SONOGASHIRA COUPLING OF QUINOXALINE-O-SULFONATES LEADING TO HETEROCYCLIC COMPOUNDS WITH POTENTIAL MEDICINAL PROPERTIES AGAINST TB

by

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DISSERTATION

Submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

in

CHEMISTRY

in the

FACULTY OF SCIENCE AND AGRICULTURE

(School of Physical and Mineral Science)

at the

UNIVERSITY OF LIMPOPO

Supervisor: Dr W Nxumalo

June 2015

Declaration

I (Herman Dikosha Makgoathana) hereby declare and certify that the information in this booklet is true and original, the result of my own investigations under the supervision of Dr W Nxumalo. I submit this dissertation for the master's degree in the chemistry department at the University of Limpopo. This work has not previously been submitted; all sources that were used or quoted have been duly acknowledged.

Makgoathana H. D (Mr) **Date**

…… **31/03/2015**

Dedication

I am dedicating this project to the following people:

To my mother (Mmaphuti Rosinah Makgoathana), who deserves the most credit for this work for her sacrifices, continuous love, support, comfort and her influence throughout my life.

To my brothers (Tebogo Cosley, John Blue Mamoloko, Mahlodi Boss-Mike Michael) and my younger sister (Lucy Mokgadi). Their love and trust has always encouraged me to achieve my dream.

To Jane Choene Segolela, who struggled with me during the hard times of my study. I thank her for giving me strength, hope throughout my studies and everything she has done.

To my whole family, friends for their support and love they have contributed and also believing in me that I can do it no matter what comes my way.

Acknowledgments

I would like to give thanks to the almighty God for being there for me throughout the year, for helping me to survive all the challenges that I've met in my studies, for giving me wisdom and courage.

First of all, I must express my appreciation to my research supervisor and advisor Dr Winston Nxumalo for giving me the opportunity of doing my master's under his group and for giving me constant encouragement, support and involvement in the course of my scientific research. His supervision and guidance throughout my research project is worth more than a piece of gold.

I am very grateful to the Head of Chemistry Department, Dr Mary S Thomas and the leadership under faculty of Science and Agriculture at University of Limpopo Turfloop campus for providing the necessary equipment's and facilities for my project. I would like to offer my sincere gratitude and appreciation to the below mentioned people for their respective contributions, assisting me and being there for me in times of need during the course of my research programme; Dr R Mampa, Dr KD Modibane and Ms TG Ramakadi for guiding, helping, trusting me throughout the entire years of successful research.

Chemistry department (staff and other postgraduates) and my laboratory colleagues for providing help and support when needed during my research. Their contribution, guidance, motivation, inspiration and completeness of this project is highly appreciated.

To my parents and the entire extended family, my deepest thanks for all the love, support and encouragement that they have given me throughout the years of my studies. I would like to give a lot of thanks to many people who have contributed toward this research. Without them, the chemistry I have done could have been impossible. I would like to thank Jane for appreciation, unconditional love, guidance, motivation, support and sacrifice she made for me throughout the course of my research.

Special thanks and deepest appreciation is expressed to Prof Mokhonoana, Dr Hato, Mr Mulaudzi, Mr Mulalo, Mr Maswanganyi and Mr Murulana for their contribution, guidance and active support. I would like to acknowledge the presence of all my

friends that were there for me during the difficult and happy times of my studies. I express my gratitude for the invaluable collaboration of Dr Mbazima from Biochemistry Department for anti-cancer activity. The department of chemistry at University of Cape Town (H3-D centre) for *M tuberculosis* activity test. This research was funded by National Research Foundation (NRF) and Sasol Inzalo Foundation.

Table of Contents

List of Abbreviations

A

A549 cells	adenocarcinomic human alveolar basal epithelial cells
AIDS	Acquired immune deficiency syndrome

C

C-C	Carbon-carbon bond
δ	Chemical shift
conc	Concentrated

D

^{o}C	Degree Celsius
DNA	Deoxyribonucleic acid
DCM	Dichloromethane
DHPM	Dihydropyrimidinone
DMAP	4-Dimethylamino pyridine
DMF	*N,N*-Dimethylformamide
DMSO	Dimethyl sulfoxide
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
DOTS	Directly observed treatment short-course
d	Doublet
dd	Doublet of doublets
H3-D	Drug Discovery and Development Centre

E

EC	Effective concentration
EtOH	Ethanol
eq	Equivalents
XDR	Extensive drug resistance
F	
FQs	Fluoroquinolones
G	
g	gram
GFP	Green fluorescent protein
H	
HRMS	High-resolution mass spectrometry
HTS	High-throughput screening
h	hour
HIV	Human immunodeficiency virus
J	
J	Coupling constants
I	
IR	Infra-red
IC	Inhibitory concentration
INH	Isonicotinic Hydrazide or isoniazid
L	
Lit	Literature
Lq	Liquid phase

M

0.1% (w/v)	mass/volume multiplied by 100
MHz	Mega Hertz
M.p	Melting point
MeOH	Methanol
µL	microlitre
µM	Micromolar
min	minutes
mg/ml	milligram per millilitre
mmol	Millimole
MIC_{90}	minimum inhibitory concentration of compound needed to inhibit cells growth by 90%
MIC_{99}	minimum inhibitory concentration of compound needed to inhibit cells growth by 99%
MDR	Multi drug resistance
m	Multiplet
M tuberculosis	*Mycobacterium tuberculosis*

N

ND	Not determined
NMR	Nuclear Magnetic Resonance

P

ppm	Parts per million
%	Percentage

% Yield	Percentage Yield
R	
RNA	Ribonucleic acid
RIF	Rifampicin
Rf	Retention Factor
S	
SI	Selective index
sol	Solution
SC	Sonogashira cross coupling reaction
SA	South Africa
T	
THF	Tetrahydrofuran
TB	Tuberculosis
TLC	Thin layer chromatography
t	triplet
U	
USA	United States of America
W	
WHO	World Health Organization

Abstract

Alkyne-quinoxaline compounds were successfully synthesised form the cross-coupling of 2-benzenesulfonyloxyquinoxaline with terminal alkynes. The current study demonstrates the ability of benzenesulfonyloxy as a good leaving group in Sonogashira coupling reaction, generating a number of alkyne-quinoxaline compounds. The structures of the synthesised compounds were characterised through the comprehensive analysis of spectroscopic data from NMR, HPLC-MS and others selected compounds with IR.

Several alkyne-quinoxaline compounds were synthesised in moderate to good yields. During analysis, it was observed that the highest yield was obtained when using 4-(trifluoromethyl)phenylacetylene **4e** and the lower yield was obtained when using the 3-ethynylthiophene **4s** as a substrate. The alkyne-quinoxaline compounds were investigated for 1,3-dipolar cycloaddition which was done from 2-ethylquinoxaline **6**. The 1,3-dipolar cycloaddition reactions were successful and the compounds were obtained in good yields.

The *in vitro* analysis on anti-tubercular screening against H37RvMA strains of *M tb* reveals that compounds **5e** and **5o** which contained a trifluoroanisole moiety showed promising activity amongst all the synthesised compounds. The compounds were having MIC_{90} values 11.8 µM and 12.7 µM respectively, however, they were found to be less active than rifampicin. Compounds **5a**, **5b**, **5e**, **5g**, **5h**, **5i**, **5l**, and **5r** were evaluated for anti-cancer activity on A549 cells. The results have showed that **5i** was able to retard the migration of A549 cells in the Wound-Healing Migration Assay.

Chapter 1

Chapter 1

1 INTRODUCTION

1.1 Tuberculosis

Tuberculosis (TB) is a highly contagious, infectious disease and remains a significant public health problem globally [1]. It is caused by an infection of the bacteria called *Mycobacterium tuberculosis* (*M tuberculosis*) and about one-third of the world's population is infected by this disease [2]. It is one of the world's most infectious diseases suffered by mankind in mostly low and middle income countries [1,3] and causes an increase of morbidity and mortality worldwide [3]. South Africa (SA) and other countries around the world are facing a major challenge of high TB infections [4–5], especially due to HIV.

The sub-Saharan African region has the greatest burden of TB infections [6–7] and SA has one of the largest TB burdens in Africa [1,4]. Within a population of 50 million, SA has a TB prevalence of approximately 400,000 [6]. TB is the most common opportunistic infection associated with HIV-AIDS and it is also the leading cause of death amongst HIV-infected patients in sub-Saharan Africa region [3]. The World Health Organization (WHO) estimates that within the next 20 years about 30 million people will be infected with *M tuberculosis* [6].

TB still remains a leading cause of death worldwide due to a number of factors such as: (a) long duration of treatment (6–9 months), (b) increased incidence of multi drug resistance (MDR) or extensive drug resistance (XDR), (c) co–morbidity with HIV-AIDS and (d) declined effort in anti-infective drug discovery research [4]. Despite the existence of an efficient anti-TB treatment, the emergence of MDR and XDR-TB has become a great threat to TB control [5,7].

Current treatment of TB is based on drugs that are more than 40 years old. However, there are number of side effects associated with these drugs [6]. Most of the currently used TB drugs need to be taken for a long period (from 6–9 months or even a year), especially if the disease has advanced to MDR-TB [6,9]. Drugs that are active against resistant forms of TB are less potent due to the higher drug-drug interactions, are more toxic and need to be taken for a long time (≥18 months) [8].

1.2 Current treatment for TB

The current standard regimen called directly observed treatment short-course (DOTS) for TB recommended by WHO is a combination therapy of the four mostly used drugs for a period of 6 to 9 months (or even a year) (Figure 1) [10–12]. The most effective combination used to treat TB includes isoniazid, rifampicin, pyrazinamide and ethambutol [4] for two months, then followed by isoniazid and rifampicin for a further four months [6,12]. The ineffectiveness on the present therapy may be caused by long duration of the therapy which results in poor patient compliance and which may lead to the development of resistance to these mostly used drugs [4,12]. Active TB, particularly if it is a drug-resistant strain, will require several drugs at once.

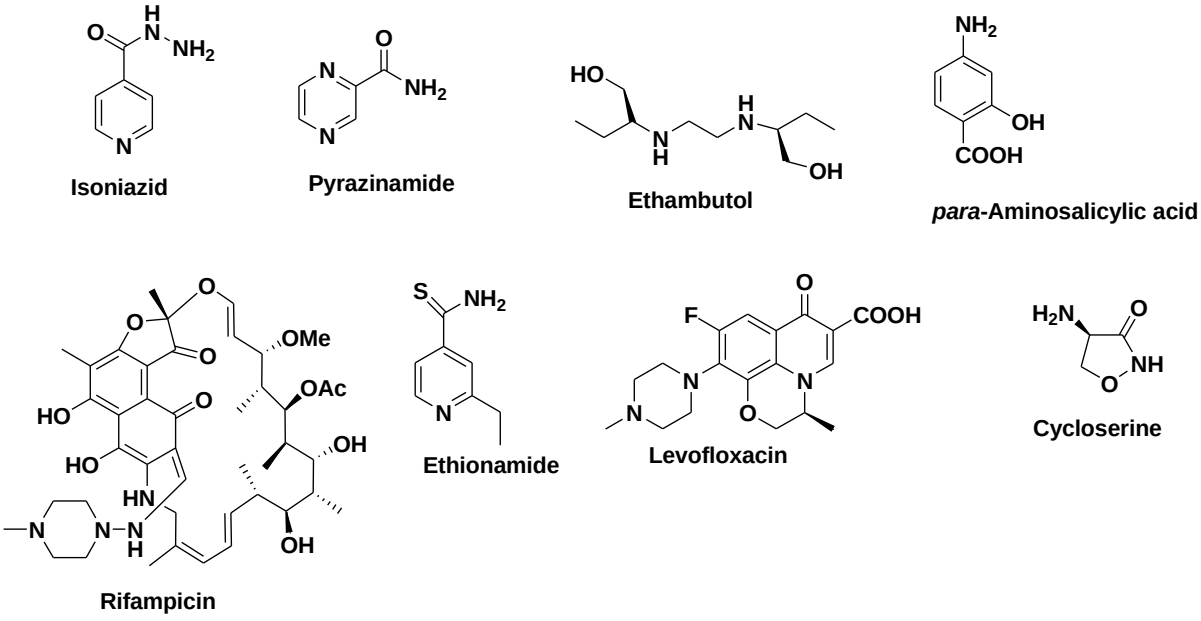


Figure 1: Structures of some anti-tuberculosis drugs [11].

About 51 million TB patients have been successfully treated and up to 20 million lives have been saved since 1995 through the basic package that underpins the Stop TB Strategy (Figure 1) [13]. The first-line drugs which are used in SA are rifampicin, isoniazid, pyrazinamide, ethambutol and streptomycin. Isoniazid also known as isonicotinyl hydrazine (INH), is an organic compound that forms part of first-line medication used in treatment of TB. Its activity against TB was first reported

in the early 1950s. Isoniazid is used to inhibit the fatty acid synthase II complex that produces mycolic acids that are unique to mycobacteria [14].

Ethambutol is an antituberculosis drug that was discovered in 1961 and was found to possess bacteriostatic action against *M tuberculosis* in an unknown mode of action and it is active against the *M tuberculosis*, *Mycobacterium kansasii* and *Mycobacterium scrofulaceum* [15]. Pyrazinamide, first synthesised in 1952 from quinoxaline, is the nitrogen analog of nicotinamide and was found to exhibit hepatotoxicity [16]. It inhibits mycobacterial fatty acid synthesis by targeting fatty acid synthase I complex [17]. It is used in SA's standard regimens for the treatment of TB and has been shown to improve prognosis in treating MDR and XDR-TB in patients that are still responsive to the medicine [18].

Rifampicin (RIF) also known as rifampin in the United States (US) is a bactericidal drug which inhibits bacterial ribonucleic acid (RNA) polymerase during RNA synthesis [21] and is highly active against *M tuberculosis* [19]. It is the most effective drug for treating pulmonary and non-pulmonary forms of TB, including TB meningitis [18–20]. Recently, the efforts made on treatment of TB are being threatened and weakened by the disease resistant (MDR and XDR-TB) to current proven drugs [4].

Current proven treatment is not effective enough to overcome the development of resistant strains leading to MDR and XDR-TB. Some of the risk factors associated with this disease are TB/HIV co-infection which needs urgent medical attention. Moreover, there is greater concern on how drug-resistant TB should be managed in order to control the spread of this resistance. The current challenge on this disease is to improve treatment compliance and managing the spread of it.

Even though this disease (TB) can be prevented and curable, the challenges associated with the current TB treatment suggests an urgent need for newly synthesised compounds with more effective anti-TB properties. New active compounds against drug-resistant TB are desperately needed to inhibit *M tuberculosis* strains and this challenge can be addressed through the field of synthetic medicinal chemistry.

1.3 Heterocyclic compounds and drug discovery

N-heterocyclic compounds, such as quinolones, quinoxalines, benzodiazepines, dihydropyrimidinones, and quinazoline, are very useful intermediates for the development of molecules of pharmaceutical (Medicinal chemistry). N-heterocyclic-framework have contributed in the field therapeutic agent against various diseases and assist in restoring health to humans, through drug discovery processes [14,25]. It is also known as a discipline with a traditional focus on organic synthetic chemistry with the broad goals of drug discovery and optimization [22]. Synthetic organic chemistry plays a significant role in contributing to chemotherapy against various diseases [23]. Chemistry of heterocyclic compounds is one of the most complex and interesting branches of organic chemistry which deals with drug discovery through organic synthesis [19].

Recently, the nitrogen contained compounds have attracted much interest due to their biological properties against various diseases. Many heterocyclic compounds have been used as inhibitors of various diseases and are synthesised from the field of synthetic medicinal chemistry [23–24]. Over the past decade, heterocyclic compounds have been known to be one of the largest areas of research in the field of synthetic organic chemistry for drug discovery [24]. It has been established that heterocyclic compounds play an important role in designing new class of structural entities for medicinal applications in various fields [25,26].

Nitrogen containing compounds have been reported to play an important role in the field of synthetic medicinal chemistry [27] and these compounds are desirable for their structural units in both chemistry and biochemistry applications [28]. Quinoline and its derivatives are considered as an important class of bioactive molecules in the field of drugs and pharmaceuticals [29]. Quinolones and fluoroquinolones (FQs) have shown activity against *M tuberculosis* and these compounds are among the nitrogen-containing heterocyclic compounds (Figure 2) [29–30].

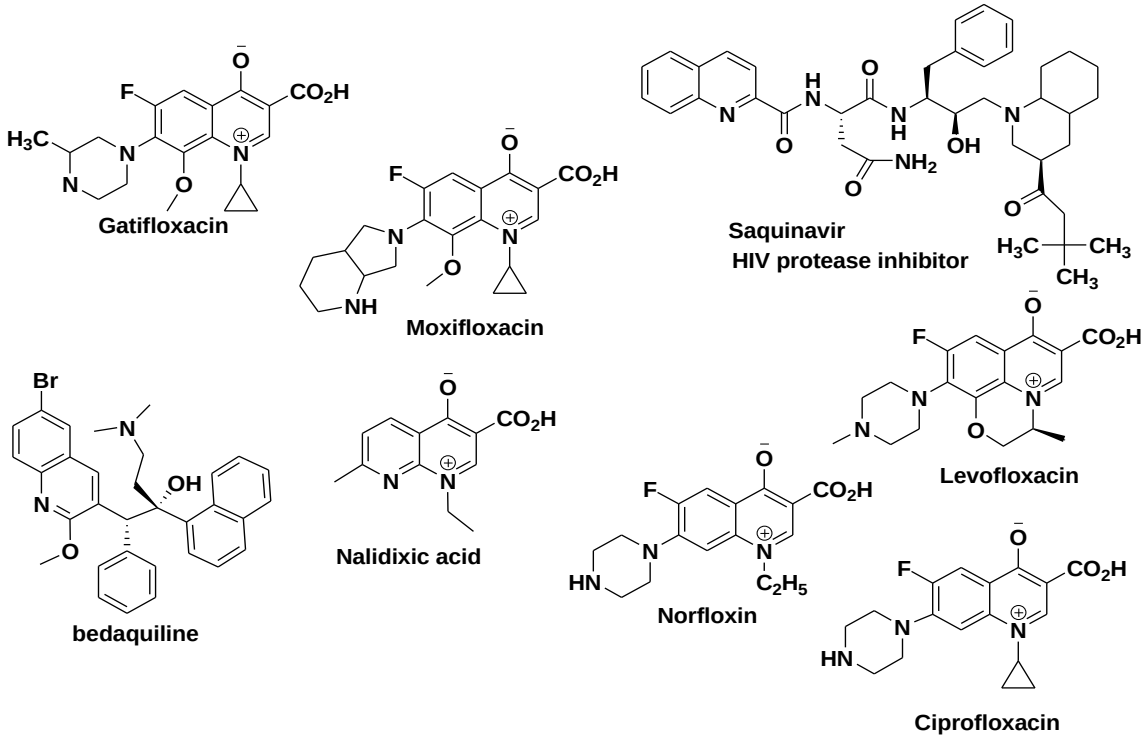


Figure 2: Structures of quinolones and fluoroquinolones

The need for novel chemical compounds to treat TB disease is ever increasing because of challenges such as MDR and XDR-TB [30]. FQs have been extensively used to treat bacterial infections such as bacterial keratitis. Some of the FQs such as ciprofloxacin, gemifloxacin, proquin XR, floxin and noroxin are already on market for the treatment of antimicrobial activity like *Mycoplasma* and *Chlamydia spp* [31]. Most recent approved anti-TB drugs from FQs are derived from nalidixic acid, discovered in the early 1960's for its antibacterial activity (Figure 2) [21].

