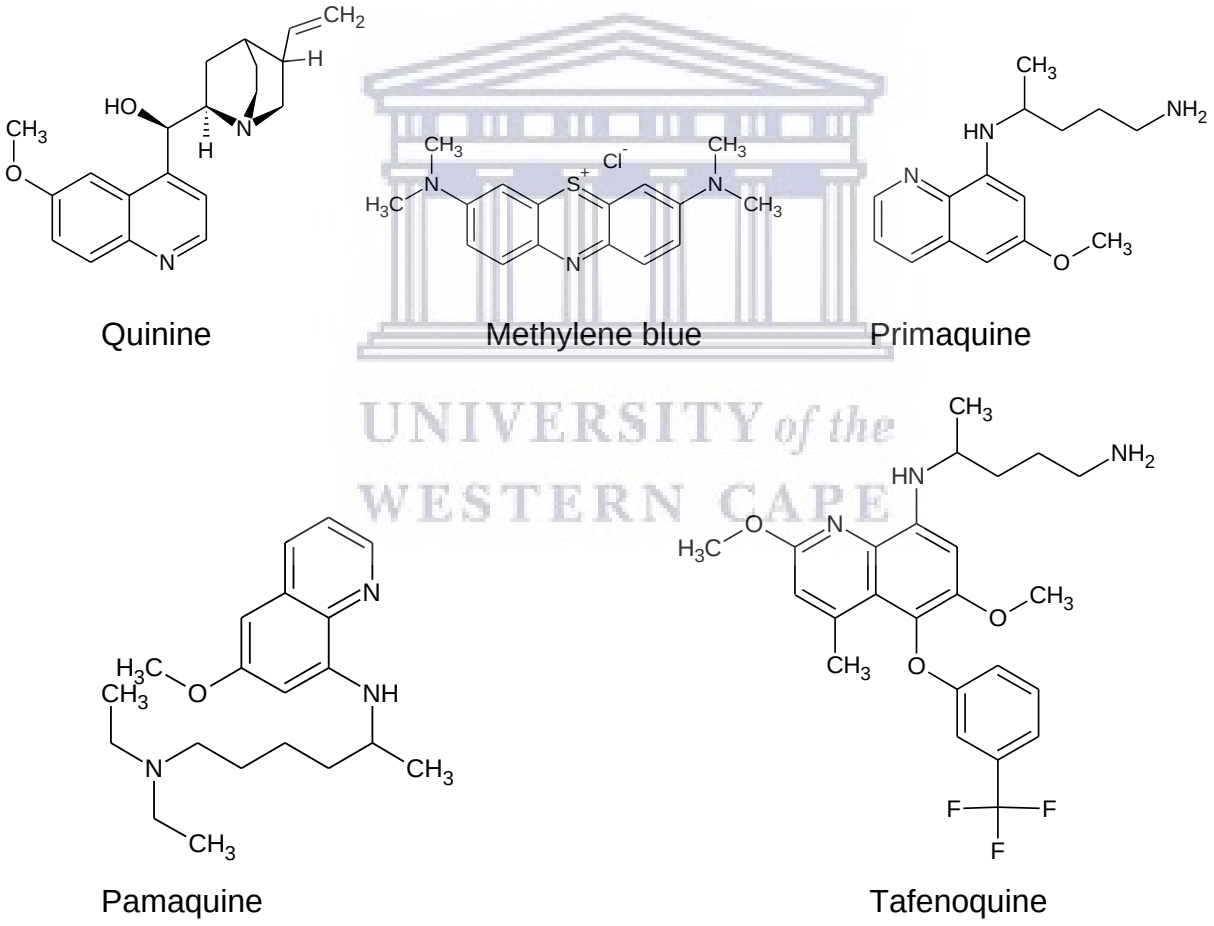


Figure 2.2: Quinine and related compounds.

Quinacrine is antimalarial drug discovered in Elberfeld laboratories by German scientists. It was developed by conjugation of a diethylaminoisopentylamino side-

chain of pamaquine to the acridine heterocyclic ring system of methylene blue. Quinacrine was commercialised under the name of Ateberin® and it has proven to be active against the blood stages of *P. falciparum*. Unfortunately its toxicity restricted its use (Coatney, 1963).

The 4-aminoquinoline, resochin, was synthesised by structural modification of methylene blue. It has shown strong antiplasmodial activity during clinical trials, but was ignored for years because of its toxicity. After re-evaluation of resochin and a realated analogue sontaquine during the 2nd world war, it was found to be safe and to have far greater activity than quinacrine. Resochin was later renamed to chloroquine (figure 2.3) (Turner *et al.*, 1953; Loeb *et al*., 1946).

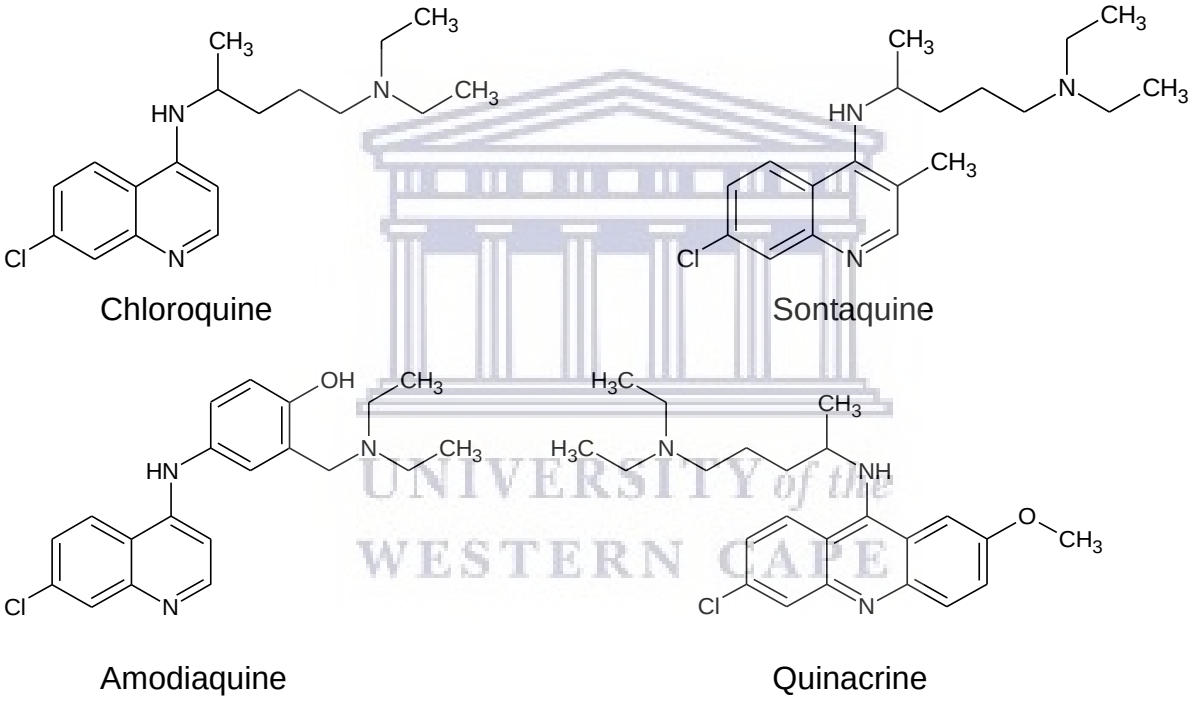


Figure 2.3: 4-Aminoquinolines.

Chloroquine (CQ) has been the antimalarial drug of choice for a very long time due to its cost, safety profile and excellent clinical efficacy. CQ exerts its antimalarial activity by inhibiting the formation of inert haemozoin leading to build up of toxic free haem in the acidic digestive food vacuole of the parasite (Coban, 2020). It has been prescribed and used for decades in many parts of the world where the *P. falciparum* is prevalent to treat malaria. Unfortunately, CQ became ineffective and almost obsolete due to the widespread resistance of the malaria parasite to the drug in Africa, South America and

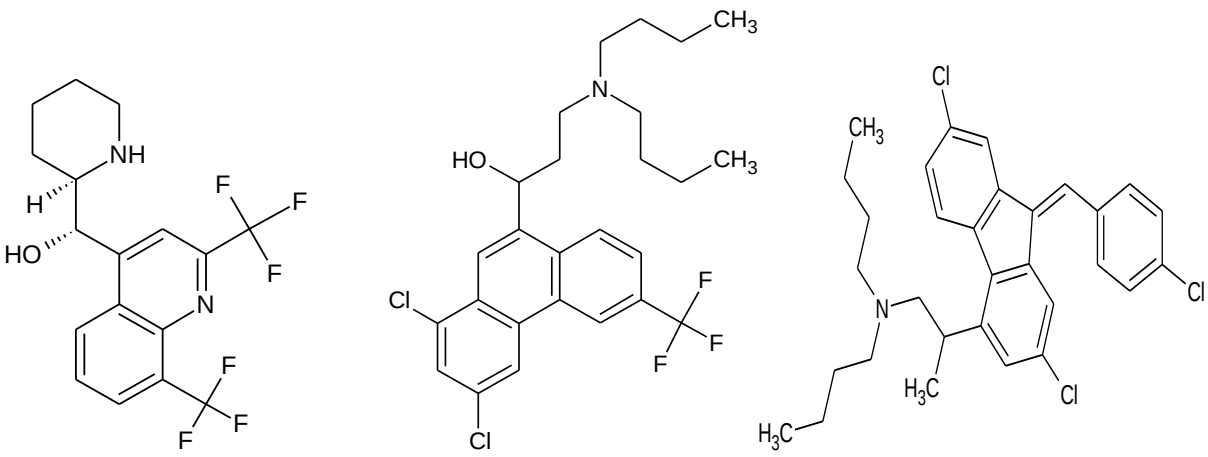
South-East Asia (Kouznetsov *et al*., 2009; Trape *et al*., 1998). The resistance of *P. falciparum* to CQ became a global problem and urgent solutions needed to be found.

Amodiaquine, a 4-aminoquinoline derivative was developed by the substitution of the alkylamino side chain of CQ with an aniline group. Amodiaquine has shown better antimalarial activity than chloroquine, but its high toxicity has been a problem. The toxicity of amodiaquine has been found to be dose-dependent and the side effects are lessened when the correct dose of the drug is given (O'Neill *et al*., 1994).

In an effort to solve the issue of malarial resistance against chloroquine, different structural analogues to quinine were developed (figure 2.4). Among the synthesised derivatives a 4-quinolinemethanol derivative, called mefloquine, was found to be the most promising compound. Mefloquine has demonstrated potent activity against chloroquine resistant *falciparum* and *vivax* malaria infections. However a problem regarding its toxicity became apparent, thus limiting its use (Trenholme *et al*., 1975; Lutz *et al*., 1971).

Furthermore, the Walter Reed Army Institute for Research conducted additional screening of compounds which led to the discovery of halofantrine, by the replacement of the quinoline scaffold of the 4-quinolinemethanol with various aromatic ring systems. Halofantrine is an oral blood shizonticide like mefloquine and it has shown good activity against chloroquine-resistant *falciparum* malaria (Bryson & Goa 1992; Watkins *et al*., 1988). However, the seriousness of cardiotoxicity reported in the treatment of uncomplicated *falciparum* malaria limits its use (Nosten *et al*., 1993). In addition, both mefloquine and halofantrine are quite expensive as drugs.

Lumefantrine is a schizonticide used with artemether as combination therapy. The treatment against malaria is enhanced with this combination through the rapid activity of the artemisinin derivative and the slow acting activity of lumefantrine (Van Vugt *et al*., 2000; Lefevre *et al*., 2001). Even though this combination therapy is quite effective, in many parts of the world and especially in Africa it remains inaccessible due to its cost.



Mefloquine Halofantrine Lumefantrine

Figure 2.4: 4-Quinolinemethanols and related compounds.

2.1.3.2 Antifolates

Antifolates also known as folate inhibitors are drugs that inhibit the folate biosynthetic pathway, which is necessary to malaria parasite survival. This group of drugs act in a synergistic manner through their different combinations and they target two essential enzymes known as dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) involved in folate metabolism. DHPS is not present in humans and thus represents a worthy drug target for the development of antimalarial compounds because of its uniqueness (Nzila *et al*., 2005).

In the folate biosynthetic pathway (figure 2.5), dihydropteroate disphosphate with p-aminobenzoic acid (PABA) is catalysed by DHPS to from dihydropteric acid which will be converted later in the pathway to dihydrofolic acid. DHFR reduce dihydrofolic acid to tetrahydrofolic acid (Wang et al., 1999; Gregson *et al*., 2005). The combination of antifolate drugs that inhibit both DHFR and DHPS enzymes has shown to be effective against the $plasmodium$ parasite through their synergistic antimalarial activity (Nzila, 2006).

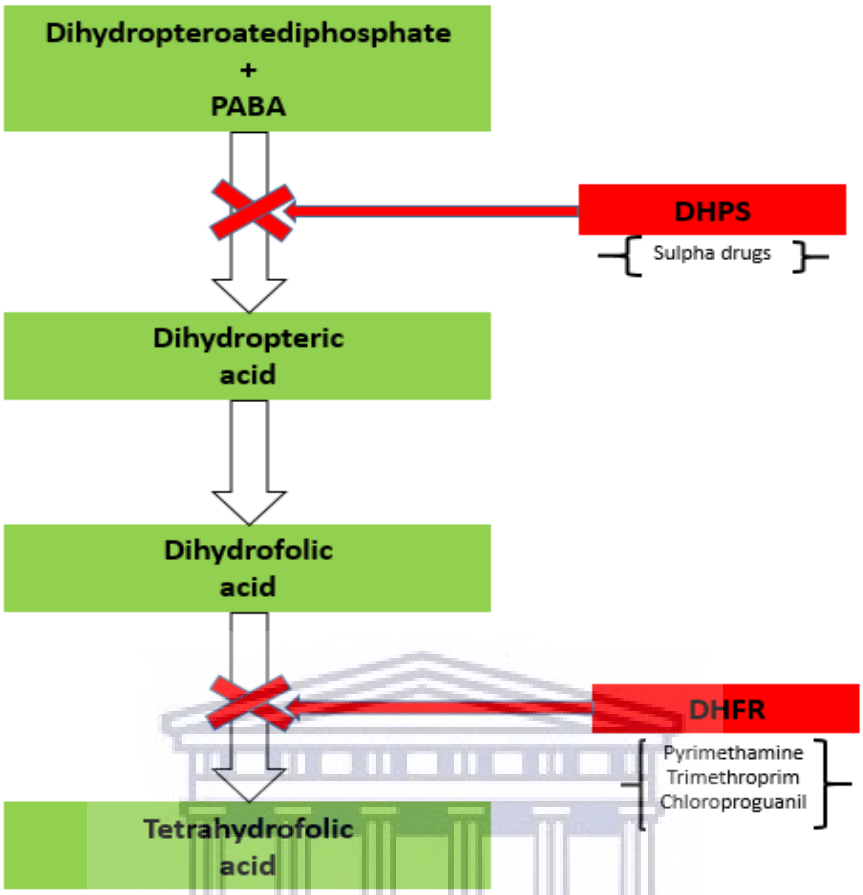


Figure 2.5: Summarized folate biosynthetic pathway with folate inhibitors.

Proguanil is a prodrug metabolised to its active metabolite, a tricyclic triazine called cycloguanil, which is a potent DHFR inhibitor (Carringtone *et al*., 1951; Crowther & Levi 1953). In efforts to develop effective drugs, in-depth studies of proguanil led to the discovery of chlorproguanil a structurally related compound and pyrimethamine.

Prontosil is an azo dye metabolised to its active metabolite form called sulphanilamide, which inhibits the DHPS enzyme (Hakulinen *et al*., 1993; Gingell *et al*., 1971). There are various combinations of antifolates (figure 2.6) available on the market. Fansidar® which consist of sulfadoxine and pyrimethamine is one of the prefered antifolate combinations used in sub-Sahara Africa because it is affordability. Fansidar® remains an effective alternative to choloroquine resistant *falciparum* strains (Chulay *et al*., 1984). Unfortunately, the recurrence of resistance limit is use in Africa (Gregson *et al*., 2005). An alternative combination that is effective against Fansidar® resistant parasites, comprising chlorproguanil and dapsone, has been developed (Nzila-Mounda *et al*., 1998). Malarone® which combines proguanil and atovaquone, is an

effective drug used for prophylaxis and treatment of malaria, but remains inaccessible because this drug combination is still very expensive.

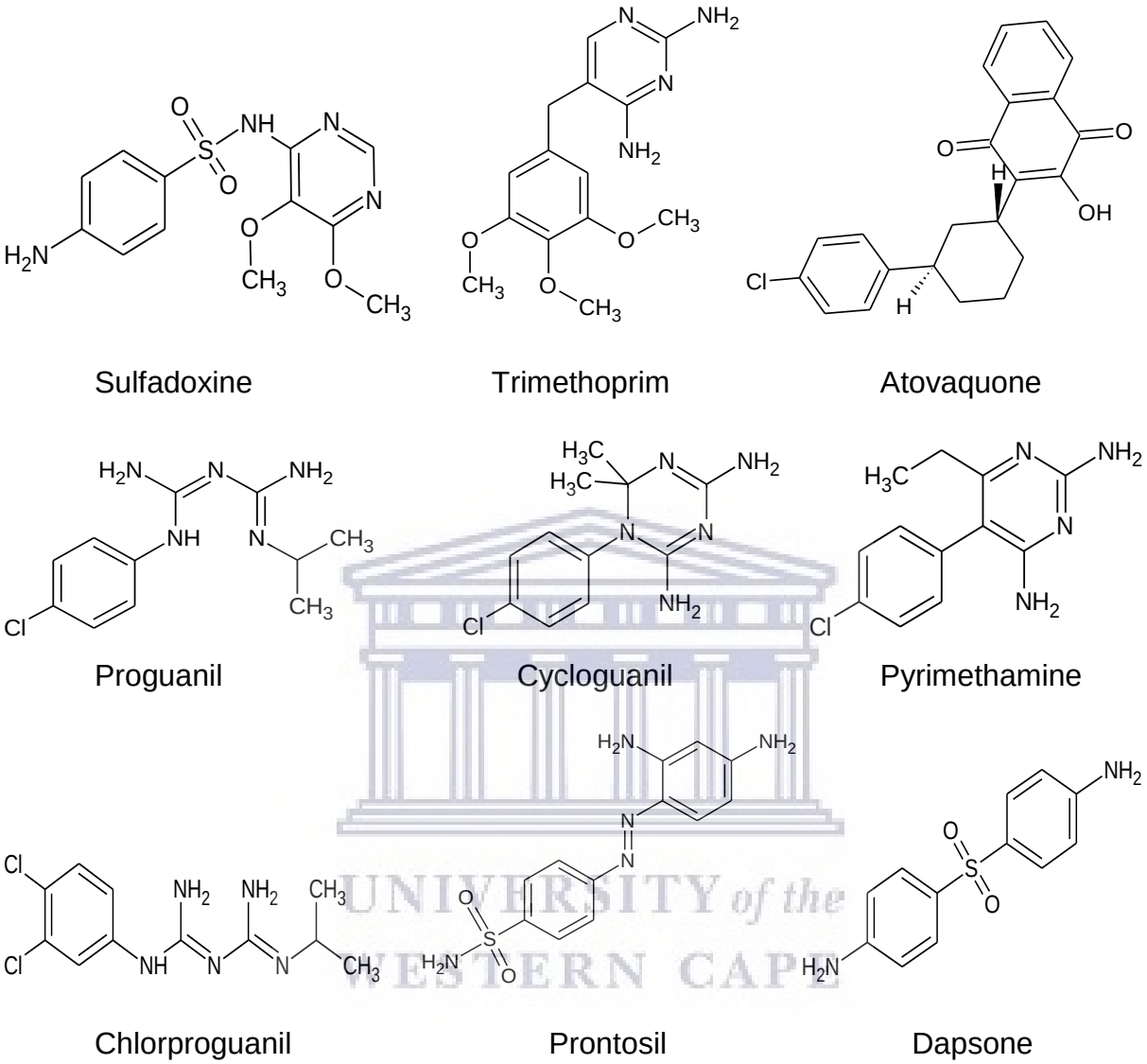


Figure 2.6: Folate inhibitors

2.1.3.3 Artemisinin and related agents

In 1967 an ambitious program for the discovery and development of new antimalarial drugs was launched by the Chinese government. Numerous indigenous plants used in Chinese medicine were systematically studied, which led to the discovery of a compound named *qinghaosu* (artemisinin) in 1972. The source of artemisinin was the plant *Artemisia annua* (Sweet wormwood) and it was isolated by extraction at

low temperature by Chinese researchers (O'Neil *et al*., 2004; Klayman *et al*., 1985; Butler *et al*., 1992).

Although artemisinin was found to treat multidrug resistant *P. falciparum* malaria faster than chloroquine in China, a problem concerning its low solubility in both oil and water was encountered, thus limiting its therapeutic value. Chinese researchers prepared a number of derivatives to the parent drug (artemisinin) in the quest to find more potent and soluble drugs (Ravindranathan, 1994).

Researchers proceeded to structurally modify artemisinin which led to formation of dihydroartemisinin and further optimisation yielded a series of semisynthetic first-generation analogues that include artemether and arteether (figure 2.7). It was found that artemether and arteether were more potent than the parent drug, artemisinin, but had short plasma half-lives and produced fatal central nervous system (CNS) toxicity in animal models (Brewer *et al*., 1994; Kamchonwongpaisan *et al*., 1997).

A water-soluble derivate of artemisinin is necessary for treatment of advanced and life-threatening cases of *P. falciparum* malaria infection because intravenous drug administration is a more efficient method for rapid drug delivery compared to intramuscular injection. Thereby quickly reducing parasitaemia and curing cerebral malaria (Lin *et al*., 1989). Unfortunately, these analogues are rapidly metabolised to dihydroartemisinin, which is associated with short plasma half-life and high rate of recrudescence i.e. recurrence of asexual parasitaemia after treatment of the infection (Waste *et al*., 1994; Chaturvedi *et al*., 2010).

Because of the high recrudescence rate, these antimalarial drugs are generally given as a combination therapy, by co-administering the rapid action of artemisinins with non-artemisinin drugs (long action). The WHO recommend the used of artemisinin combination therapies (ACTs) such as artemether-lumefantrine (Coartem®), dihydroartemisinin-piperaquine, artesunate-amodiaquine and artesunate-mefloquine for the treatment of uncomplicated malaria (WHO, 2015).

The mechanism of action of the artemisinins is still under investigation, but it is suggested that artemisinin and related analogues act by a Fe(II) mediated cleavage of endoperoxide linkage of the compound leading to the formation of oxygen free radicals. The latter re-arrange to carbon free radicals and target the parasite proteins, eventually leading to parasite death (Van Agtmael *et al*., 1999).

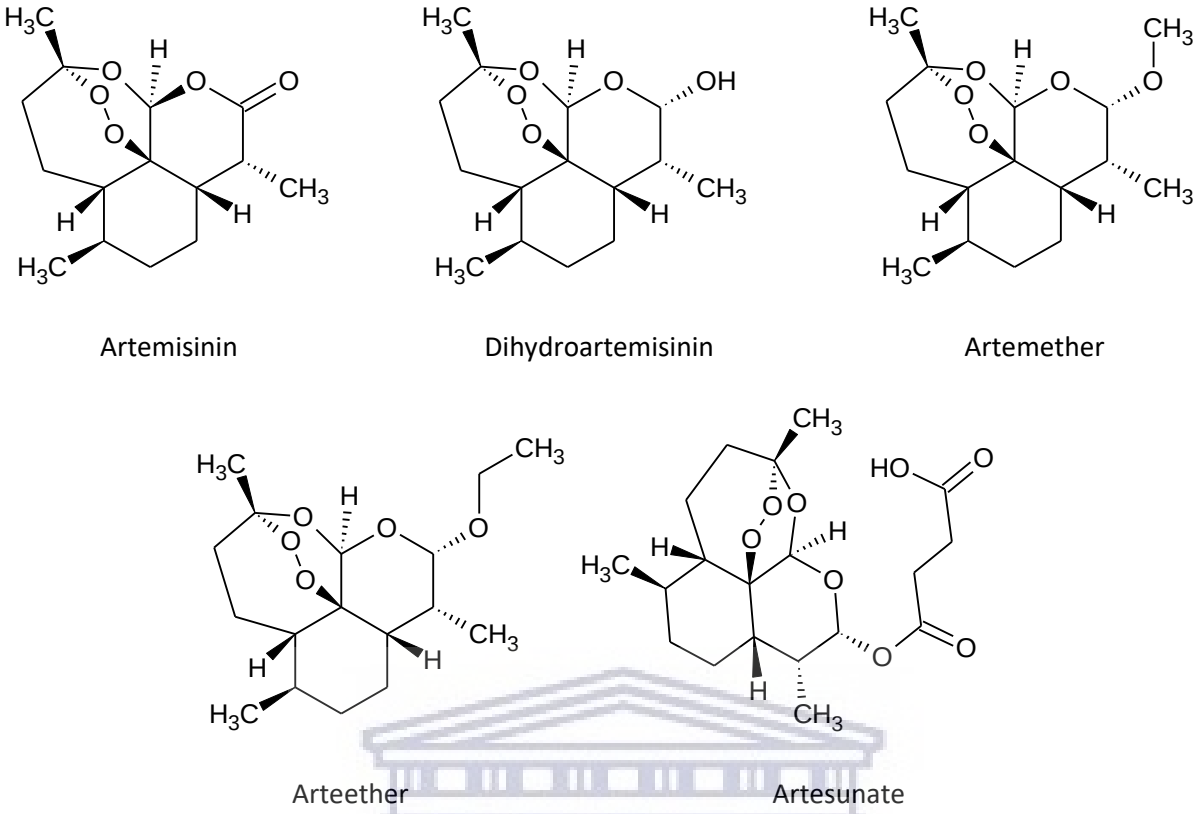


Figure 2.7: Artemisinin and its derivatives.

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2.2 Cheminformatics

New classes of medicine to combat malaria are urgently needed due to the surge in resistance of the parasite to antimalarial drugs. Even more recent therapies (ACTs) recommended by the WHO have shown emerging *P. falciparum* resistance in South-East Asia (WHO, 2017). The identification of new scaffolds from natural products with *in vitro* antiplasmodial activities (NAA) can be an ideal starting point for the design and synthesis of novel antimalarial drugs with new mechanism(s) of action able to circumvent the problem of resistance. One interdisciplinary field which can aid in the discovery of novel chemical entities and ultimately the design of new drug-like molecules is cheminformatics.

Cheminformatics is the interdisciplinary field which could be helpful for the discovery and design of new molecules. The main application of cheminformatics is to analyse, simulate, model and manipulate chemical information which can be represented either in a 2D or 3D structure using a proper database (Begam *et al*., 2012). One area where

new chemical entities can be explored to find potential novel synthetic drugs is natural products.

Natural products (NP) and their derivatives have been a powerful part in the fight against malaria and NP scaffolds have been the basis of the majority of current anti-malarial drugs (Soh *et al.*, 2007; Wells *et al.,* 2011). Drugs such as quinine and artemisinin which were originally isolated from herbal medicinal products have significantly contributed to the antimalarial arsenal, so natural products with antiplasmodial activities (NAA) may represent a source of potentially new pharmacophores, with probable new mechanisms of action to circumvent the resistance of the malaria parasite (Wells *et al.,* 2011).

Since the molecular scaffolds as well as the pharmacophore features of a compound define the uniqueness of a compound, exploration of scaffolds of NAA may lead to identification of new antimalarial chemotypes (Egieyeh *et al.,* 2016). The limitation on the application of plant natural products is that they possess highly complex structures, consequently it is often difficult to synthesize these compounds on an industrial scale. Even where a total synthesis procedure has been established, this method is frequently not economically viable to produce the target natural product on a commercial scale (Yun *et al*., 2012).

As a result, there is a need to find a way to design compounds which have similar pharmacophoric groups, look like and behave like natural products with antiplasmodial activities, but with simpler structures which would enable industrial scale synthesis. One way of designing chemical compounds with the previously stated characteristics is to design virtual compound (VC) libraries with structural features of natural products, but with simpler structures. Previously, computational approaches were used to design virtual compounds from NAA (Egieyeh *et al.,* 2016). Virtual compound (VC) libraries are useful only if individual molecules can practicably be selected from them, which constitutes a general starting point for molecule prioritization (Cramer *et al.,* 1998). The main implication with the design of VC is that a very large amount of molecules is generated. This makes it impossible to synthesise and test all compounds from the VC library. Therefore molecule selection is of utmost importance (Cramer *et al.,* 1998).