Among all the antibacterial FQs developed, ciprofloxacin, ofloxacin and levofloxacin are the most widely used to stop the replication of bacteria by inhibiting the reproduction and repair of their genetic material [21,43]. The mechanism of action shows that FQs target the deoxyribonucleic acid (DNA) gyrase which is the sole type II topoisomerase in bacteria.

Villemagne *et al*., (2012) reported that the FQs, gatifloxacin and moxifloxacin are being evaluated in Phase 3 of clinical trial of TB, while oxazolidinone, linezolid, nitroimidazole and metronidazole in Phase 2 of TB. These drugs have been repurposed for TB treatment [6,21,43]. FQs are already used as bactericidal agents and have shown activity against TB.

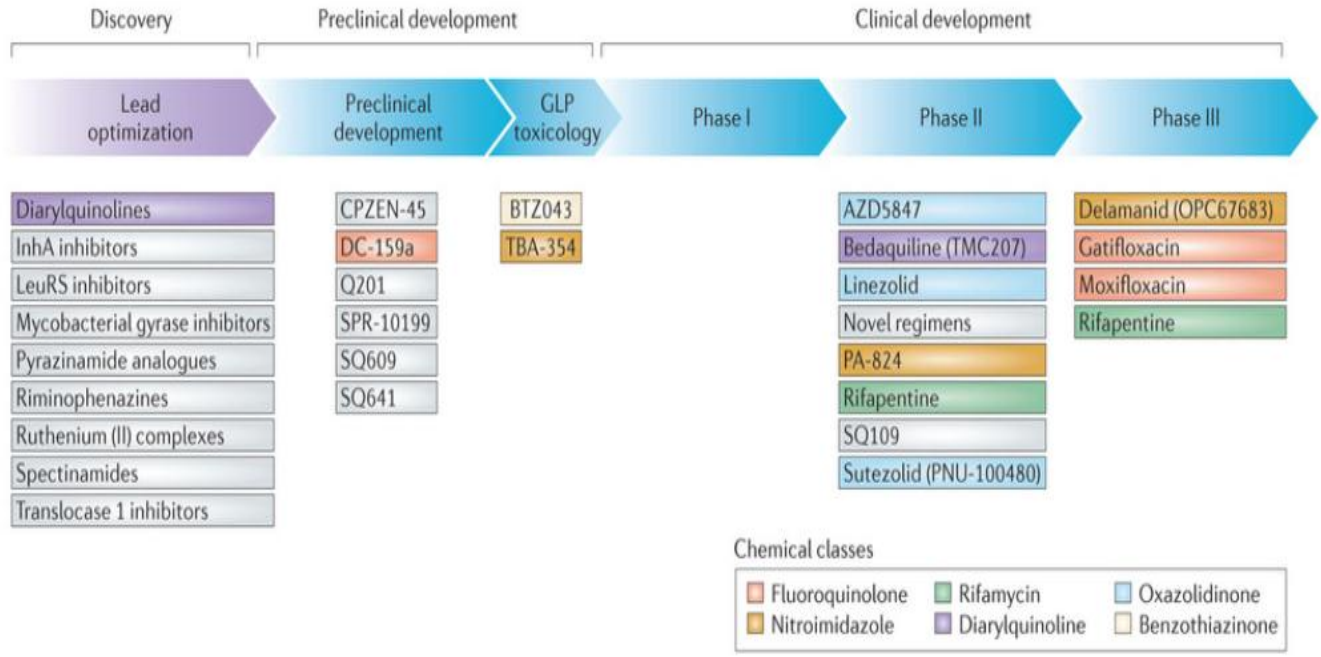


Figure 3: Current global pipeline of new tuberculosis drugs [32].

New TB drugs are needed because of the challenges of complexity and toxicity of the current TB drug regimens and the major problem of TB drug resistance [32]. For new compounds to be regarded as active compounds against TB, it must follow the regular drug discovery process (Figure 3) which summarises some of the most important attributes that a new drug must have to contribute to a future regimen [33].

The current TB drug pipeline is still not sufficient enough to address the challenges inherent in the current proven drugs, because they are still failing to fulfil the requirements for the treatment of the MDR and XDR-TB therapy [32–35]. Developing a new drug product is a complex process which can take 12–15 years and is costly. The process of finding a new drug against a chosen targeted disease usually involves high-throughput screening (HTS), wherein large libraries of chemical compounds are tested for their ability to inhibit the target disease.

The processes involved have to undergo through some stages, such as discovery (which include lead identification and lead optimization), preclinical development, and clinical development (which include phases 1, 2 and 3). Drug discovery involves the identification of screening hits and optimization of these, is to increase the affinity, selectivity, efficacy and metabolic stability of the active compounds [36].

Once a compound fulfils all of these requirements mentioned above, it will begin the process of drug development prior to clinical trials (Figure 3). An active compound undergoes clinical stages for further investigation and determination on targeted disease. Clinical investigations of a new drug candidate start with phase 1 testing with the aim of finding active compounds that may be safely administered to patients [37].

Phase 1, studies are carried out in volunteers (normally healthy young males) who don't have the targeted disease such as TB and involves a small number of 20 to 100 patients [36]. These studies include initial single-dose studies, dose escalation and short-term repeated-dose studies. The main goal is to determine what the drug's most frequent side effects are and often, how the drug is metabolized and excreted in the body [36,38].

In Phase 2, studies are carried out on 100 to 250 volunteered patients in order to evaluate and monitor drugs preliminary efficacy and side-effect profile. The clinical trials in this phase provide more detailed information about the safety and the effectiveness in patients with the targeted disease. Additional safety and clinical pharmacology studies are also included in this category [37]. These studies concentrate on safety and dosing of the new drug with an increased focus on its efficacy [37,39].

Phase 3 of clinical trial include studies that determine the effectiveness of a new compound in treating the targeted disease [37]. This phase is commonly known as a final confirmation of safety and efficacy on a new drug which involves the testing of large groups of volunteered patients (1000 to 3000) to confirm its effectiveness. Phase 3 of clinical trials concentrate on patients with a specific disease, they typically involve patients of different ages, ethnicities, and both genders so that the results may be relevant to a large number of people [36,37,39].

In addition to these three phases, phase 4, also known as Post Marketing Surveillance is also carried out once the drug is approved and marketed. This stage consists of some of the drugs that are currently used against TB such as ethambutol, pyrazinamide, *etc*. Phase 4 of trial is done after the drug has been shown to work and has been granted a licence and is ready to be used [36].

In recent years, medicinal chemists have modified the quinolone scaffold to develop novel heterocyclic compounds with fascinating antitubercular activity in order to optimize its pharmacology [29]. Quinolones have been identified as potential compounds for treatment of TB because these compounds have shown antibacterial activity and exhibit anti-TB properties. In addition to quinolones, quinoxaline derivatives have been synthesised with the aim of obtaining biologically active compounds. There are some quinoxaline compounds that have been reported to be active against *M tuberculosis* and most of those promising active compounds are 1,4-di-*N*-oxide quinoxaline derivatives [24,27].

1.4 Quinoxaline compounds and their derivatives

Quinoxaline compounds are naphthalene isosteres where carbon atoms 1 and 4 have been replaced with nitrogen atoms. They are a class of nitrogen containing compounds which constitutes useful intermediates in organic synthesis and have gained considerable attention in recent years due to their pharmaceutical and industrial applications [40]. Quinoxaline structures, also called benzopyrazine are a class of heterocyclic compounds which consist of a fused ring made up of benzene and pyrazine ring (Figure 4) [41].

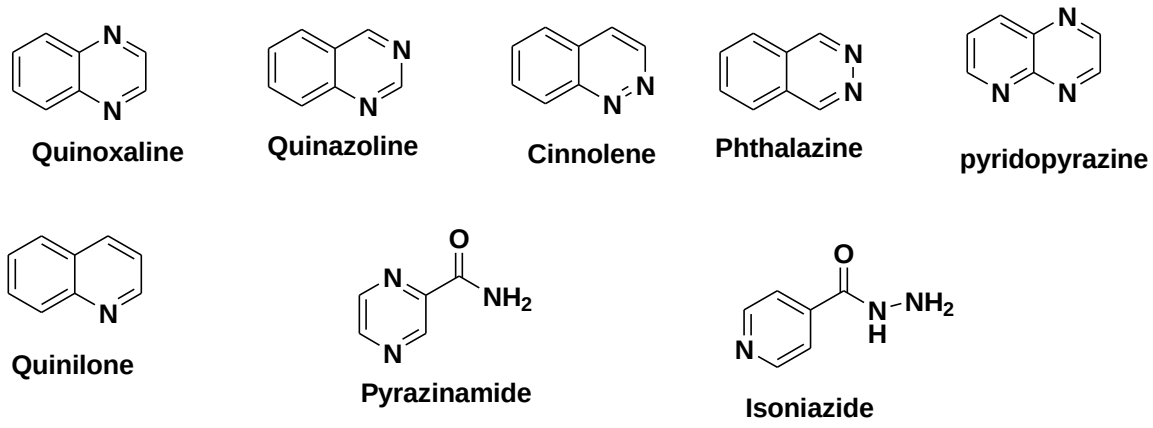


Figure 4: Structure of *N*–heterocyclic isomeric and isosteric compounds

Due to the wide range of applications, quinoxaline compounds have been a centre of interest in scientific fields and their nucleus is acting as a precursor to assembly a large number of new compounds with different applications [42]. Quinoxaline compounds are isomers of cinnolines, phthalazines and quinazolines (Figure 4) [43].

Quinoxaline compounds are also used as potential building blocks for the synthesis of organic semiconductors [44]. Most of the quinoxaline derivatives are synthetic and natural quinoxaline derivatives are rare. Examples of natural compounds containing a quinoxaline ring which form the core moiety are echinomycin, leromycin, actinomycin and triostin-A (Figure 5) [45].

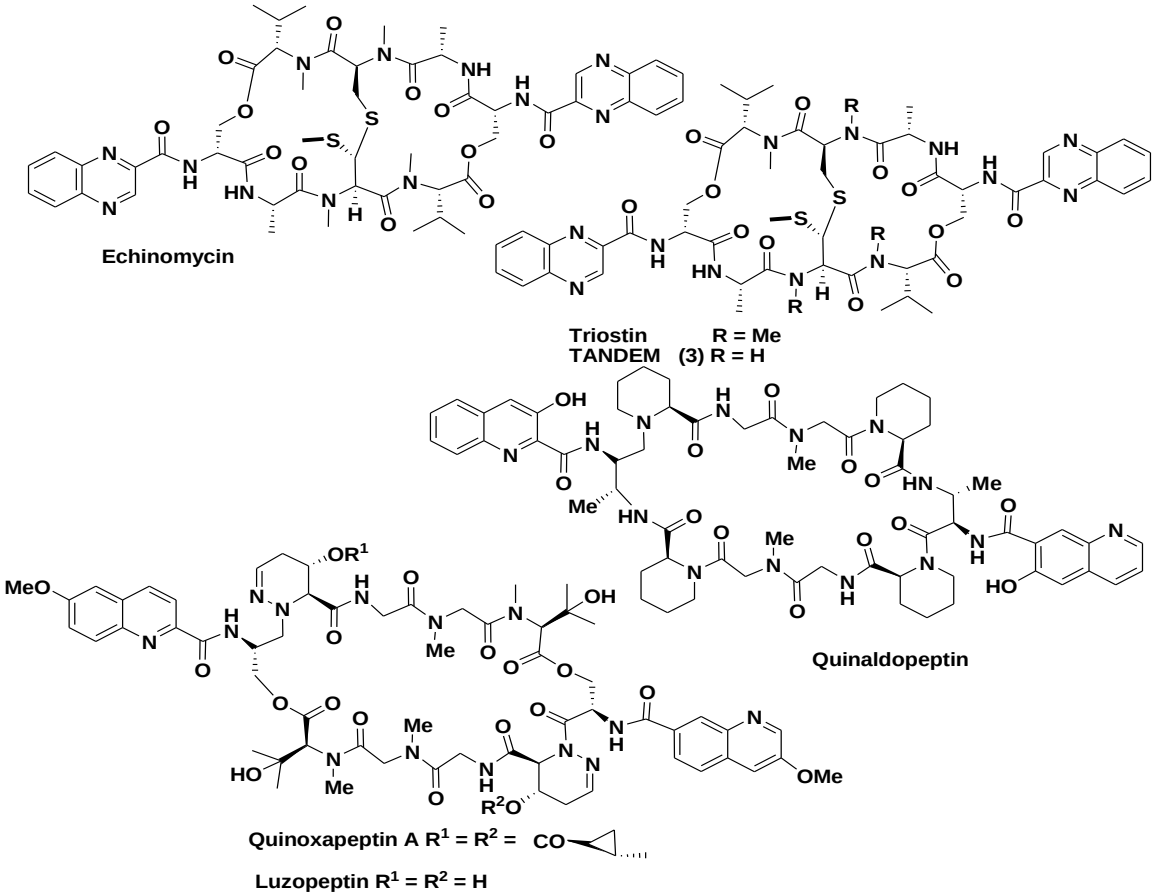


Figure 5: Structures of natural occurring quinoxaline derivatives [45]

Echinomycin is isolated from various bacteria such as *Streptomyces lasalienis* and it belongs to a family of quinoxaline antibiotics used as antitumor agents. The quinoxaline motif is known to represent a class of medicinally important compounds which are effective as antibacterial [24], antifungal [27], anticancer [46], antitubercular [27] and antiviral agents [47].

Quinoxaline derivatives constitute the basis of many insecticides, fungicides and herbicides [49,50]. Currently, the chemistry of quinoxaline and its related derivatives continue to attract considerable attention due to their potential biological activity [49]. The quinoxaline moiety is found in a number of antibiotics which are known to inhibit

the growth of Gram-positive bacteria and also active against various transplantable tumours [51].

Many studies have been conducted concerning synthesis and biological activity of a large number of quinoxalines [50]. Quinoxaline derivatives constitute an important class of heterocycles in drug discovery and agrochemicals. Examples are pyrazoloquinoxaline which have showed a relatively promising antibacterial activity and imidazo quinoxalines ribonucleosides which are used as antiviral agents(Figure 6) [42,52].

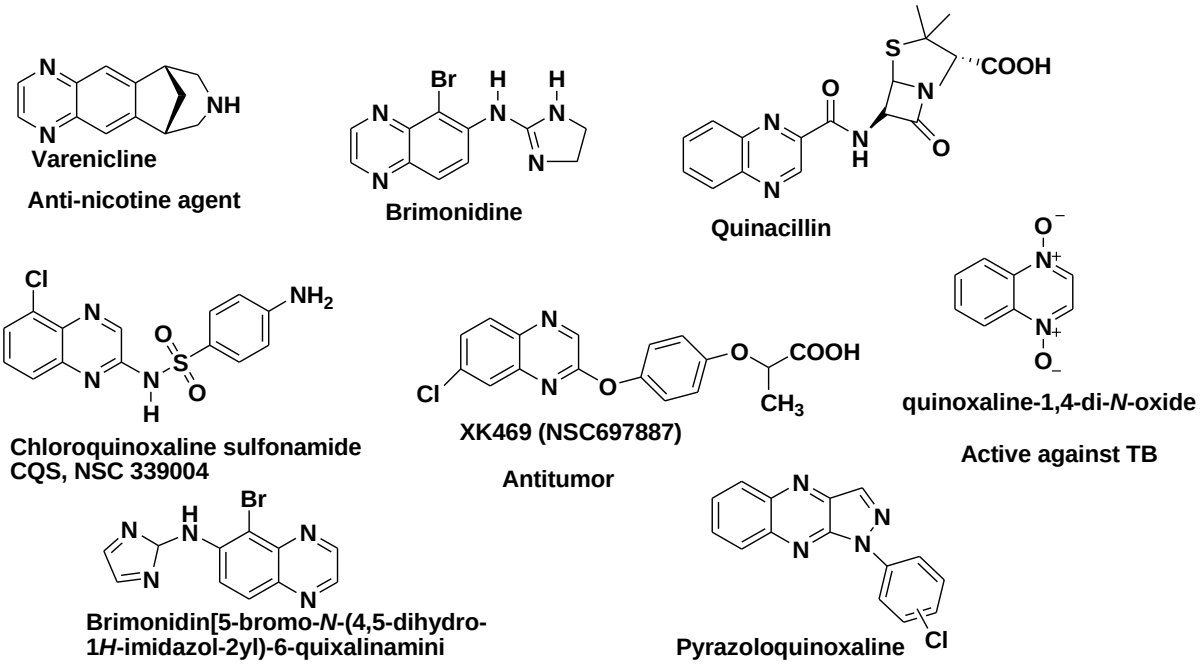


Figure 6: Biologically active quinoxaline molecules [52,54].

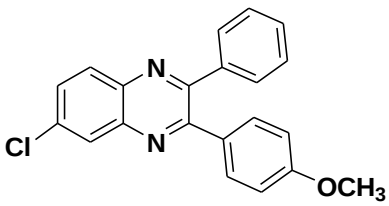
The significance of quinoxalines derivatives as pharmaceutical agents was recognised through brimonidine [42]. Modification of brimonidine revealed a possibility of obtaining a wide variety of compounds with different biological properties such as varenicline, pyrazoloquinoxaline, brimonidine, chloroquinoxaline sulphonamide, *etc* (Figure 6).

Vacenicline is a medicinal compound which consists of quinoxaline moiety and is used to treat nicotine addiction. Brimonidine is a quinoxaline compound used in the treatment of glaucoma, by reducing the body's production of aqueous humour and increases the flow of aqueous humour out of the eye. Chloroquinoxaline

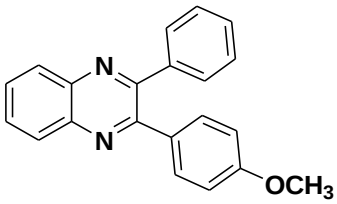
sulphonamide (Figure 6) is an active quinoxaline compound used against rats and human solid tumours [53].

Quinoxalines are also described as a bioisostere of quinoline and benzothiophene which have shown very good biological activity against TB [44,54]. Quinoxaline compounds have the potential of improving the TB treatment when considering the advantage of previously reported data on biological activity against antibacterial and antitubercular [14,56].

There are some quinoxaline compounds which are used against leishmaniasis, tuberculosis, cancer, depression and neurological activities [56]. Cogo *et al.,* (2015) reported that quinoxaline derivatives are promising as chemotherapeutic agents against *Leishmania* sp. and *T. cruzi* (Figure 7). Quinoxaline derivatives such as 4-substituted pyrrolo[1,2-*a*] quinoxalines have been reported to exhibit antiprotozoal and antileishmanial activity [57].



6-chloro-3-(4-methoxyphenyl)-2-phenylquinoxaline



2-(4-methoxyphenyl)-3-phenylquinoxaline

Figure 7: Active quinoxaline derivatives against antileishmanial and antitrypanosomal [57].

There is little data about quinoxaline derivatives used as inhibitors of *M tuberculosis*, but there are some compounds that have been shown to be active against TB (Figure 8) [58]. Ramli *et al.,* (2013) reported that quinoxaline derivatives have been identified as one of the most promising compounds in the development phase of new drugs for various diseases (Figure 8) [58,59] and these compounds have the potential in medical and pharmacological applications.

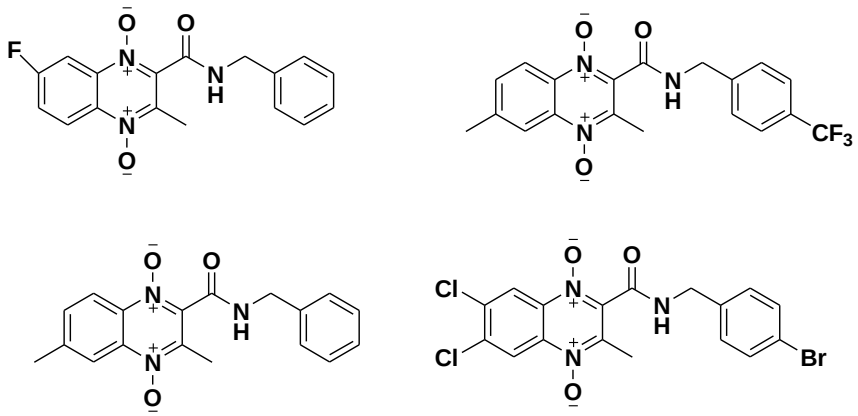


Figure 8: Structures of antitubercular quinoxaline derivatives [59]

1.5 Acetylene moiety

The C–C alkyne bond containing compounds are widely used as medicinal compounds against numerous diseases [60–61]. Rao *at el*., (2013) reported that the introduction of terminal alkyne functionalised substituents via Sonogashira coupling reaction on heterocyclic compounds has gained considerable attention on synthetic medicinal chemistry for drug development [62–63].

The alkyl substituted dihydropyrimidinone (DHPM) framework (Figure 9) appeared as a new scaffold for the development of novel inhibitors of chorismate mutase and are currently investigated as potential drugs against *M tuberculosis* [64]. It was observed that acetylene moiety is an important component found in many compounds that are of pharmaceutical and biological interest [65].

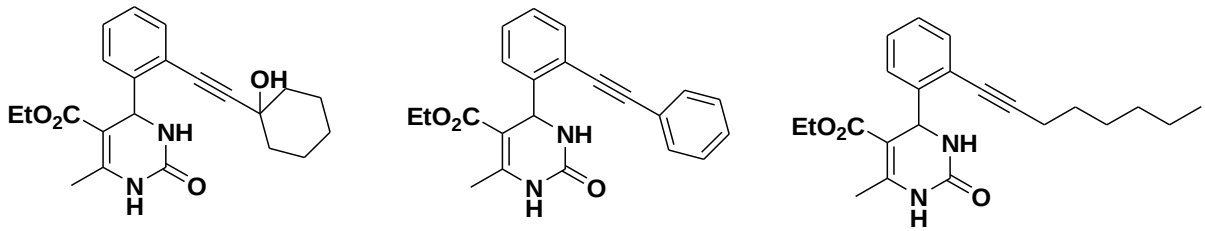


Figure 9: Some of DHPM based novel inhibitors of chorismate mutase [64].

Zheng *at el.,* (2012) reported that trifluoromethylated acetylenes ($RC{\equiv}CCF_3$) are used as building blocks in pharmaceuticals and as functional materials. As a result, it has been a topic of increasing importance in organic synthesis towards new compounds development [66]. The analogues of the natural antibiotic thiolactomycin

with acetylene-based side chains have been reported to have *in vitro* inhibitory activity against the recombinant *M tuberculosis*, with the IC_{50} value of 7±0.5 μM and 7±0.2 μM [67].

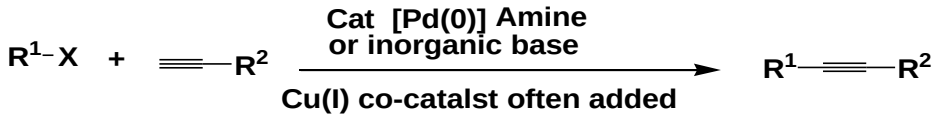
The acetylene moiety is used as an inhibitor for some diseases and the quinoxaline compounds are commonly found in a number of biologically active molecules [68]. In addition the importance of quinoxaline is highlighted in section 1.4, which suggests that the possibility of combinations of the two advantages (quinoxaline and acetylene moiety) may lead to fruitful active compound against TB. Moreover, quinoxaline and its derivatives may serve as novel active compounds against TB, resistant TB and other diseases. The acetylene moiety can be introduced by the commonly used process in synthetic organic chemistry called Sonogashira cross coupling reaction.

1.6 Sonogashira coupling reaction

The Sonogashira coupling reaction is a cross-coupling reaction used in organic synthesis to form carbon-carbon bonds formation. It employs a palladium catalyst to form a carbon-carbon bond between a terminal alkyne and an aryl or vinyl halide [69]. It has become an important tool for C-C alkyne bond formation reaction (Scheme 1) and is one of the most important and widely used sp^2–sp C-C alkyne bonds forming reactions [70].

Today, the Sonogashira cross-coupling reaction between haloaryl derivatives and terminal alkynes has become the most commonly used reaction for functionalizing aromatic scaffolds with alkynyl groups [71]. Sonogashira coupling reaction has recently become the third most popular organic transformation based on the number of publications [72]. This reaction is frequently employed in the synthesis of biologically active molecules, molecular electronics, conjugated polymers or nanostructures [73]. It is also applied in areas such as natural product synthesis and materials science [74].

The Sonogashira coupling reaction offers an extremely useful route into aryl- and alkenyl-alkynes. The alkyne moiety is usually introduced via its copper salt [71,74]. The reaction normally requires a co-catalyst, Copper (I) iodide (CuI) which plays a significant role in the transmetalation process [74].



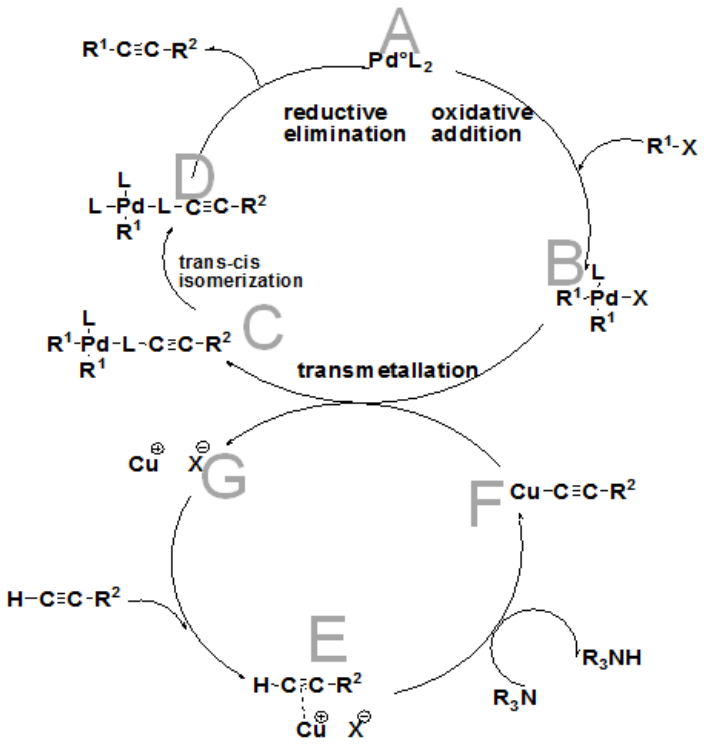
R^1 = Aryl, Vinyl

R^2 = Aryl, Alkyl, SiR_3

X = I, Br, Cl, OTf, etc

Scheme 1: General approach of Sonogashira cross coupling reaction [60,74]

Sonogashira cross-coupling reaction between haloaryl derivatives and terminal alkynes with functional aromatic scaffold groups, proceed under mild conditions [69,75]. Thus makes it most compatible with a number of functional groups and can easily be extended to a number of commercially available terminal alkyne compounds [74].



Scheme 2: Mechanism of the Sonogashira coupling reaction [76]

The Sonogashira reaction uses both palladium and copper catalysts simultaneously, as depicted in Scheme 2. The mechanism of action is believed to revolve around a palladium cycle and a copper cycle (Scheme 2) [76]. There are many factors that influence the Sonogashira cross coupling reaction such as leaving groups, amount of

catalyst used and nature of solvent and base. The effect of the leaving group determines the rate of reaction because, the better the leaving group the faster reaction, resulting in better yield [77].

1.6.1 Effect of the leaving groups on Sonogashira coupling reaction

The leaving groups that are commonly used on Sonogashira cross coupling reaction are usually the halogens [chloride (Cl), bromide (Br) and iodide (I)] and the sulfonate (tosylate, triflate and mesylate). The ideal leaving group is Cl, since organic chlorides are cheaper and most of the Sonogashira reaction uses the halides as leaving groups. Iodine is one of the most commonly used leaving groups in Sonogashira reactions based on the number of publications (Table 1) [78].

Table 1: Leaving groups used in Sonogashira cross coupling reaction

Leaving group	$–OSO_2CF_3$	$–OSO_2Ph$	$–OSO_2Tol$	$–OSO_2Me$	–I	–Br	–Cl
Pka of conjugate acid	-14.9	-5.9	-5.3	-1.9	-10	-8	-6
Number of reported SC*	13235	5	127	8	6313	1611	2521

*Sonogashira cross coupling reactions found by a scifinder search Nov 2014 [78,79].

Triflates and bromides were found to give comparable results on Sonogashira coupling reactions. Triflates are very useful for the activation of phenols when the corresponding aryl halide is not available [80]. The problem with the triflates is that they are expensive. The halides such as aryl or vinyl halides are hard to prepare and require different reaction conditions like different base, temperature, *etc*. As a result, the current study designs an efficient synthetic method for the incorporation of the acetylene moiety using benzenesulfonyl as a leaving group via Sonogashira coupling reaction.

The hydroxyl group is not a good leaving group, there is a need to convert the hydroxyl group into a better leaving group and hence it was important to incorporate

benzenesulfonyl chloride as a leaving on quinoxaline-2-ol. Benzenesulfonyl chloride is chosen over triflate substrates because they are much cheaper and easy to handle. It is important to understand the concept of benzenesulfonate anion as a leaving group in coupling reactions. There is less data reported on Sonogashira coupling reactions when benzenesulfonate anion is used as a leaving group for coupling [78–79].

1.7 Purpose of the study

1.7.1 Aim

The aim of the study was to synthesise quinoxaline-containing compounds and evaluate their biological activity against TB.

1.7.2 Objectives

The objectives of this study are to:

I. synthesise a library of compounds with different alkyne substituents on the quinoxaline ring

II. Develop a general approach on Sonogashira cross coupling reaction for the integration of quinoxaline and alkyne derivatives

III. test the synthesised compounds against TB

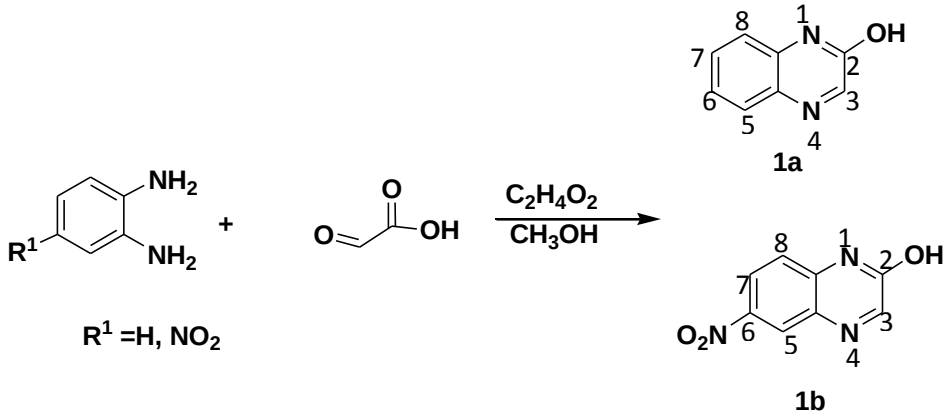
Chapter 2

Chapter 2

2 RESULTS AND DISCUSSION

2.1 Synthesis of the quinoxaline ring

We started by synthesising quinoxalin-2-ol **1a** as the starting material which was prepared by the condensation reaction between *o*-phenylenediamine and glyoxylic acid, dissolved in methanol and acetic acid (Scheme 3), using a method reported by Nxumalo (2011) [78]. The reaction was monitored by thin-layer chromatography (TLC) analysis and after the completion of the reaction, the mixture was filtered, then washed with water and methanol. The obtained crude was recrystallized from dimethylformamide (DMF).



Scheme 3: Synthesis of quinoxaline-2-ol (**1a**)

Compound **1a** was recovered as a cream white solid material, which was characterised by nuclear magnetic resonance (NMR) spectroscopy recorded in DMSO-d_6. The proton (^{1}H) NMR spectrum showed a singlet peak in the downfield region at 8.16 ppm which attributable to one (1) proton resonance of the –N=C-H group from the pyrazine ring attached to carbon number 3 (Scheme 3).

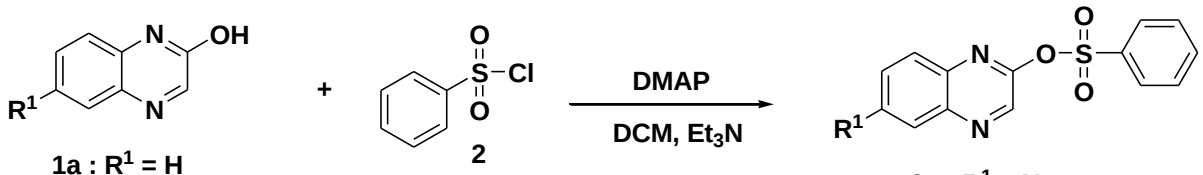
There were two multiplet peaks (which were supposed to be doublet of a doublet due to J^3 and J^4 coupling constant on carbon 5, 6, 7 and 8) at 7.32–7.30 ppm and 7.80–7.76 ppm assigned to the quinoxaline ring. Both peaks were integrating for two (2) protons each. The carbon (^{13}C) NMR spectrum showed a total number of eight (8) carbons peak around the aromatic region. The melting point was found to be 265–268 °C (Lit. 266–267 °C) and the results were consistent with those reported in the literature [78]. Compound **1a** was achieved in 80% yield.

The 6-nitroquinoxalin-2-ol **1b** was prepared in a similar way as **1a**. After the completion of the reaction, the crude product was recrystallizated from DMF to yield a dark grey solid material and was characterised by NMR. The ^{1}H NMR spectrum showed a doublet peak at 8.00–7.97 ppm which integrates for one (1) proton. There was multiplet peak at 8.08–8.03 ppm (which was supposed to be a doublet of doublets due to J^{3} and J^{4}) integrating for two (2) protons. A characteristic singlet peak was observed at 8.34 ppm integrating for one (1) proton which was due to proton resonances of the –N=C-H group from the pyrazine ring. The ^{1}H NMR results were in agreement with the proposed compound.

The ^{13}C NMR spectrum showed a total number of eight (8) carbons around the aromatic region. The significant intermediates along the reaction pathway have been observed with the assignment of pyrazine benzene ring bonded together, were in agreement with ^{13}C shift, which were conform by the bicyclic structure. The melting point was 222–225°C (Lit 224 °C–226 °C). The synthesised compounds data was consistent with literature reports [81]. The target compound **1b** was obtained as a brown solid material obtained in 74% yield. Disappearance of the one (1) proton at the quinoxaline ring being replaced by –NO_2 substituent confirmed the formation of **1b**.

2.2 Incorporation of benzenesulfonyl group

Quinoxalin-2-ol **1a** was treated with benzenesulfonyl chloride **2** in the presence of dry dichloromethane (DCM) and 4-dimethylaminopyridine (DMAP) in order to produce a suitable starting material for the coupling reaction. The 2-benzenesulfonyloxyquinoxaline **3a** compound was synthesised following a method reported by Nxumalo (2011) [78]. The incorporation of **2** in **1a** was achieved according to the Scheme 4 below.



Scheme 4: Synthesis of 2-benzenesulfonyloxyquinoxaline **3a**

The reaction mixture was monitored by TLC analysis and after the completion of the reaction. The reaction mixture was quenched with a saturated solution of sodium hydrogen carbonate ($NaHCO_3$) and the crude product was purified by column chromatography. The product was analysed and characterised by NMR spectrometry recorded in $CDCl_3$. The ^1H NMR spectrum showed a triplet peak at 7.72–7.65 ppm integrating for two (2) protons and a multiplet peak at 7.80–7.72 ppm integrating for three (3) protons, both peaks were assigned to the phenyl ring.

The appearance of a doublet peak at 7.92 ppm integrating for one (1) proton and a doublet of doublets at 8.19 ppm integrating for three (3) protons were both assigned to the quinoxaline ring. A characteristic singlet peak was observed at 8.70 ppm integrating for one (1) proton, due to a proton resonance of the –N=C-H group from the pyrazine ring. This confirmed successful incorporation of benzesulfonyl group.

The ^{13}C NMR spectrum was expected to give fourteen (14) carbons but only twelve (12) carbons were observed around the aromatic region, which means that other carbons are equivalent due to the symmetry at the phenyl ring. Compound **3a** was achieved in 68% yield with the melting point of 89–92 °C (Lit 91 °C). The NMR and melting point analysis supported the formation of **3a** and the results were consistent with those reported in the literature [78].

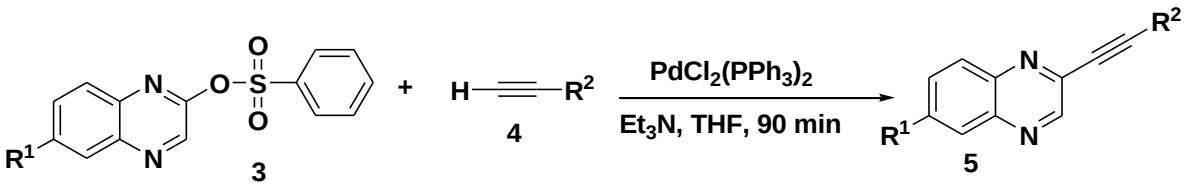
Compound **3b** was synthesised in a similar way as compound **3a**. It was difficult to purify by column chromatography even when the mobile phase was changed from 5:95 to 40:60 ethyl acetate/*n*-hexane and 5:95 to 40:60 MeOH/DCM. The compound was recrystallized from petroleum ether and *n*-hexane but still the compound was not clean enough. The ^1H NMR spectrum showed a triplet peak at 7.77 ppm integrating for two (2) protons and a doublet peak at 8.06 integrating for one (1) proton.

Two triplet peaks at 8.56 ppm and 8.81 ppm were integrating for two (2) and three (3) protons respectively. There were two extra peaks observed as a doublet at 8.22 ppm and multiplet peak at 7.65–7.74 ppm, both integrating for four (4) protons each. These peaks might be due to the excess of benzenesulfonyl protons, which means that the reagent was still present. This indicates that the substrate had not been completely consumed. There were some peaks on the ^1H NMR which were corresponding to the substrate **2**. Although we could not get **3b** in a pure form, the

compound was used in subsequent Sonogashira coupling reaction in the following section.

2.3 Sonogashira cross coupling reactions with different acetylene substrates

The second objective was to develop a general route for the synthesis of alkyne-quinoxaline derivatives using Sonogashira cross coupling reaction. The exploration on synthetic approaches of quinoxaline compounds was further evaluated when **3a** and **3b** were used as a starting material for Sonogashira coupling reactions. The synthetic routes on preparation of the quinoxaline compounds and its application from Sonogashira reaction took place according to Scheme 5.