The computational tool KNIME (Konstanz Information Miner) (figure 2.8) can be used for the prioritization and selection of the most promising compounds from a library of

VC designed from NAA using predefined parameters. KNIME is a widely used open source data mining tool developed at University of Konstanz and it is available online (Germany) (Berthold *et al.,* 2009).

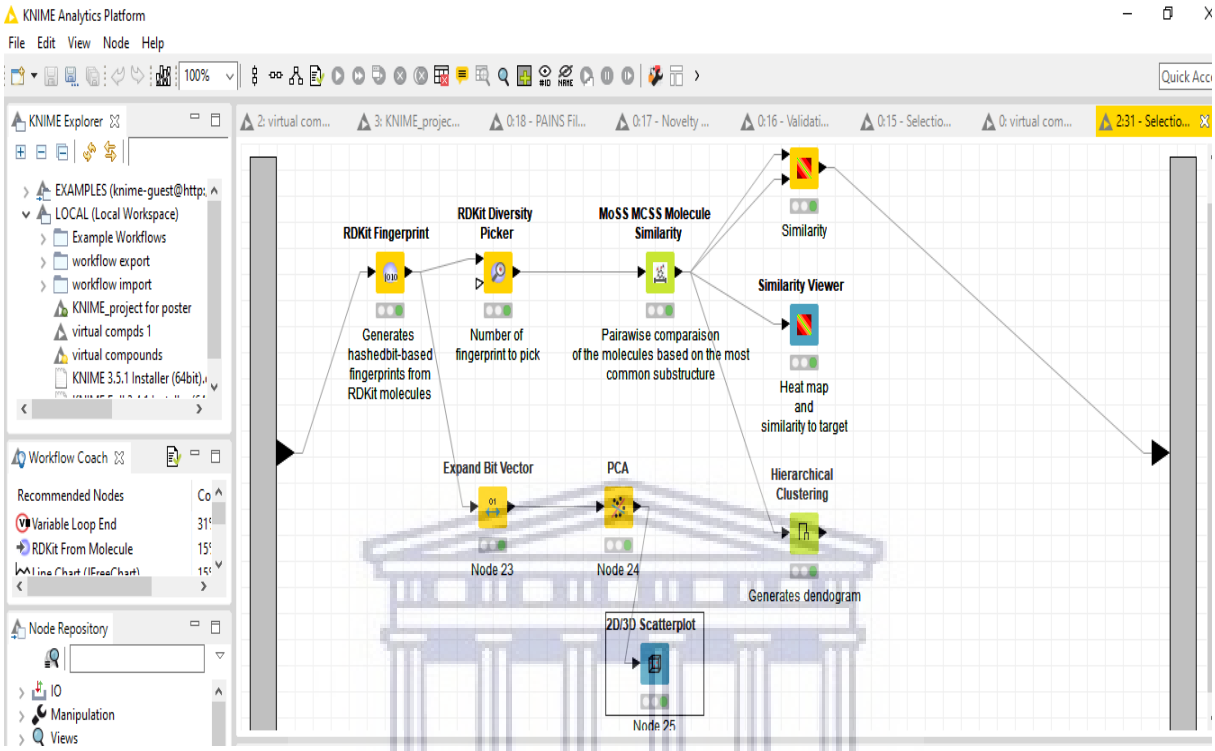


Figure 2.8: Screenshot of the standard KNIME workbench with a small example workflow.

Given the challenges associated with the alarming increased resistance of the *P. falciparum* to current available chemotherapies and the high cost of the treatment, it is imperative to explore new avenues to combat the malaria parasite. Artificial intelligence (computational approaches) offers an opportunity for the design, prioritization, selection and ultimately the synthesis of novel compounds with potentially new mechanisms of action against malaria.

CHAPTER 3

USE OF CHEMINFORMATICS FOR PRIORITIZATION AND SELECTION OF COMPOUNDS FROM A VIRTUAL COMPOUND LIBRARY

3.1 Introduction

Cheminformatics is a relatively new interdisciplinary field that may be used for the characterization, prioritization and selection of compounds from virtual or *in vitro* screening platforms. Here, cheminformatic techniques were used to discover new chemical entities from an antiplasmodial virtual compound library with the ultimate goal to select the most promising potential anti-malarial compounds for synthesis and biological evaluation. The antiplasmodial virtual compound library (https://drive.google.com/drive/u/0/folders/1Pc9zGn-29IVShsVUgDsDBEQ4YM88LIMS) was generated from natural products with antiplasmodial activities (NAA) using an evolutionary virtual compound enumeration algorithm (Egieyeh, 2016). NAA were retrieved from published articles, MSc and PhD theses, textbook chapters, collaborative drug discovery databases, ChEMBL and PubChem (Egieyeh *et al*., 2016).

In this chapter, the process and results of characterization, prioritization and selection of virtual compounds (VC) from the antiplasmodial virtual compound library for synthesis and biological evaluation are discussed. Virtual compounds (VC) with greater chance of success as novel antiplasmodial lead compounds in the drug discovery pipeline were identified for synthesis and biological evaluation.

3.2 Characterization, prioritization and selection of virtual compounds

A Konstanz Information Miner (KNIME) workflow (Figure 3.1), which consist of six major steps, was developed for the characterization, prioritization and selection of virtual compounds (VC) with the greater chance of success as a novel antiplasmodial lead compounds. KNIME is an analytical platform which enable easy visual assembly and interactive execution of a data pipeline using predefined parameters. KNIME

workflow consist of nodes that process data from a data source, which are transported *via* connection between those nodes (Berthold *et al.,* 2009).

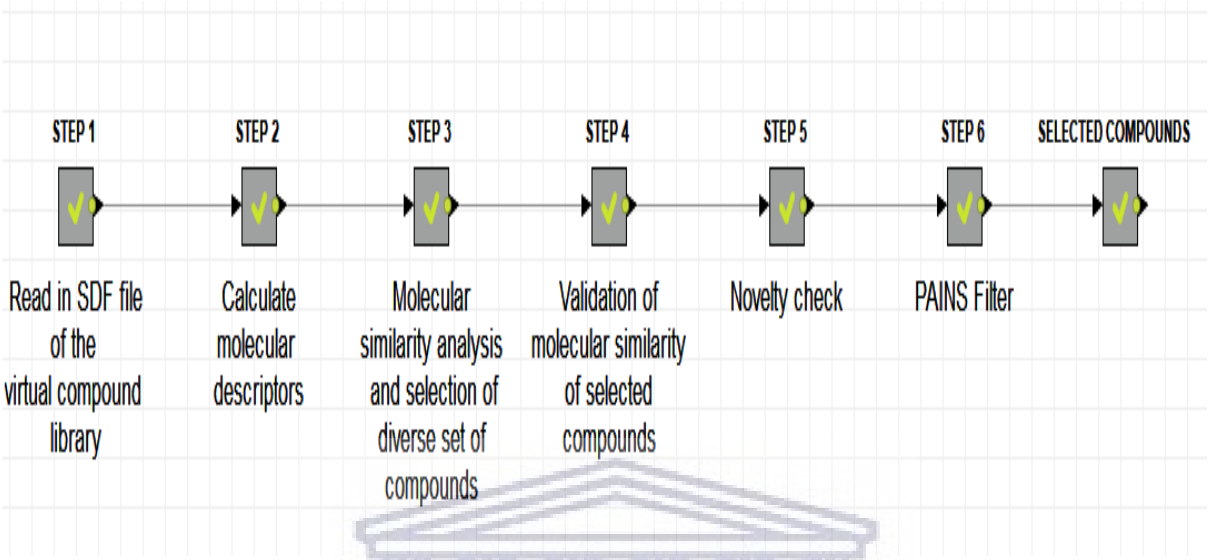


Figure 3.1: KNIME workflow overview

Using the computational tool KNIME and the workflow developed (Figure 3.1), the following will explain each of the 6 steps that was required for the characterization, prioritisation and selection of the most promising VC.

3.2.1 Read in SDF file of the virtual compound library

In the first step, the virtual compound library of 164530 (VCs) in SDF format, generated as previously described (Egieyeh, 2016), was read in with a SDF reader node in KNIME. The SDF reader node creates columns encoded in the SDF file with each molecule in a new row (Figure 3.2). Faulty molecules (e.g. with incorrect bonds) are excluded.

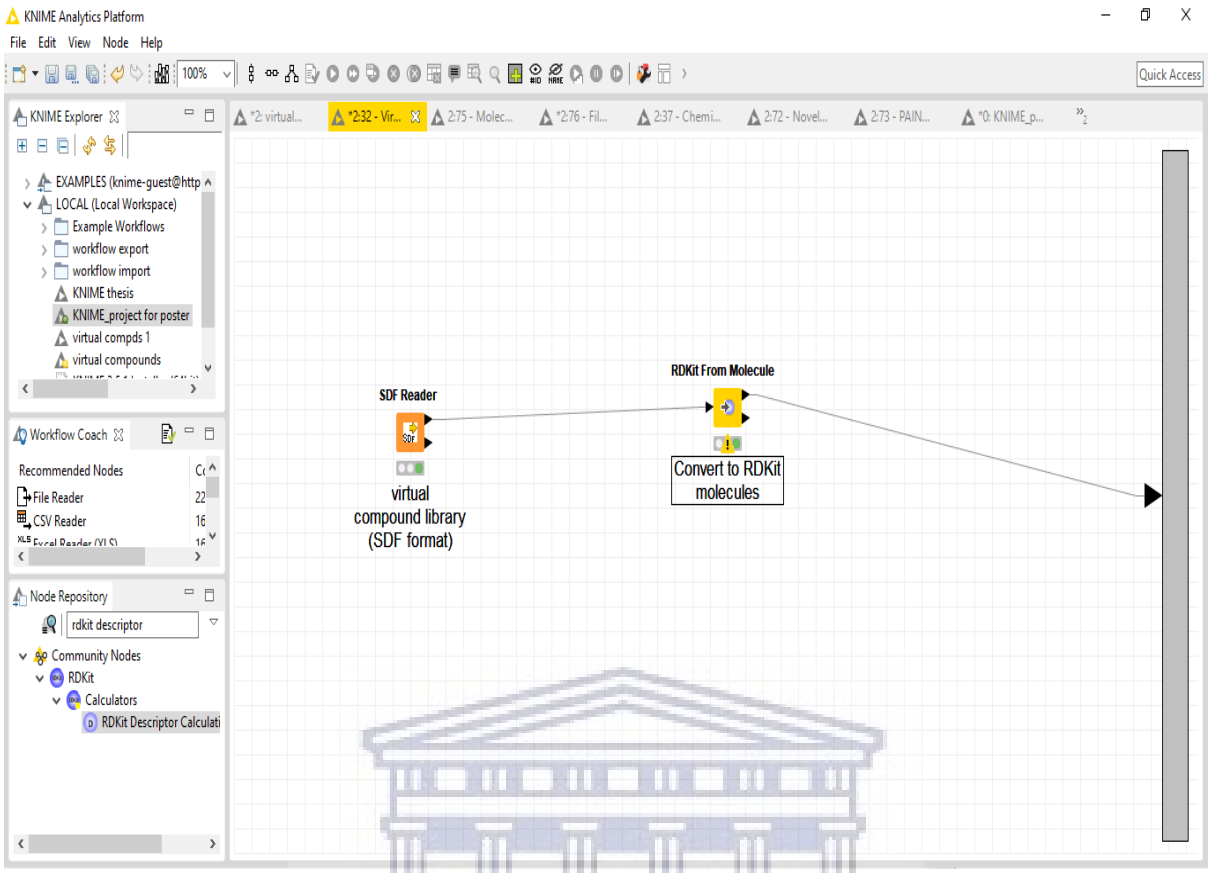


Figure 3.2: KNIME workbench consisting of SDF reader node to read the VC library. RDKit from molecule node to convert the molecule to RDKit molecules.

3.2.2 Calculate molecular descriptors

In second step, molecular descriptors included in the Lipinski's rule of five (Ro5) were calculated for each molecule in the compound set. Lipinski's rule of five is an aid to characterise the drug-likeness and bioavailability profile of the VCs (Lipinski *et al*., 2004). Each molecular descriptor that define the Ro5 was used as filtering and selection criteria with cut-off values such as *H-bond donors ≤ 5, H-bond acceptors ≤ 10, 350 ≤ MW ≤ 600, 2 ≤ log P≤ 5* (Figure 3.3). Molecules that fell within the Ro5 filtering criteria cut-off values were retained (1805 molecules).

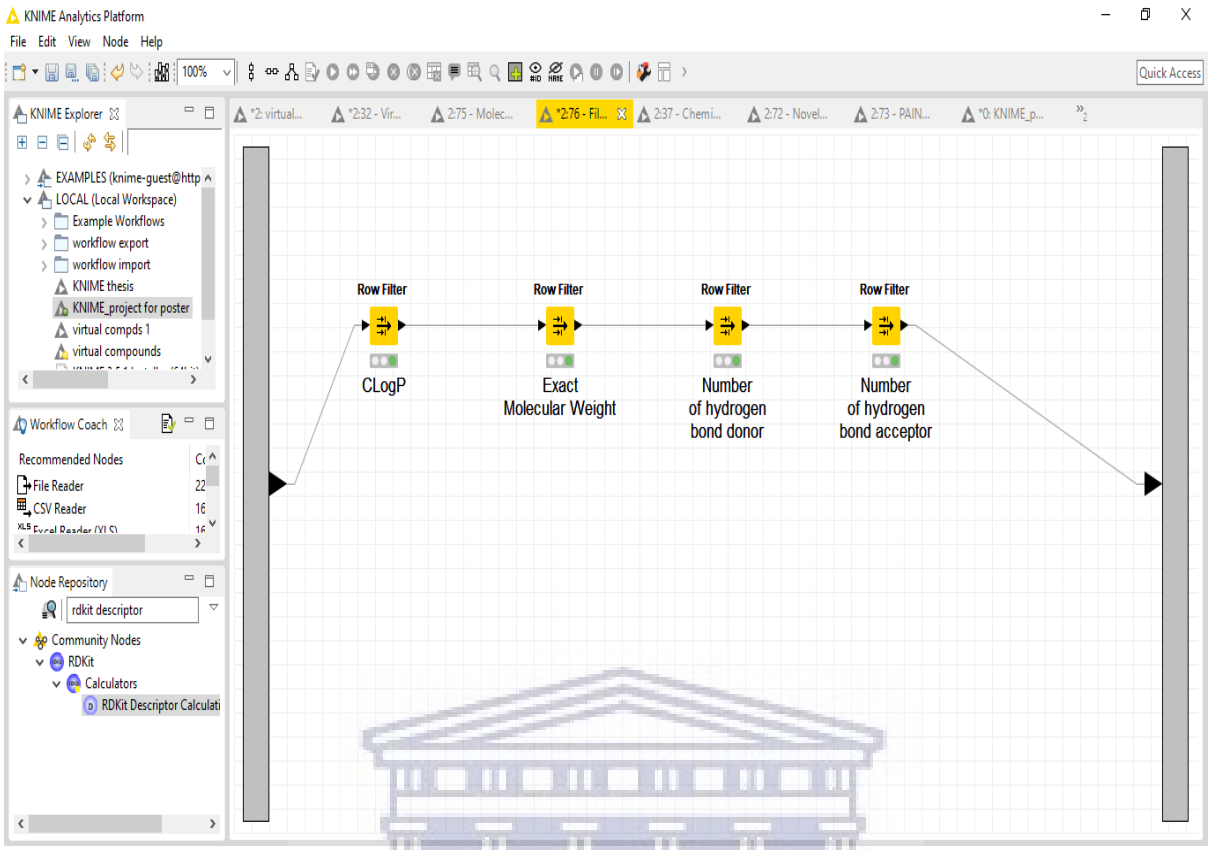


Figure 3.3: KNIME workbench consisting of molecular descriptors nodes defining the Ro5

3.2.3 Molecular similarity analysis and selection of diverse set of compounds

In third step, exploration of the similarity amongst the compound set was performed with the aim of selecting diverse compounds from the compound set. A pairwise comparison of molecules based on the most common substructure (MCSS) was done amongst the 1805 retained VC to assess the extent of similarity or diversity of the compound set. The results from the foregoing (Figure 3.4) showed that the retained compounds were sufficiently diverse (i.e. low similarity amongst the compound set). A highly diverse chemical library increases the hit rate from such a library (Shi & von Itzstein, 2019). Therefore, the diversity observed in the retained VC suggests that they may have a high hit rate in antimalarial screening.

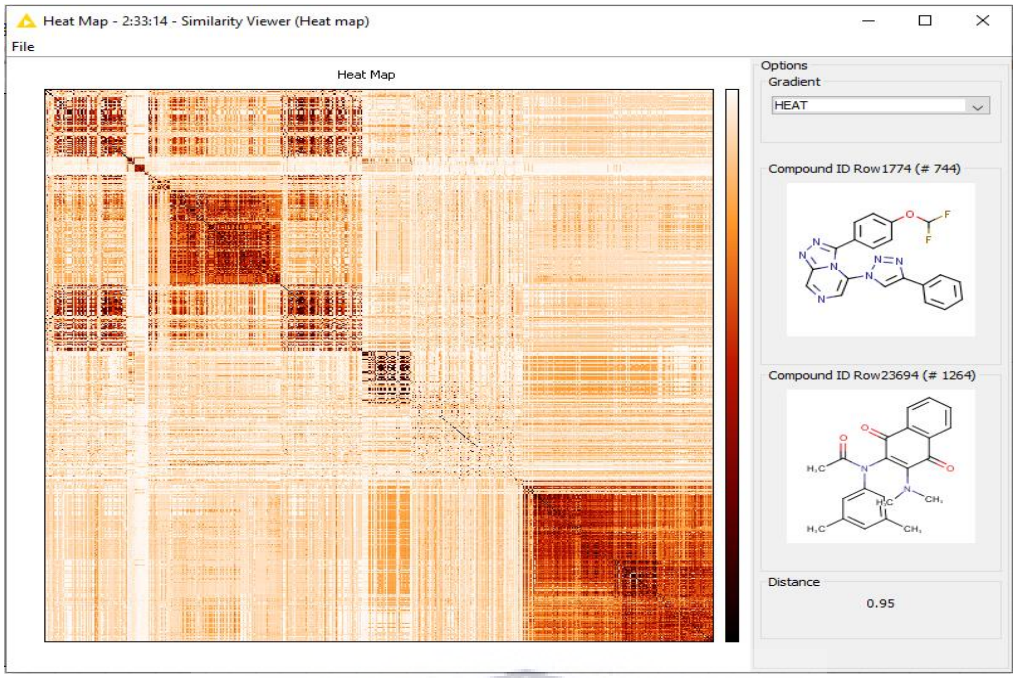


Figure 3.4: The heat map from the MCSS analysis of the 1805 compounds, using the similarity viewer node. The dark red regions (short distance) on the heat map represent compounds that are structurally close to each other or highly similar.

Furthermore, the compound set retained were displayed in a three dimension chemical space (Figure 3.5). The three dimensions were the first three principal components from principal component analysis (PCA) performed on the retained compound set (using the structural fingerprints of the molecules as the descriptive features). This was done to avoid the selection of compound sets that are in the same region or coordinates in the chemical space. Exploration of the chemical space of the retained 1805 VC showed five main clusters (Figure 3.5).

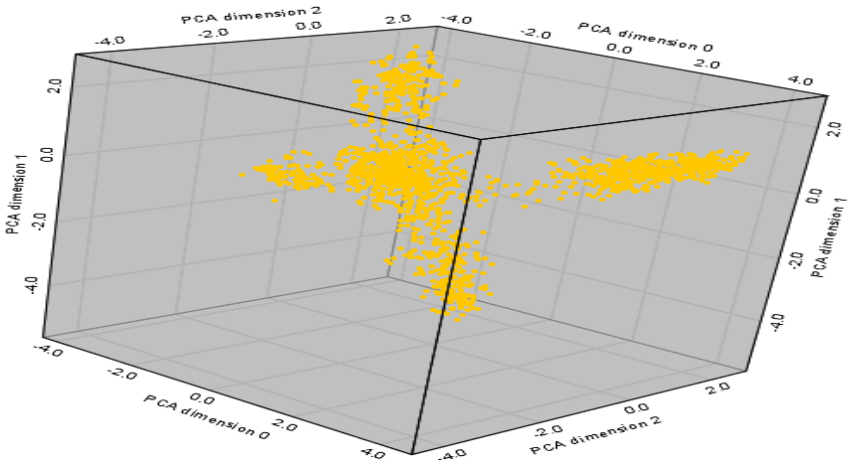


Figure 3.5: Chemical space coordinates of compounds sets (in yellow the 1805 VC).

A total of 25 molecules were selected (five from each cluster) for further profiling and filtering (Figure 3.6). Rational selection of diverse compounds from chemical libraries maximizes the hit rates from such selected compounds and makes them amenable to hit to lead optimization (Huggins *et al*., 2011).

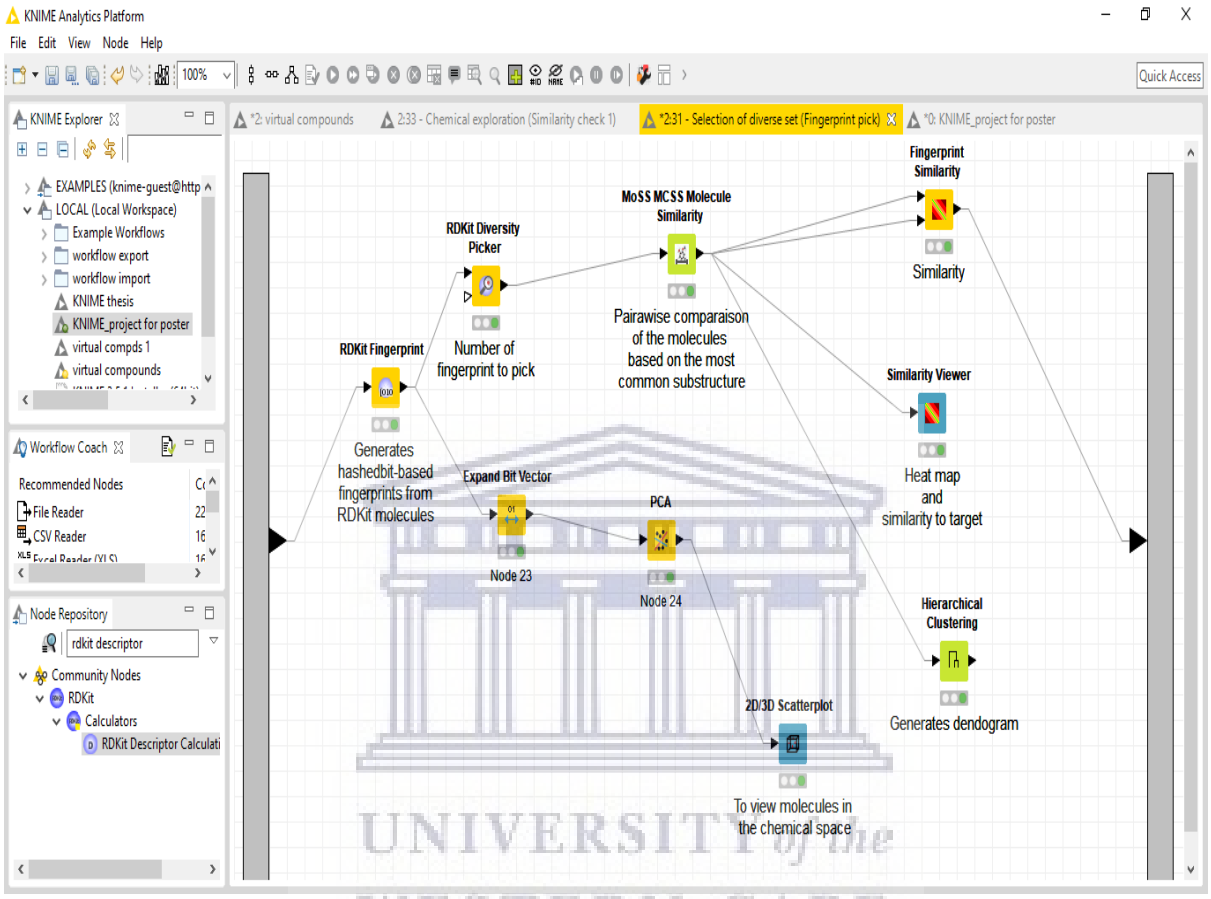


Figure 3.6: KNIME workbench containing nodes used for the Molecular similarity analysis and selection of diverse set of compounds

3.2.4 Validation of molecular similarity of selected compounds

In the fourth step, a second pairwise comparison of the 25 selected molecules was performed to assess their similarity based on their most common substructure (MCSS). The results were presented as a heat map (Figure 3.7) and dendrogram (Figure 3.8). This enabled the visualisation of the distance between two molecules. The relationship between distance and similarity of two virtual compounds is given by Eq. (1) (Bajusz *et al.,* 2015).

Eq. (1) Similarity = 1/(1+distance)

When the distance between two molecules is equal to zero the VC have similar structures, as the distance increases to 1 between the VC, their similarity decrease suggesting structural diversity.

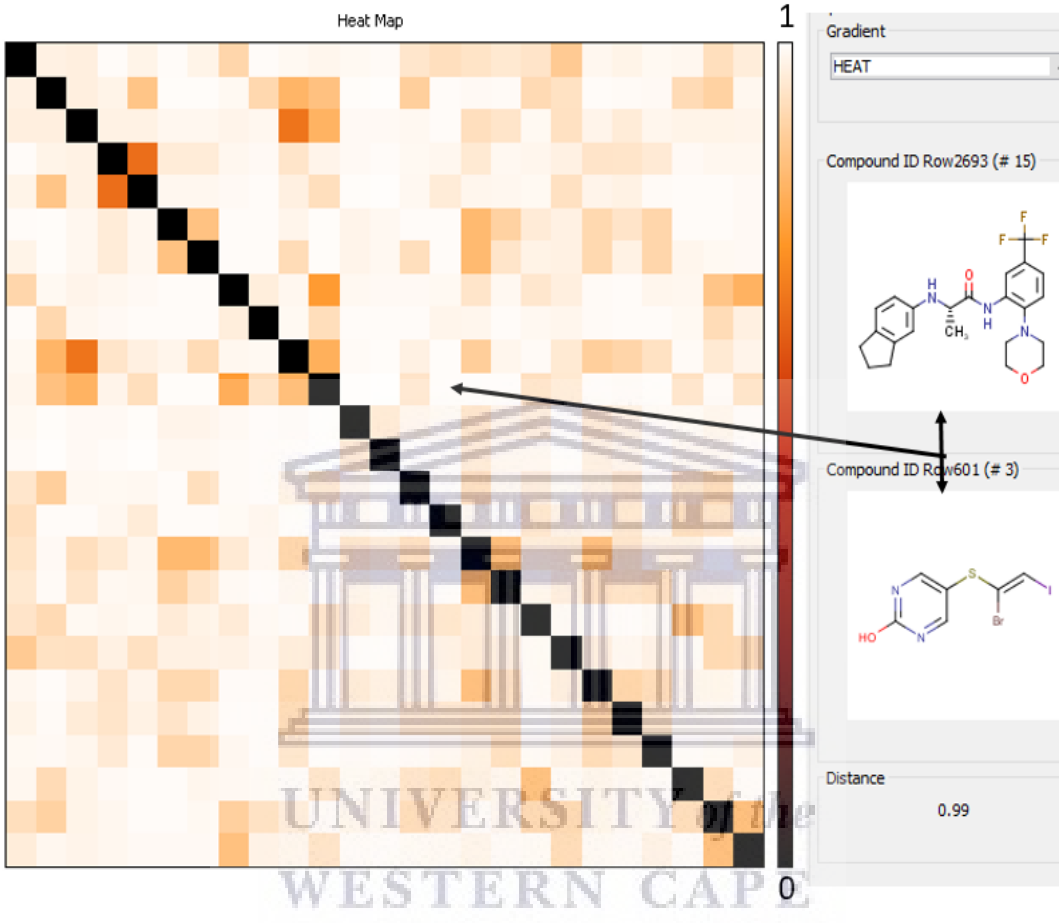


Figure 3.7: Heat map of the 25 selected virtual compounds (diverse set) based on most common substructure in KNIME. The light/white colour (high distance) on the map represent compounds that are structurally far apart or highly diverse.