3a : R^1 = H
3b : R^1 = -NO_2
R^2 = aryl, alkyl, SiR

Scheme 5: General approach to the Sonogashira cross coupling reaction

Several substrates (**4a-4k**) were evaluated in this study and the results are summarised in Table 1 and 2. The reactions were refluxed at 55-60 °C in a freshly distilled dry THF for 1–24 h. The progress of the reactions was monitored by TLC analysis. The synthesised compounds were purified by column chromatography. The NMR spectra of the coupling products were recorded in $CDCl_3$, where tetramethylsilane (TMS) was used as an internal standard. The results of the proposed compounds were characterised by ^{1}H NMR, ^{13}C NMR and for selected compounds by HPLC-MS spectroscopy.

2.3.1 Sonogashira coupling reaction of different acetylene substrates using 3a as a starting material for coupling

The first attempt on the Sonogashira coupling reaction was between 2-benzenesulfonyloxyquinoxaline **3a** coupled with 3,3-dimethyl-1-butyne **4a** following a method reported by Nxumalo (2011) [78]. The coupling reaction occurs in the

presence of bis(triphenylphosphine)palladium(II) chloride [$PdCl_2(PPh_3)_2$]. After 4 h, the TLC was showing the spot for the quinoxaline **3a** and the reaction mixture with the same Rf values and ^{1}H NMR spectrum was showing the presence of the starting material.

The reaction between **3a** and **4a** was repeated with an increase in reaction time from 4h to 24h, but still there was no improvement, the ^{1}H NMR spectrum was showing the presence of the starting material. The coupling condition was changed from using a normal THF to a fresh distilled dry THF which was prepared over sodium/ benzophenone.

The crude product was obtained as a dark brown solid material which was purified by column chromatography and characterised by NMR. The ^{1}H NMR spectrum showed a characteristic singlet peak at 8.82 ppm integrating for one (1) proton assigned to the –N=C-H group from the pyrazine ring. Another singlet peak was observed at more upfield at 1.42 ppm integrating for nine (9) protons assigned to the methyl groups from 3,3-dimethyl-1-butyne moiety (Figure 10).

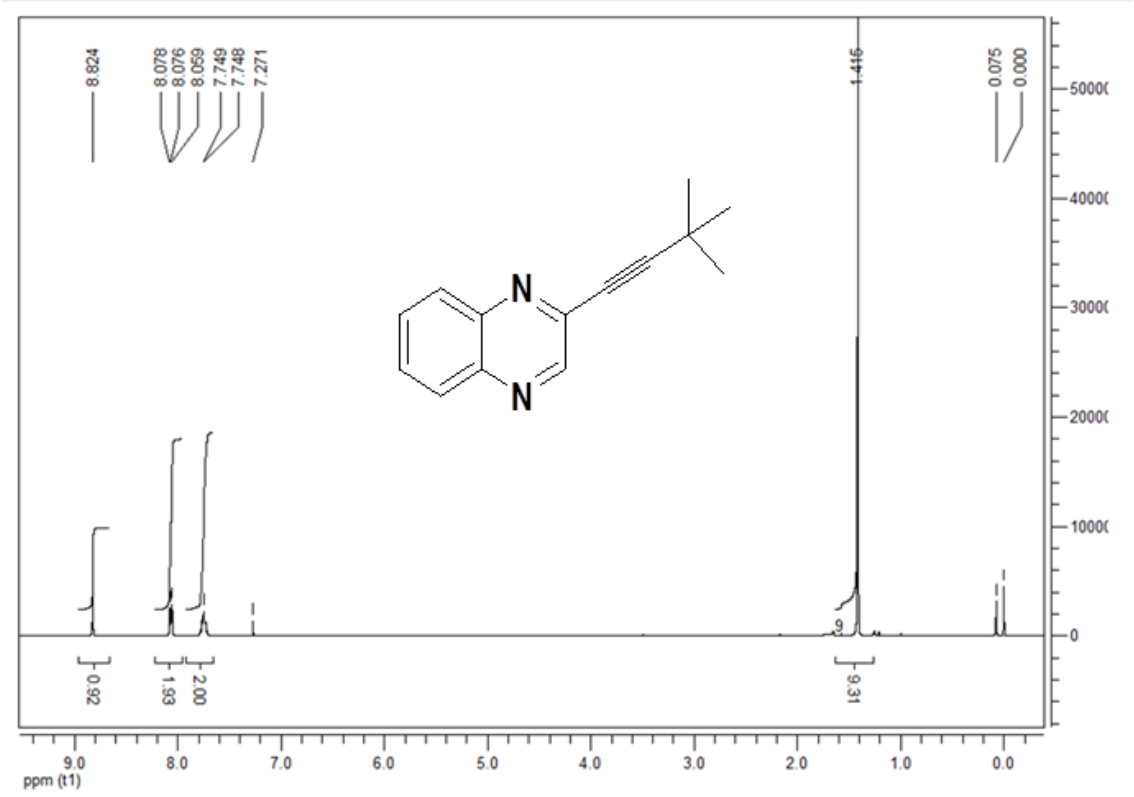


Figure 10: ^{1}H NMR spectrum of compound **5a**

The ^{1}H NMR showed the disappearance of the benzenesulfonyl protons from the aromatic region, which was replaced by methyl protons at 1.42 ppm. The doublet peak at 7.75 ppm and a triplet peak at 8.08 ppm, were integrating for two (2) protons assigned to the quinoxaline ring. The results support the formation of compound 2-(3,3-dimethyl-1-butynyl)quinoxaline **5a**. The ^{13}C NMR spectrum showed a total of twelve (12) carbons due to the symmetry at the 3,3-dimethy-1-butyne.

The ^{1}H NMR and ^{13}C NMR spectrum of compound **5a** shows that the ratios of hydrogen and carbon environments together with their chemical shifts are consistent with the expected compound **5a**. Compound **5a** was obtained in 12% yield. The mass spectrum of compound **5a** gave a peak at *m/z* 211 (M+1), which is consistent with the calculated mass (210.274 g/mol) of the desired compound **5a**.

In optimization attempts when the amount of substrate **4a** was doubled, the yield did not improve. Substrate **4a** was increased to 3 equivalents (eq.), but still there was no improvement. The base was also changed from Et_3N to aqueous ammonia. The yield was still very little (< 20%), which suggest that factors such as volatility of the substrate used at that temperature might have a major effect on the reaction since the boiling point of **4a** was very low (37–38 °C). In future, the reaction will be performed in a sealed tube.

The reaction was repeated and substrate **4a** was coupled with **3a**, the reaction occurred in freshly distilled dry THF in an inert environment (N_2) using Et_3N as the base. The yield increased from 12% to 39%, which suggest that the reaction between **3a** and **4a** was sensitive to oxygen, hence lower yield was observed in the absence of inert environment. The melting point of compound **5a** was 55–58 °C. According to our knowledge (search from Scifinder on 24/02/2015), compound **5a** has never been synthesised before.

The second substrate used was phenylacetylene **4b**, which was coupled with **3a** under freshly distilled dry THF in an inert environment and Et_3N was used as a base. After the completion of the reaction, the crude was purified by column chromatography and obtained as a yellowish solid material which was characterised by NMR.

The ^{1}H NMR showed two peaks resonances of a phenyl ring which were observed as a doublet peak at 7.45 ppm integrating three (3) protons and a quartet peak at 7.71–7.69 ppm integrating for two (2) protons. There was a multiplet peak at 7.82–7.78 ppm integrating for two (2) protons and a doublet peak at 8.12 ppm integrating for two protons (2) assigned to the quinoxaline ring (Figure 11).

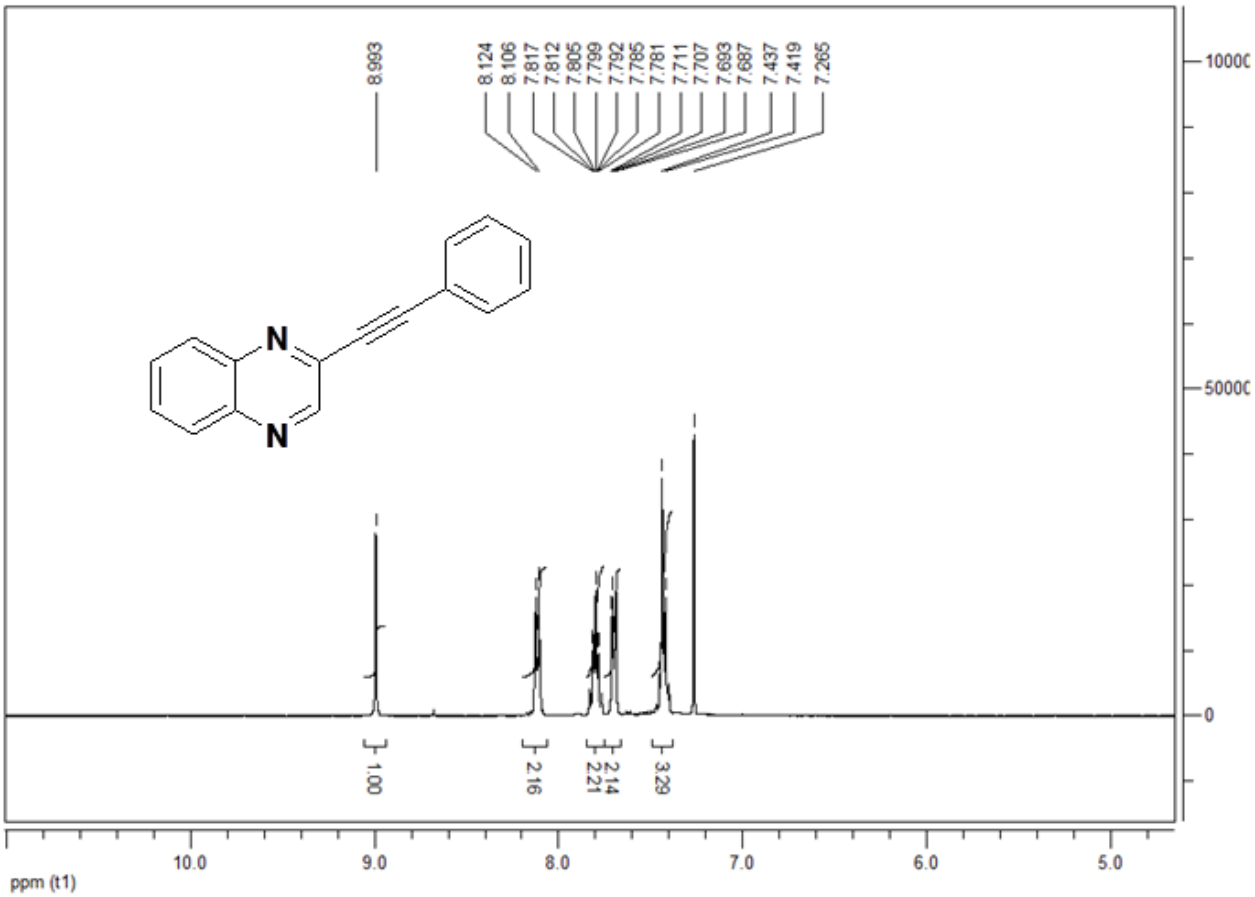


Figure 11: ^{1}H NMR spectrum of compound **5b**

A characteristic singlet peak was observed at 8.99 ppm integrating for one (1) proton assigned to the proton resonance of the –N=C-H group from the pyrazine ring. The ^{1}H NMR was having a total of ten (10) protons. The melting point was found to be 63–66 °C (Lit 65–66.5 °C) [87]. The ^{13}C NMR gave fourteen (14) carbons instead of sixteen (16), which means that other carbons are equivalent due to the symmetry at the phenyl ring.

The NMR spectrum of compound 2(2-phenylethynyl)quinoxaline **5b** gave peaks which were consistent with the coupled product and the results obtained were

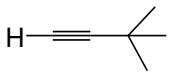
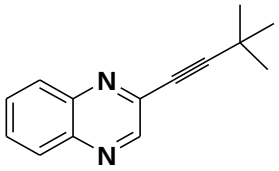
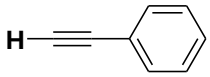
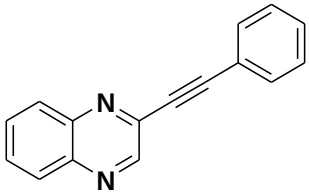
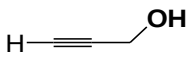
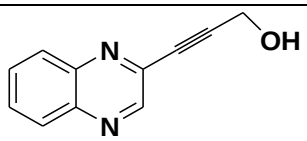
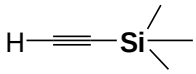
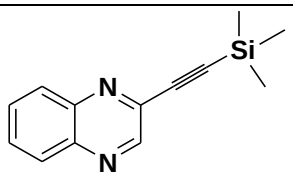
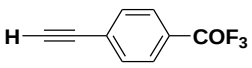
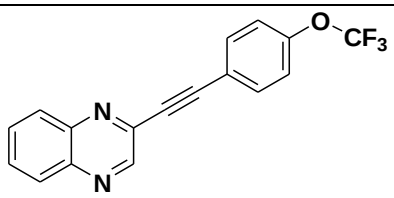
consistent with those previously reported in literature [87]. The mass spectrum of the synthesised compound **5b** gave a peak at *m/z* 231 (M+1) and was consistent with the calculated mass (230.256 g/mol) of the desired compound **5b**.

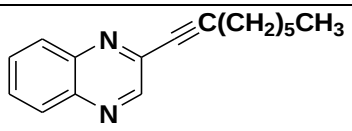
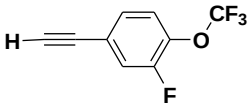
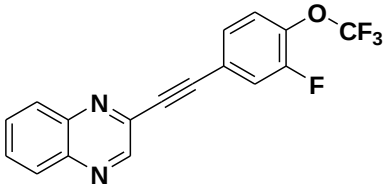
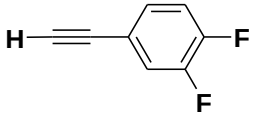
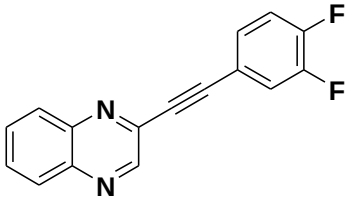
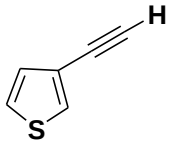
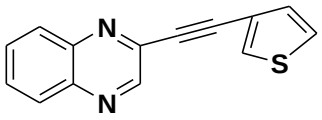
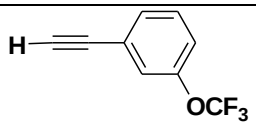
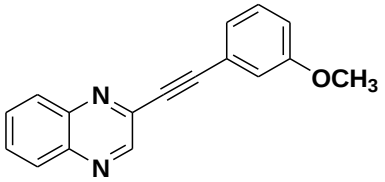
Propargyl alcohol **4c** was coupled with **3a** under the same conditions as **5b**, after completion of the reaction, the crude was recrystallized from acetone and then characterised by NMR. The ^{1}H NMR spectrum showed a characteristic singlet peak which was observed at 8.74 ppm integrating for one (1) proton assigned to the –N=C-H group from the pyrazine ring. Another singlet peak was observed for the alcohol proton which was found at 2.20 ppm integrating for one (1) proton, it was revealed that there was a slight shift from 2.20 ppm to 2.96 ppm when compared to the previous report [78].

The resonance for the methylene protons (–CH_2–) appeared as a singlet peak at 3.66 ppm integrating for two (2) protons. The chemical shift was slightly deshielded due to the influence of the alkyne bond and a strongly electronegative oxygen atom. There was a multiplet peak at 7.84–7.81 ppm integrating for two (2) protons and a triplet peak at 8.02 ppm and a quartet at 8.19–8.16 ppm, both integrating for one (1) proton each, assigned to the quinoxaline ring. The disappearance of the benzenesulfonyl protons being replaced by the prop-2-yl-1-ol protons supports the formation of **5c**.

The ^{1}H NMR showed a total of eight (8) protons and the ^{13}C NMR spectrum showed a total of ten (10) carbons instead of eleven (11) carbons, which means that other carbons are overlapping at the quinoxaline ring. The melting point was found to be 140–143 °C (Lit 141–142 °C) and was consistent with the previous results [78]. The 3-(quinoxaline-3-yl)prop-2-ny-1-ol **5c** was obtained in 64% yield. The mass spectrum of compound **5c** gave a peak at *m/z* 185 (M+1) and was consistent with the calculated mass (184.194 g/mol) of the desired compound **5c**.

Table 1: Pd-catalyzed coupling reaction of quinoxaline compounds with different acetylene substrates.

Entry	Aryl sulfonates	Alkyne	Product	Yield
1	**3a**	 **4a**	 **5a**	39
2	**3a**	 **4b**	 **5b**	61
3	**3a**	 **4c**	 **5c**	64
4	**3a**	 **4d**	 **5d**	72
5	**3a**	 **4e**	 **5e**	92

6	**3a**	$CH_3(CH_2)_5C{\equiv}CH$ **4f**	 **5f**	33
7	**3a**	 **4g**	 **5g**	68
8	**3a**	 **4h**	 **5h**	59
9	**3a**	 **4i**	 **5i**	42
10	**3a**	 **4j**	 **5j**	91

Another coupling reaction was between **3a** and ethynyltrimethylsilane **4d** as the coupling partners. After the completion of the reaction, the crude was purified by column chromatography to give a brown oily residue which was characterised by NMR. The ^{1}H NMR spectrum showed a characteristic singlet peak at 8.70 ppm integrating for one (1) proton assigned to the proton resonance of the –N=C-H group from the pyrazine ring (Figure 12).

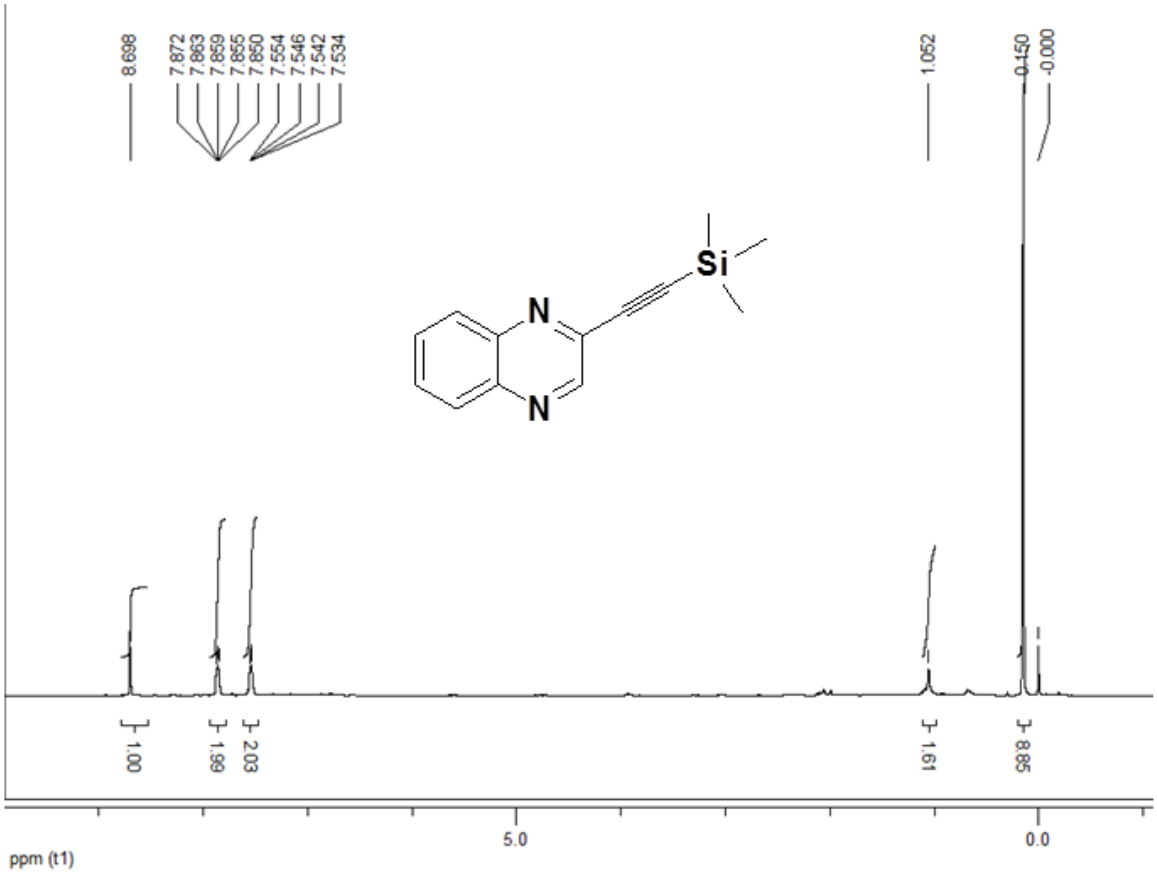


Figure 12: ^{1}H NMR spectrum of compound **5d**

There were two quartet peaks at 7.55–7.53 ppm and 7.87–7.85 ppm respectively which were attributed to the protons of the quinoxaline ring and both were integrating for two (2) protons each around the aromatic region. There was a singlet peak at more upfield region at 0.15 ppm assigned to the protons resonance of the trimethylsilyl group integrating for nine (9) protons.

The ^{13}C NMR spectrum showed a total of eleven (11) carbons instead of thirteen (13), which means that other carbons were equivalent due to the symmetry at the trimethylsilyl group. The NMR spectrum gave peaks which were consistent with the 2-(2-(trimethylsilyl)ethynyl)quinoxaline **5d,** which was obtained in 72% yield. The NMR data was strongly agreeing with the previous reported results [78]. The mass spectrum of compound **5d** gave a peak at *m/z* 227 (M+1) and was consistent with the calculated mass (226.337g/mol) of the proposed compound **5d**.

Substrate 4-(trifluoromethoxy)phenylacetylene **4e** was coupled with **3a** and after the completion of the reaction, the crude was obtained as a cream white to light brown

solid material and was characterised by NMR. The ^{1}H NMR spectrum showed two triplet peaks at 7.31 ppm and 7.47 ppm, which were both integrating for two (2) protons assigned to the 4-(trifluoromethoxy)phenylacetylene moiety.

There was a triplet peak at 7.76 ppm and a multiplet peak at 8.07–8.04 ppm, integrating for two (2) protons each assigned to the quinoxaline ring. The presence of a characteristic singlet peak was observed at 8.92 ppm integrating for one (1) proton assigned for the –N=C-H group from the pyrazine ring. The ^{1}H NMR spectrum of the synthesised compound supports the formation of 2-(2-(trifluoromethoxy)phenylethynyl)quinoxaline **5e**.

The ^{13}C NMR showed a total of fourteen (14) carbons instead of seventeen carbons which means that other carbons are equivalent due to the symmetry at the phenyl ring. The ^{13}C shift around at 88.31 ppm was assigned to the –CΞC– group from phenylacetylene substrate. The $–OCF_3$ group were supposed to be observed around 55 ppm region, but we did not manage to characterise the compound by proton and fluorine decoupled ^{13}C NMR. Compound **5e** was obtained with the highest isolated in 92% yield. The NMR data was consistent with the proposed compound. The melting point of the synthesised compound **5e** was 100–103 °C.

The mass spectrum of compound **5e** gave a peak at *m/z* 315 (M+1), which was consistent with the calculated mass (314.261 g/mol) of compound **5e**. According to our knowledge and search (Scifinder on 24/02/2015), compound **5e** is novel. All novel compounds will be further characterised by HRMS spectrometry and IR spectroscopy to confirm the structure of the synthesised compound.

Oct-1-yne **4f** was coupled with **3a** and after the completion of the reaction, a dark brown solid material was obtained and characterised by NMR. The ^{1}H NMR spectrum showed two multiplet peaks at 7.70–7.63 ppm and 7.99–7.96 ppm, both peaks were integrating for two (2) protons each, assigned to the quinoxaline ring. A characteristic singlet peak was observed at 8.75 ppm integrating for one (1) proton assigned to the –N=C-H group from the pyrazine ring.

A triplet peak was observed at 0.91ppm integrating for three (3) protons assigned to the methyl group. There was a quartet peak at 1.49–1.42 ppm which was integrating for two (2) protons. There was a multiple peak at 1.32–1.09 ppm integrating for two

(2) protons. The appearance of triplet peaks at 1.62 ppm integrating for two (2) protons followed by a quartet peak at 1.49–1.42 ppm integrating for two (2) protons. A triplet peak was observed at 2.49 ppm integrating for two (2) protons and these peaks were assigned to the oct-1-yne moiety.

The ^{1}H MNR data support the formation of the compound 2-(oct-1-ynyl)quinoxaline **5f** which was isolated in 33% yield. The ^{13}C NMR spectrum showed a total of fifteen (15) carbons instead of sixteen (16) which means that other carbons at the oct-1-yne moiety and are overlapping. The melting point of compound **5f** was 132–135 °C. The NMR strongly agreed with the proposed compound. According to our knowledge and search (Scifinder on 24/02/2015), compound **5f** is novel.

3-Fluoro-4-(trifluoromethoxy)phenylacetylene **4g** substrate was coupled with **3a**, the crude product was isolated as a light brown to yellowish solid material and was characterised by NMR. The ^{1}H NMR spectrum of compound 2-(2-(3-fluoro-4-(trifluoromethoxy)phenyl)ethynyl)quinoxaline **5g** was compared with **5e** because these compounds were similar, but in **5g** one of the hydrogen in the phenyl ring was replaced by a fluorine atom.

The ^{1}H NMR of **5g** showed a quartet peak at 7.39–7.35 ppm which integrates for one (1) proton since the other proton was replaced by fluorine. There was an appearance of a triplet peak at 7.55 ppm integrating for two (2) protons assigned to the (trifluoromethoxy)phenyl ring.

Another triplet peak at 7.84 ppm integrating for two (2) protons and a quartet peak were observed at 8.14–8.12 ppm integrating for two (2) protons and were assigned to the quinoxaline ring. A characteristic singlet peak was observed at 8.99 ppm integrating for one (1) proton. The NMR results were consistent with the proposed compound **5g** which was isolated in 68% yield.

The compound was not characterised by proton and fluorine decoupled ^{13}C NMR to identify the C–F bond. The ^{13}C NMR spectrum gave a total of fourteen (14) carbons instead of seventeen (17) which means that other carbons are overlapping at the phenyl and quinoxaline ring. The melting point of compound **5g** was found to be 119–122 °C. The mass spectrum of compound **5g** gave a peak at *m/z* 333 (M+1) and was consistent with the calculated mass (332.252 g/mol) of the compound **5g**.

According to our knowledge and search on Scifinder on 24/02/2015, compound **5g** is novel.

3,4-difluorophenylacetylene **4h** substrate was coupled with **3a** and after the completion of the reaction, the crude was obtained as brown solid material which was characterised by NMR. The ^{1}H NMR spectrum showed two multiplet peaks at 7.38–7.33 ppm and 7.82–7.75 ppm integrating for two (2) and three (3) protons respectively. A quartet peak was observed at 8.12–8.09 ppm integrating for two (2) protons followed by a singlet at 8.97 ppm integrating for one (1) proton.

The ^{1}H NMR spectrum showed a total of eight (8) protons and was consistent with the proposed compound. The ^{13}C NMR spectrum of compound 2-(2-(3,4-difluorophenyl)ethynyl)quinoxaline **5h** showed a total of ten (10) carbons instead of sixteen (16) carbons, which could be due to the overlapping of peaks at the 3,4-difluorophenyl moiety and quinoxaline rings. The compound was not characterised by proton and fluorine decoupled ^{13}C NMR to identify the C–F bond.

The compound was consistent with the expected compound. The melting point of compound **5h** was 164–167 °C with 59% yield. The mass spectrum of compound **5h** gave a peak at *m/z* 267 (M+1) and was consistent with the calculated mass (266.245 g/mol) of the compound **5h**. According to our knowledge and search (Scifinder on 24/02/2015), compound **5h** is novel.

Substrate 3-ethynylthiophene **4i** was coupled with **3a** and the reaction occurred to yield a dark brown solid material which was characterised by NMR. The ^{1}H NMR spectrum of the compound showed two a multiplet peaks at 7.40–7.33 ppm and at 7.81–7.77 ppm integrating for two (2) protons and integrating for three (3) protons respectively.

A triplet was observed at 8.12 ppm integrating for two (2) protons and a singlet characteristic peak at 8.87 ppm integrating for one (1) proton assigned to the quinoxaline ring (Figure 13). The ^{13}C NMR spectrum showed ten (10) carbons instead of fourteen (14), which could be due to the overlapping of peaks at the 3-ethynylthiophene moiety and quinoxaline ring.

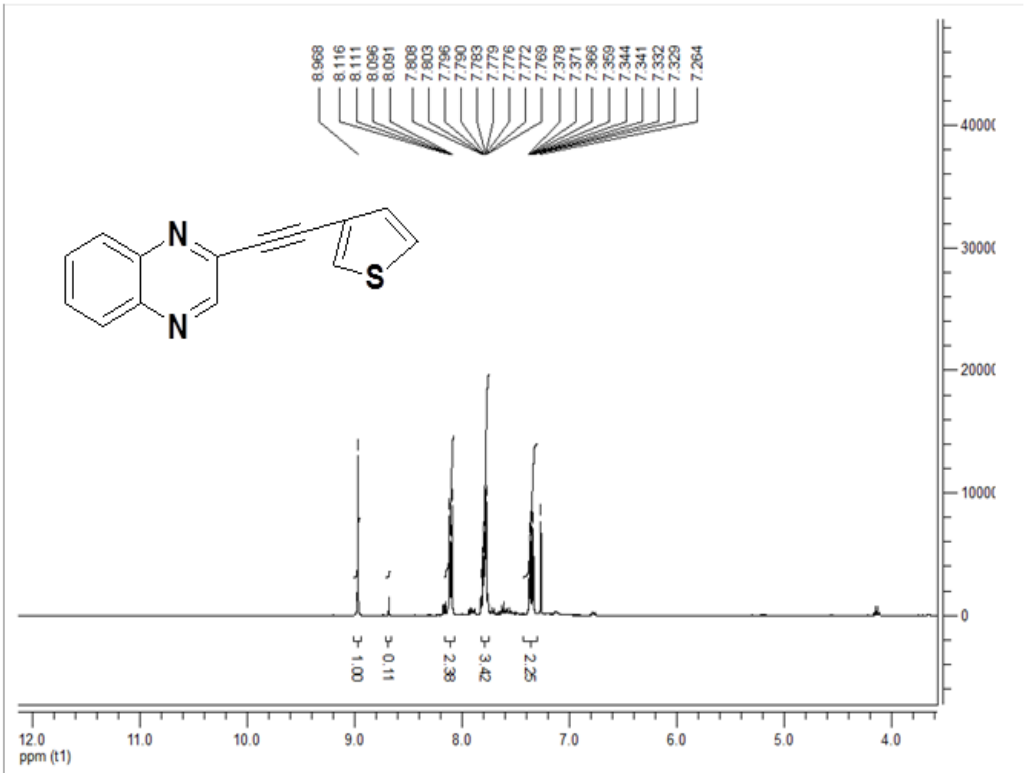


Figure 13: ^{1}H NMR spectrum of the compound **5i**

The melting point of compound **5i** was found to be 80–84 °C. The mass spectrum of compound 2-(2-(thiophen-3-yl)ethynyl)quinoxaline **5i** gave a peak at *m/z* 237 (M+1) and was consistent with the calculated mass (236.292 g/mol) of the desired compound **5i**. According to our knowledge and search on Scifinder on 24/02/2015, compound **5i** is novel.

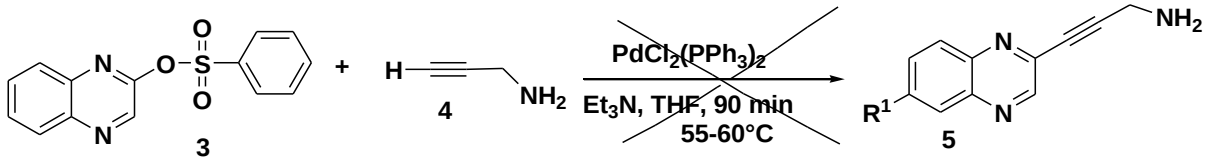
The coupling reaction between **3a** and 3-ethynylanisole **4j** occurred to yield a cream white solid material and was characterised by NMR. The ^{1}H NMR spectrum showed a characteristic singlet peak at 8.82 ppm integrating for one (1) proton assigned to the pyrazine ring in –N=C-H group. There was a doublet peak at 8.19 ppm integrating for two (2) protons and a multiplet at 7.93–7.91 ppm integrating for two (2) protons, which were assigned to the quinoxaline ring. The appearance of two doublet peaks at 7.53 ppm and 7.44 ppm were integrating for two (2) protons each assigned to the 3-methoxyphenyl moiety.

The presence of singlet peak at 3.80 ppm integrating for three (3) protons assigned to the methoxy group confirms the formation of compound 2-(2-(3-methoxyphenylethynyl)ethynyl)quinoxaline **5j**. The ^{13}C NMR spectrum showed a total

of seventeen (17) carbons, the methoxy moiety (–OCH_3) carbon was observed at 55.6 ppm.

The melting point of compound **5j** was found to be 63–66 °C with 91% yield. The mass spectrum of compound **5j** gave a peak at *m/z* 261 (M+1) and was consistent with the calculated mass (260.391 g/mol) of the proposed compound **5j**. According to our knowledge and search (Scifinder on 24/02/2015), compound **5j** is novel.

Coupling reaction involving propargylamine as a substrate and **3a** did not occur when a Pd(II)-containing catalysts was used. The first attempt was on propargylamine and **3a** as a coupling partner which was done under the same condition as **5b** (Scheme 6), but the ^{1}H NMR showed the presence of the starting material. The reaction time was prolonged beyond 24 h to 48 h but there was still no improvement, the ^{1}H NMR showed only starting material.



Scheme 6: Attempted on Sonogashira coupling reaction using proparylamine as a substrate.

Even when the reaction was prolonged beyond 76 h, there was no sign of product formation, which means that the reaction conditions have to be changed. It was reported that there are some problems in Sonogashira coupling reactions when using free amines as substrates in the presence of Pd(II) catalyst, because they coordinate with the palladium catalyst during coupling [82]. The results have indicated that amines were not favoured during the proposed coupling conditions.

2.3.2 Sonogashira coupling reactions of different acetylene substrates using 3b as a starting material

The coupling reaction between **3b** and **4a** occurred in similar conditions as **5b** above. The reaction was monitored by TLC analysis. After the completion of reaction, the obtained crude was characterised by NMR. The ^{1}H NMR spectrum of the compound showed a triplet peak at 8.07 ppm, two multiplet peaks at 8.40–8.33

ppm and 8.87–8.82 ppm, integrating for one (1) proton each. The trimethylsilyl peak was observed at 0.17 ppm as a singlet peak integrating for four (4) protons instead of nine (9) protons, which suggests that there was a mixture of products.

The ^{13}C NMR spectrum showed a total of thirteen (13) carbons. The melting point of the synthesised compound **5k** was 157–160 °C. The mass spectrum of *2-(2-*trimethysily)ethynyl)-6-nitroquinoxaline **5k** gave a peak at *m/z* 272 (M+1) and was consistent with the calculated mass (271.347) of the proposed compound **5k**. The synthesised compound **5k** was in good agreement with the proposed compound. According to our knowledge and search on Scifinder on 24/02/2015, compound **5k** is novel.

The coupling reaction between **3b** and **4b** occurred to yield a yellowish solid material which was characterised by NMR. The ^{1}H NMR spectrum showed the presence of a characteristic singlet peak of pyrazine ring in –N=C-H group at 9.10 ppm integrating for one (1) proton. There a doublet at 8.25 ppm and a quartet peak at 8.58–8.83 ppm followed by a doublet peak at 9.00 ppm which was integrating for one (1) proton each, assigned to the quinoxaline ring.

There was a multiplet at 7.50–7.43 ppm integrating for three (3) protons and a doublet peak at 7.72 ppm integrating for two (2) protons, both assigned to the phenyl ring. The ^{13}C NMR spectrum showed a total of thirteen (13) carbons instead of sixteen (16), which means that other carbons are equivalent due to the symmetry at the phenyl ring (Figure 14). The melting point of the synthesised compound was 77–80 °C.