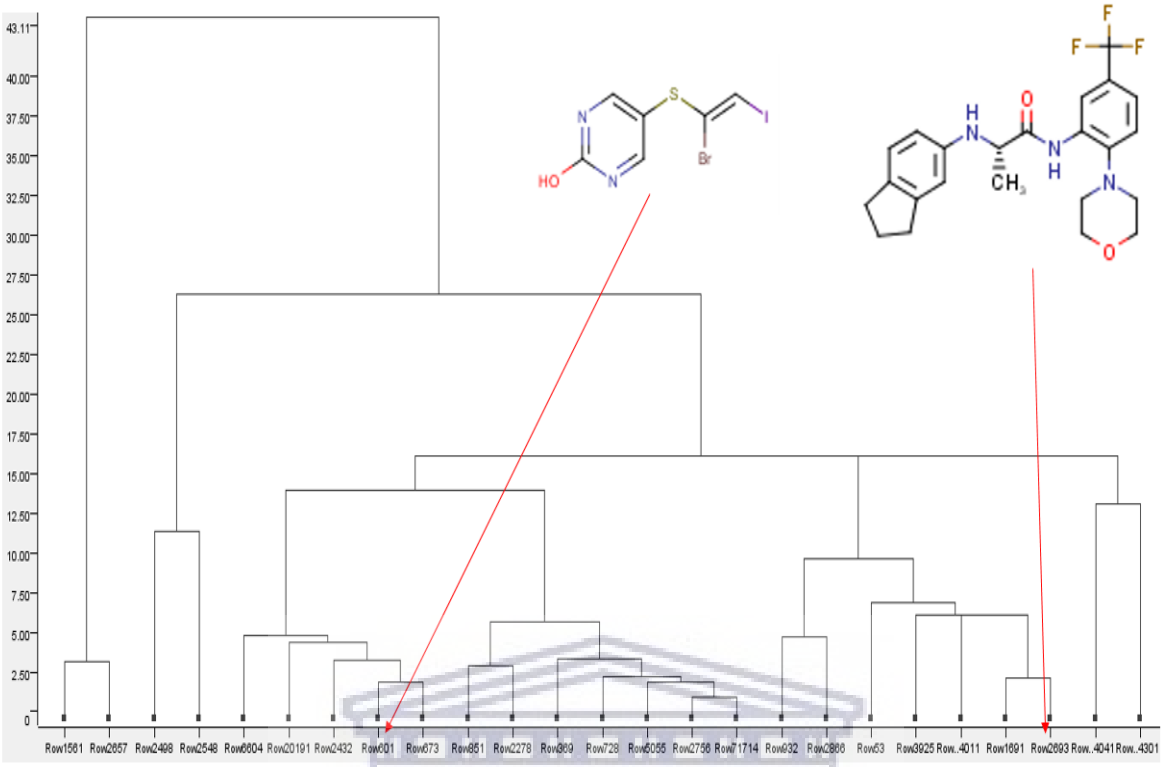


Figure 3.8: Dendrogram of the 25 selected VC generated in KNIME. It shows the hierarchical relationship amongst the compounds. It allocates compounds to clusters based on most common substructure (MCSS). The horizontal axis of the dendrogram represents the distance or dissimilarity amongst the compounds.

3.2.5 Novelty check

In the fifth step, the selected VC were used to perform similarity searches against molecules found in ChEMBL, which is a chemical database of bioactive molecules. Virtual compounds with more than 70% similarity to molecules found in ChEMBL were identified (Figure 3.9). A number of molecules in ChEMbl were found to have varying percentage similarity to our VC (Figure 3.10). Excitingly, a number of the VC did not show similarity (> 70%) to existing compounds. These VC that are not similar to compounds in the ChEMBL database are novel compounds with potential novel antimalarial activities. The discovery of novel compounds that may be active against malaria creates a chance to get a return on investment from antimalarial drug discovery process. Any existing patents do not cover such compounds and they provide a platform for optimization into novel analogues.

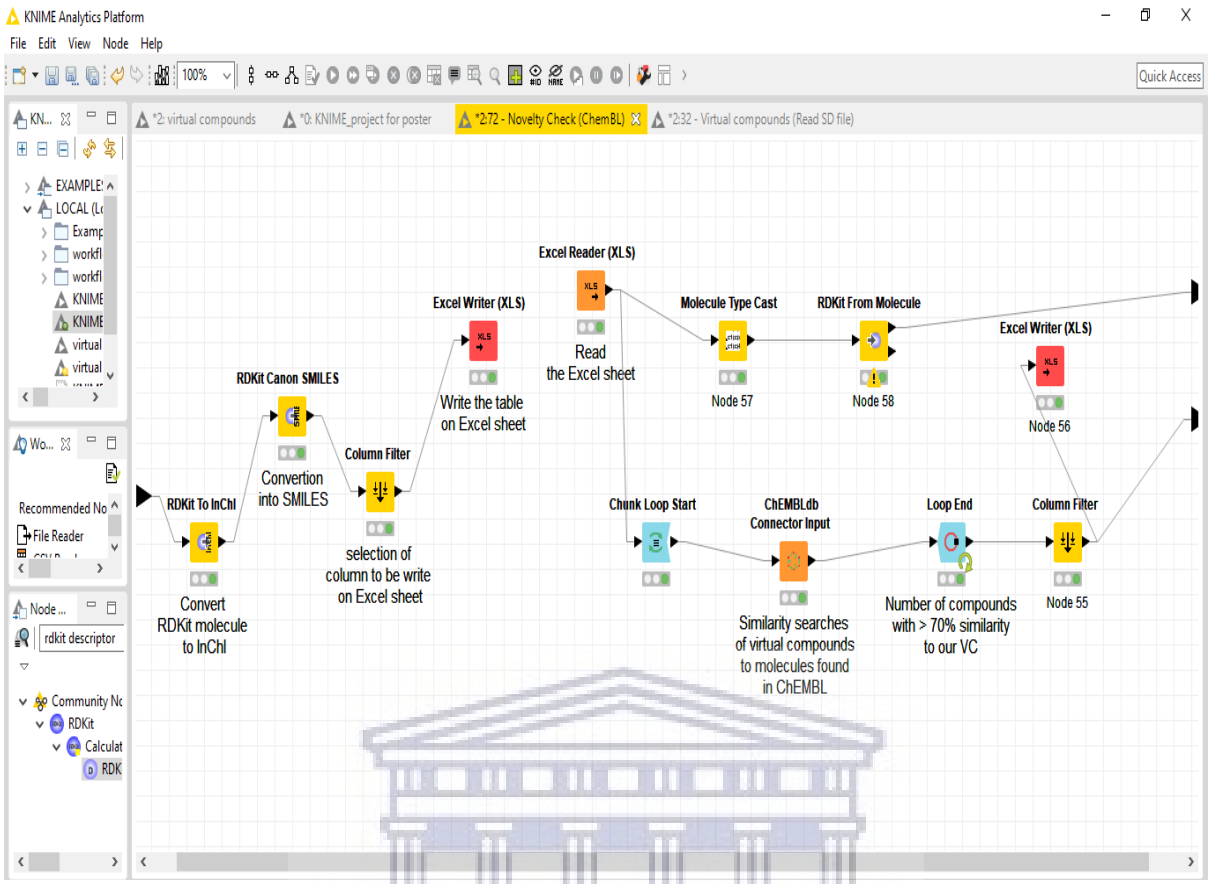


Figure 3.9: KNIME workbench containing nodes for similarity searches of our VC against molecules found in ChEMBL.

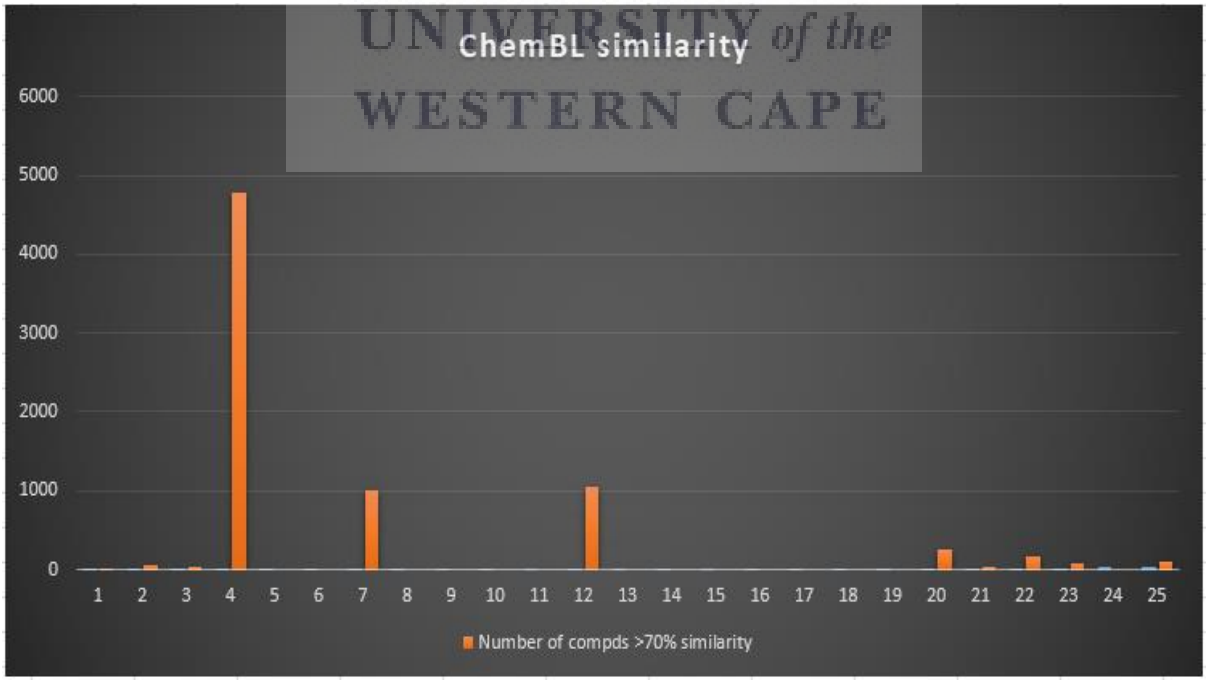


Figure 3.10: Number of compounds in ChEMBL with more than 70% similarity to the 25 diverse set of VC (In orange the number of compounds found in ChEMBL).

3.2.6 PAINS filter

In the last step, PAINS filter was used to identify VC that could have nonspecific interaction with multiple biological targets. PAINS (pan assay interference compounds) are compounds that contain substructures that tend to react non-specifically with numerous biological targets rather than specifically affecting one desired target. PAINS are a prominent source of false positives. Literature has reported 960 PAINS substructures amongst which 50 PAINS substructures were found in the selected 25 VC (Figure 3.11). Fourteen out of the 25 VC had two PAINS substructures; two VC had four PAINS substructures; one VC had six and another one had eight PAINS substructures. Seven of the selected VC did not have any known PAINS substructure (Figure 3.12). Compounds with PAINS substructures may show exciting bioactivities, they typically turn out to be non-progressive within the drug discovery pipeline. This suggests that follow-up on these compounds (with PAINS substructures) may lead to loss of millions of dollars on dead-end research and hundreds, if not thousands, of hours of research time.

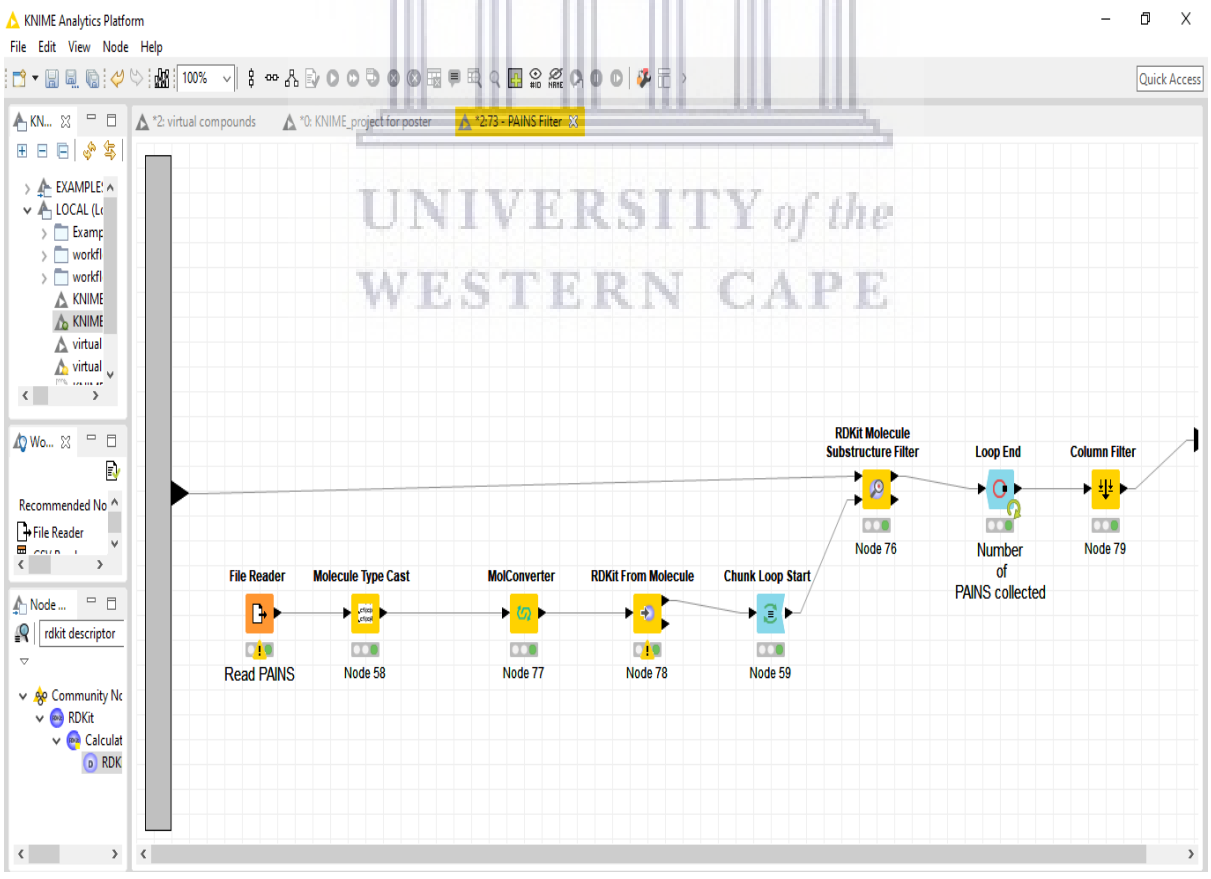


Figure 3.11: KNIME workbench containing nodes for PAINS identification.

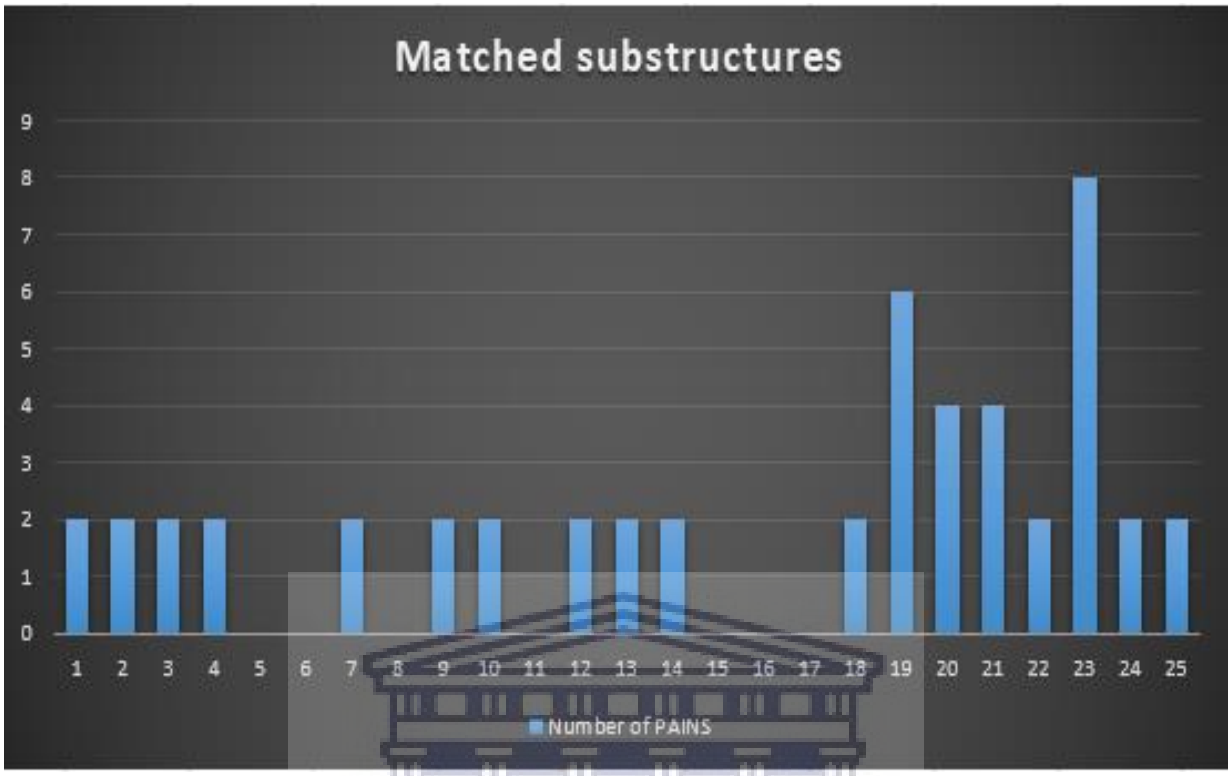
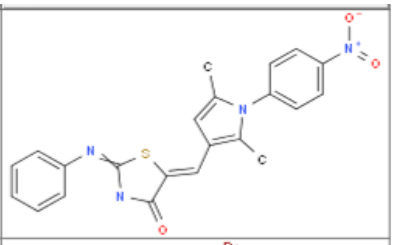
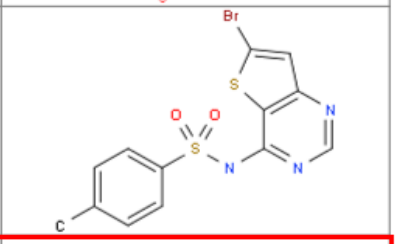
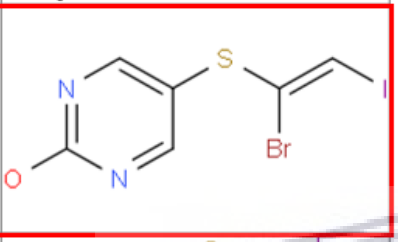
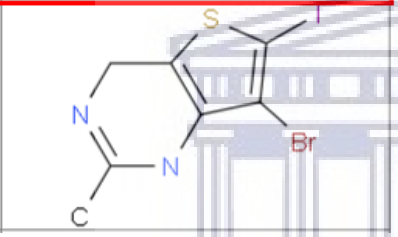
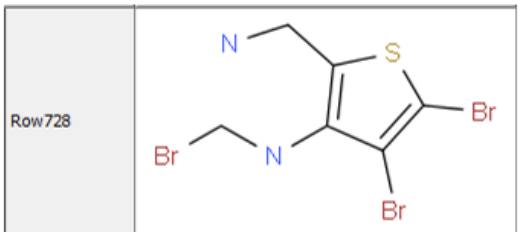
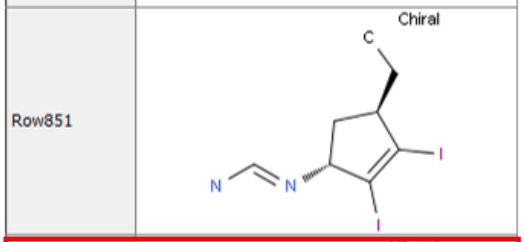
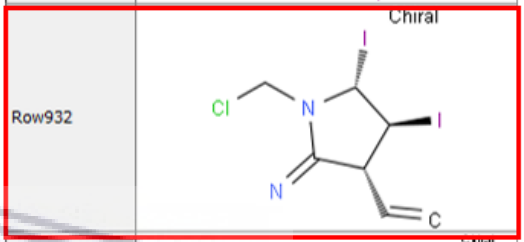
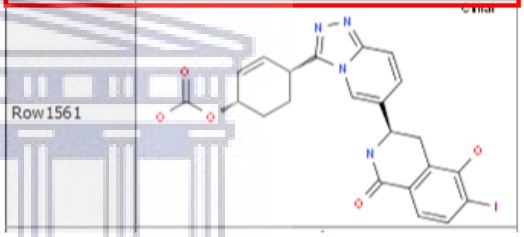
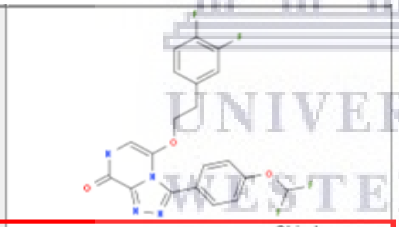
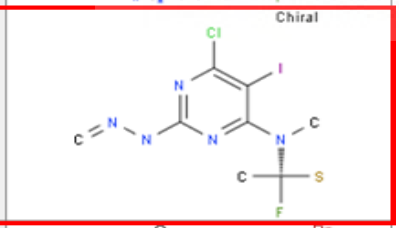
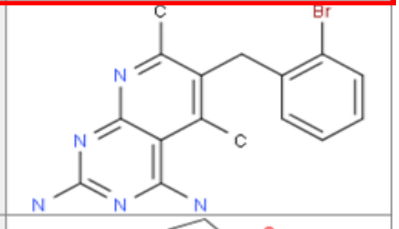
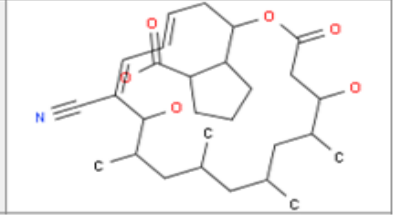


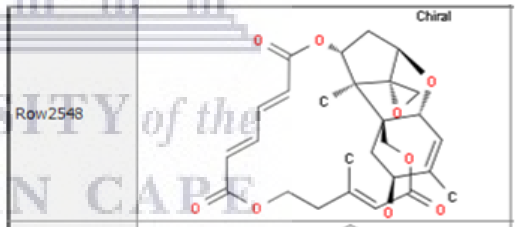
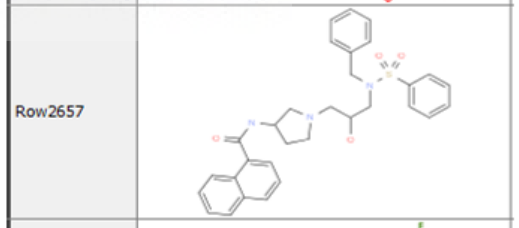
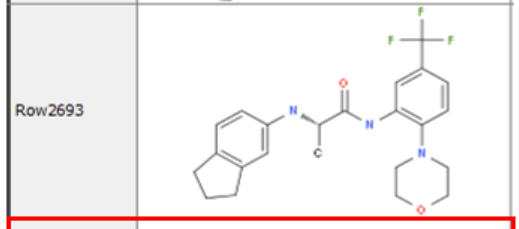
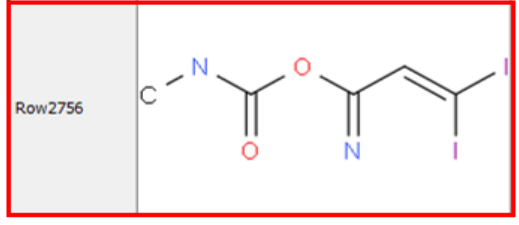
Figure 3.12: Number of PAINS fond in each of 25 diverse set of VC.

The synthetic feasibility of each of the diverse 25 VC (Figure 3.13 and 3.14) were identified using the website SWISSADME (http://www.swissadme.ch/). This web platform is used in drug discovery to predict ADME (absorption, distribution, metabolism, excretion) parameters and evaluate the pharmacokinetic, drug-likeness and medicinal chemistry friendliness of molecules (Diana *et al*., 2017). The ease of synthesis of molecules can be predicted by the evaluation of the synthetic accessibility score generated by SWISSADME. The difficulty in synthesis of a selected molecule increases as the synthetic accessibility score increases from 1 to 10 (1 very easy to synthesise, 10 very difficult to synthesise). Therefore it expected for a compound having a score of 6 to be difficult to synthesise compared to a compound with a score of 2.

Row53	
Row369	
Row601	
Row673	

Row728	
Row851	
Row932	
Row1561	

Row1691	
Row2278	
Row2432	
Row2498	

Row2548	
Row2657	
Row2693	
Row2756	

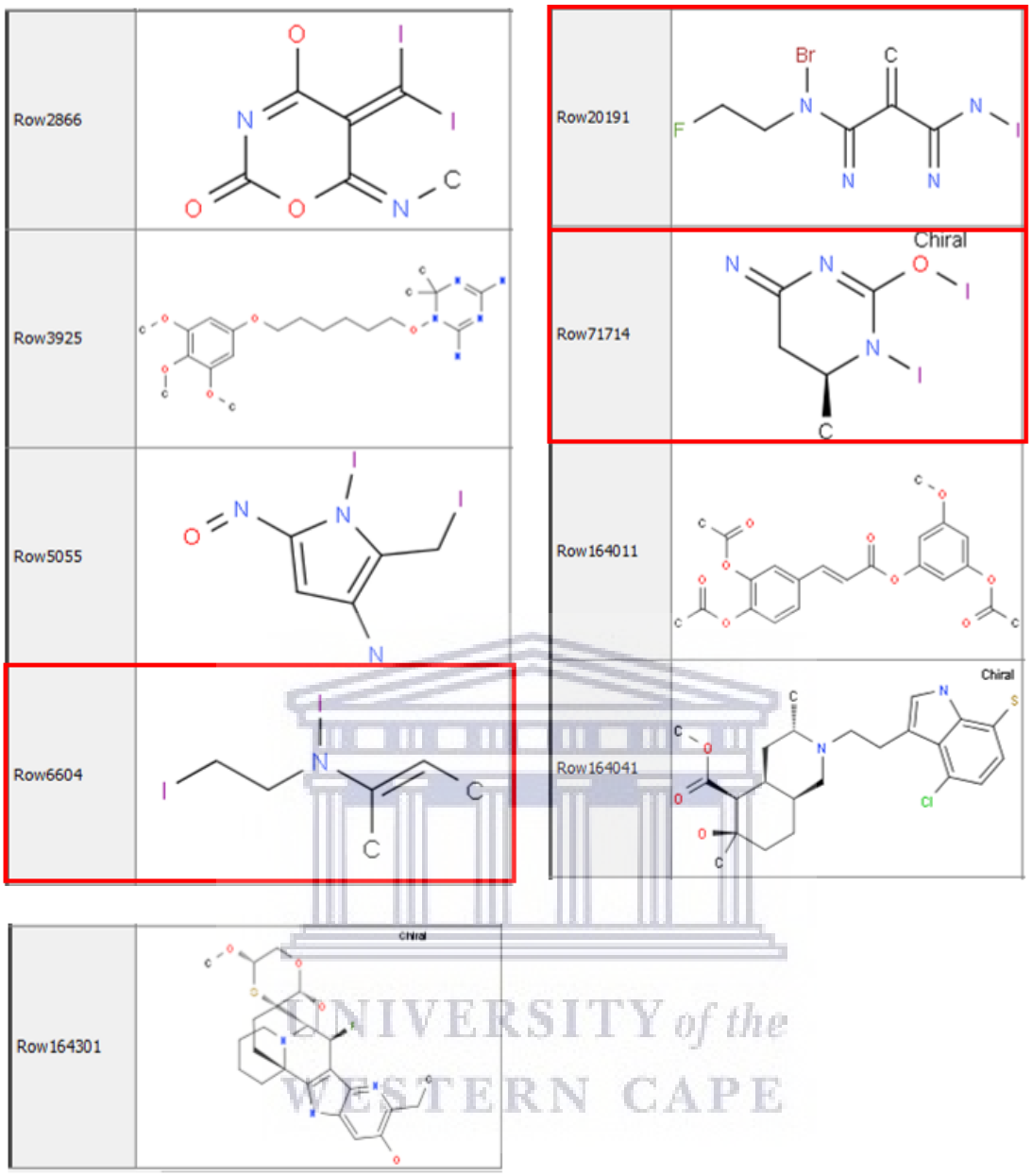


Figure 3.13: Structural overview of the diverse 25 VC (compounds with no PAINS are highlighted in red)

Row No	Row No in virtual compound	Row No of PAINS	No compds>70% similarity in ChemBL		No of PAINS	Synthetic accessibility score
1	1561	418; 898	11	0,145138 %	2	4,49
2	2657	418; 898	49	0,646523 %	2	4,53
3	2498	418; 898	37	0,488191 %	2	6,8
4	2548	418; 898	4783	63,10859 %	2	7,36
5	6604	None	0	0 %	None	3,56
6	20191	None	0	0 %	None	3,34
7	2432	418; 898	1008	13,29991 %	2	2,82
8	601	None	0	0 %	None	2,67
9	673	418; 898	0	0 %	2	3,31
10	851	418; 898	0	0 %	2	3,96
11	2278	None	0	0 %	None	3,44
12	369	418; 898	1054	13,90685 %	2	3,08
13	728	418; 898	0	0 %	2	3,01
14	5055	418; 898	0	0 %	2	2,52
15	2756	None	0	0 %	None	2,54
16	71714	None	0	0 %	None	3,87
17	932	None	0	0 %	None	3,87
18	2866	464; 944	0	0 %	2	3,31
19	53	95; 418; 430; 575; 898;910	0	0 %	6	3,98
20	3925	411; 418; 891; 898	263	3,470115 %	4	4,72
21	164011	411; 418; 891; 898	33	0,435414 %	4	3,31
22	1691	418; 898	158	2,084708 %	2	3,53
23	2693	161; 418; 430; 477; 641; 910; 957; 898	82	1,081937 %	8	3,61
24	164041	418; 898	0	0 %	2	4,21
25	164301	418; 898	101	1,33263 %	2	6,32
		TOTAL	7579	100 %	50	

Figure 3.14: Summary of the findings for the diverse 25 VC.