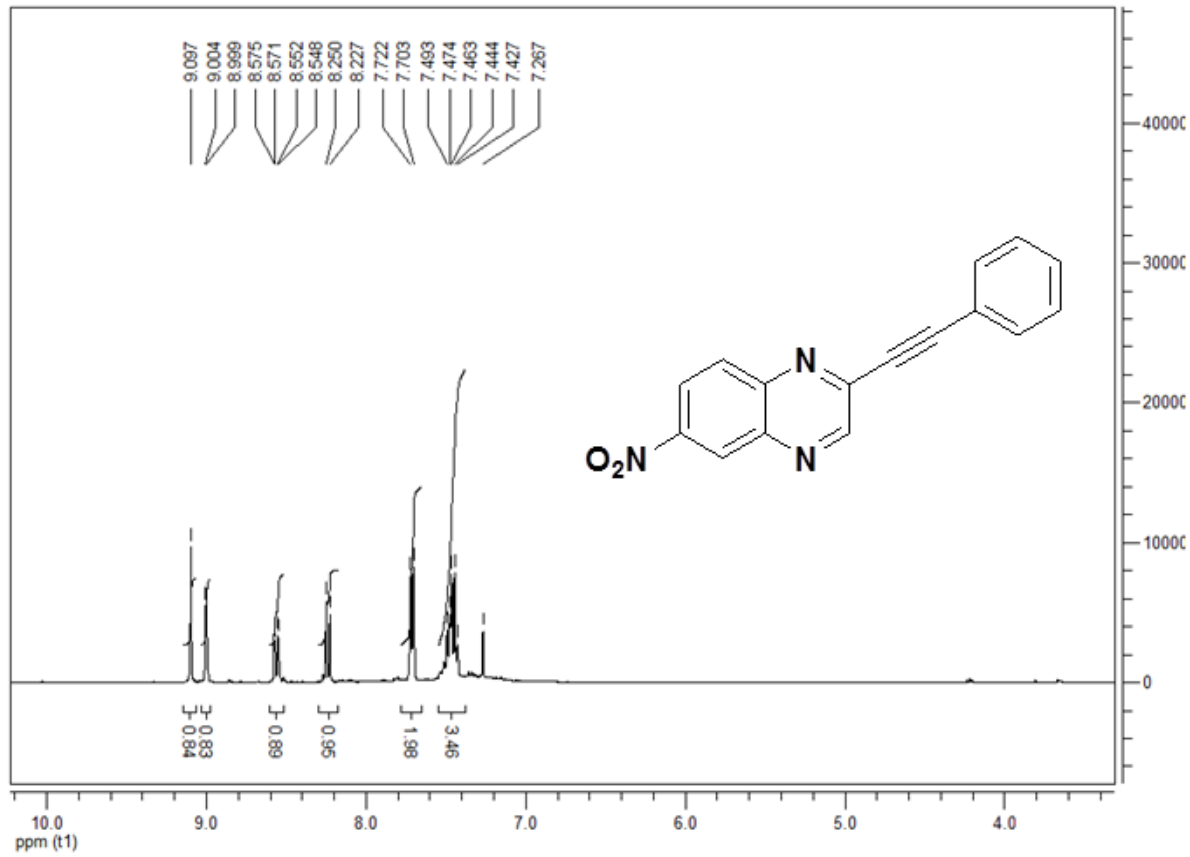
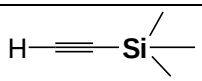
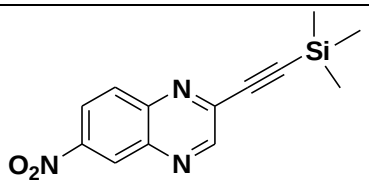
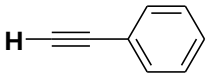
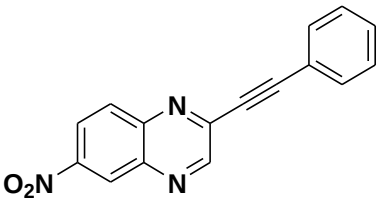
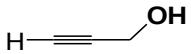
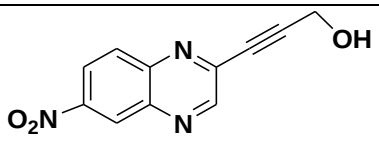
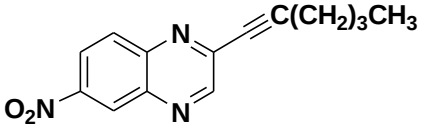
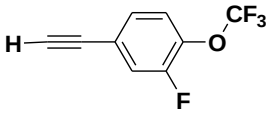
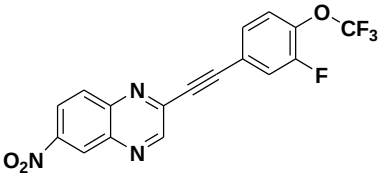
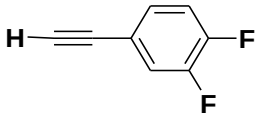
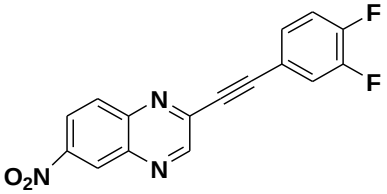
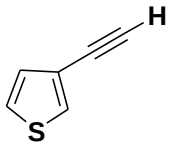
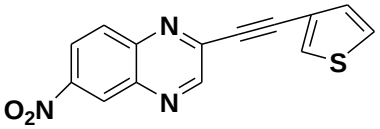
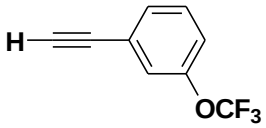
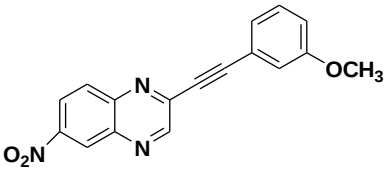


Figure 14: ^{1}H NMR spectrum of compound **5l**

The final product was obtained as a yellowish solid material, 6-nitro-2-(2-phenylethynyl)quinoxaline **5l**. Compound **5l** was isolated in 77% yield and the NMR data was strongly agreeing with the expected compound. The mass spectrum of compound **5l** gave a peak at *m/z* 276 (M+1) and was consistent with the calculated mass (275.262 g/mol) of the proposed compound **5l**. According to our knowledge and search on Scifinder on 24/02/2015, compound **5l** is novel.

Table 2: Pd-catalyzed coupling reaction of quinoxaline compounds with different acetylene substrates.

Entry	Aryl sulfonates	Alkyne	Product	Yield (%)
1	**3b**	 **4a**	 **5k**	52
2	**3b**	 **4b**	 **5l**	77
3	**3b**	 **4c**	 **5m**	62
4	**3b**	$CH_3(CH_2)_3C\equiv CH$ **4k**	 **5n**	42
5	**3b**	 **4g**	 **5o**	44
6	**3b**	 **4h**	 **5p**	50

7	**3b**	 **4i**	 **5q**	32
8	**3b**	 **4j**	 **5r**	50

Propargyl alcohol **4c** was coupled with **3b** and the reaction was monitored by TLC. After completion of the reaction, the crude was recrystallized in acetone and characterised by NMR. The ^{1}H NMR spectrum showed a little bit of some impurities on the compound, it was difficult to obtain a clean compound from recrystallization. Compound 3-(6-nitroquinoxalin-2-yl)prop-2-yl-1-ol **5m** was recrystallized from *n*-hexane followed by acetone, but the compound was not clean enough. It was revealed that the compound was not clean after being characterised by NMR.

The appearance of a characteristic singlet peak at 8.91 ppm integrating for one (1) proton was assigned to the –N=C-H group from the pyrazine ring. A singlet peak for the alcohol proton was observed at 2.79 ppm integrating for one (1) proton. The appearance of a singlet peak at 3.39 ppm integrating for two (2) protons was assigned to the methylene (–CH_2–) group.

There was a triplet peak at 7.61–7.52 ppm and a doublet peak at 7.81–7.78 ppm, both integrating for one (1) and two (2) protons respectively assigned to the quinoxaline ring. There was an extra singlet peak at 7.99 ppm, which was integrating for one (1) proton. Moreover, the ^{1}H NMR spectrum showed the formation of compound **5m** with a little bit of impurities.

The ^{13}C NMR spectrum showed a total of ten (10) carbons instead of eleven (11), which means that other carbons in the quinoxaline ring are overlapping. The melting point of compound **5m** was found to be 187–190 °C. According to our knowledge and search (Scifinder on 24/02/2015), compound **5m** is novel.

The reaction between **3b** and **4k** was coupled together, to yield the crude which was recovered as a brown solid material and was characterised by NMR. The ^{1}H NMR spectrum showed a triplet peak at 0.94–0.91 ppm integrating for three (3) protons assigned to the methyl group and a doublet peak at 1.18 ppm integrating for two (2) protons.

The doublet peak was observed at 1.48 ppm and a triplet peak at 1.66 ppm, both integrating for two (2) protons each assigned to the methylene groups. There were three multiplet peaks at 8.16–8.10 ppm, 8.48–8.41 ppm and 8.90-8.86 ppm, which were integrating for one (1) proton each assigned to the quinoxaline ring. A characteristic singlet peak was observed at 9.00 ppm assigned to the –N=C-H group from the pyrazine ring.

The ^{13}C NMR spectrum gave a total of fourteen (14) carbons. The melting point of compound **5n** was found to be 120–123 °C. The NMR data of the compound 2-(hex-1-ynyl)-6-nitroquinoxaline **5n** was in agreement with the structure of compound **5n**. According to our knowledge and search on Scifinder on 24/02/2015, compound **5n** is novel.

3-Fluoro-4-(trifluoromethoxy)phenylacetylene **4g** was coupled with **3b** and a brown solid material was obtained and characterised by NMR. The ^{1}H NMR spectrum of the compound showed two doublet peaks at 7.36 ppm and 7.90 ppm integrating for one (1) proton each. A singlet was observed at 7.83 ppm integrating for one (1) proton. These peaks were assigned to the 3-fluoro-4-(trifluoromethoxy)phenylacetylene moiety. The appearance of three doublets at 8.26 ppm, 8.51, 8.98 ppm and characteristic singlet peak at 9.07 ppm, they were integrating for one (1) proton each assigned to the quinoxaline ring.

The ^{13}C NMR spectrum showed a total of thirteen (13) carbons instead of seventeen (17) carbons, which means that other carbons are equivalent due to the overlapping of peaks at 3-fluoro-4-(trifluoromethoxy)phenylacetylene moiety and quinoxaline ring. The NMR results consistent with the expected compound 2-(2-(3,4-(trifluoromethoxy)phenyl)ethynyl)-6-nitroquinoxaline **5o** which was isolated in 44% yield. The melting point of compound **5o** was 95–97 °C. According to our knowledge and search on Scifinder on 24/02/2015, compound **5o** is novel.

The coupling reaction between **3b** and **4h** occurred, to yield the crude which was isolated as a light brown solid material and was characterised by NMR. The ^{1}H NMR showed a characteristic singlet peak at 9.09 ppm, followed by a triplet peak at 8.08–8.06 ppm, both integrating for one (1) proton each.

Doublet was observed at 7.89 ppm and multiplet peak at 7.64–7.58 ppm integrating for one (1) proton each assigned to the quinoxaline ring. Three multiplet peaks at 7.41–7.36 ppm, 7.33–7.30 ppm and 7.20–6.97 ppm, integrating for one (1) proton each were assigned to the phenyl ring on 3,4-difluorophenylacetylene moiety.

The ^{1}H NMR showed seven (7) protons, which were consistent with the proposed compound. The ^{13}C NMR spectrum showed a total of ten (10) carbons instead of sixteen (16), which means that other carbons could be overlapping at the 3,4-difluorophenylacetylene moiety and quinoxaline ring.

The compound was not characterised by proton and fluorine decoupled ^{13}C NMR to identify the C-F bond. The melting point of compound **5p** was 117–119 °C. The NMR spectrum gave peaks which were consistent with the coupled product and compound 2-(2-(3,4-difluorophenyl)ethynyl)-6-nitroquinoxaline **5p** was obtained in 50% yield. According to our knowledge and search on Scifinder on 24/02/2015, compound **5p** is novel.

3-Ethynylthiophene **4i** was coupled with **3b** to yield a light brown solid which was characterised by NMR. The ^{1}H NMR showed multiplet peak at 7.53–7.43 ppm and a triplet peak at 7.82 ppm integrating for two (2) protons each. There was a multiplet peak at 8.13–8.10 ppm integrating for two (2) protons and a characteristic singlet peak at 8.97 ppm assigned to the quinoxaline ring.

The ^{1}H NMR gave a total of seven (7) protons, which correspond to the expected compound. The ^{13}C NMR spectrum have showed a total of ten (10) carbons instead of fourteen (14), which could be due to the overlapping of peaks at the 3-ethynylthiophene moiety and quinoxaline ring. The melting point of compound **5q** was 84–88 °C. The NMR data was strongly agreeing with the proposed compound. Compound 6-nitro-2(2-(thiophen-3-yl)ethyl)quinoxaline **5q** was isolated in 32% yield.

Substrate **4j** was coupled with **3b** to yield a light brown solid material and was characterised by NMR. The ^{1}H NMR of **5r** was showing a singlet at 3.85 ppm

integrating for three (3) protons assigned to the methoxy group, supporting the formation of **5r** (Figure 15). The appearance of multiplet was observed at 7.01–6.98 ppm integrating for two (2) protons. There was triplet at 7.32 ppm integrating for two (2) protons followed by an apparent multiplet peak at 7.81–7.77 ppm integrating for two (2) protons.

A triplet was observed at 8.12 ppm integrating for two (2) protons. A characteristic singlet peak was observed at 8.99 ppm integrating for one (1) proton assigned to the –N=C-H group from the pyrazine ring. The ^{1}H NMR showed a total of eight (8) protons and was strongly agreeing with the expected compound 2-(2-(3-methyphenyl)ethynyl)-6-nitroquinoxaline **5r**, which was obtained in 50% yield.

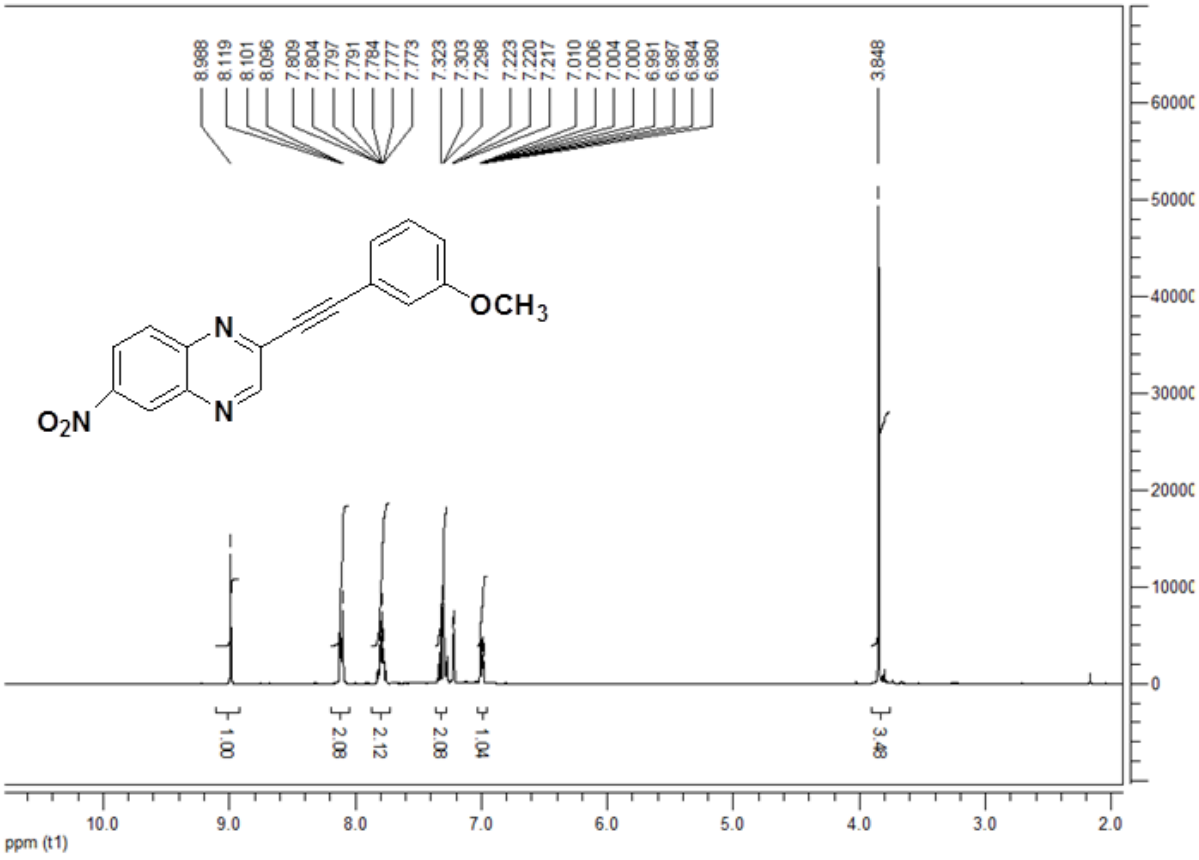


Figure 15: ^{1}H NMR spectrum of compound **5r**

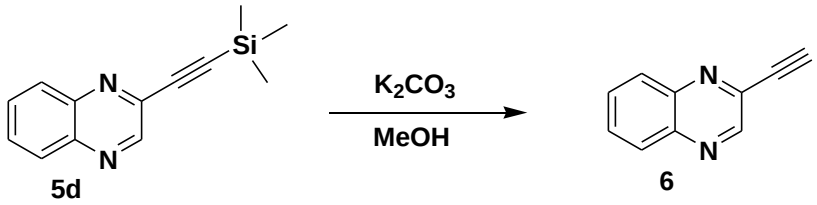
The ^{13}C NMR spectrum showed a total of twelve (12) carbons instead of seventeen (17), which means that it could be due to the overlapping of peaks. The melting point of compound was found to be 119–121 °C. The mass spectrum of compound **5r** gave a peak at *m/z* 306 (M+1) and was consistent with the calculated mass (305.279

g/mol) of the proposed compound **5r**. According to our knowledge and search (Scifinder on 24/02/2015), compound **5r** is novel.

Several alkyne quinoxaline compounds were successfully synthesised using Sonogashira cross coupling reaction. The results showed that most of the substrates with the phenyl ring substituents are mostly favoured in terms of product formation than just an open chain substrates which turned to give less than 50% yields (Table 1 and 2). The aromatic substituents might have an effect on stability of the reaction to yield satisfactory results. During analysis, it was observed that the highest yield was obtained when using 4-(trifluoromethyl)phenylacetylene **4e** and the lower yield was obtained when using the 3-ethynylthiophene **4i** as a substrate. Et_3N was found to be an optimal base for the coupling reaction.

2.4 Preparation of 1,3-dipolar cycloaddition across the alkyne compounds

The scope of our work was extended to 1,3-dipolar cycloaddition, which was investigated from the coupling products. Alkyne quinoxaline compounds are the class of compounds which may generate useful compounds in medicinal chemistry when they are further modified. The results obtained from coupling reaction were further evaluated for 1,3-dipolar cycloaddition across the alkyne bonds, using compound **6** as the starting material. Preparation of 2-ethynylquinoxaline **6** was achieved in the presence of potassium carbonate and methanol [83] (Scheme 7).



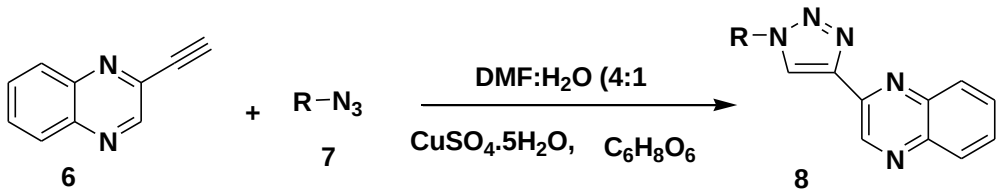
Scheme 7: Preparation of 2-ethynylquinoxaline **6** in the presence of potassium carbonate and methanol

The crude product was characterised by NMR. The ^{1}H NMR spectrum of compound **6** showed singlet peak at 3.10 ppm integrating for one (1) proton assigned to the proton attached to the alkyne group. There was a multiplet peak at 7.69–7.65 ppm integrating for two (2) protons. A triplet peak was observed at 8.32–8.29 ppm integrating for two (2) protons. These peaks were assigned to the quinoxaline ring. A

characteristic singlet peak was observed at 9.01 ppm integrating for one (1) proton assigned to –N=C-H group from the pyrazine ring.

The ^{1}H NMR spectrum confirms the formation of the 2-ethynylquinoxaline **6**, by showing the disappearance of the trimethylsilyl group from compound **5d**. The ^{13}C NMR spectrum gave a total of 10 (ten) carbons. The melting point of compound **6** was found to be 94–97 °C (Lit 95–96 °C) [83]. The synthesised compound was isolated in 76% yield.

The 2-ethynylquinoxaline **6** was selected to undergo the 1,3-dipolar cycloaddition reaction, which was achieved according to Scheme 8. The different compounds were synthesised via 1,3-dipolar cycloaddition from 2-ethynylquinoxaline **6**. Compound **6** was treated with the corresponding azide substrate **7** which was dissolved in DMF:H_2O (4:1) followed by addition of $CuSO_4.5H_2O$ and ascorbic acid solution.



Scheme 8: Functionalization of quinoxaline compound via click chemistry

The reaction mixture was heated at 80°C for 12 h. After the completion of the reaction, the reaction mixture was then quenched with concentrated ammonium chloride (NH_4Cl) solution. The compounds were purified by column chromatography to give the corresponding 1,3-cycloaddition products **8a**–**8b** (Table 3). The reaction between 2-ethynylquinoxaline **6** and 1-azidobenzene **7a** occurred to yield a cream white solid material which was characterised by NMR.