3.3 Conclusion

A library of virtual compounds designed from NAA was characterized or profiled. Prioritization and selection of compounds were conducted based on drug-like properties and parameters that defines probability for successful development into drug candidate. Using the computational platform KNIME, 1805 VC were prioritized from a library of 164532 VC and a final number of 25 diverse VC were selected for further exploration. As proof of concept, the compound found in row 12 (Figure 3.14) was selected as the most promising candidate for synthesis and biological evaluations. This was decided firstly because it is not economically viable trying to synthesise all the diverse 25 VC with their analogues, secondly the synthetic routes of those compounds were not easy to find even though some compounds shown no PAINS (no non-specific interaction with drug targets) and no molecules with more than 70% similarity in ChEMBL (novelty of the compound). Therefore the decision came to the

compound that could be synthesised with the lowest number of PAINS and smallest number of compounds with more than 70% similarity in ChEMBL – the compound found in row 12 (Figure 3.14) was chosen for this study as initial proof-of-concept.

CHAPTER 4

SYNTHETIC PROCEDURE

This chapter highlights all the experimental work carried out in this study to synthesize a series of novel compounds from a virtual compound library. Synthetic procedures of all 6 compounds as well as challenges that were encountered are reported herein. The compounds successfully synthesized were characterized by nuclear magnetic resonance (NMR), mass spectrometry (MS) and infrared (IR) spectroscopy.

4.1 Standard experimental procedures

4.1.1 Instrumentation

Nuclear magnetic resonance spectroscopy (NMR): ^{1}H and ^{13}C NMR spectra were obtained using Bruker Avance IIIHD Nanobay spectrometer equipped with a 5 mm BBO probe at resonance frequency of 400 MHz and 100 MHz, respectively. All chemical shifts were reported in parts per million (ppm) relative to the signal from tetramethylsilane (TMS; $\delta = 0$) added to an appropriate deuterated solvent. The following abbreviations are used to describe the multiplicity of the respective signals:

- s-singlet
- bs-broad singlet
- d-doublet
- dd-doublet of doublets
- t-triplet
- m-multiplet

The relevant spectra are included in the annexure.

Infrared spectroscopy (IR):

The IR spectra were recorded on a Perkin Elmer Spectrum 400 spectrometer, fitted with a diamond attenuated total reflectance (ATR) attachment. The data were analysed on a computer connected to the spectrometer. Relevant spectra are included in the annexure.

Mass spectroscopy (MS):

The MS spectra were obtained from a Perkin Elmer Flexar SQ 300 detector. The samples were dissolved in dimethyl sulfoxide, filtered through a 0.2 micron filter and diluted with 50 % (aq.) dimethyl sulfoxide before injecting 100 µl of the 50 ppm solution *via* UHPLC auto-sampler into SQ 300 MS. Relevant spectra are included in annexure.

Melting point (MP):

Melting points of all synthesised compounds were determined using a Lasec SMP-10 melting point apparatus and capillary tubes. The melting points are uncorrected.

Microwave reactor:

Microwave synthetic procedures were performed utilising a CEM Discover® focused closed vessel reactor. This method was used to shorten reaction time and the reaction yields were generally higher.

4.1.2 Chromatographic techniques

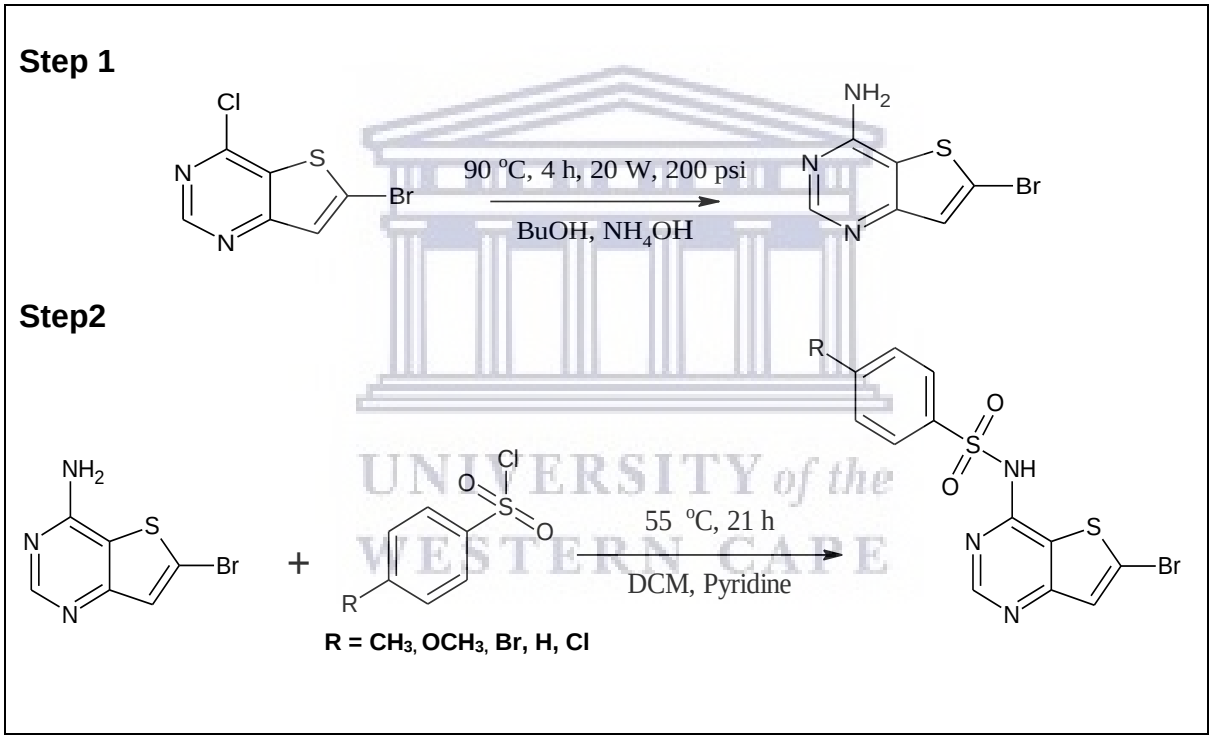
All reactions were monitored with thin layer chromatography (TLC), using 0.20 mm thick aluminium silica gel sheets (TLC Silica gel 60 F254 Merck KGaA). The mobile phases were prepared on a volume-to-volume basis, 3:7 (ethanol:hexane) and 1:2 (hexane:ethylacetate). Visualisation was achieved using UV light (254 nm and 366 nm) and/or iodine vapours. Product mixtures were purified by Column chromatography using silica gel (0.063 - 0.200 mm/70 - 230 mesh ASTM, Macherey-Nagel, Duren, Germany) as the stationary phase with hexane:ethyl acetate in the ratio of 1:2 as mobile phase.

4.1.3 Materials

Except if otherwise specified, all materials were purchased from commercial suppliers (Sigma-Aldrich, Merck) and used without further purification. Solvents were dried using standard methods.

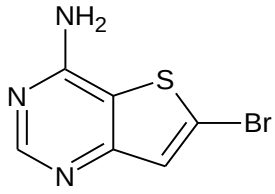
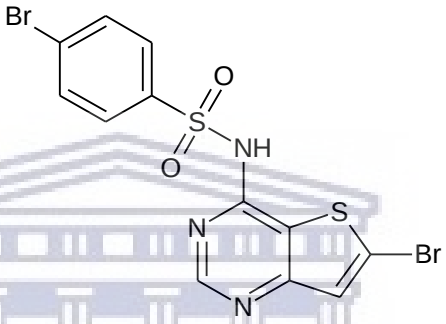
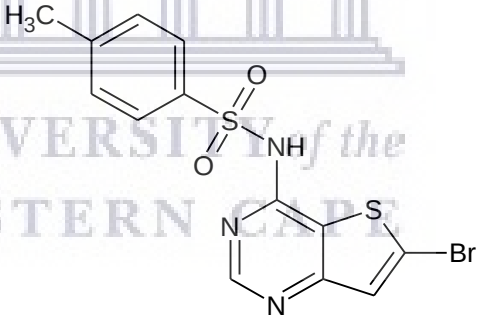
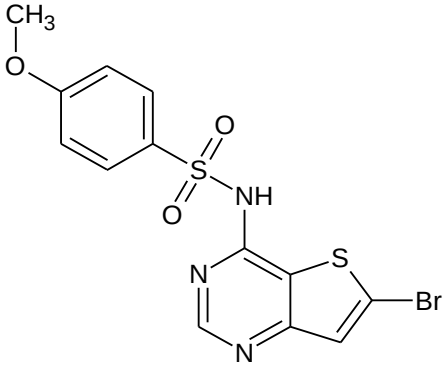
4.2 General synthesis routes

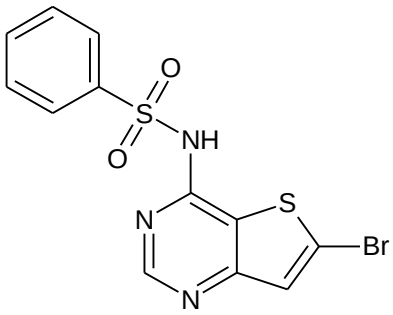
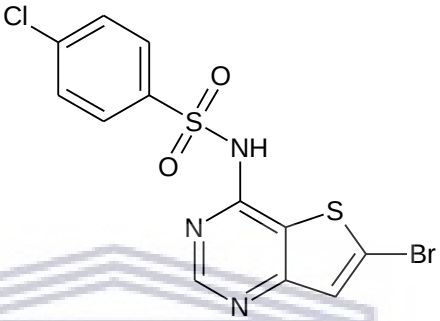
The general synthesis method is shown in figure 3.1. The preparation of *N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)-4-methylbenzenesulfonamide was accomplished using a two-step procedure. The first step consisting of dehalogenation of the commercially available 6-bromo-4-chlorothieno[3,2-d]pyrimidine using an ammonia solution to produce the intermediate 6-bromothieno[3,2-d]pyrimidin-4-amine (**1**, scheme 1: step 1, table 4.1) (McDonald *et al*., 2012). The resultant intermediate was further reacted with commercially available R-para substituted benzenesulfonyl chloride by sulphonamide formation to produce the final compounds (scheme 1: step 2, table 4.1) (Everson *et al*., 2013).



Scheme 1: The general synthesis of the final sulphonamide containing compounds.

Table 4.1: Assigned names and structures of the final compounds selected for synthesis.

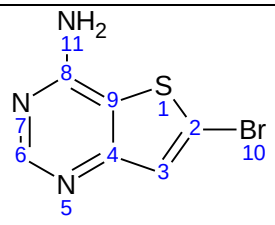
Assigned number	Structure and name of each final compound
1	 **6-bromothieno[3,2-d]pyrimidin-4-amine**
2	 ***N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)-4-bromobenzenesulfonamide**
3	 ***N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)-4-methylbenzenesulfonamide**
4	 ***N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)-4-methoxybenzenesulfonamide**

5	 ***N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)benzenesulfonamide**
6	 ***N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)-4-chlorobenzenesulfonamide**

4.3 Synthesis of selected compounds

All NMR, MS and IR spectrum results for each compound are found in annexure and labelled accordingly in text.

4.3.1 6-bromothieno[3,2-*d*]pyrimidin-4-amine (compound 1)

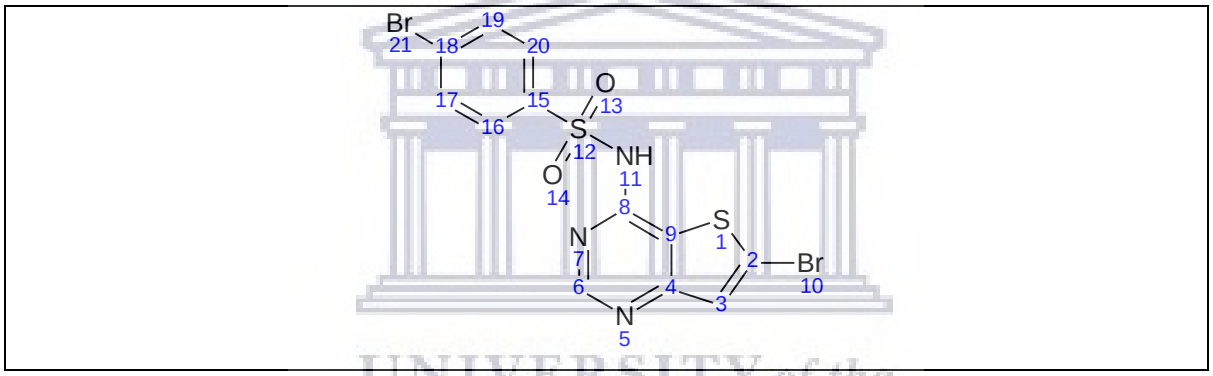


Synthesis: In a sealed microwave compatible glass-vessel was added 6-bromo-4-chlorothieno[3,2-*d*]pyrimidine (0.1 g, 0.4 mmol) and ammonia solution (0.63 ml, 16.25 mmol) in butanol (0.35 ml). Using a microwave (MW) irradiation method, the reaction mixture was heated to 90 ^{o}C while stirring for 4 hours, with a pressure of 200 psi and power of 20 W. Thereafter the reaction mixture was allowed to cool to room

temperature. The reaction mixture was filtered and the resulting white precipitate was washed with cold butanol and collected to give 6-bromothieno[3,2-*d*]pyrimidin-4-amine (0.076 g, 76 % yield).

Physical data: $C_6H_4BrN_3S$; **mp**: 240 - 243 ^oC; **^{1}H NMR** (400MHz, $CDCl_3$-d) δ (Spectrum 1): 8.56 (s, 1 H, H-6), 7.45 (s, 1 H, H-3); **^{13}C NMR** (100 MHz, DMSO-d) (Spectrum 2): 159.56, 157.02, 155.36, 127.58, 122.43, 115.42; **MS** (ESI-MS) m/z, (Spectrum 3): 229.93 $[M+H]^+$, 231.93 $[M+H]^+$+2; **IR** (ATR, cm^{-1}) Vmax (Spectrum 4): 3089, 1529, 1514.

4.3.2 *N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)-4-bromobenzenesulfonamide (compound 2)

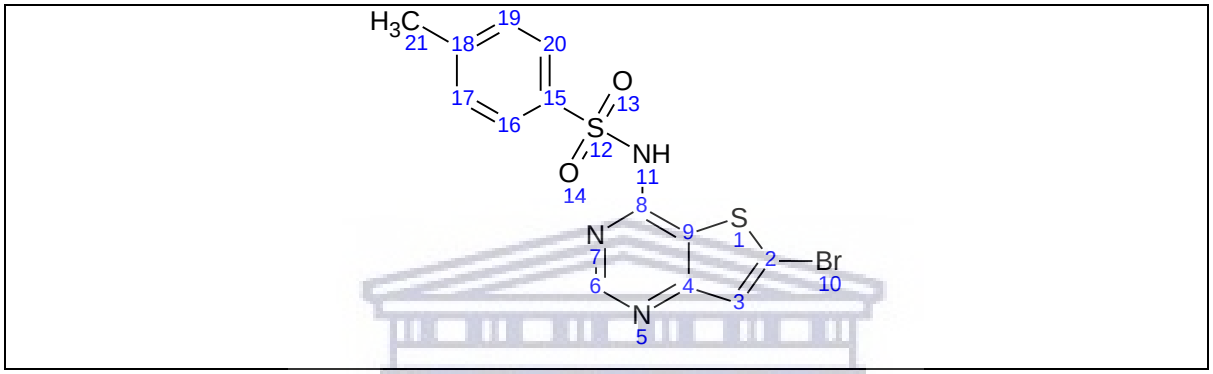


Synthesis: 4-Bromobenzenesulfonyl chloride (56.1 mg, 0.22 mmol) and 6-bromothieno[3,2-*d*]pyrimidin-4-amine (50 mg, 0.22 mmol) were weighed and added to a 50 ml round-bottomed flask equipped with a magnetic stir bar. Dry dichloromethane (0.57 ml) was then poured into the vessel, followed by pyridine (0.02 ml). The reaction mixture was then heated to reflux temperature (55 ^oC) for 21 h. The reaction mixture was monitored by TLC and considered complete when the 4-bromobenzenesulfonyl chloride spot on the TLC plate was no longer visible. The reaction mixture was purified by column chromatography using hexane:ethyl acetate in a ratio of 1:2 as eluent to obtain 4-bromo-*N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)benzene sulfonamide as a white powder (yield: 0.003 g, 3 %).

Physical data: $C_{12}H_7Br_2N_3O_2S_2$; **mp**: 150-152 ^oC; **^{1}H NMR** (400MHz, DMSO-d) δ (Spectrum 5): 8.11 (s, 1 H, H-6), 7.84-7.82 (d, 2 H, J = 8.6 Hz, H-20,16), 7.64-7.62 (d,

2H, J = 8.7 Hz, H-19,17), 7.41 (s, 1 H, H-3); **^{13}C NMR** (100 MHz, DMSO-d) (Spectrum 6): 157.56, 151.84, 147.71, 138.68, 130.61, 127.76, 125.69, 124.44, 121.57, 115.55; **MS**(ESI-MS) m/z, (Spectrum 7): 447.84 $[M+H]^+$, 449.84, 451.83 $[M+H]^+$+2; **IR** (ATR, cm^{-1}) Vmax (Spectrum 8): 3222, 3098, 2922, 2853, 2253, 1620, 1572.

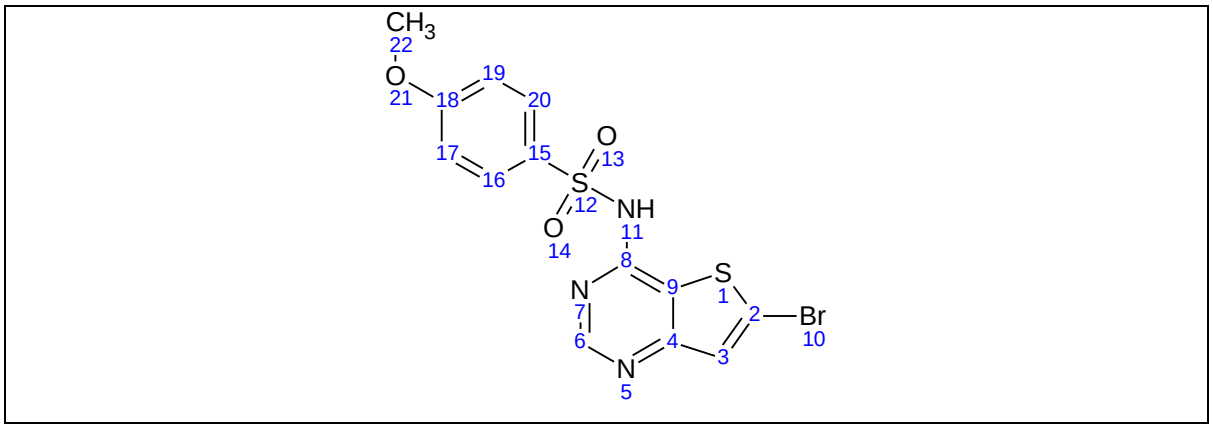
4.3.3 *N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)-4-methylbenzenesulfonamide (compound 3)



Synthesis: 4-Methylbenzenesulfonyl chloride (41.8 mg, 0.22 mmol) and 6-bromothieno[3,2-*d*]pyrimidin-4-amine (50 mg, 0.22 mmol) were weighed and added to a 50 ml round-bottomed flask equipped with a magnetic stir bar. Dry dichloromethane (0.57 ml) was then poured into the vessel, followed by pyridine (0.02 ml). The reaction mixture was then heated at reflux temperature (55 ^{o}C) for 21h. The reaction mixture was monitored by TLC using hexane/ethyl acetate in the ratio 1:2 as mobile phase and considered complete when the 4-methylbenzenesulfonyl chloride spot on the TLC plate was no longer visible. The reaction mixture was purified by column chromatography using hexane:ethyl acetate in a ratio of 1:2 as eluent to obtain *N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)-4-methylbenzene sulphonamide as pale yellow solid (yield: 0.005 g, 6 %).

Physical data: $C_{13}H_{10}BrN_3O_2S_2$; **mp**: 160 - 163 ^{o}C; **^{1}H NMR** (400MHz, $CDCl_3$-d) δ (Spectrum 9): 8.09 (s, 1 H, H-6), 7.86-7.84 (d, 2 H, J = 8.3 Hz, H-20,16), 7.38 (s, 1 H, H-3), 7.30-7.28 (d, 2 H, J = 7.9 Hz, H-19,17); 2.41 (s, 3 H, H-21);; **^{13}C NMR** (100 MHz, DMSO-d) (Spectrum 10): 157.81, 150.12, 145.73, 137.60, 128.04, 127.27, 126.89, 125.49, 122.89, 115.61, 20.78 ; **MS**(ESI-MS) m/z, (Spectrum 11): 383.94 $[M+H]^+$, 385.94 $[M+H]^+$+2; **IR** (ATR, cm^{-1}) Vmax (Spectrum 12): 3086, 2920, 1716, 1702, 1566.

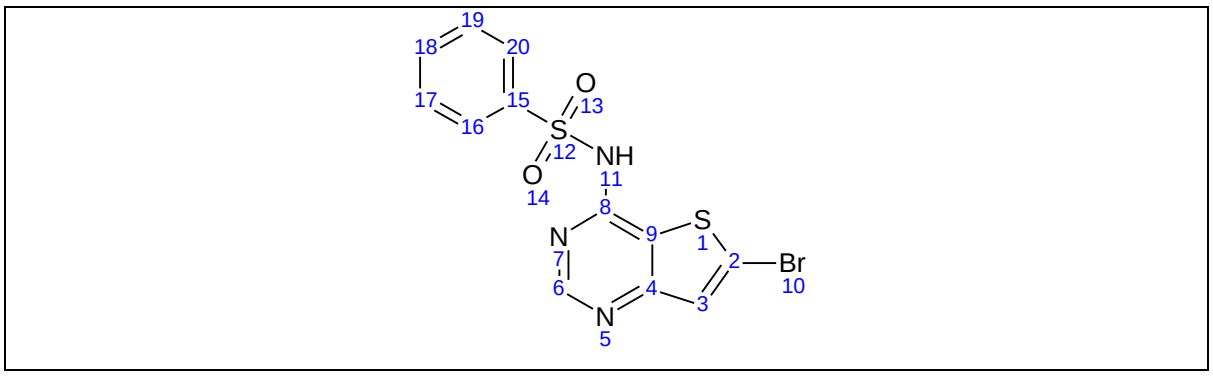
4.3.4 *N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)-4-methoxybenzenesulfonamide (compound 4)



Synthesis: 4-Methoxybenzenesulfonyl chloride (45.4 mg, 0.22 mmol) and 6-bromothieno[3,2-*d*]pyrimidin-4-amine (50 mg, 0.22 mmol) were weighed and added to a 50 ml round-bottomed flask equipped with a magnetic stir bar. Dry dichloromethane (0.57 ml) was then poured into the vessel, followed by pyridine (0.02 ml). The reaction mixture was then heated at reflux temperature (55 ^{o}C) for 21h. The reaction mixture was monitored by TLC and considered complete when the 4-methoxylbenzenesulfonyl chloride spot on the TLC plate was no longer visible. The reaction mixture was purified by column chromatography using hexane: ethyl acetate in a ratio of 1:2 as eluent to obtain *N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)-4-methoxybenzene sulphonamide as a yellow amorphous solid (yield: 0.006 g, 7 %).

Physical data: $C_{13}H_{10}BrN_3O_3S_2$; **mp**: 162 – 165 ^{o}C; **^{1}H NMR** (400MHz, $CDCl_3$-d) δ (Spectrum 13): 8.11 (s, 1 H, H-6), 7.91-7.89 (d, 2 H, J = 8.97 Hz, H-20,16), 7.37 (s, 1H, H-3), 6.97-6.95 (d, 2 H, J = 8.96 Hz, H-19,17), 3.85 (s, 3H, H-22); **^{13}C NMR** (100 MHz, DMSO-d) (Spectrum 14): 159.15, 157.57, 152.19, 151.26, 141.09, 127.02, 124.36, 125.75, 115.54, 112.70, 55.12; **MS** (ESI-MS) m/z, (Spectrum 15): 399.94 $[M+H]^+$, 401.94 $[M+H]^+$+2; **IR** (ATR, cm^{-1}) Vmax (Spectrum 16): 3242, 2922, 2852, 1574.

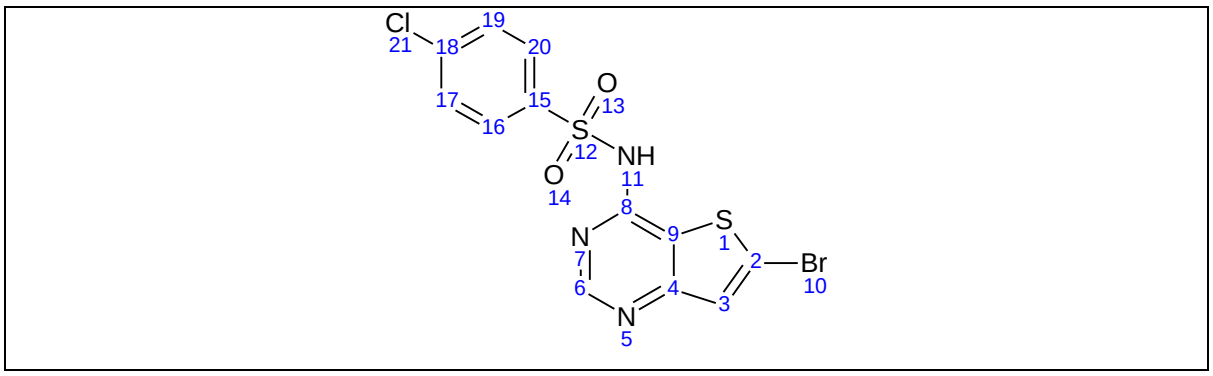
4.3.5 *N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)benzenesulfonamide (compound 5)



***Synthesis**:* The commercially available benzenesulfonyl chloride (28 µl, 0.22 mmol) and 6-bromothieno[3,2-*d*]pyrimidin-4-amine (50 mg, 0.22 mmol) were weighed and added to a 50 ml round-bottomed flask equipped with a magnetic stir bar. Dry dichloromethane (0.57 ml) was then poured into the vessel, followed by pyridine (0.02 ml). The reaction vessel was then topped with a plastic stopper before stirring (500 rpm) at room temperature (25 ^{o}C) for 10h. The reaction mixture was monitored by TLC plate and considered complete when the benzenesulfonyl chloride spot was no longer visible on the TLC plate using UV light. Thereafter the reaction mixture is then poured in a 100 ml separatory funnel, extracted with dichloromethane (20 ml x 2), washed with distilled water (20 ml) and brine (20 ml). The organic layer was collected in a 100 ml round-bottomed flask and the solvent was evaporated using a rotary evaporator. A yellow wax was collected without further purification (yield: 0.070 g, 86 %).

Physical data: $C_{12}H_8BrN_3O_2S_2$; **mp**: wax; **^{1}H NMR** (400MHz, $CDCl_3$-d) δ (Spectrum 17): 8.97 (s, 1 H, H-6), 8.96 (s, 1 H, H-3), 7.97-7.90 (m, 3 H, H-20,16,18), 7.38-7.36 (m, 2H, H-19,17); **^{13}C NMR** (100 MHz, DMSO-d) (Spectrum 18): 157.99, 149.43, 148.19, 146.57, 142.10, 128.49, 127.68, 125.48, 122.25, 115.62; **MS** (ESI-MS) m/z, (Spectrum 19): 369.93 $[M+H]^+$, 371.92 $[M+H]^+$+2; **IR** (ATR, cm^{-1}) Vmax (Spectrum 20): 3090, 2813, 2570, 1653, 1578.