The ^{1}H-NMR spectrum showed a sharp singlet peaks at 8.83 ppm which was attributed to a proton assigned to –N=C-H group from the pyrazine ring. Another singlet peak was observed at 9.83 ppm which was attributed to a proton assigned to –N-C=CH group from the triazole group. Both peaks were integrating for one (1) proton each. There were two apparent triplet peaks in the region of 8.17 ppm and 8.10 ppm integrating for one (1) proton each around the aromatic region (Figure 16).

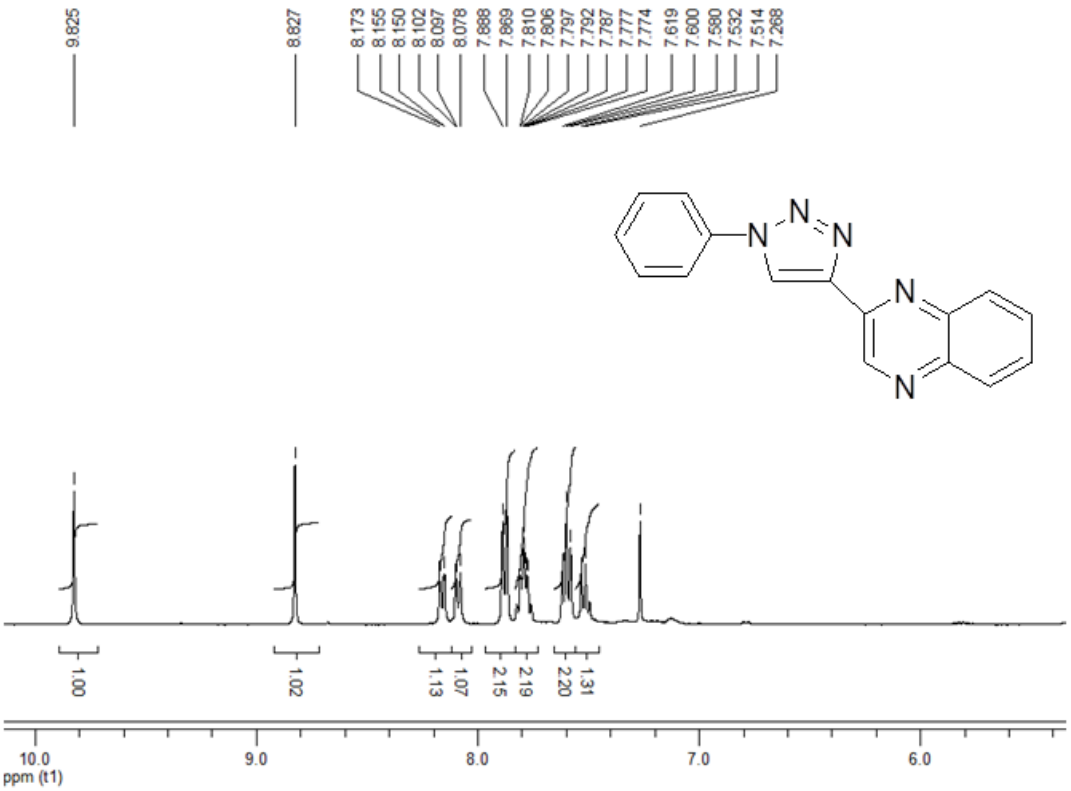


Figure 16: ^{1}H NMR spectrum of compound **8a**

There was a doublet peak at 7.90 ppm and an apparent multiplet peak at 7.81–7.77 ppm, both were integrating for two (2) protons each. The ^{1}H NMR showed an a triplet peak at 7.61 ppm integrating for two (2) protons followed by a doublet peak at 7.53–7.51 ppm integrating for one (1) proton. The ^{1}H NMR result was consistent with the expected compound 2-(1-phenyl-1*H*-1,2,3-triazol-4-yl)quinoxaline **8a**.

The ^{1}H NMR spectrum of **8a** displayed a total of eleven (11) protons and these protons were found to be in a good agreement with the number of hydrogen atoms expected in the compound. The ^{1}H NMR spectrum confirms the appearance of the alkene proton attributed to the triazole ring and supports the formation of **8a**.

Compound **8a** was isolated in 71% yield. The ^{13}C NMR spectrum gave a total of sixteen (16) carbons. The melting point of compound **8a** was found to be 151–155 °C. According to our knowledge and search on Scifinder on 24/02/2015, compound **8a** has never been synthesised before. Further characterisation such as HRMS spectrometry and IR spectroscopy are still required to confirm the structure of the synthesised compound.

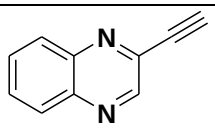
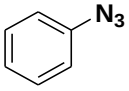
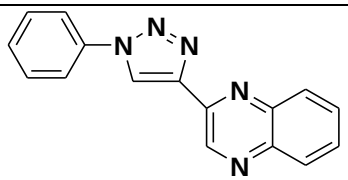
Compound 2-(1-(4-fluorophenyl)-1*H*-1,2,3-triazol-yl)quinoxaline **8b** was synthesised using a similar procedure as in **8a**, but the substrate used was 4-fluoro-1-azidobenzene **7b**. The reaction was monitored by TLC analysis and after the completion of the reaction, the crude was isolated as a light brown to yellowish solid and was characterised by NMR.

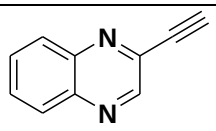
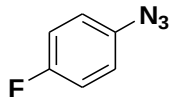
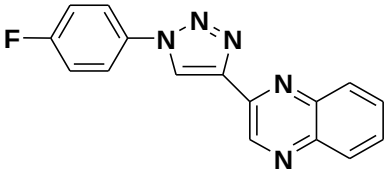
The ^{1}H NMR spectrum of **8b** showed a triplet peak at 7.32 ppm and a multiplet peak at 7.82–7.78 ppm, integrating for two (2) protons each and was assigned to the 4-fluorophenyl moiety. A quartet was observed at 7.88–7.84 ppm integrating for two (2) protons and two triplet at 8.10 ppm and 8.20 ppm integrating for one (1) proton each were assigned to the quinoxaline ring.

A characteristic singlet peak was observed at 8.78 ppm integrating for one (1) proton assigned for –N=C-H group from the pyrazine ring. A singlet peak was observed at 9.82 ppm attributed to a one (1) proton which was assigned to –N-C=CH group from a triazole group. The ^{1}H-NMR spectrum of **8b** displayed a total of ten (10) protons.

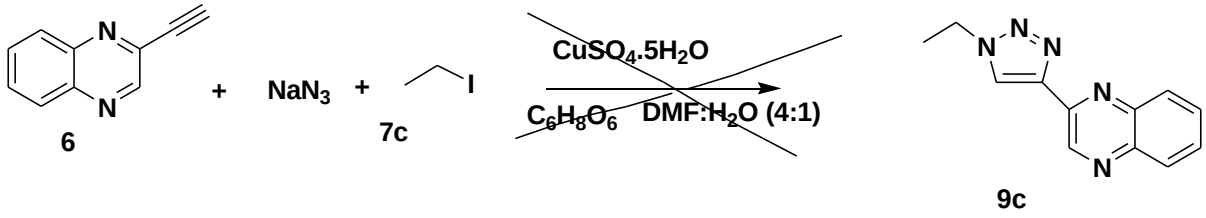
The ^{1}H NMR spectrum confirms the appearance of the alkene proton attributed to the triazole ring and supports the formation of **8b** and it was obtained in 67% yield. The ^{13}C NMR spectrum gave a total fifteen (15) carbons instead of sixteen (16), which means that other carbons are equivalent due to the symmetry at the phenyl ring. The melting point of compound **8b** was 167–170 °C. According to our knowledge and search on Scifinder on 24/02/2015, compound **8b** is novel. It was concluded that the 1,3-dipolar cycloaddition reactions were successfully synthesised, since the compounds were obtained in good yields.

Table 3: Synthesis of 1,3-dipolar cycloaddition across the alkyne compounds

Entry	2-ethynylquinoxaline	Azide substrate	Product	% yield
1	 **6a**	 **7a**	 **8a**	71

2	 **6a**	 **7b**	**8b** 	67

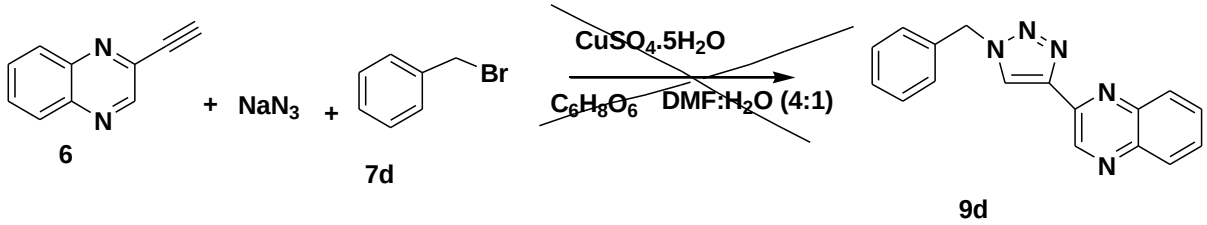
In an attempt to determine the 2-(1-ethyl-1H-1,2,3-triazol-4-yl)quinoxaline **9c** following similar procedure for **8a** for the reaction of 1,3-dipolar cycloaddition across the alkyne compound **6** using iodoethane **7c** in the presence of sodium azide used as a substrate (Scheme 9). The reaction was monitored by TLC analysis. After the completion of the reaction, the crude was purified by column chromatography and analysed by ^{1}H-NMR, which revealed the presence of the starting material. It was observed that the reaction between **6** and **7c** failed to occur



Scheme 9: An attempt on 2-(1-ethyl-1H-1,2,3,-trial-4-yl)quinoxaline compound **9c** via click chemistry

. The reaction between 6 and 7c was repeated with the change in solvent, reaction time and an increase in temperature, but there was no improvement. In an attempt to determine the 2-(1-benyl-1*H*-1,2,3,-trial-4-yl)quinoxaline **9d** following similar procedure for **8a** for the reaction between **6** and benzylbromide **7d** was investigated (Scheme 10).

After the completion of the reaction, the crude was purified by column chromatography and analysed by ^{1}H-NMR, which revealed the presence of the starting material. It was observed that the reaction between **6** and **7d** have failed to occur. The reaction between **6** and **7d** was repeated with the change in solvent, reaction time and an increase in temperature, but there was no improvement.

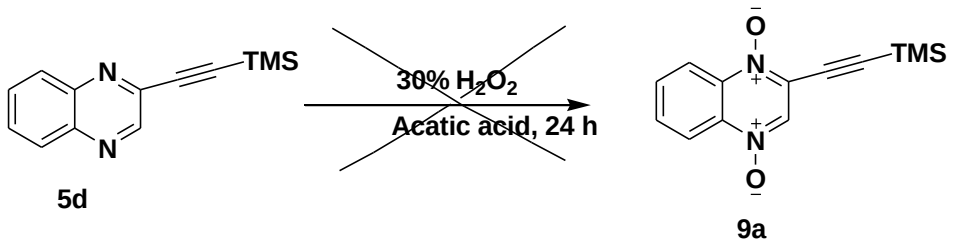


Scheme 10: An attempt on 2-(1-benyl-1*H*-1,2,3,-trial-4-yl)quinoxaline compound **9d** via click chemistry

2.5 Preparation on quinoxaline 1,4-di-*N*-oxide derivative.

After successfully synthesising alkyne-quinoxaline derivatives, another attempt was to perform the synthesis of 1,4-di-*N*-oxide quinoxaline derivatives (Scheme 11 and 12). These compounds which were hoping to have an impact on selectivity, stability and sensitivity in their antimycobacterial activity, were further investigated to undergo 1,4-di-*N*-oxide quinoxaline derivative.

The two coupled compounds such as 2-(2-(trimethylsilyl)ethynyl)quinoxaline **5d** compound and 2-2(3-methoxylphenyl)ethynyl)quinoxaline **5j** compound were selected to undergo the oxidation reaction to give 1,4-di-*N*-oxide quinoxaline derivatives. Compound **5d** was treated with 30% H_2O_2 to form nitric oxide on the pyrazine ring (Scheme 11). The TLC showed a complete consumption of the starting material and formation of a new spot which might correspond to the desired product.



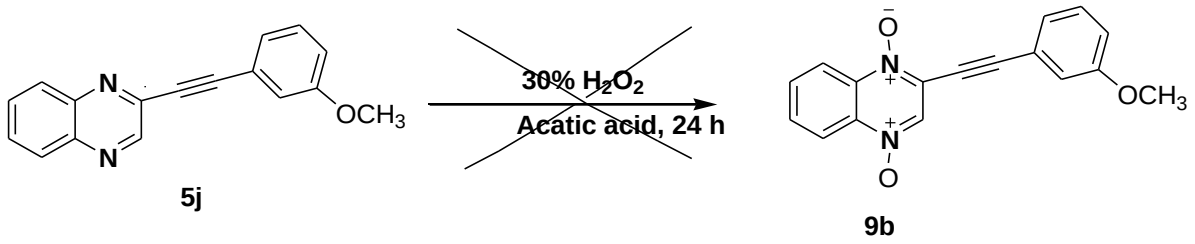
Scheme 11: Preparation of a 2-(2-(trimethylsilyl)ethynyl)quinoxaline 1,4-di-*N*-oxide derivative.

The crude was purified by column chromatography and was further purified by Preparative Thin Layer Chromatography (Prep TLC), then characterised by NMR According to the ^{1}H NMR of **9a**, there was a mixture of compounds since the spectrum gave more protons than expected which might correspond to a mixture of mono and di-*N*-oxide products.

Most of the 1,4-di-*N*-oxide quinoxaline derivatives are characterised by IR in order to observe the possible spectral changes in the compounds when compared to the reactants with the aim of monitoring the presence of the –NO group. The synthesised compound was further characterised by IR. The IR spectrum was supposed to show an absorption peak between 1330 to 1674 cm^{-1} to indicate the presence of –NO group on the pyrazine ring, unfortunately we did not manage to observe the expected peak (1330 to 1674) in the characterised spectrum.

We did not manage to synthesise the 1,4-di-*N*-oxide quinoxaline derivatives. The IR spectrum was showing the broader peak around 3640–3610 cm^{-1} which suggests the presence of O–H stretch. The ^{1}H NMR of compound **9a** was still showing the presence of TMS group. The LC characterisation of **9a** was not showing a good separation which might be due to the presence of charges on the compound and the LC-MS also did not give the expected mass of the compound.

The second attempt was to synthesise 2-2(3-methoxylphenyl)ethynyl)quinoxaline **9d** (Scheme 12). The reaction was monitored by TLC analysis and was showing the formation of new spot. After the completion of reaction, the crude product was characterised by NMR and IR. The NMR and IR results revealed that the reaction did not occur.



5j **9b**

Scheme12: Preparation of a 2-2(3-methoxylphenyl)ethynyl)quinoxaline 1,4-di-*N*-oxide derivative.

The ^{1}H NMR spectrum of **9b** showed more protons than expected which might correspond to a mixture of mono and di-*N*-oxide products. The IR spectrum did not show an absorption peak between 1330 to 1674 cm^{-1} for the presence of –NO group and the reason might be because of the moisture from the water during material isolation process.

It was observed that 1,4-di-*N*-oxide compounds could not be purified to the purity level desired for further use. The reaction mixture was repeated using a freshly prepared starting material and the reactions was monitored by TLC analysis. The ^{1}H NMR showed more protons than expected and IR spectrum did not show any absorption peak at the expected region (1330 to 1674 cm^{-1}), then the reaction mixture was regarded incomplete after 120 h with no improvement. Due to the poor supporting information from NMR, HPLC-MS and IR, the reaction of 1,4-di-*N*-oxide quinoxaline derivatives were not formed. No further attempt was made on 1,4-di-*N*-oxide quinoxaline derivatives. Additional studies are still required to advance the reaction of 1,4-di-*N*-oxide quinoxaline derivatives.

2.6 Biological activity against *M tuberculosis*

The biological activity of the synthesised compounds against TB was done at the University of Cape Town, drug discovery and development centre (H3-D). The antibacterial activities of the compounds (**5a**-**5r**) were evaluated by means of the minimum inhibitory concentrations (MIC) which are MIC_{90} and MIC_{99} against *M tuberculosis* (Table 4). MIC_{90} and MIC_{99} are defined as the minimum concentration of the compound that inhibits growth of bacteria by 90% and 99% respectively [84].

Preliminary investigations of *in vitro* anti-TB activity of the alkyne-quinoxaline compounds were done for the antimycobacterial activity against *M tuberculosis* H37RvMA strain. Some of the compounds have showed promising inhibition percentages against *M tuberculosis* when compared with rifampicin which was used as a positive control.

The most promising results were compounds with the trifluoroanisol alkyne-quinoxaline derivatives, 2-(2-(trifluoromethoxy)phenylethynyl)quinoxaline **5e** and 2-(2-(3,4-(trifluoromethoxy)phenyl)ethynyl)-6-nitroquinoxaline **5o**. They have displayed promising activity with MIC_{90} when compared with the known drug rifampicin. The results have shown that most of the compounds were exhibiting higher MIC_{90} and MIC_{99} values. The results were considered to be inactive when they were having >20.0 valve and were considered to have a poor activity against *M tuberculosis* strain.

Table 4: Antimycobacterial activity of compounds (**5a-5r**) against *M tuberculosis* on $H_{37}Rv$ strain using rifampicin as a standard

Entry number	Compound ID	MIC_{90}	MIC_{99}
1	Rifampicin	0.00386	0.0139
2	**5a**	>20.0	>20.0
3	**5b**	>20.0	>20.0
4	**5c**	>20.0	>20.0
5	**5d**	>20.0	>20.0
6	**5e**	11.8	>20.0
7	**5f**	>20.0	>20.0
8	**5g**	>20.0	>20.0
9	**5h**	>20.0	>20.0
10	**5i**	>20.0	>20.0
11	**5j**	>20.0	>20.0
12	**5k**	>20.0	>20.0
13	**5l**	>20.0	>20.0
14	**5m**	>20.0	>20.0
15	**5n**	>20.0	>20.0
16	**5o**	12.7	>20.0
17	**5p**	>20.0	>20.0
18	**5q**	>20.0	>20.0
19	**5r**	>20.0	>20.0
20	**5s**	>20.0	>20.0

Active compounds (**5e** and **5o**) which were exhibiting efficacy activity were selected for further investigations and follow up studies are still underway in the laboratory to support these compounds. Despite the fact that most of the quinoxaline compounds are used as inhibitors for various diseases, the current alkyne-quinoxaline compounds have shown a promising activity TB, but further investigated are still required on alkyne-quinoxaline.

The activity of the **5e** and **5o** in comparison to other synthesised compounds can be attributed to the trifluoromethoxy moiety in these compounds. It seems like the trifluoromethoxy moiety in **5e** and **5o** have the influence on the activity against *M tuberculosis*. These compounds are selected as a promising antimycobacterial compounds against *M tuberculosis* and further investigations are still required. The present results (Table 4) show that modifications of quinoxaline compound can provide analogues with enhanced activity against *M tuberculosis* H37RvMA strain [85].

2.7. Evaluation of anti-cancer activity of quinoxaline derivative, in human lung A549 cancer cells

The biological activity of the synthesised compounds against A549 human lung cells was done at the University of Limpopo, biochemistry department. Compounds **5a**, **5b**, **5e**, **5g**, **5h**, **5i**, **5l**, and **5r** were evaluated for anti-cancer activity on A549 cells. Compounds **5a**, **5b**, **5e**, **5g**, **5h**, **5i**, **5l,** and **5r** were further subjected to MTT assay for viability on A549 cells in order to check the cell growth inhibition. Compound **5i** has shown a promising anti-cancer activity against A549 cells when compared to other compounds, the results were tabulated as shown in Table 5.