4.3.6 *N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)-4-chlorobenzenesulfonamide (compound 6)



***Synthesis**:* The commercially available 4-chlorobenzenesulfonyl chloride (46.4 mg, 0.2197 mmol) and 6-bromothieno[3,2-*d*]pyrimidin-4-amine (50 mg, 0.22 mmol) were weighed and added to a 50 ml round-bottomed flask equipped with a magnetic stir bar. Dry dichloromethane (0.57 ml) was then poured into the vessel, followed by pyridine (0.02 ml). The reaction vessel was then topped with a rubber stopper and 21-gauge needle to vent the reaction to air before stirring (500 rpm) at room temperature (25 ^{o}C) for 9h. At different time interval (t = 3h, t = 6h), 0.15 ml of DCM was added to the reaction mixture. The reaction mixture was monitored by TLC plate and considered complete when the 4-chlorobenzenesulfonyl chloride spot was no longer visible on the TLC plate using UV light. The reaction mixture was then filtered and the precipitate was collected. The filtrate was poured in a 100ml separatory funnel and extracted with dichloromethane (20 ml x 2), washed with distilled water (20 ml) and brine (20 ml). The organic layer was collected in a 100 ml round-bottomed flask and the solvent was evaporated using a rotary evaporator to obtain the *N*-(6-bromothieno[3,2-*d*]pyrimidin-4-yl)-4-chlorobenzenesulfonamide. A brown solid was collected without further purification (yield: 0.018 g, 20 %).

Physical data: $C_{12}H_7BrClN_3O_2S_2$; **mp**: 165 - 168 ^{o}C; **^{1}H NMR** (400MHz, $CDCl_3$-d) δ (Spectrum 21): 8.13 (s, 1 H, H-6), 7.91-7.89 (d, 2 H, J = 8.7 Hz, H-20,16), 7.48-7.45 (d, 2 H, J = 8.7 Hz, H-19,17), 7.40 (s, 1 H, H-3); **^{13}C NMR** (100 MHz, DMSO-d) (Spectrum 22): 150.35, 147.33, 144.99, 143.18, 132.88, 127.67, 127.46, 126.79, 123.05, 115.87; **MS** (ESI-MS) m/z, (Spectrum 23): 403.89 $[M+H]^+$, 405.89, 407.88 $[M+H]^+$+2; **IR** (ATR, cm^{-1}) Vmax (Spectrum 24): 3088, 2920, 1596, 1580.

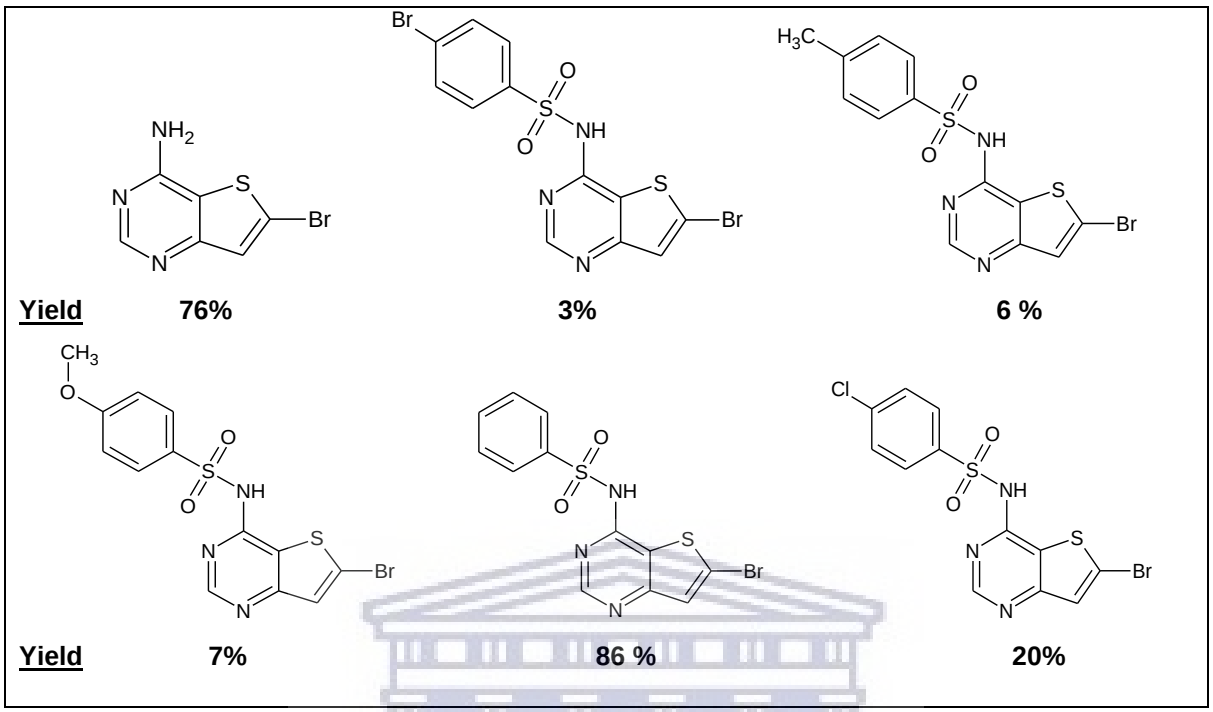
4.4 Structure elucidation

The intermediate (compound **1**) was confirmed with ^{1}H-NMR, IR, MS. In the proton NMR, the 6-bromothieno[3,2-*d*]pyrimidin-4-amine (**1**) moiety showed two proton peaks down-field in the aromatic region (9 – 6 ppm), characterised by a two singlet (s) pattern (McDonald *et al*., 2012). In the ^{13}C-NMR for compound **1**, the six aromatic carbon peaks were still found down-field at around 160 – 140 ppm. On the IR spectra, a broad peak characteristic of the primary amine group of compound 1 was observed at 3089 cm^{-1}, compared to the sharp peak observed from the IR spectra of the 6-bromo-4-chlorothieno[3,2-*d*]pyrimidine (starting material). The MS of compound **1** was similar to the calculated molecular mass and the Bromine isotope peak at the correct place also confirmed the mass, thus the structure of compound **1**. All other novel compounds synthesised were analysed and confirmed with NMR, MS and IR. For instance, the final compounds **2**, **3**, **4** and **6** were characterised by the presence of 2 singlets integrating for 1 proton each between 8.81 – 7.33 ppm of their ^{1}H NMR. This could be differentiated from compound **5** by the presence of 2 singlets overlapping and integrating for 2 protons between 8.97 – 8.96 ppm of their ^{1}H NMR and this represent the protons that make up the 6-bromothieno[3,2-d]pyrimidine moiety. The presence of a para substitution on the benzenesulfonamide moiety of the final compounds **2**, **3**, **4** and **6** caused the formation of two doublets integrating for 2 protons each between 7.91 and 6.95 ppm of their ^{1}H NMR spectrum. In contrast, the absence of substitution at the para position on the benzenesulfonamide moiety of compound **5**, caused the formation of two multiplets integrating for 3 and 2 protons between 7.97 – 7.90 ppm and 7.38 – 7.36 ppm respectively. Compound **3** and **4** were also characterised by the presence of a singlet integrating for 3 protons at 2.41 ppm and 3.85 ppm respectively of their ^{1}H NMR. The MS isotope peaks of compound **2**, **3**, **4**, **5**, and **6** also confirmed their structures.

4.5 Conclusion

A total of six compounds were synthesised with varying degrees of success (scheme 2). The compounds with low percentage yields can be attributed to unreacted starting materials or by-product formation during the synthesis and/or material lost during the purification steps. Optimization of the reactions and improved solubility of reactants

would be essential in further studies to increase the yield and reduce the amount of contamination in some of these novel compounds.



Scheme 2: Structures synthesised for biological evaluation

CHAPTER 5

BIOLOGICAL EVALUATION AND RESULTS

5.1 Introduction

The aim of this study was to discover novel compounds derived from natural products with in-vitro antiplasmodial activities (NAA) and potential for a new mechanism of action against malaria. Artificial intelligence (KNIME) was used as a tool for the prioritization and selection of compounds from a library of virtual compounds designed from NAA (Egieyeh, 2016). This chapter focuses on the most promising compounds that were synthesised and evaluated in *in vitro* assays for their antimalarial activity against both chloroquine sensitive (NF54) and resistant (K1) strains of *P. falciparum* using a modified method of the parasitic lactate dehydrogenase (pLDH) assay (Makler *et al*., 1993). From the dose-inhibition data collected, the 50 % inhibitory concentration (IC_{50}) values for the test compounds were calculated and compared with chloroquine (Penna-Coutinho *et al*., 2011). The novel synthesised compounds were tested for their *in vitro* cytotoxicity against a Chinese hamster ovarian (CHO) cell line using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tertrazoliumbromide (MTT) colorimetric assay. The MTT assay identifies the growth and survival of the CHO cell line used (Mosmann, 1983), based on the ability of the viable cells to modify the colour of the yellow MTT into water-insoluble purple-blue formazan product (Twentyman & Luscombe, 1987).

5.2 Materials and methods

5.2.1 Cells and *P. falciparum* parasite

All stock parasite cultures were maintained using the method of Trager and Jensen (1976). The chloroquine sensitive (CQ^S) NF54 and chloroquine resistant (CQ^R) strain K1 of *P. falciparum* and normal type A human red blood cells (2 % hematocrit) suspended in complete tissue culture medium (RPMI 1640 containing 25 mM HEPES buffer, 20 µg/ml of gentamicin, 27 mM bicarbonate and 10 % normal type A human serum) were used for the assay.

5.2.2 Parasite Lactate Dehydrogenase (pLDH) Assay procedure

The assay was initiated by adjusting the initial parasitemia to 1-2 % with the normal type A human red blood cell suspension. The test samples were prepared to a 20 mg/ml stock solution in 100 % DMSO and sonicated to enhance solubility. Samples were tested as a suspension if not completely dissolved. Stock solutions were stored at -20 ºC. Further dilutions were prepared on the day of the experiment. Chloroquine (CQ) was used as the reference drug in all experiments. The diluted parasite suspension was dispensed in triplicate at 0.2 ml/well into a 96-well, flat-bottomed microtiter plates. At a starting concentration of 1000 ng/ml, test samples were serially diluted 2-fold in complete medium to give 10 concentrations; with the lowest concentration being 2 ng/ml. The same dilution technique was used for all samples. The cultures were incubated at 37 °C for 72 hours in 3 % O_2, 6 % CO_2 and 91 % N_2. At the conclusion of the incubation period the cultures were carefully re-suspended and aliquots were removed for spectrophotometrical analysis of pLDH activity (Makler *et al.,* 1993). The IC_{50} values were obtained using a non-linear dose-response curve fitting analysis with Graph Pad Prism v.4.0 software.

5.2.3 Cytotoxicity (MTT) assay procedure

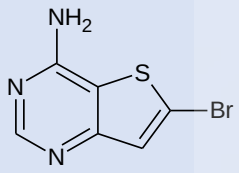
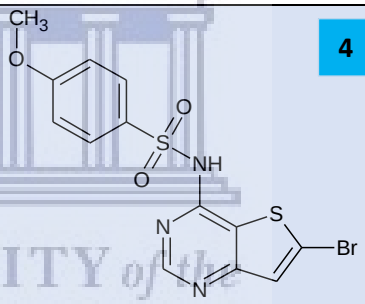
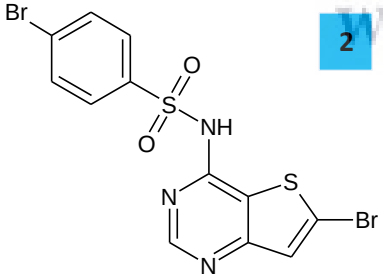
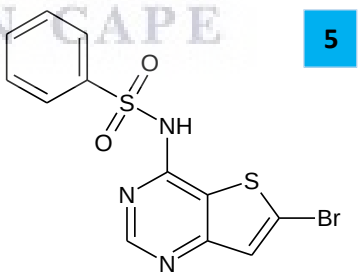
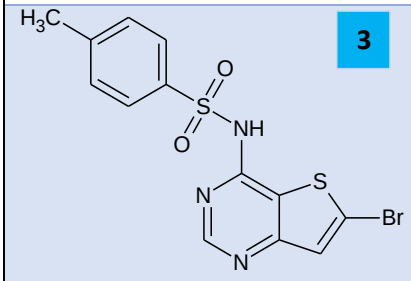
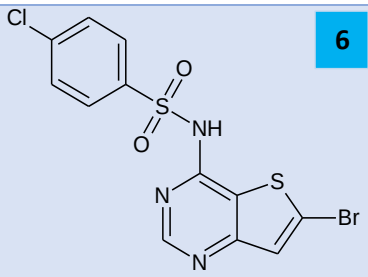
Test samples were screened for *in vitro* cytotoxicity against a CHO cell line using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) MTT-assay. The MTT-assay is used as a colorimetric assay for cellular growth and survival, and compares well with other available assays (Mosman 1983; Rubinstein *et al.,* 1990). The tetrazolium salt MTT was used to measure all growth and chemosensitivity. The test samples were tested in triplicate. The same stock solutions prepared for antimalarial testing were used for cytotoxicity testing. Test compounds were stored at -20 ºC until use. Dilutions were prepared on the day of the experiment. Emetine was used as the reference drug in all experiments. Initially the stock solution was thawed and starting from a 100 µg/ml concentration, it was serially diluted in 10-fold dilutions to give 6 different concentrations in complete medium and the lowest concentration was 0.001 µg/ml. The reference drug was also serially diluted with the same dilution technique as above. Lastly, the 50% inhibitory concentration of cell line growth were obtained using non-linear dose-response curve fitting analysis with GraphPad Prism v.4 software from full dose-response curves (Vreese *et al*., 2017).

5.3 Results and discussion

5.3.1 Cytotoxicity study

One objective of this chapter for this study was to determine the cytotoxicity of the synthesised compounds. Therefore, all synthesised compounds were subjected to cytotoxicity evaluation using the Chinese Hamster Ovarian (CHO) cell line. From the results collected and presented in Table 4.2 it can be seen that all tested compounds (**1**, **2**, **3**, **4**, **5** and **6**) have high CHO IC_{50} values (IC_{50} > 10000 nM) compared to the cytotoxic reference drug emetine (IC_{50} = 60 nM). This high IC_{50} values indicate that all synthesised compounds have a very low toxicity towards non-parasitic cells and as such are much safer than the cytotoxic agent emetine.

Table 4.2: Cytotoxicity assay IC_{50} values of the novel compounds.

Compounds synthesised	CHO IC_{50} (nM)	Compounds synthesised	CHO IC_{50} (nM)
1 	>10000	4 	>10000
2 	>10000	5 	>10000
3 	>10000	6 	>10000
Emetine	60		

CHO = Chinese Hamster Ovarian.

5.3.2 Antimalarial activity

The second objective of this chapter was to evaluate the antimalarial activity of the synthesised compounds against CQ-sensitive (NF54) and CQ-resistant (K1) strains. The synthesised compounds are expected to possess antimalarial activity because they originated from a library of virtual compounds designed from natural products with *in vitro* antiplasmodial activities (NAA). The antimalarial activity of the synthesised compounds were defined as follows: IC_{50} < 100 nM is highly active; IC_{50} < 1000 nM is active and IC_{50} > 1000 nM is inactive. The work of Makler and Hinrichs (1993) has demonstrated that the IC_{50} values are inversely proportional to the potency of the compounds.

The synthesised compounds (**1**, **2**, **3**, **4**, **5** and **6**) were not as potent as chloroquine (CQ) against the CQ-sensitive strain (NF54 IC_{50}: 7 nM), but compounds **2**, **3**, **4** and **6** still displayed statistically significant antimalarial activity against the same strain (NF54 IC_{50}: 320 - 930 nM), categorising them as active antimalarials (Table 4.3). The activity of chloroquine was significantly reduced against the CQ-resistance strain (K1 IC_{50}: 300 nM). Synthesised compounds **2**, **3**, **4** and **6** exhibited similar antimalarial activity against chloroquine resistance strain (K1 IC_{50}: 410, 960, 900 and 910 nM respectively) when compared to drug reference chloroquine. The low antimalarial activity of compounds **1** and **5** were retained against the chloroquine resistance strain (K1 IC_{50}: >10000 and 2830 nM respectively). The retention of activity of all synthesised compounds in the *P. falciparum* chloroquine resistance strain (K1) could be attributed to the presence of 6-bromothieno[3.2-*d*]pyrimidin-4-amine moiety. Another mechanism of action could thus be involved in the parasite-killing and further investigation into this must be done as the synthesised compounds do not have a similar resistance pattern to CQ. Compound **1** displayed very weak activity against the CQ-sensitive strain (NF54 IC_{50}: 7780 nM) compared to the other test compounds (Table 4.3) and this could be attributed to the absence of the para substituted phenylsuphonyl moiety in its structure.

Table 4.3: *In vitro* IC_{50} values for antiplasmodial activity and cytotoxicity of compounds **1**-**6** and reference compounds.

Compound	NF54 IC_{50} (nM)	K1 IC_{50} (nM)	CHO IC_{50} (nM)	RI	SI
1	7780	>10000	>10000	ND	>1.28
2	320	410	>10000	1.28	>31.25
3	590	960	>10000	1.63	>16.95
4	930	900	>10000	0.97	>10.75
5	1110	2830	>10000	2.55	>9.01
6	540	910	>10000	1.69	>18.52
CQ	7	300	ND	38.46	ND
Emetine	ND	ND	60	ND	ND

Selectivity index (SI) = IC_{50} CHO/IC_{50} NF54; Resistance index (RI) = IC_{50} K1/IC_{50} NF54. CHO = Chinese Hamster Ovarian; ND = not determined.

The selectivity index (SI) was calculated to estimate the potential of the novel compounds to selectively inhibit *P. falciparum* growth (Table 4.3) and is defined as the ratio of the IC_{50} on the CHO cell line to the IC_{50} on CQ-sensitive NF54 strain. When the SI value is very low (below 25), it is an indication that the antimalarial activity of the compound could be due to cytotoxicity rather than its antimalarial activity against the parasite. In the same line, when the SI value is high (above 25), it suggests that the selectivity of the compounds is towards *P. falciparum* (Valdés *et al*., 2010; Soh & Benoit-Vical. 2007). Therefore as shown in Table 4.3, it can be concluded that compound **2** showed significant selectivity towards the CQ-sensitive strain NF54 (SI: >31.25).

After confirmation that the novel compounds have antimalarial activities, the next step was to evaluate their potential to retain enough activity in a CQ-resistant strain to overcome the *P. falciparum* CQ resistance. From the available antimalarial activity data (table 4.3), it was possible to calculate the resistance index (RI) which is the ratio of the IC_{50} of the CQ-resistant strain K1 to that of CQ-sensitive strain NF54. Thus, the higher the RI value, the higher the IC_{50} K1 and the higher the level of resistance (Nzila & Mwai, 2010). When compared to CQ (RI = 38.46) compounds **2**, **3**, **4**, **5** and **6** have smaller RI ranging from 0.97 to 2.55. This implies that the novel compounds

retained their antimalarial activity against *P. falciparum* CQ resistant strain and could overcome the problem of *P. falciparum* CQ resistance. This however needs to be confirmed in future studies using, for instance, *in vivo* models.

5.4 Conclusion

The synthesised compounds were not as potent as chloroquine against the CQ-sensitive NF54 strain, however they displayed significant antimalarial activities against the CQ-resistant K1 strain when compared to the activity of CQ. The results revealed that the test compounds (**1**, **2**, **3**, **4**, **5** and **6**) do not have the resistance pattern as chloroquine against *P. falciparum*. Although compound **2** didn't show the best activity against the CQ-resistant strain K1, it displayed the best activity against CQ-sensitive strain NF54 amongst the synthesised compounds. Compound **2** also had the highest selectivity index (SI > 31.25) and the second lowest resistance index (RI = 1.28). This implies that compound **2,** when compared to the other compounds within this series, is the most promising candidate. This could be due to a different mechanism of action or that the substances are not substrates of the Chloroquine Resistance Transporter (*Pf*CQRT). Further work should however be done to explore and confirm the mechanism of action of these compounds.

CHAPTER 6

SUMMARY AND CONCLUSION

6.1 Introduction

Malaria is an infectious disease that threatens the lives of 40% of the world's population in many areas and especially in Africa, which account for 90% of malaria cases and 91% of all malaria deaths (WHO, 2017). There is an alarming increase in resistance of *P. falciparum* to existing antimalarial drugs. Thus, new classes of medicine to combat malaria are urgently needed due to the surge in resistance of the parasite to available treatment (WHO, 2017). One approach to circumvent the problem of *P. falciparum* resistance to anti-malarial drugs could be the discovery of novel compounds with unique scaffolds with potential new mechanisms of action. A source where unique scaffolds can be identified is natural products, which provide a wide diversity and unique chemotypes. Therefore a library of virtual compounds (VC) designed from natural products with antiplasmodial activities (NAA) can be a worthy starting point.

Cheminformatics which is an interdisciplinary field was used as a computational approach for the discovery of novel chemical entities and ultimately the design of new drug-like molecules (Begam *et al.,* 2012). Thus, this study sought to develop an automated computerized prioritizing workflow method (as presented in Figure 1.3.1) using the computational tool KNIME (Konstanz Information Mimer) based on available data (virtual compounds from NAA) for selection of new sets of compounds to be synthesised and evaluated as potential new antimalarial agents with unique chemical scaffolds. A series of compounds were selected for synthesis based on the VC results, using multi-step procedures. The synthesised novel compounds were expected to exhibit good to moderate activity against CQ sensitive and CQ resistance strains of *P. falciparum* and could potentially be added to the antimalarial armoury.

6.2 Use of cheminformatics for prioritization and selection of compounds from a virtual compound library

A library of virtual compounds designed from NAA was characterized or profiled. Prioritization and selection of compounds were conducted based on drug-like properties and parameters that defines probability for successful development into a drug candidate. Using the computational tool KNIME, 1805 VC were prioritized from a library of 164532 VC and a final number of 25 diverse VC were selected for further exploration. As proof of concept, the compound found in row 12 (Figure 3.14) was selected as the most promising candidate for synthesis and biological evaluations. The decision came to the compound that could be synthesised with the lowest number of PAINS (non-specific interaction with drug targets) and smallest number of compounds with more than 70% similarity in ChEMBL (novelty of the compound).

6.3 Synthesis

Synthesis of the novel compounds were accomplished using a two-step procedure. Firstly, the intermediate 6-bromothieno[3,2-d]pyrimidin-4-amine (compound **1**) was synthesised by the amination of the starting material 6-bromo-4-chlorothieno[3,2-d]pyrimidine with ammonia solution using microwave irradiation resulting in a yield of 76%. Secondly, the resultant intermediate was further reacted with the relevant commercially available benzenesulfonyl chloride to produce compounds **2**, **3**, **4**, **5** and **6** using a sulphonamide formation reaction with varying percentage yields ranging between 3 % and 86 %. The compounds were purified using column chromatography with hexane:ethyl acetate as mobile phase in a 1:2 ratio. The lower yield of some compounds can be attributed to unreacted intermediate (6-bromothieno[3,2-d]pyrimidin-4-amine) and starting materials (para substituted benzenesulfonyl chloride). NMR and IR were used to characterise significant signals and MS confirmed the molecular masses of synthesised compounds.

6.4 Biological evaluation

Cytotoxicity studies were performed on all synthesised compounds against the Chinese Hamster Ovarian (CHO) cell line using the MTT-assay. As presented in the Table 4.2 all tested compounds (**1**, **2**, **3**, **4**, **5** and **6**) had very low toxicity toward non-parasitic cells. The antimalarial activities of the synthesised compounds were conducted on both CQ sensitive (NF54) and CQ resistant strains (K1) of *P.falciparum* using the lactate dehydrogenase assay (Makler *et al.,* 1993). The results obtained are shown in Table 4.3 in the previous chapter.

The synthesised compounds were not as potent as chloroquine (CQ) against the CQ-sensitive strain (NF54), however these compounds still displayed statistically significant antimalarial activity against this strain. All synthesised compounds maintained their antimalarial activity against the CQ-resistance strain (K1) compared to chloroquine. Compounds **2**, **3**, **4**, **5** and **6** had lower resistance index (RI: from 0.97 to 2.55) compared to CQ (RI = 38.46) and this was an indication that the synthesised compounds retained their activities against the CQ-resistance strain (K1) which was not the case for chloroquine. The results presented in Table 4.3 illustrated that compound **2** retained a similar activity against the *P.falciparum* resistant strain (K1) and showed significant selectivity towards the CQ-sensitive strain NF54 (SI: >31.25). Thus, compound **2** stood out as the most promising candidate to overcome the problem of *P.falciparum* CQ resistance among all synthesised compounds.

6.5 Conclusion

A library of virtual compounds (VC) designed from natural products with antiplasmodial activities (NAA) was used for the prioritization and selection of compounds. This was possible by using computational approaches which allowed us to generate, prioritize and develop new compounds with the most promising predicted antimalarial activities. Using the computational platform KNIME, 1805 VC were prioritized from a library of 164532 VC and a total of 25 molecules were selected for further profiling. As proof of concept, the compound found in row 12 (Figure 3.14) was selected as the most promising candidate for synthesis and biological evaluations. This was decided firstly because it is not economically viable trying to synthesise all of the diverse 25 VC with

their analogues, secondly the synthetic routes of those compounds were not easy to find even though some compounds shown no PAINS (no non-specific interaction with drug targets) and no molecules with more than 70% similarity in ChEMBL (novelty of the compound). Future studies should look at synthesising further compounds identified.

Results from the biological studies (Table 4.3) indicated that among all synthesised compounds (**1**, **2**, **3**, **4**, **5** and **6**), compound **2** had the best activity against CQ-sensitive strain (NF54) and the second lowest resistance index (RI). This was an indication that it retained its activity against the CQ-resistance strain (K1) compared to chloroquine where a 30-fold reduction in activity was observed. It was thus identified as the most promising candidate. Therefore, the next step will be to do further work in the optimisation of the structure and reactions to produce better yields and to investigate the mechanism(s) of action involved in parasite-killing and resistance to the *Pf*CQRT efflux effect. The role of the para substituted phenylsuphonyl moiety of the structure should also be investigated further to build on the SARs of the compounds. Additional work could also be done on the remaining 24 compounds identified from the virtual library. This could include evaluation of the 24 remaining compounds in *in vitro* assays to assess their antimalarial activity against both sensitive and resistant strains of *P.falciparum* and for their *in vitro* cytotoxicity against a Chinese hamster ovarian cell line. Lead optimisation of promising structures could then potentially deliver additional novel antimalarial medicines.

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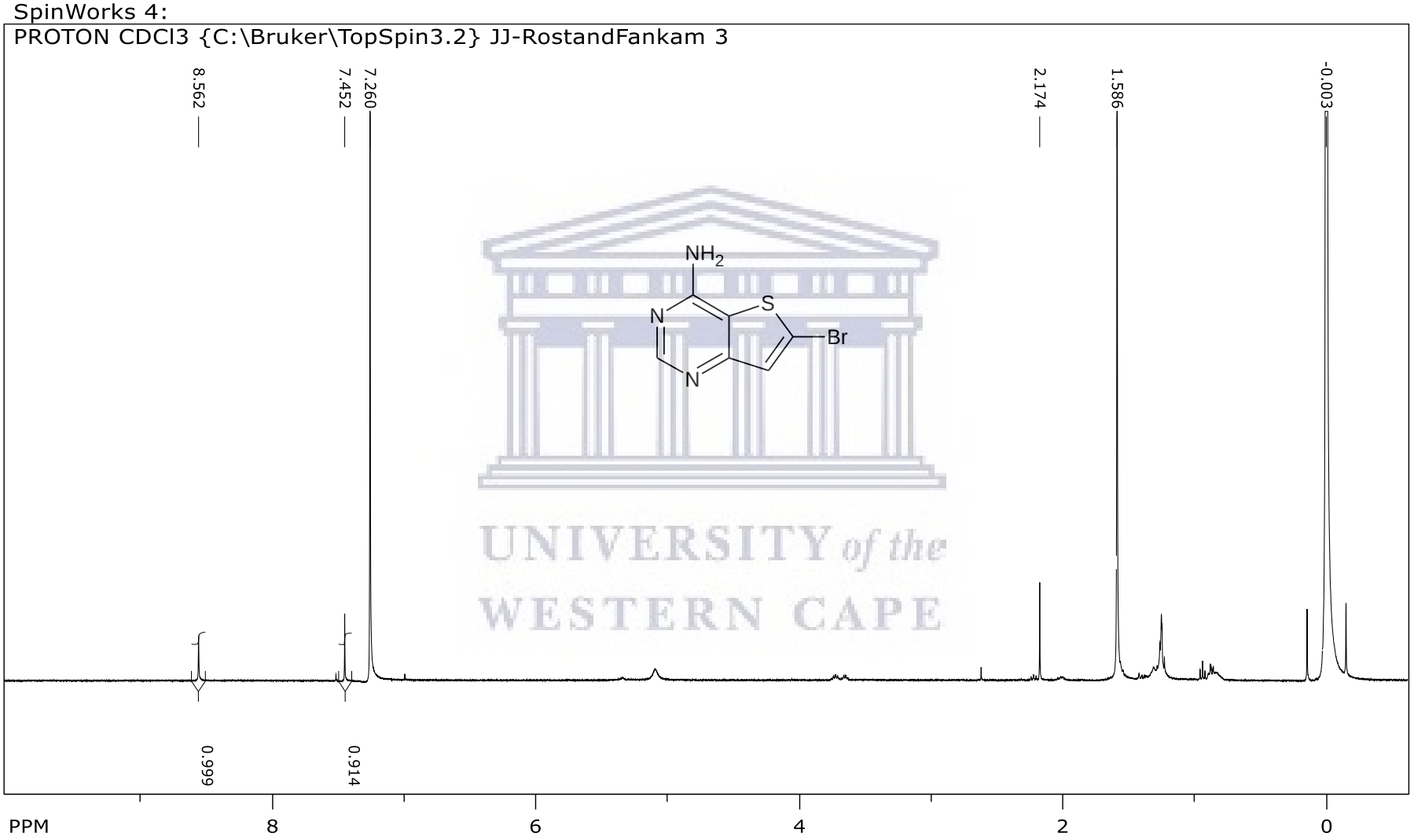
ANNEXTURE

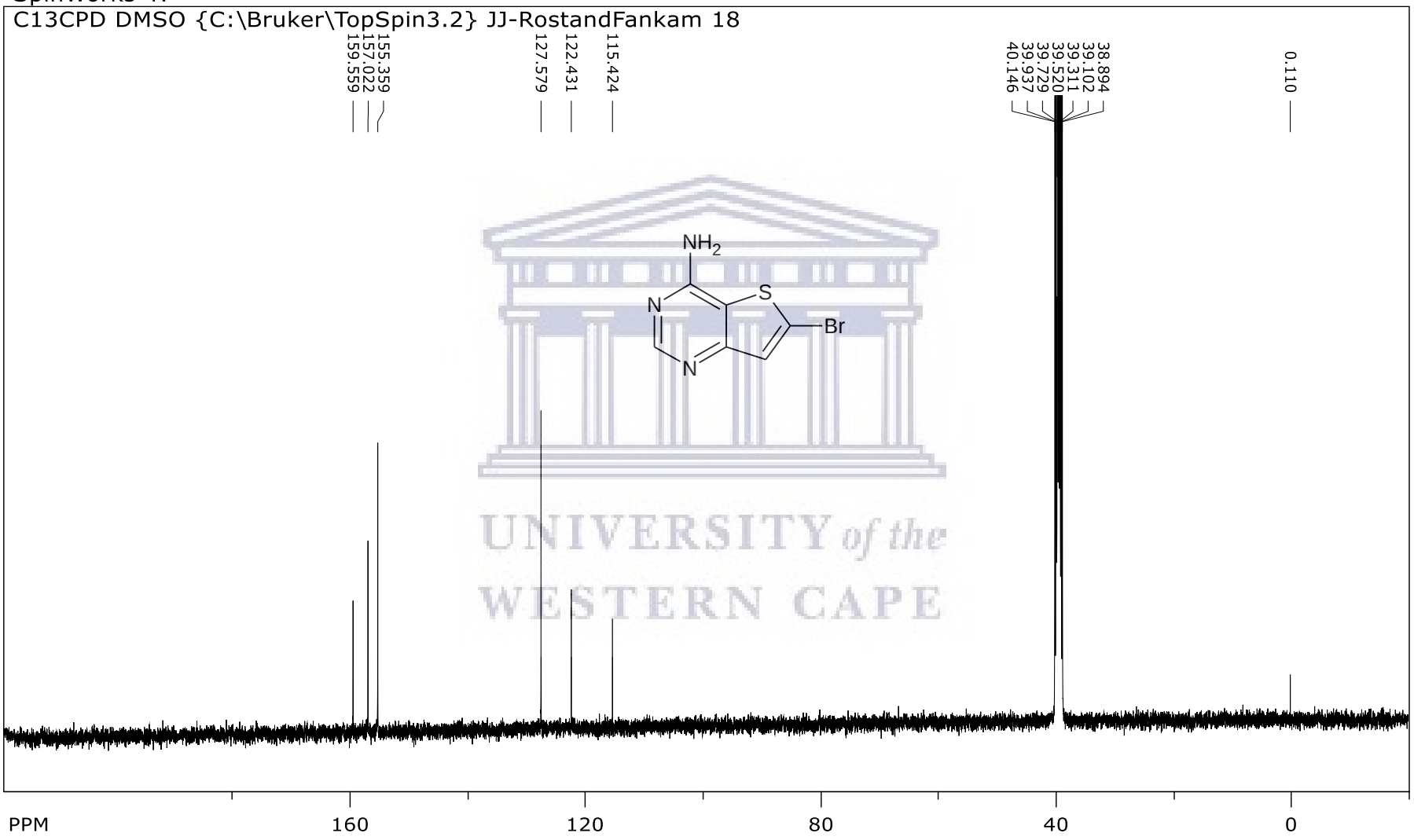
Spectral data:

^{1}H NMR, ^{13}C NMR, IR, MASS SPECTROSCOPY

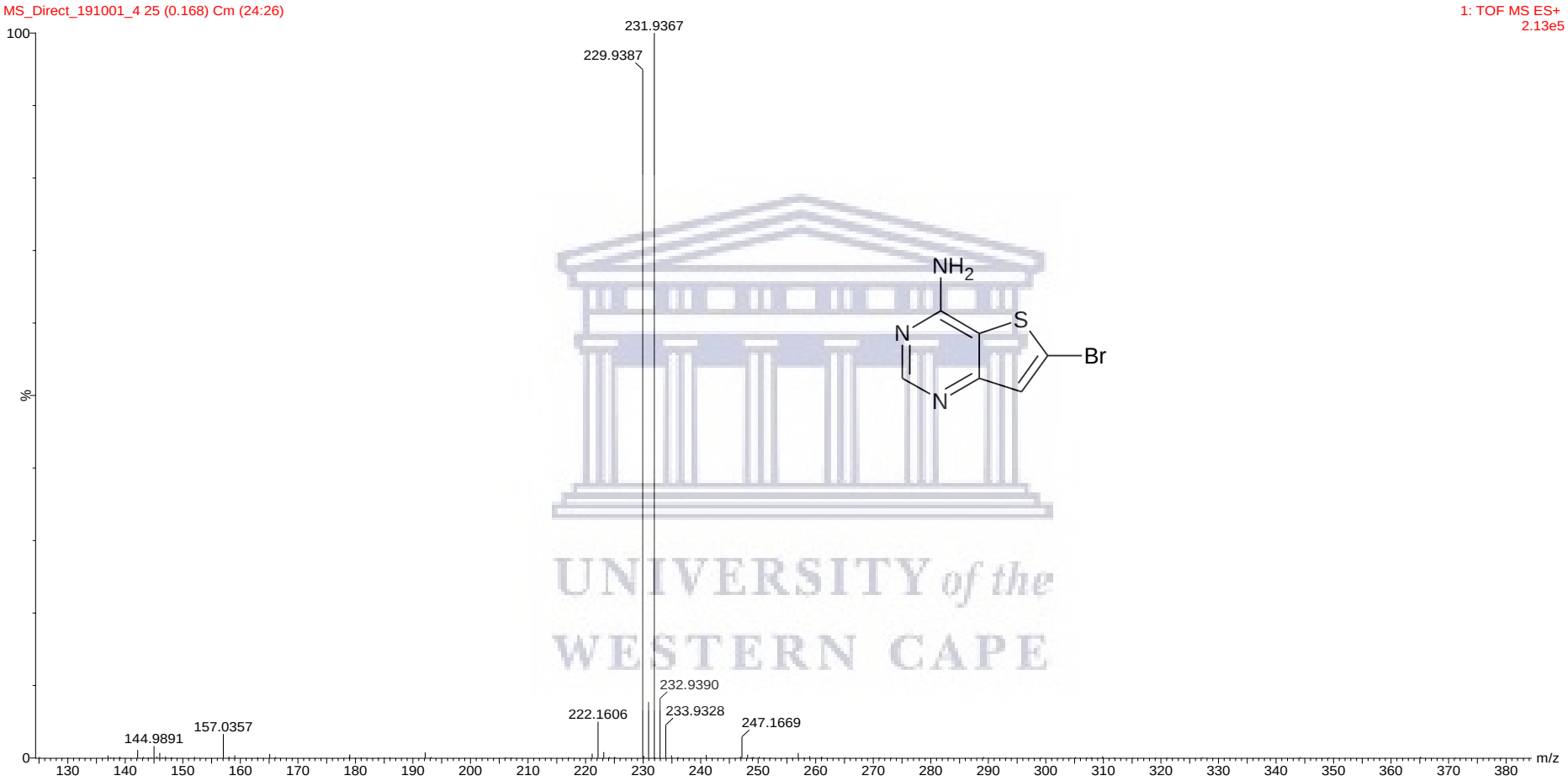
Spectrum 1 ^{1}H NMR of compound 1.



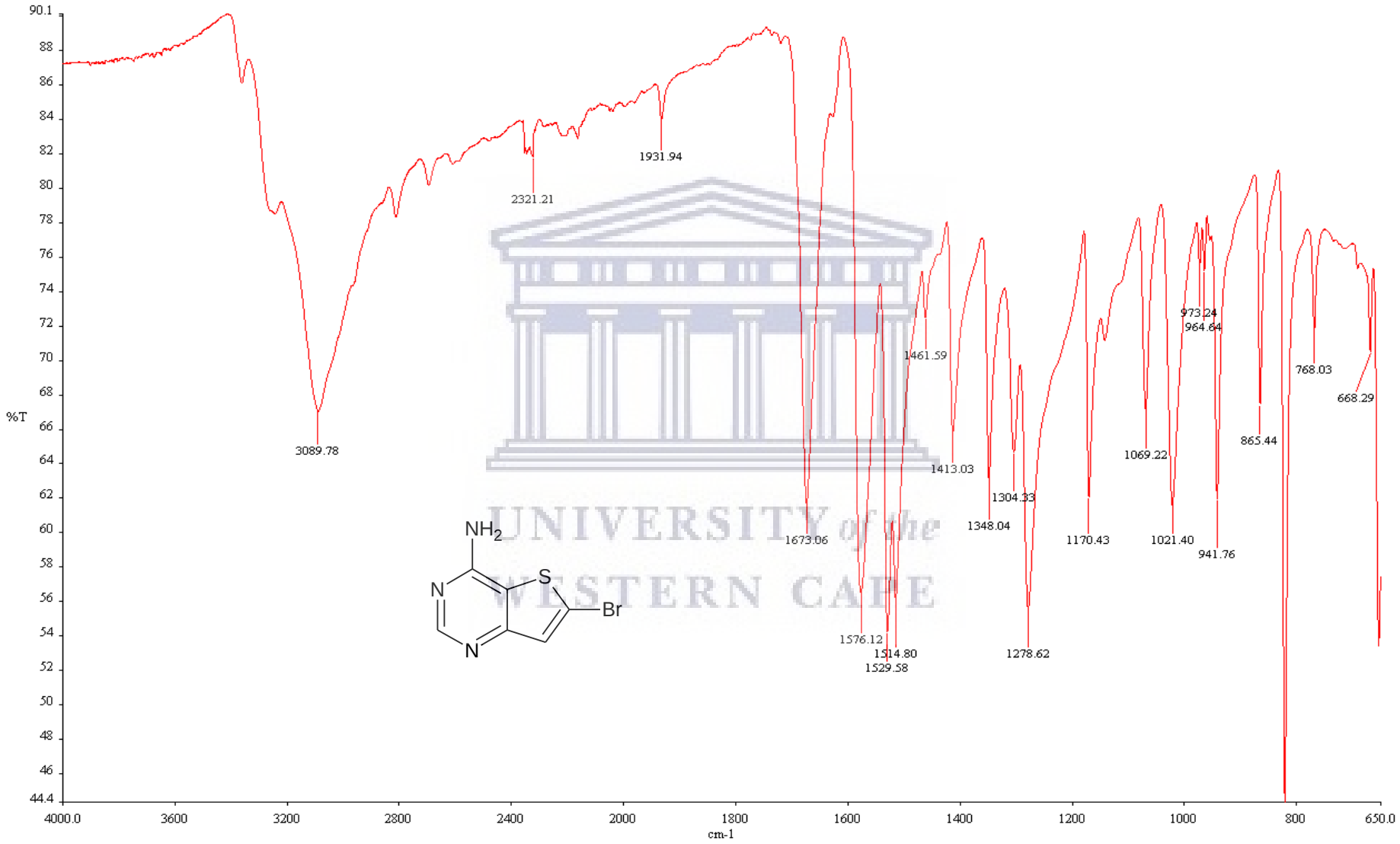
Spectrum 2 ^{13}C NMR of compound 1.



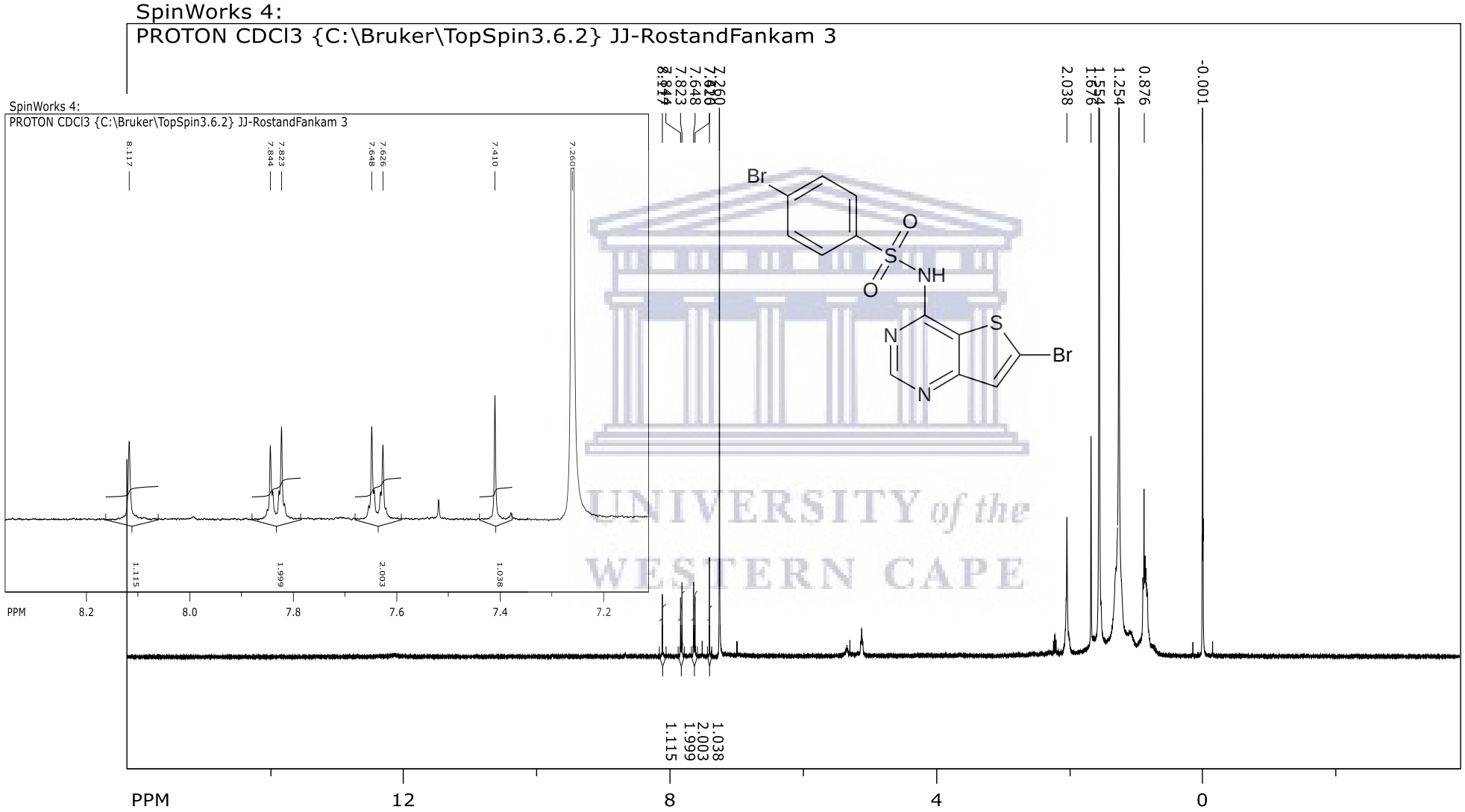
Spectrum 3 MS of compound 1.



Spectrum 4 IR of compound 1.



Spectrum 5 ^{1}H NMR of compound 2



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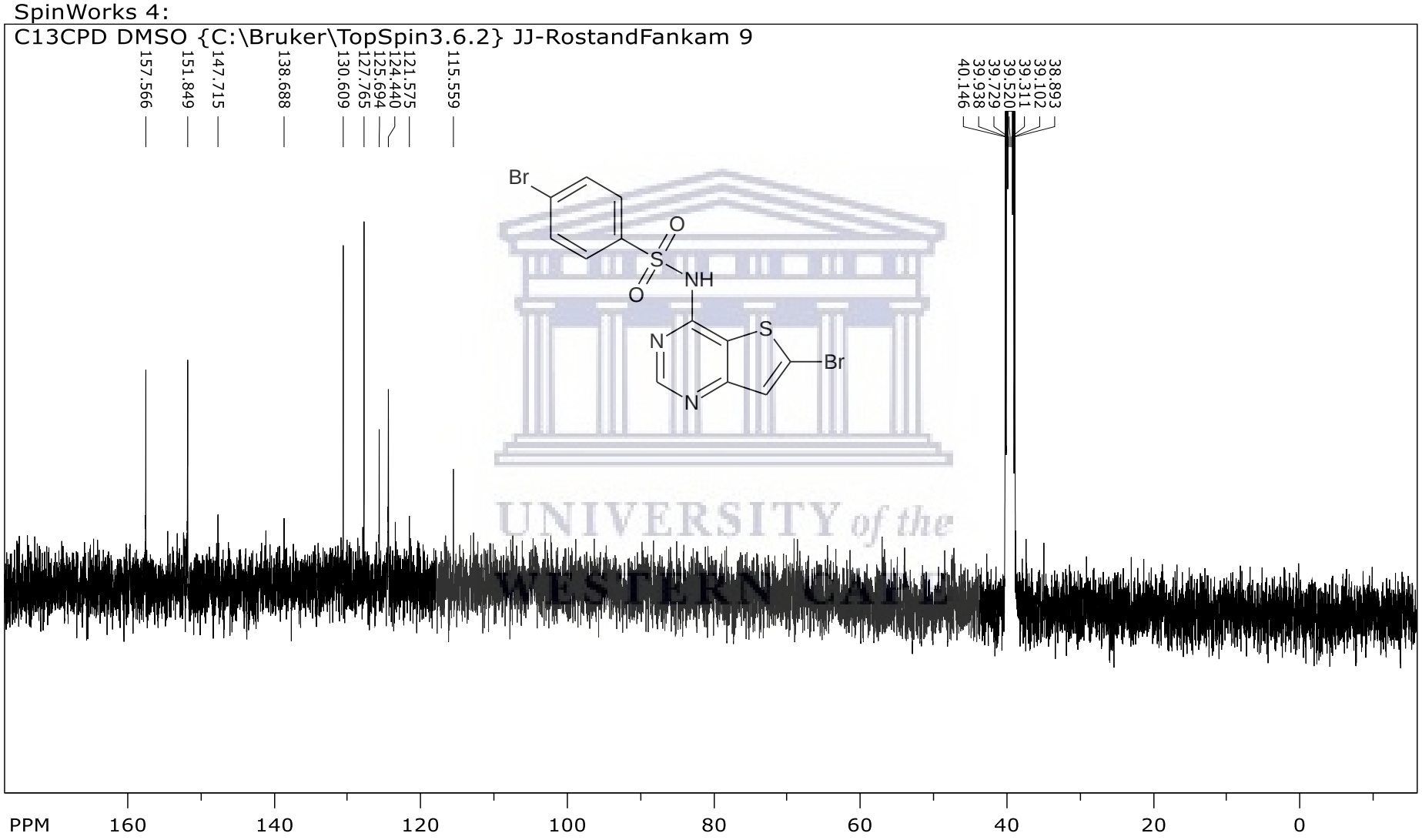

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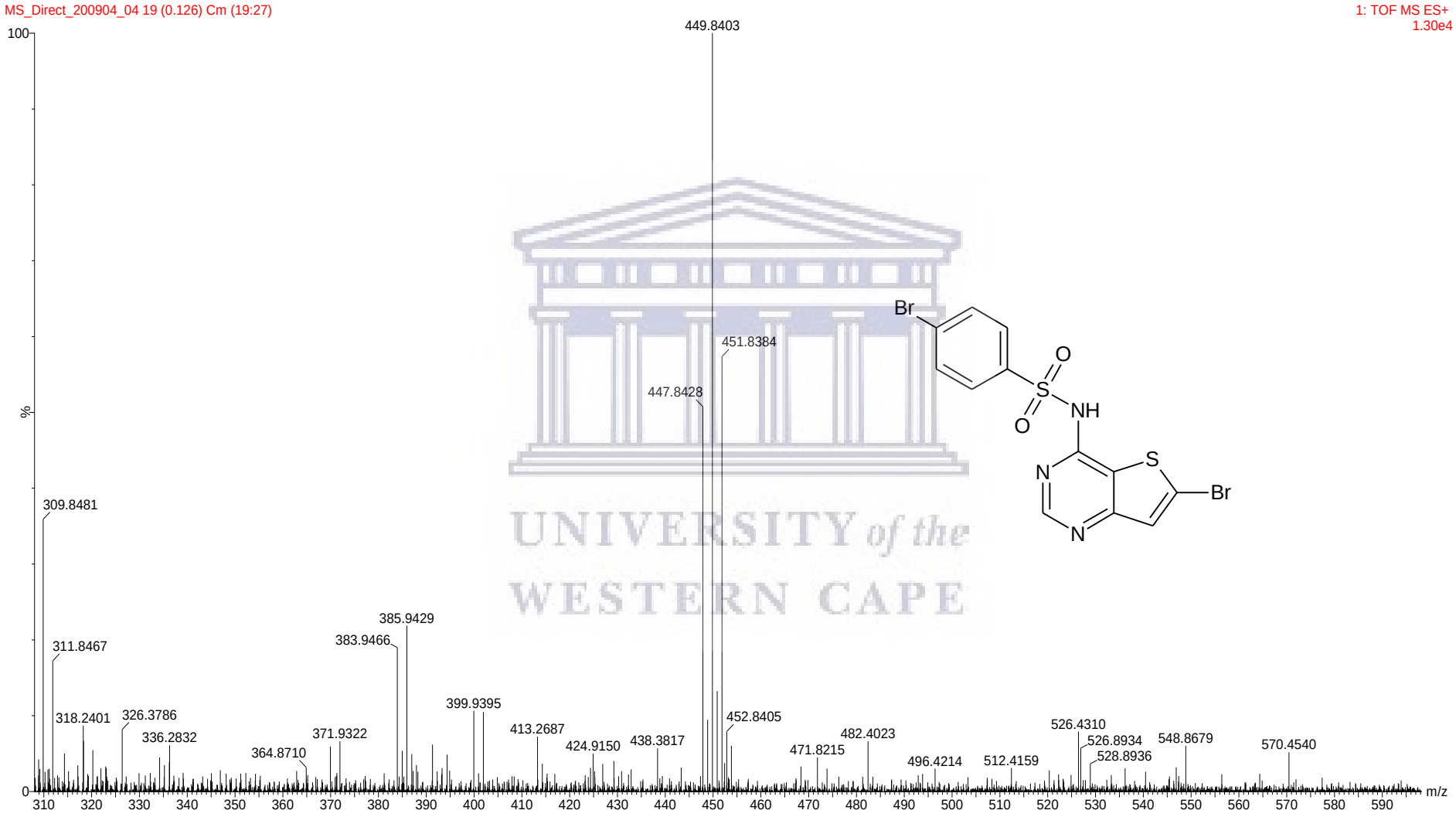
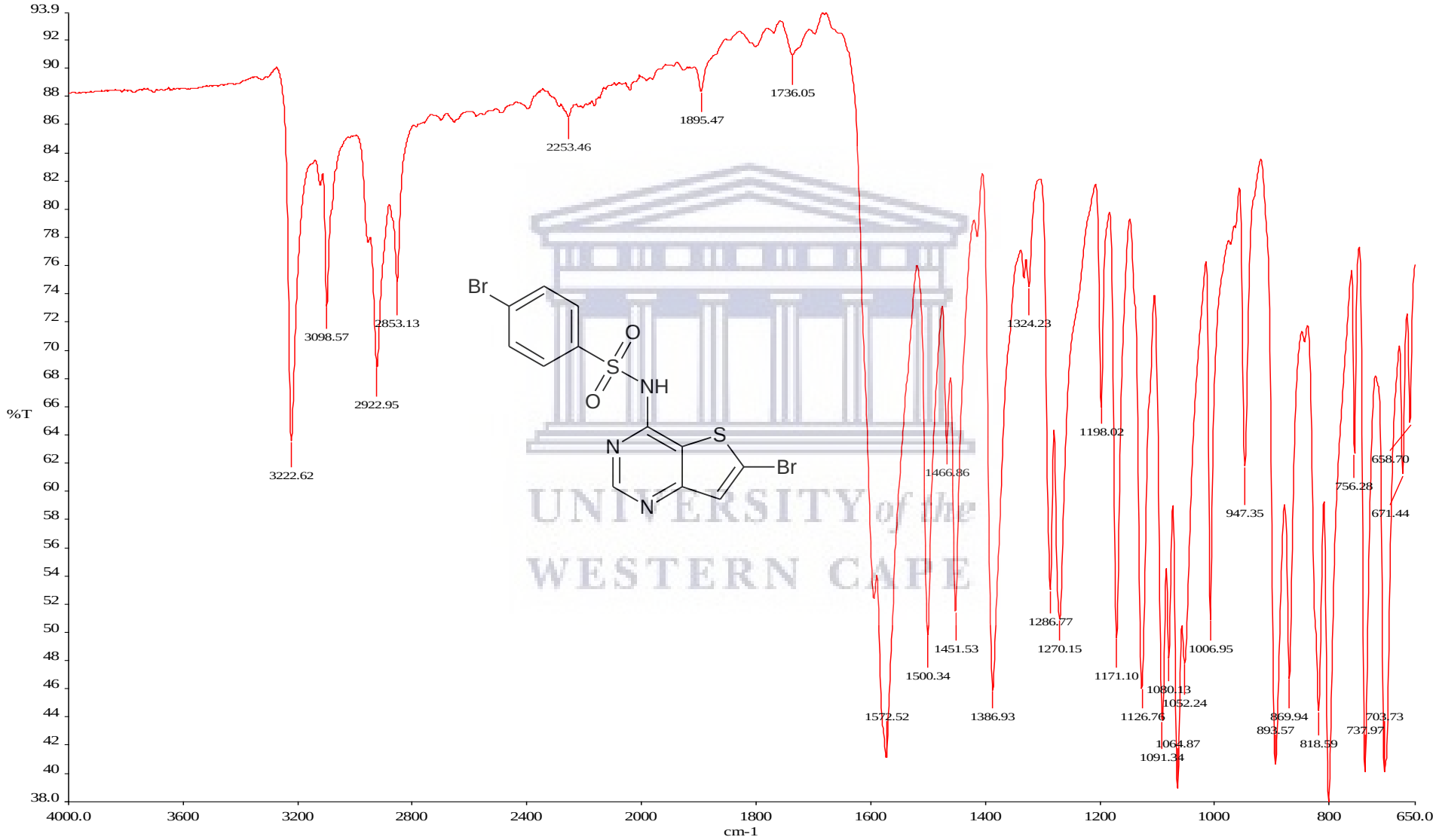
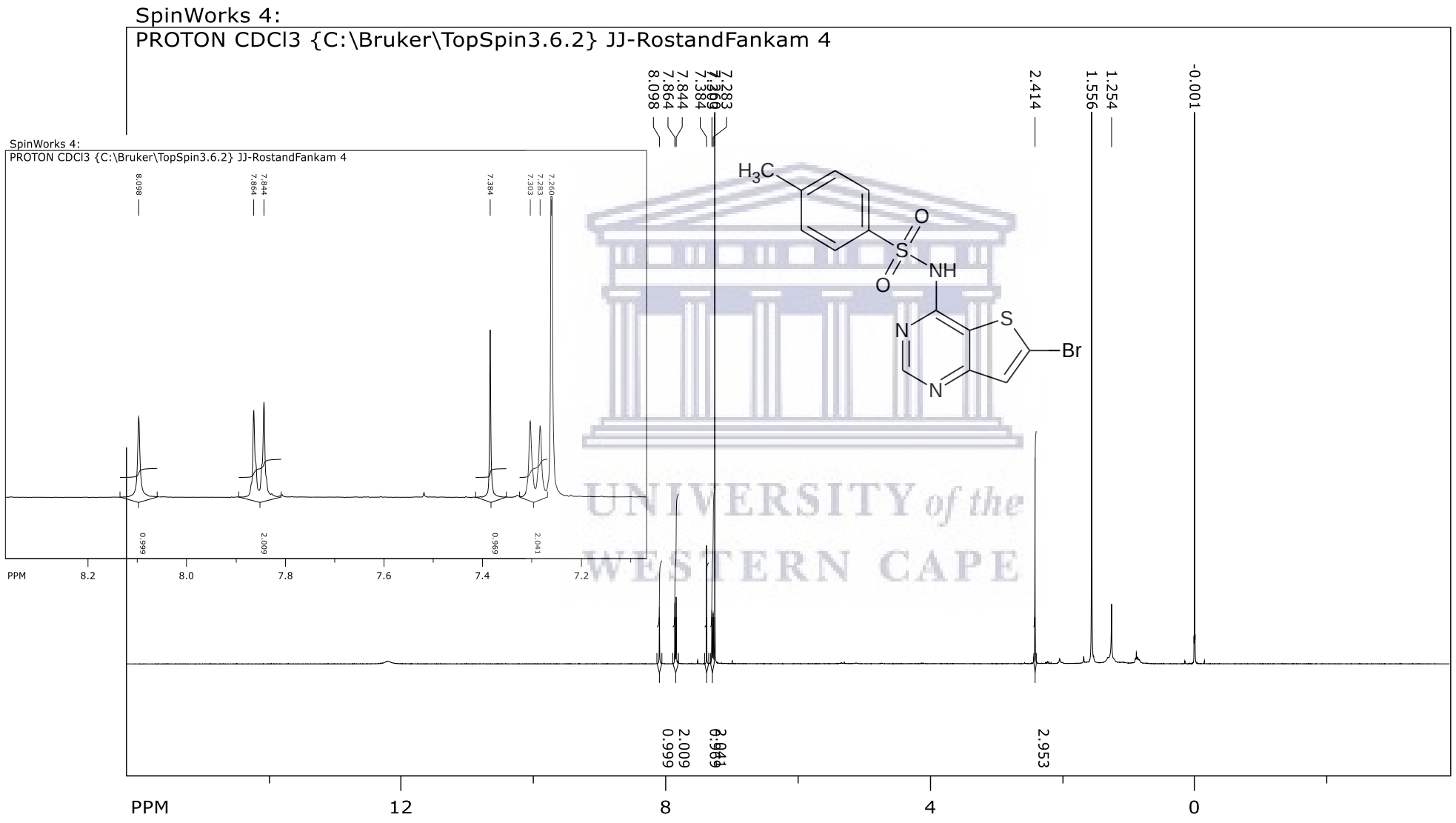


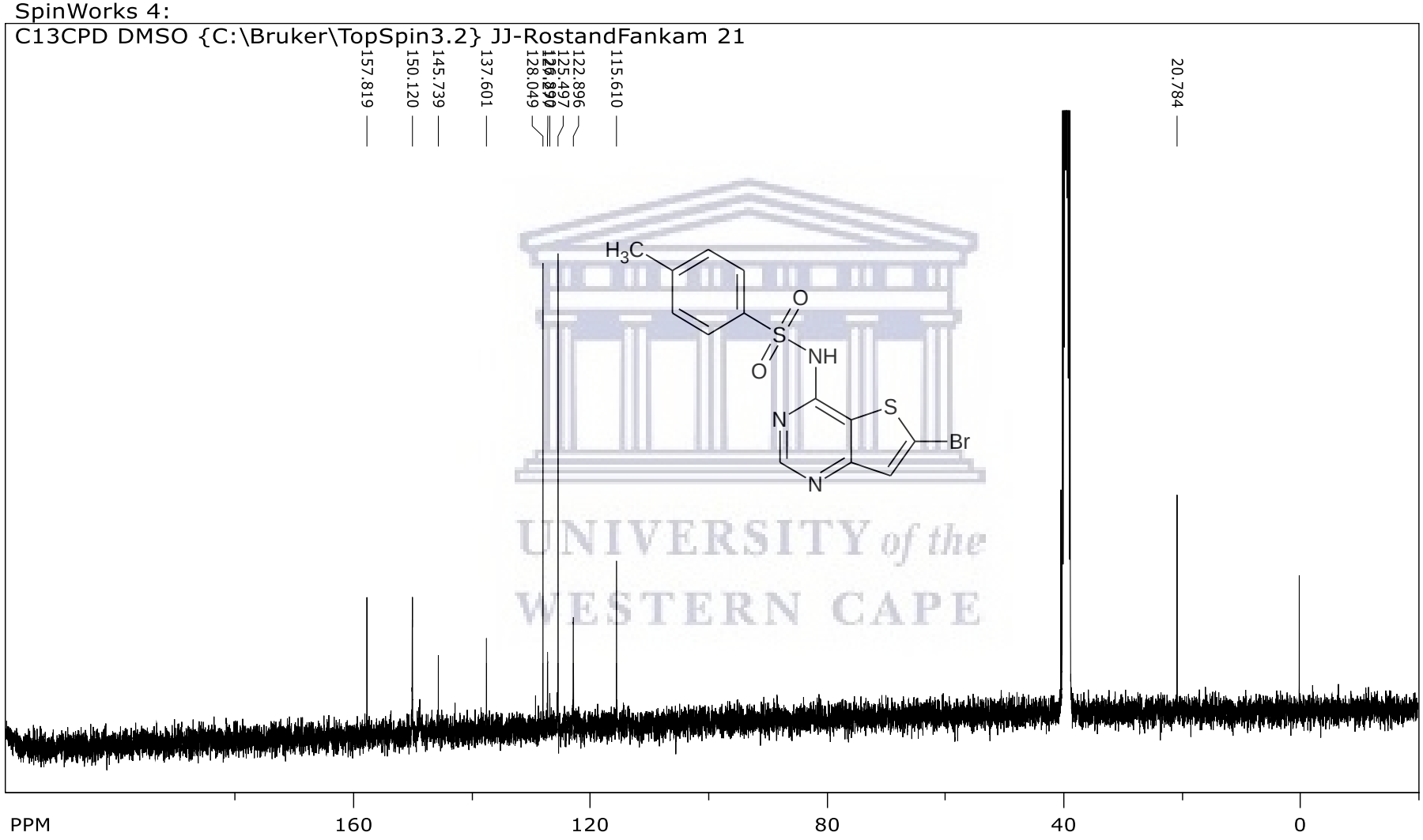
Spectrum 8 IR of compound 2



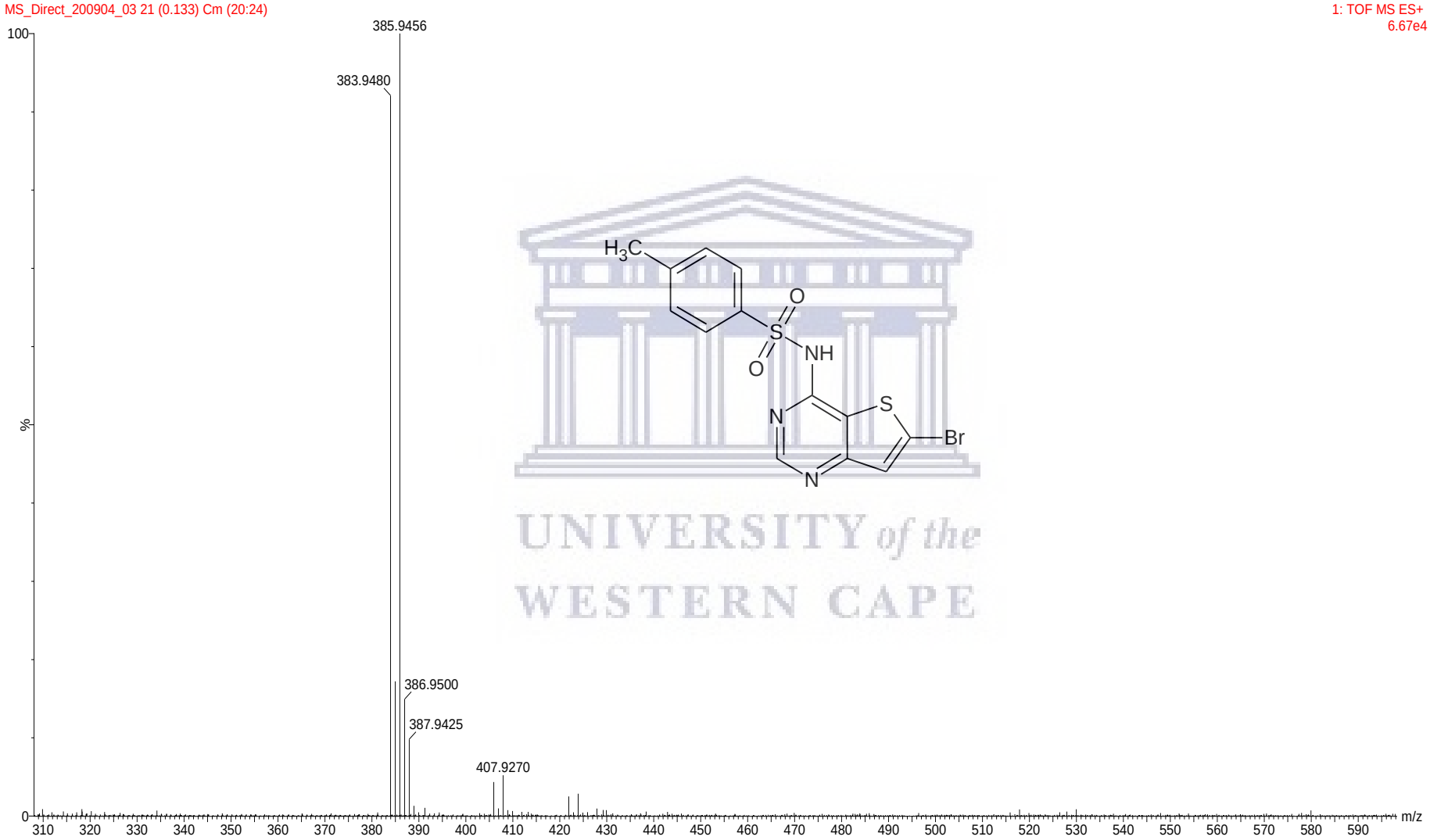
Spectrum 9 ^{1}H NMR of compound 3



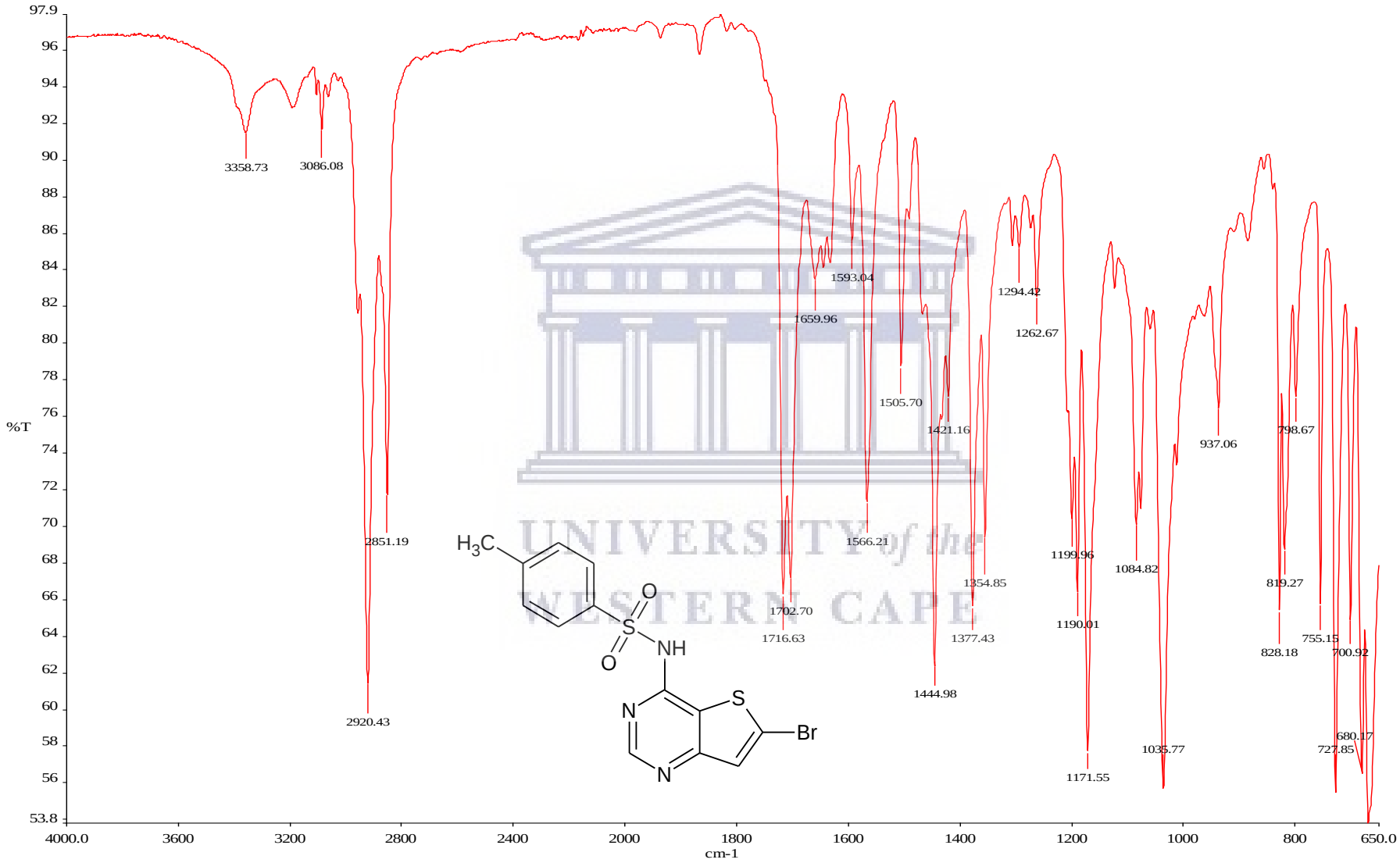
Spectrum 10 ^{13}C NMR of compound 3.



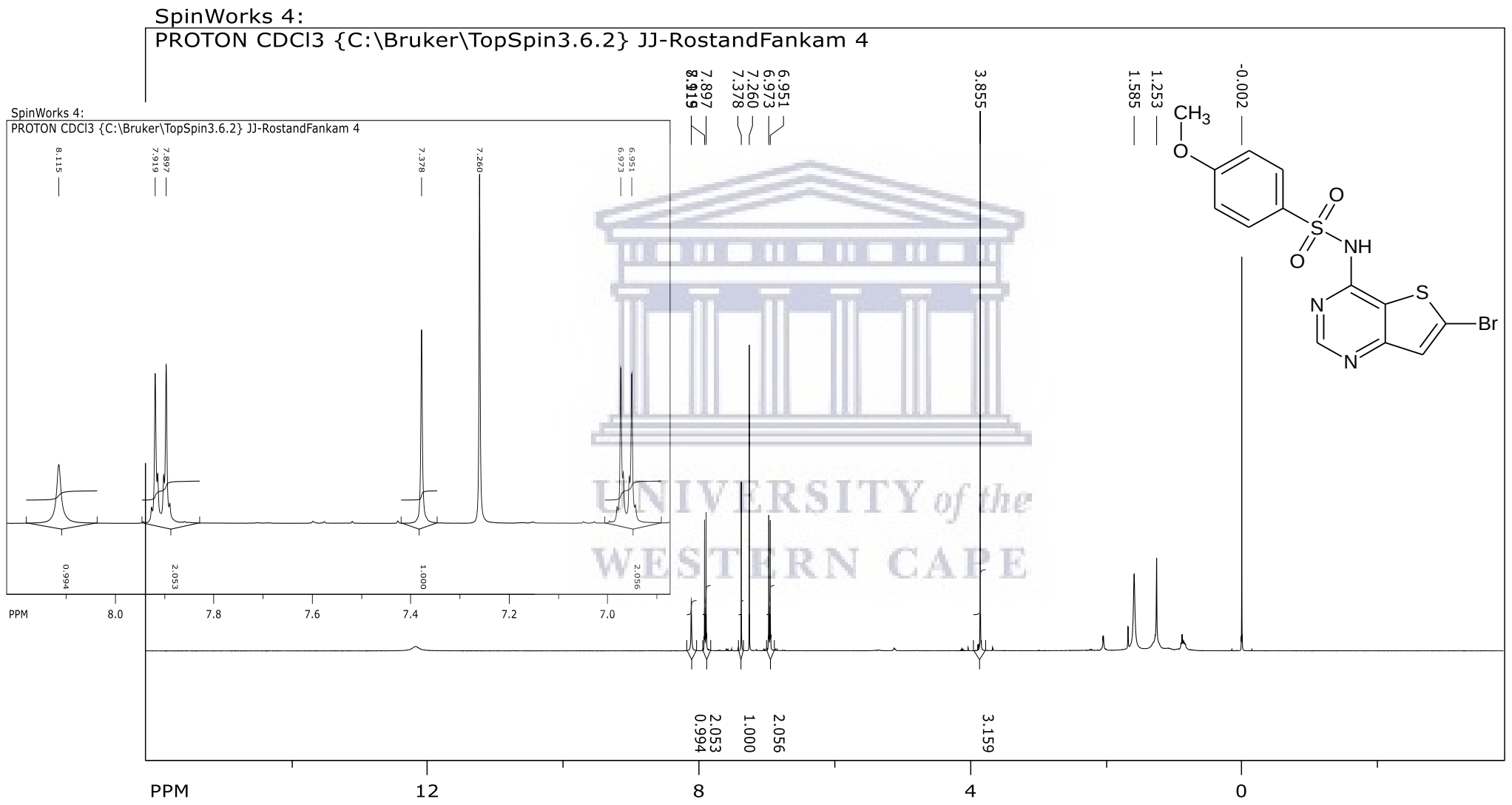
Spectrum 11 MS of compound 3.



Spectrum 12 IR of compound 3.

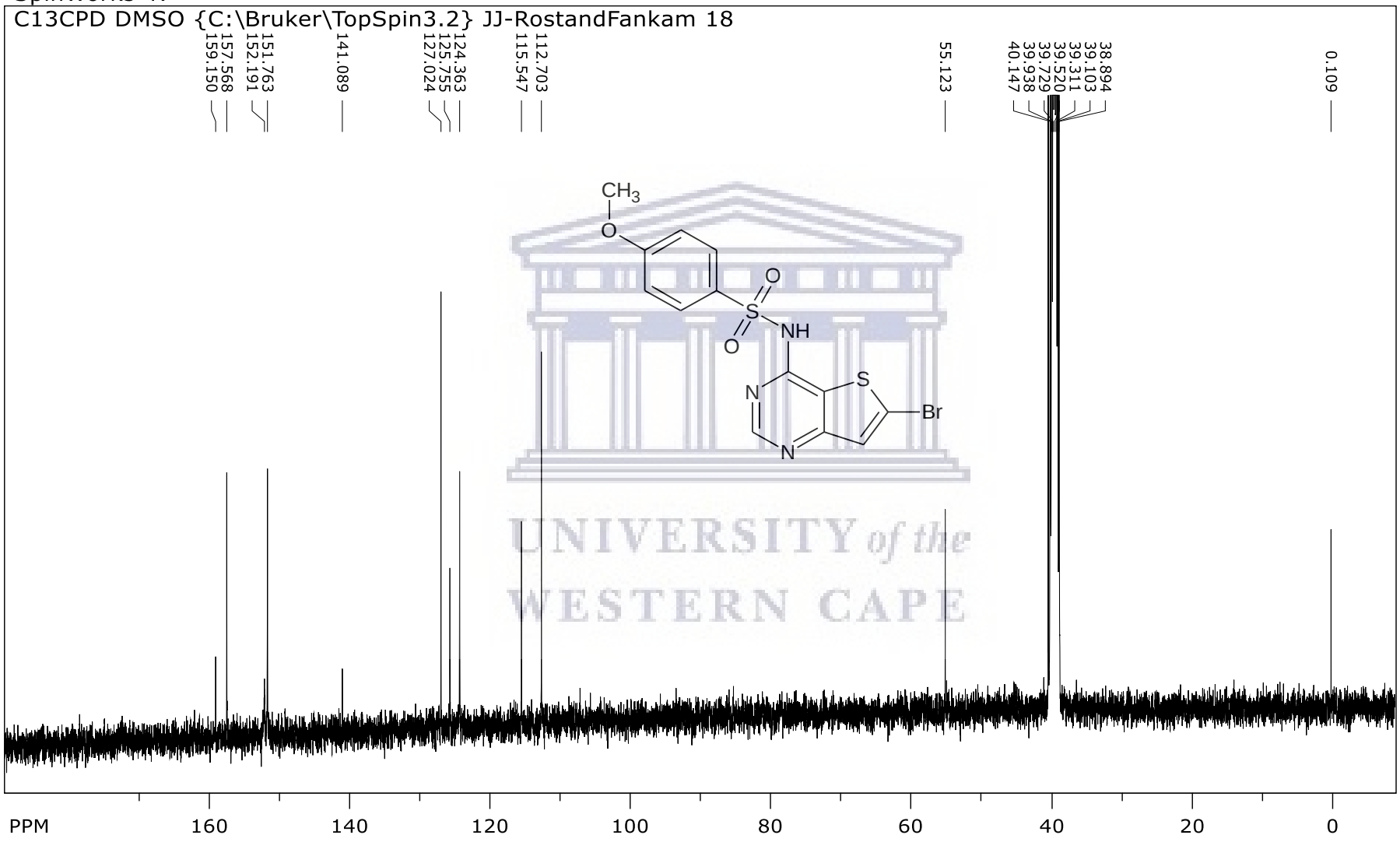


Spectrum 13 ^{1}H NMR of compound 4

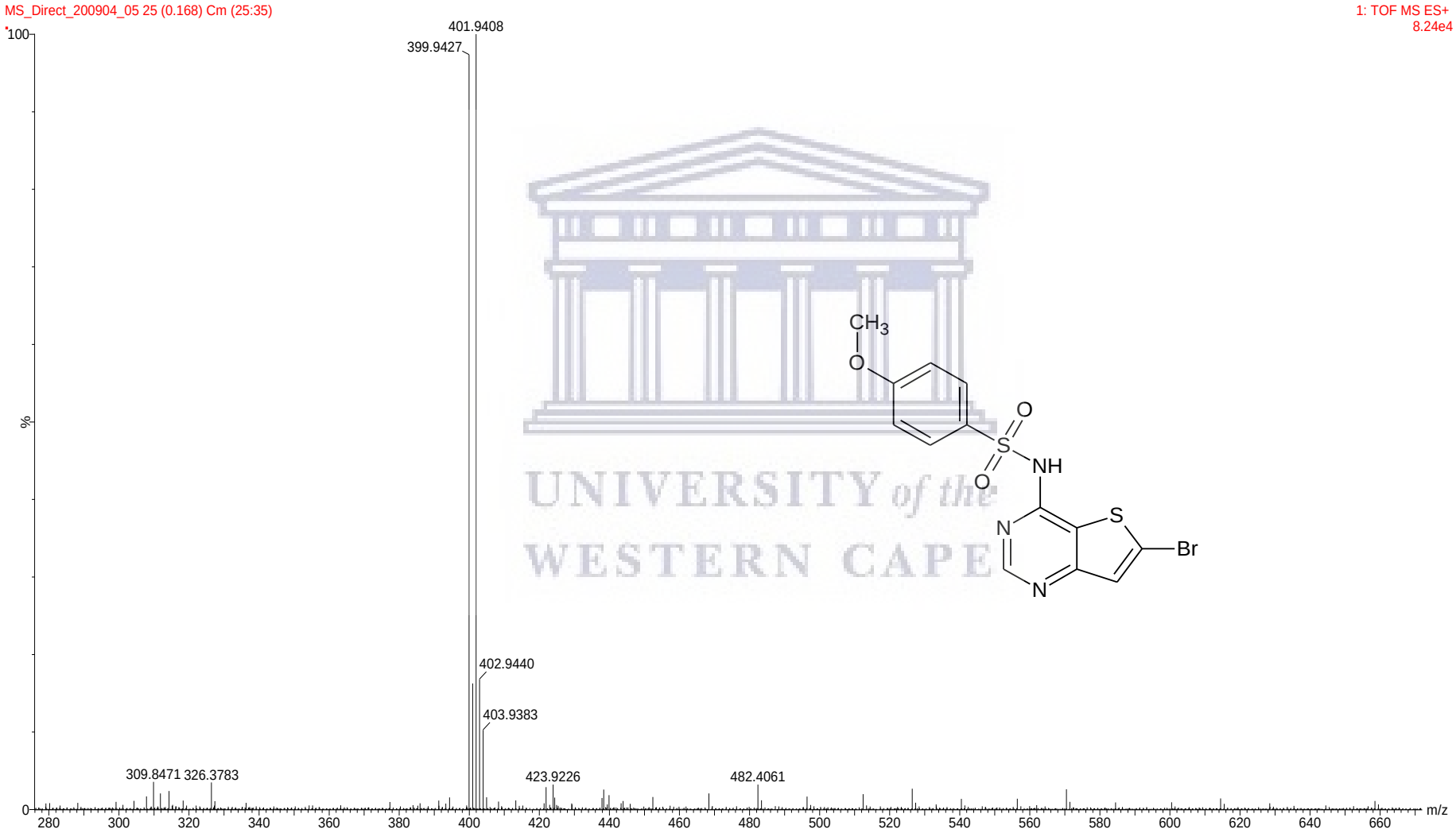


Spectrum 14 ^{13}C NMR of compound 4.

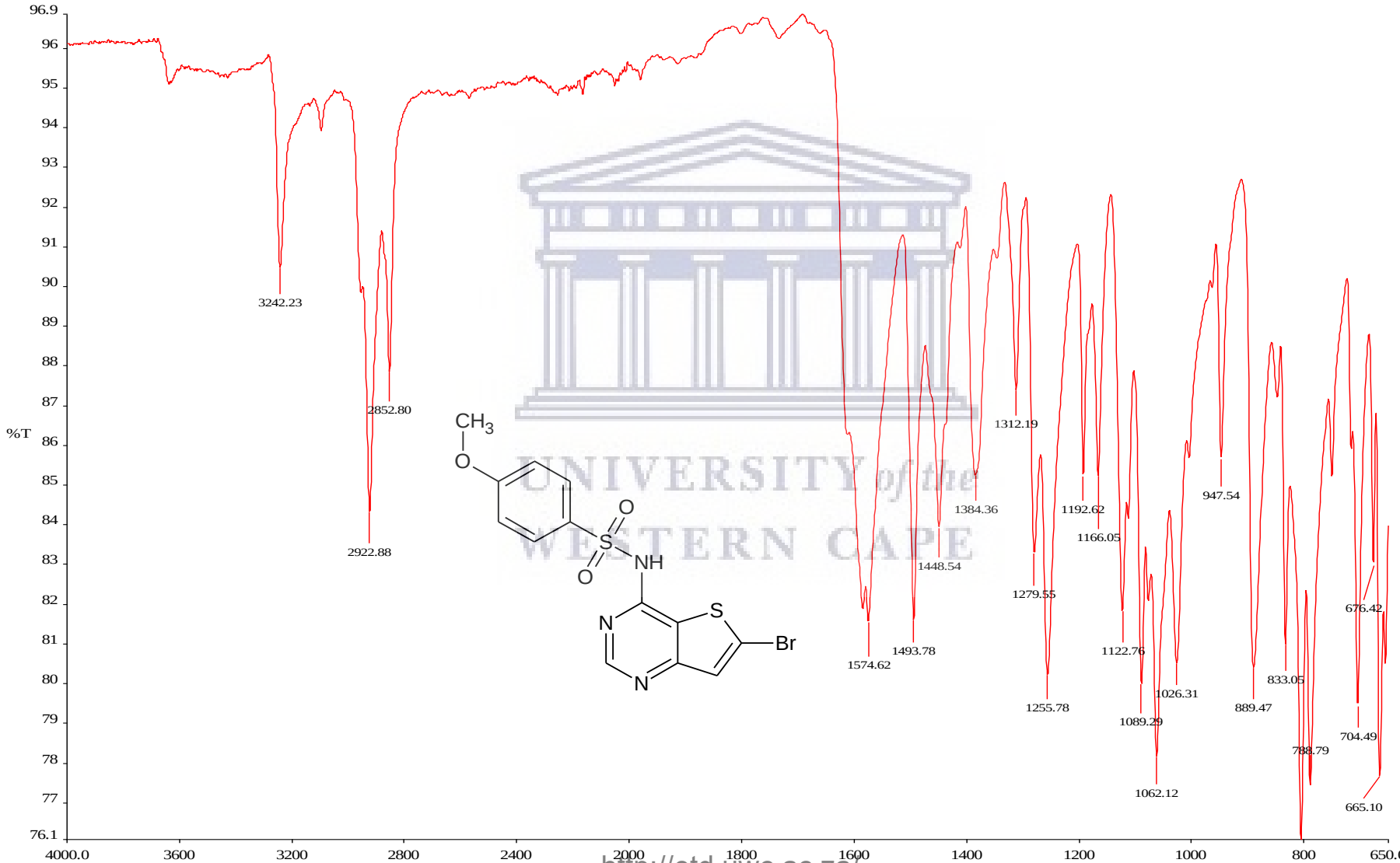
SpinWorks 4:



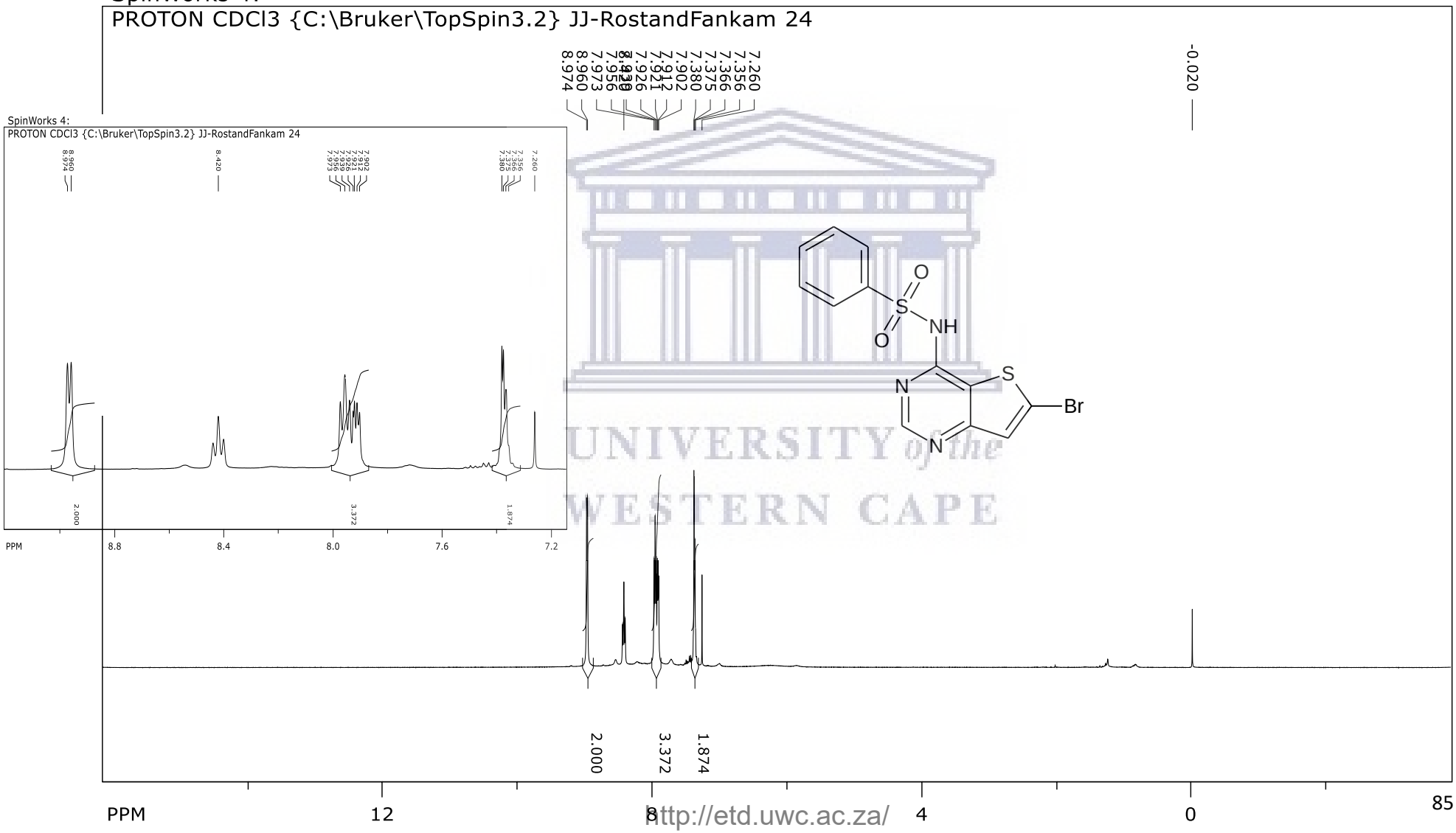
Spectrum 15 MS of compound 4



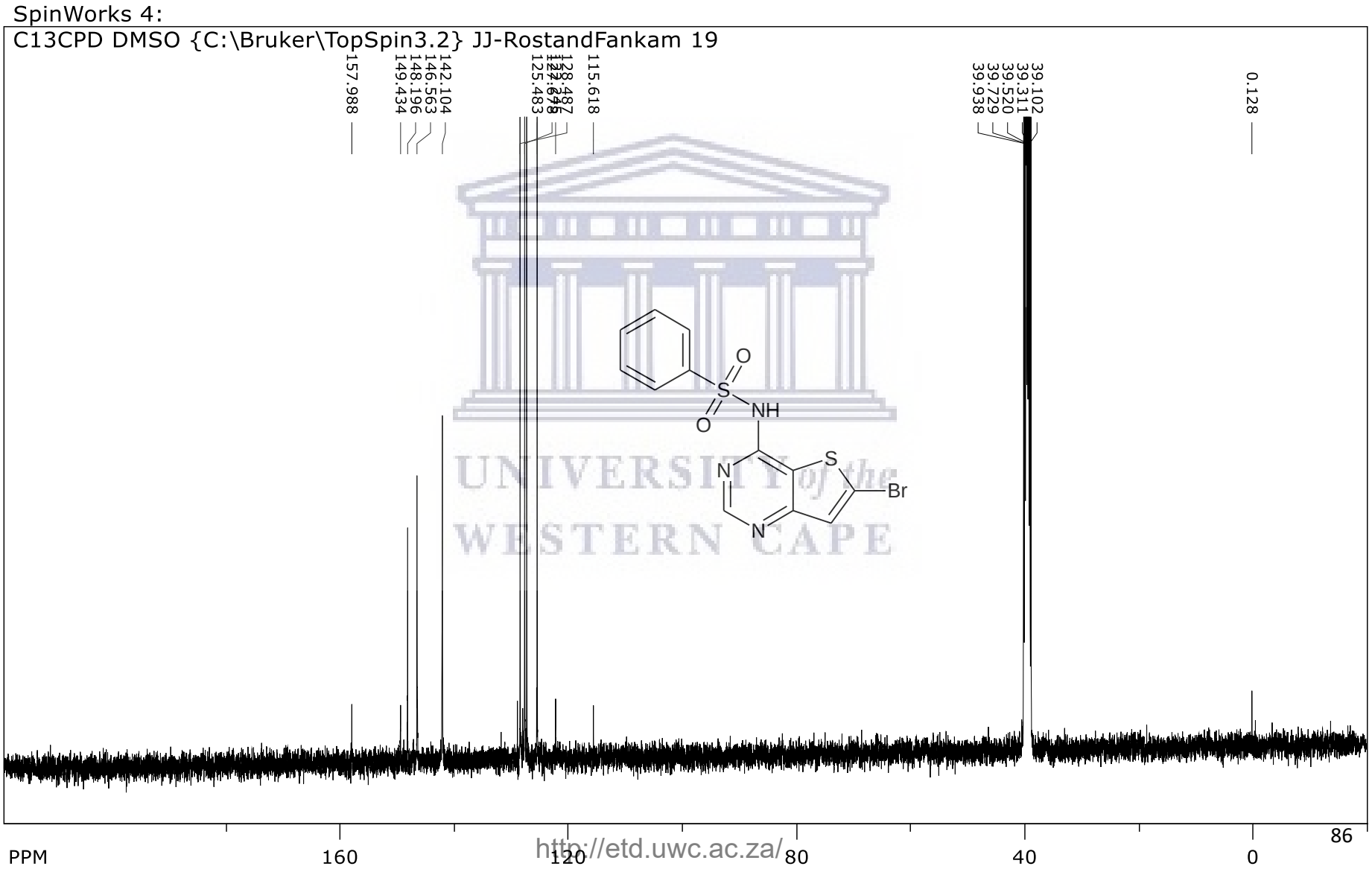
Spectrum 16 IR of compound 4



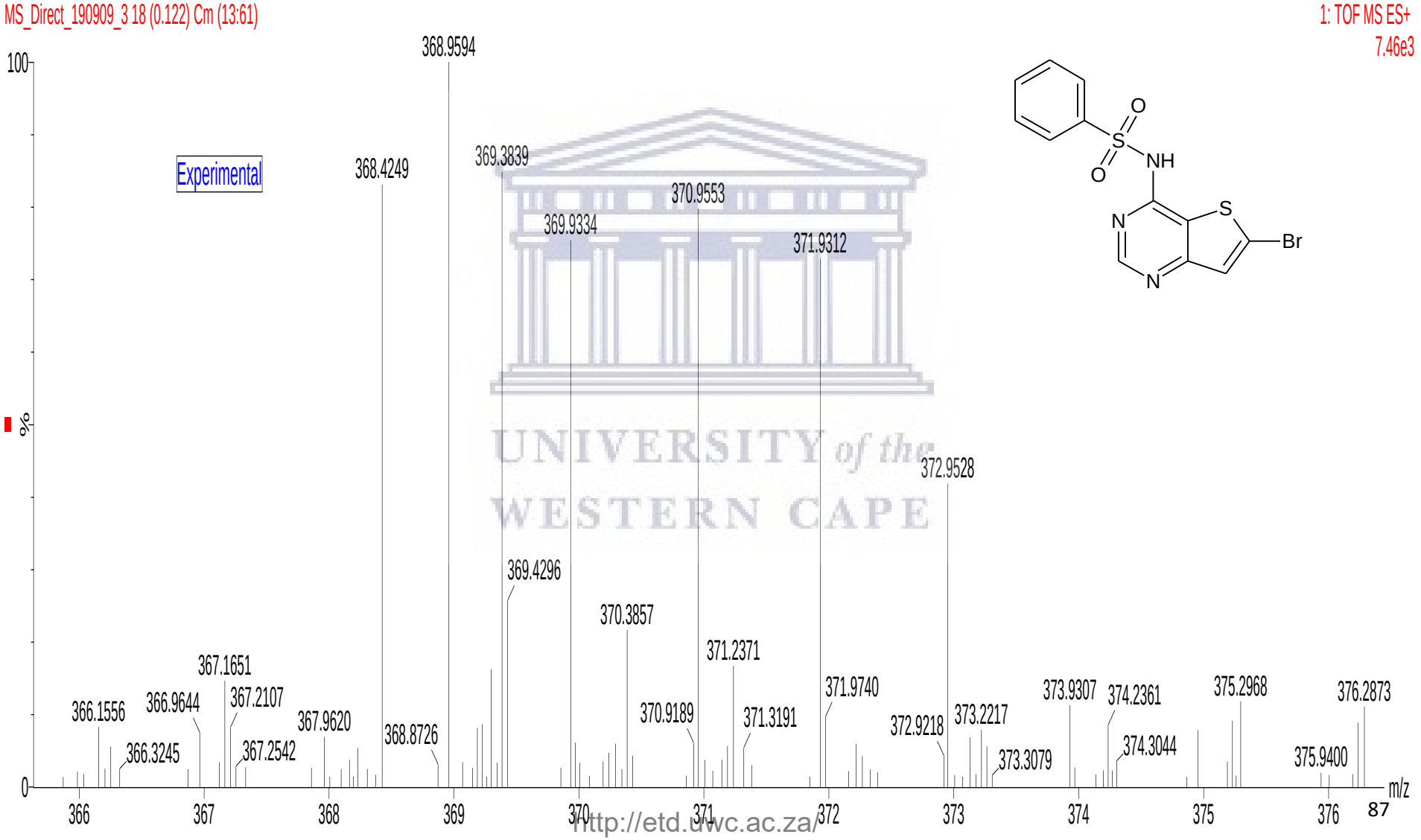
Spectrum 17 ^{1}H NMR of compound 5.



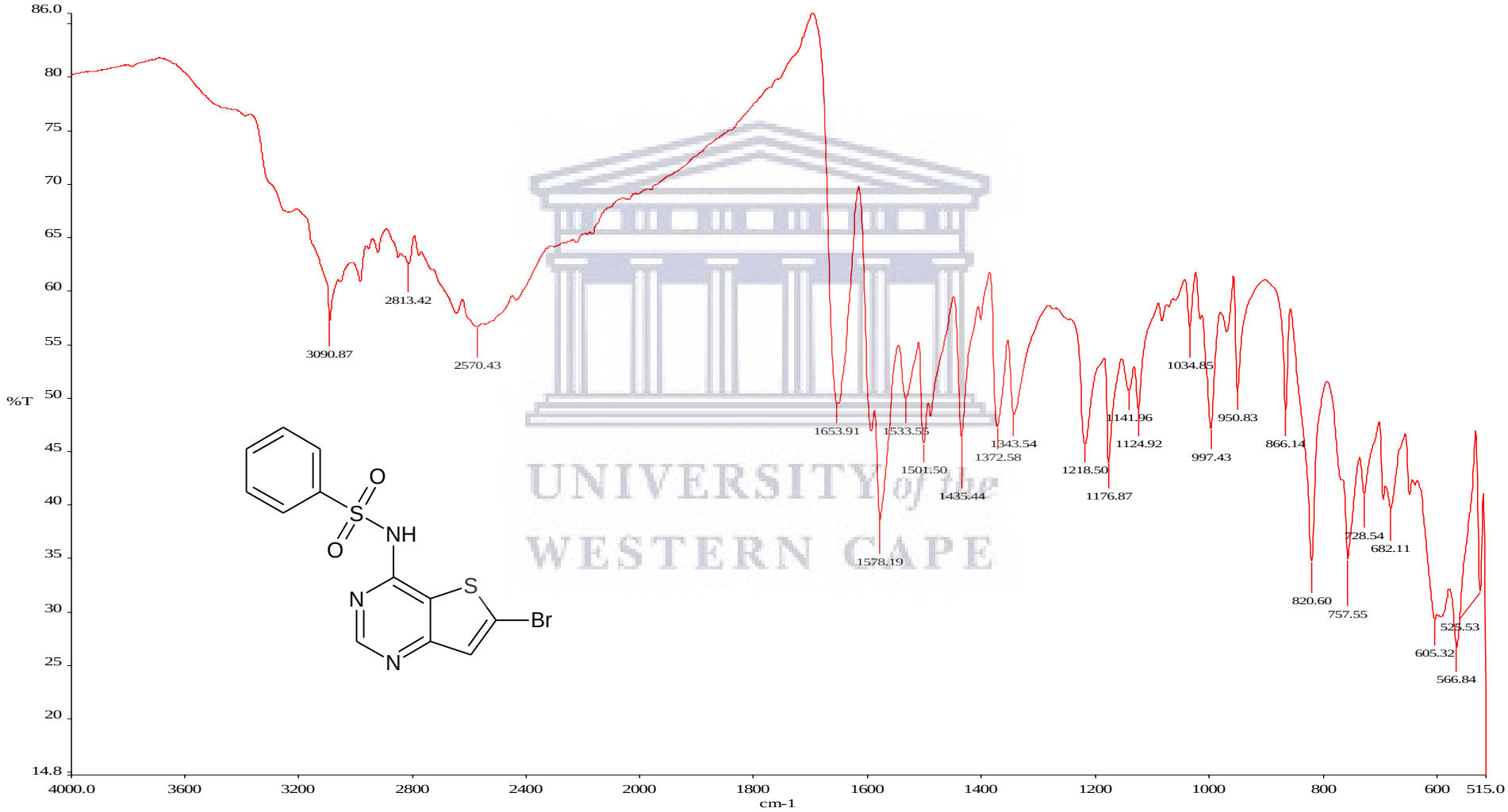
Spectrum 18 ^{13}C NMR of compound 5.



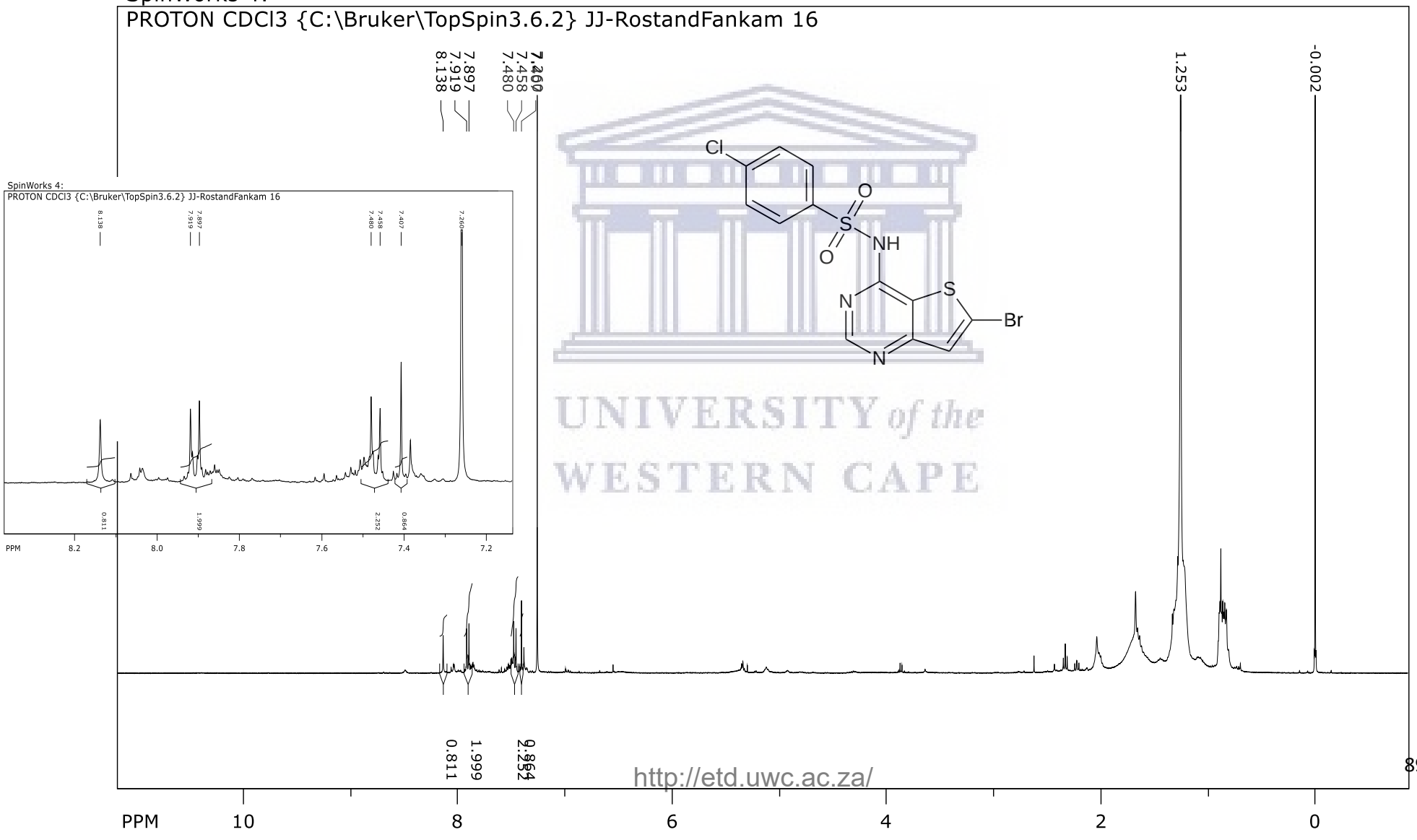
Spectrum 19 MS of compound 5.



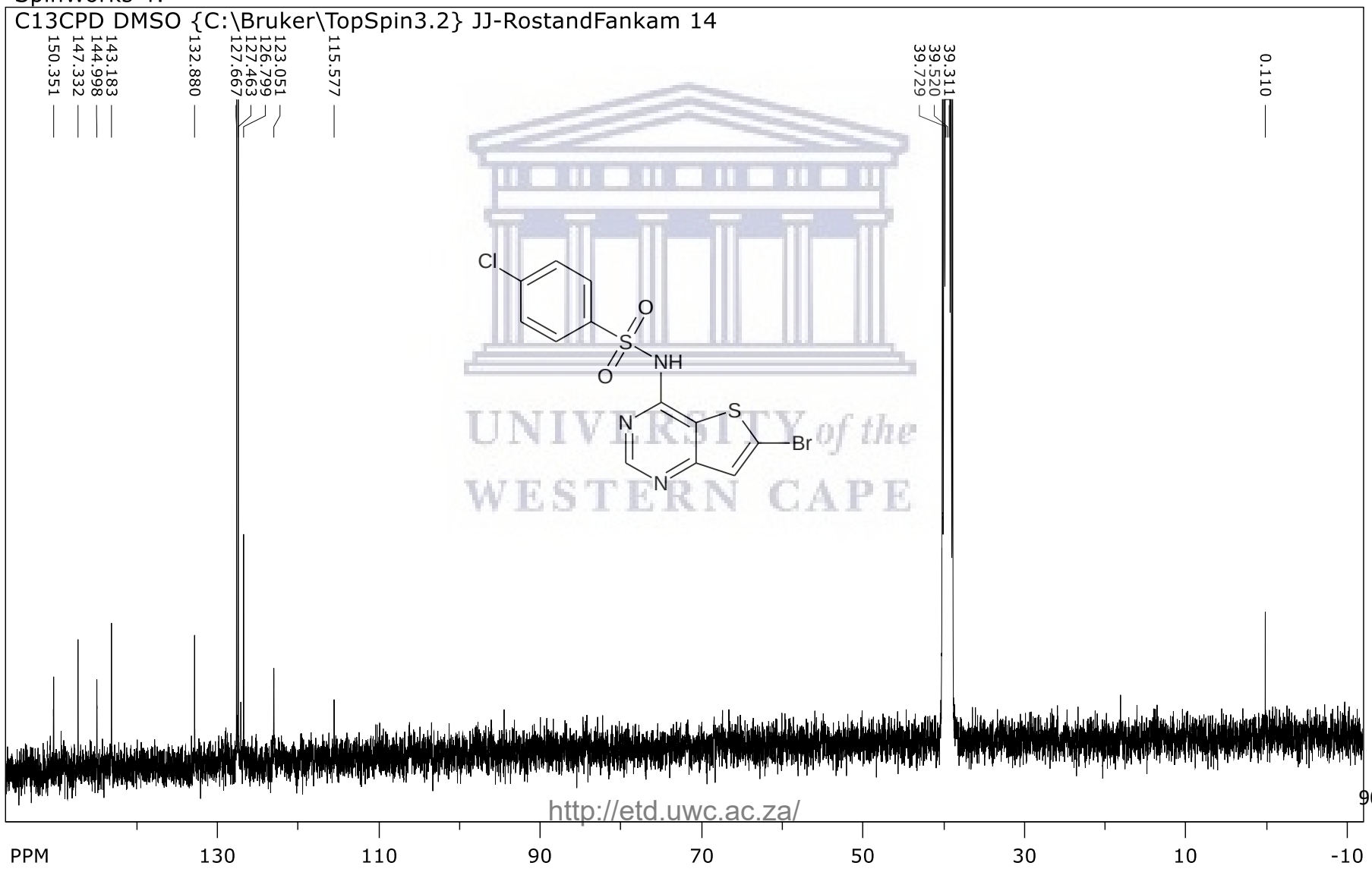
Spectrum 20 IR of compound 5.



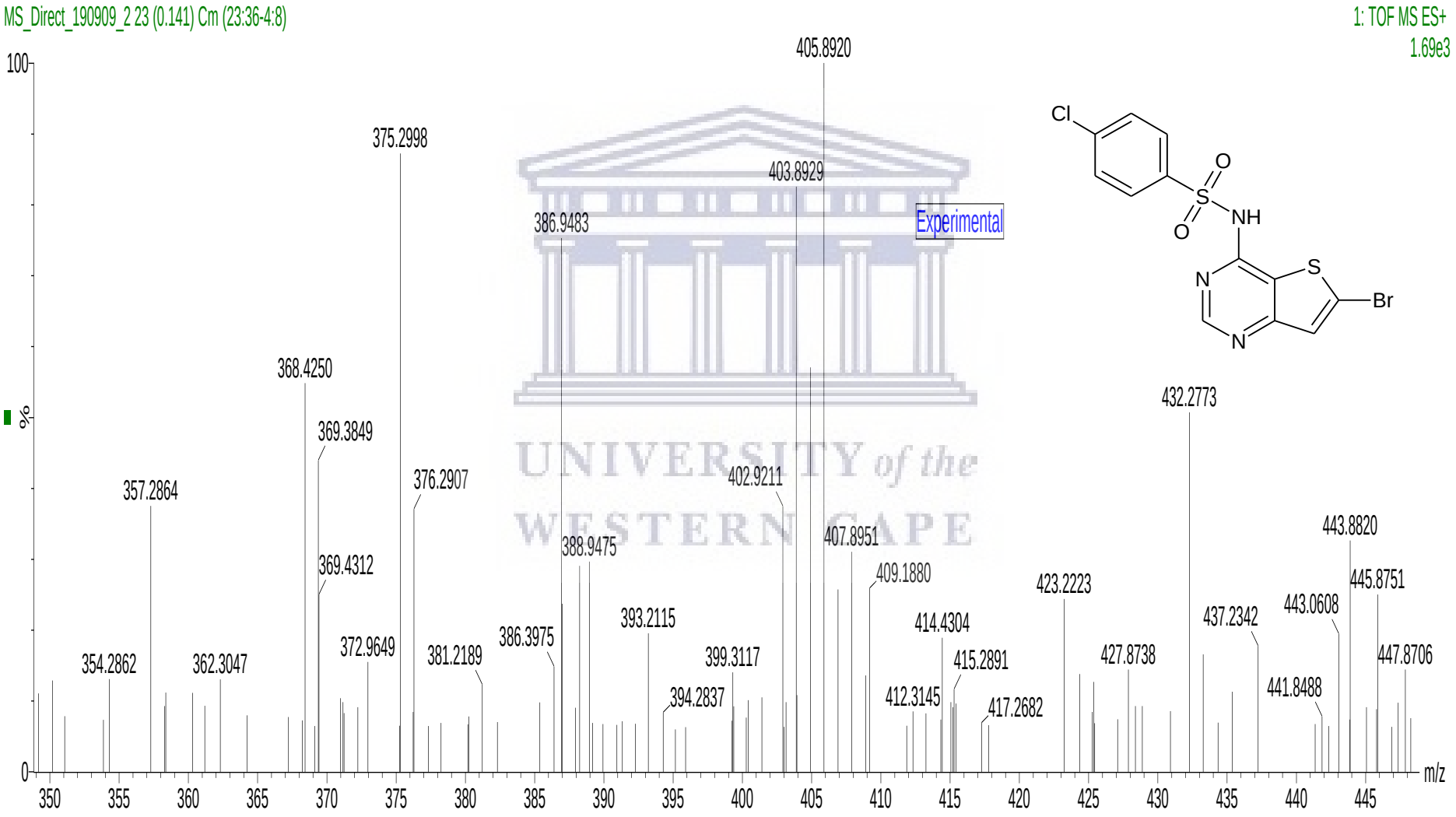
Spectrum 21 ^{1}H NMR of compound 6.



Spectrum 22 ^{13}C NMR of compound 6.



Spectrum 23 MS of compound 6.



Spectrum 24 IR of compound 6.