Table 5: Percentage viability of A549 cells on compounds **5a**, **5b**, **5e**, **5g**, **5h**, **5i**, **5l,** and **5r** using MTT assay.

Entity	Compound name	ant-cancer activity (+/-)
1	**5a**	**–**
2	**5b**	**–**
3	**5e**	**–**
4	**5g**	**–**
5	**5h**	**–**
6	**5i**	**+**
7	**5l**	**–**
8	**5r**	**–**

Key words: (+) positive means active and (–) negative means inactive

The experiments were performed in order to determine the effect of these compounds on viability of A549 cells. Compound **5i** has shown a significant reduction on viability of A549 cells when the concentration was increased with time and was considered promising as an anti-cancer agent.

The results have shown that compound **5a**, **5b**, **5e**, **5h**, **5l** and **5r** were inactive as anti-cancer agents (data not shown). It was observed that **5i** was showing a very promising activity against A549 cells and evaluated by MTT assay, adhesion and wound healing assay. Compound **5i** was assessed and investigated if it were able to inhibit the viability of A549 cells. Cells were treated with 0–50 µM of **5i** for 0–24 hours whereby cell viability was determined via the MTT assay (Figure 17).

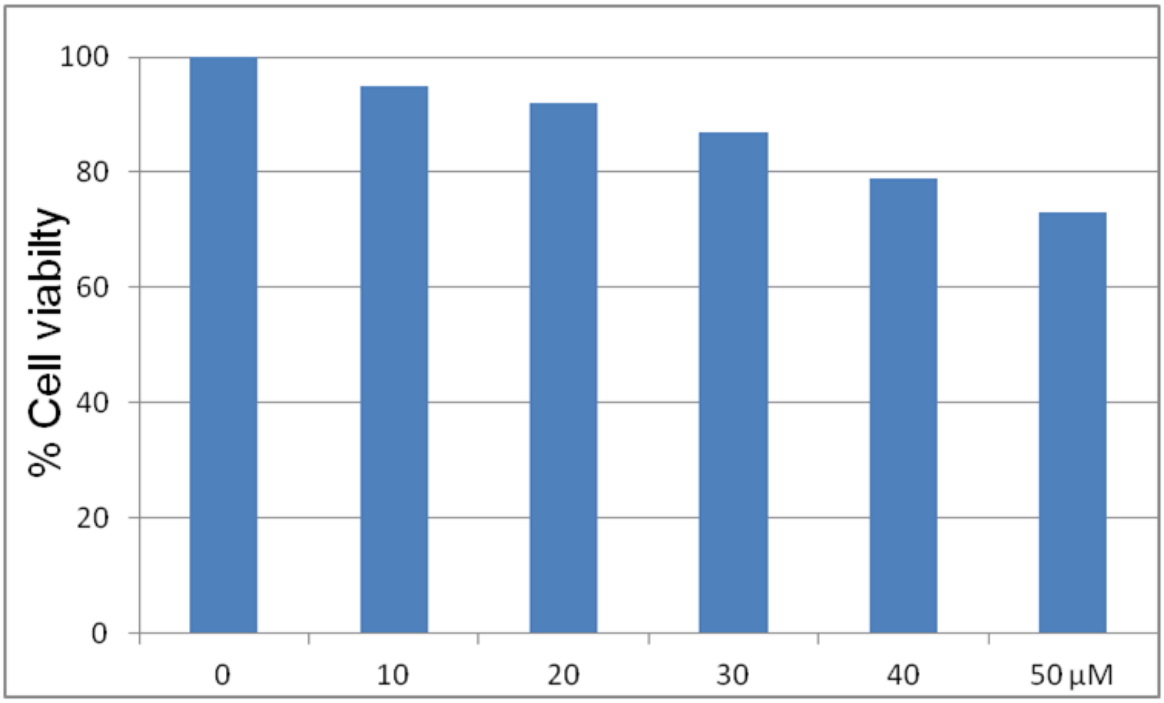


Figure 17: The effect of **5i** on cell viability in A549 cells was evaluated using MTT assay where cells without treatment were considered 100% viable.

As shown in the MTT assay, the growth of the A549 cells was inhibited in a dose dependent manner when treated with various concentrations (0, 10, 20, 30, 40 and 50 µM) of compound **5i**. As the concentration of compound **5i** increases, the percentage of the cell viability decreases which means that compound **5i** is able to inhibit the growth of the cancer cells. The results have indicated that **5i** have the promising anti-cancer activity against A549 cells.

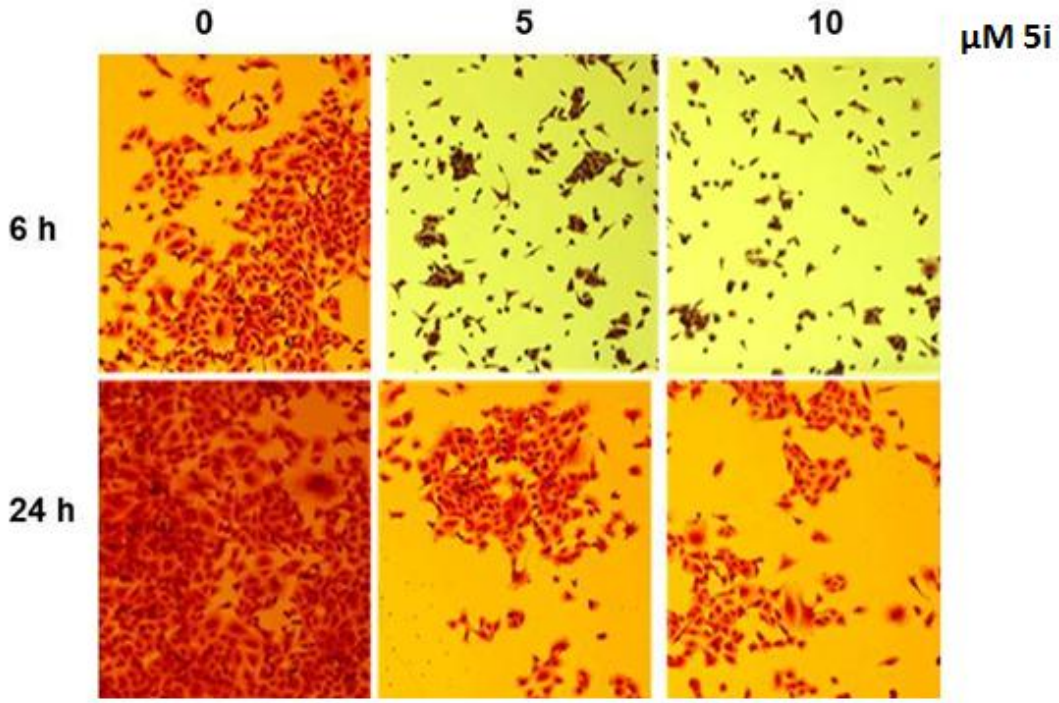


Figure 18: The viability of A549 cells decreases significantly when treated with **5i** at concentration between 0–10 µM for 6–24 h.

To confirm whether **5i** is effective against viability of A549 cells, the results were further investigated on A549 cell adhesion (Figure 18). This assay relies on the capability of the cells to adhere to the surface when they are viable and floats when they are dead. The results have demonstrated that the viability of A549 cells was decreased significantly when treated with **5i** at different concentrations (0–10 µM) from 6–24h.

It was observed that when the concentration of the compound **5i** increases together with time, the viability of the A549 cells decrease. The results were found to be consistent with the MTT results (Figure 17). To further determine if **5i** was indeed effective against A549 cell, it was necessary to investigate the A549 cell migration as shown in Figure 19.

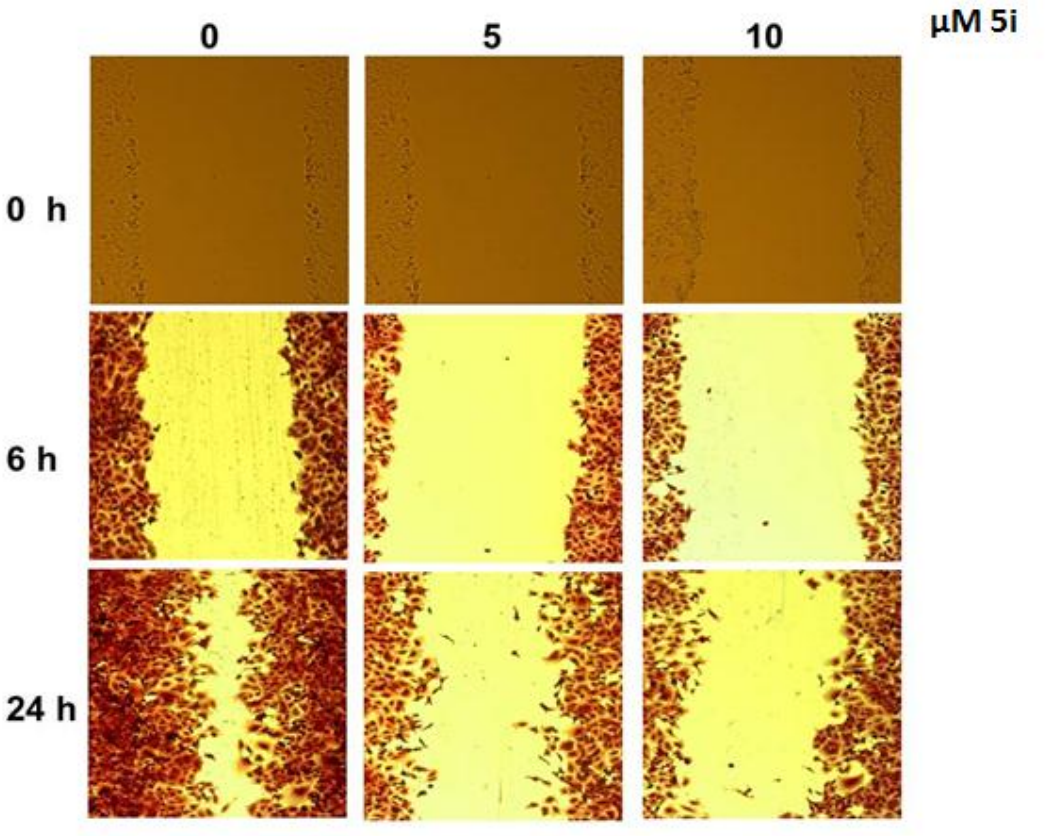


Figure 19: Determination of the number of cells migrating into the wound healing area closed at time 0–24 h.

During the investigation it was observed that the number of cells migrating through wound healing area was significantly decreasing with an increase in concentration of the **5i** (Figure 19). When the concentration and time increases on compound **5i**, it prevents the migration of cancer cells from one area to another, which means that **5i** might be a good candidate to inhibit migration of cancer cells.

The results have showed that the expression of **5i** was able to retard the migration of A549 cells in the wound healing migration assay. There was better inhibitor from 5 and 10 µM of **5i**, which means that this compound give promising results because the migration of cells is an important step for cancer cells metastasis [86]. Supabphol *et al.,* (2009) reported that the new compound possessing potential to inhibit any step or more than one step of metastasis has been the subject of interest, mostly if the substance is producing non-serious side effects [87]. Varieties of chemical compounds have been reported to be active and been used against chemical carcinogenesis, thus are considered to be cancer chemo preventive agents.

Quinoxaline derivatives are among these compounds and have so far produced some interesting biological active compounds against TB and cancer.

2.8 Conclusion

A series of alkyne-quinoxaline derivatives were successfully synthesised by Sonogashira coupling reaction using various alkyne substrates and were obtained in moderate to good yields. The purity of the isolated cross coupling products was found to agree with the expected compounds which were confirmed by NMR and HPLC-MS spectroscopy.

The ^{1}H NMR and ^{13}C NMR data analysis of the compounds were found to be in good agreement with the synthesised compounds. Other synthesised compounds have similar results when compared to previously reported data as discussed above. The synthesised compounds differ from substrate to substrate, due to various reasons such as the effect of atoms and size distribution which may play a major significant role in stability of the quinoxaline compound. From the results obtained, benzenesulfonyl would serve as a good leaving group in the Sonogashira coupling reactions for the generation of alkyne-quinoxaline containing compounds.

The synthesised alkyne-quinoxaline functionalised compounds could contribute to the new class of alkyne-quinoxaline derivatives. The 1,3-diplar cycloaddition reactions were successfully performed and products obtained in good yields. The functionalized 1,2,3-triazole-quinoxaline derivatives need to be tested against TB. Due to the poor results obtained from NMR, HPLC-MS and IR, the 1,4-di-*N*-oxide quinoxaline derivatives were not achieved thus far.

The synthesised compounds were investigated as potential inhibitor against M *tb*. The most promising results were compounds with the trifluoroanisol quinoxaline derivatives **5e** and **5o**. It was observed that the trifluoromethoxy moiety in the **5e** and **5o** have an influence on the activity of *M tuberculosis* H37RvMA strain. After investigating the alkyne-quinoxaline derivatives and evaluating their structural properties, the compounds with trifluoroanisol have been identified as a potential inhibitor of *M tuberculosis* H37RvMA strain. Based on the results obtained and other biological properties as highlighted previously in the literature, alkyne-quinoxaline compounds are promising to be considered as alternative therapy for anti-TB

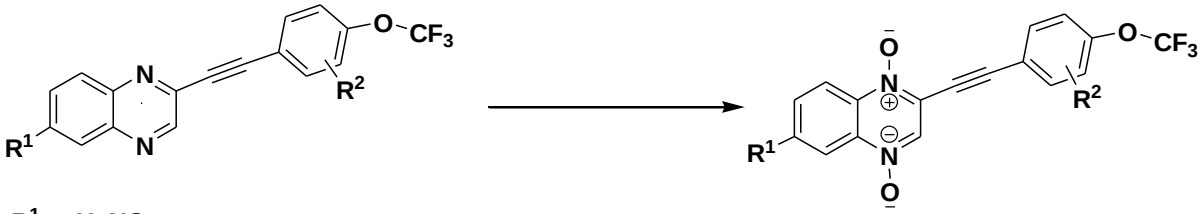
treatment. Modification of quinoxaline compounds can provide analogues with enhanced activity against *M tuberculosis* H37RvMA.

Alkyne-quinoxaline derivatives (**5a**, **5b**, **5e**, **5g**, **5h**, **5i**, **5l** and **5r**) were evaluated for anti-cancer activity on human lung A549 cancer cells. It was observed that **5i** was showing a very promising activity against A549 cells when compared to the other synthesised compounds and evaluated by MTT assay, adhesion and wound healing assay. The results have showed that **5i** was able to retard the migration of A549 cells in the wound healing migration assay

The MTT assay, wound healing assay and cell adhesion results have demonstrated significant decrease in cell viability of the A549 human lung cancer cells when treated with compound **5i**. Compound **5i** has a potent anti-cancer activity and might be used as anti-cancer inhibitor for A549 lung cancer cells. Quinoxaline compounds are showing interesting biological activity against TB and cancer.

2.9 Future work

Further studies are still required to elucidate the significant role of alkyne-quinoxaline as a potential inhibitor for TB and cancer. Quinoxaline and its derivatives will be tested as new potential treatment which might have promising activities against resistant *M tuberculosis* strains. Other detailed analysis on biological results and future applications of the synthesised compounds still require further investigation.



R^1 = H, NO_2
R^2 = F, OCH_3

Preparation of quinoxaline 1,4-di-*N*-oxide derivatives using different conditions are still required. Further evaluation, optimization of alkyne-quinoxaline compounds are still required to enlarge the knowledge of these compounds.

Chapter 3

Chapter 3

3 MATERIALS AND METHODS

3.1 Reagents and solvents

All reactions involving moisture-sensitive reactants were performed under nitrogen (N_2) atmosphere using oven-dried glassware. Tetrahydrofuran (THF) was freshly distilled over sodium/benzophenone under N_2 atmosphere for 4–6 h before use. Commercially available reagents and solvents were purchased from Sigma Aldrich, Merck, Fluka analytical, Educhem, Saarchem, Unilab, Analtech and Rochelle chemicals.

All chemicals were used directly as received, unless otherwise stated. All measurements were performed at room temperature if not otherwise mentioned. Anhydrous solvents were achieved by using standard desiccation/drying methods and stored over activated molecular sieves [94]. Column chromatography was used as a method to isolate pure products and was performed using Merck silica gel 60 on a particle size of 0.04–0.063mm (230–400 mesh ASTM).

Mobile phase solvents were prepared using different concentration of EtOAc/*n*-hexane (5:95, 20:80) and DCM/MeOH (95:5). Some of the Preparative Thin Layer Chromatography (Prep TLC) plates were prepared using Prep silica gel (200g) and water (500ml), the mixture were poured on the glass plates and were dried to air for overnight and were activated in the oven for four hours. Other was bought from Analtech for further purification process.

3.2 Physical and spectroscopic properties compounds

The structural properties of the compounds were recorded and confirmed by nuclear magnetic resonance (NMR) (Bruker Ascend 400 MHz Topspin 3.2), HPLC-MS (LCMS-2020-Shimadzu Scientific Instruments), IR (Agilent technologies cary 600 series FTIR spectroscopy). ^{1}H NMR and ^{13}C NMR spectra were referenced internally using solvent signals, ^{1}H NMR: 7.26 ppm for $CDCl_3$, 2.50 ppm for DMSO-d_6; ^{13}C NMR: 77.0 ppm for $CDCl_3$, 39.4 ppm for DMSO-d_6, respectively which were used as the solvents at room temperature. Chemical shifts are expressed in δ-values parts

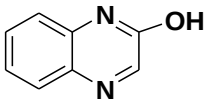
per million (ppm) and the coupling constants (*J*) in Hertz (Hz). Multiplicity of the signals is given as follows: br. = broad, s = singlet, d = doublet, t = triplet, q = quartet and m = multiplet.

3.3 Analytical methods

Melting points were measured on a Lasec/SA-Melting point apparatus from Lasec Company, SA and were obtained in open-end capillary tubes and are uncorrected. TLC was carried out on TLC Silica gel F_{254} 25 Aluminium sheet ready foils from the Merck Company and Silica gel on TLC Aluminium foils silica gel matrix, with fluorescent indicator 254 nm ready foils from Fluka analytical company.

3.4 Synthesis

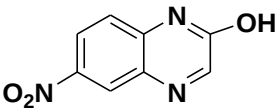
Preparation of quinoxalin-2-ol ***1a***



quinoxalin-2-ol

According to method reported by Nxumalo [78], in a typical reaction, *o*-phenylenediamine (10.04 g, 0.092 mol) was dissolved in a mixture of acetic acid (5 mL) and methanol (5 mL). The reaction mixture was stirred at room temperature for 10 min. A solution of glyoxylic acid (4.25 g, 0.046 mol) in water (10 mL) was added to the solution, drop-wise for 30 min, while stirring at 0°C, the solution was further stirred for 90 min. The solvent was removed by filtration then the filtrate was washed with water (15 mL) followed by methanol (15 mL) and air dried to give a cream white solid. The resulting cream material was purified by recrystallization using dimethylformamide (DMF), giving the quinoxaline-2-ol **1a** as a cream white solid (10.72 g, 80%). M.p 265-268 $^{\circ}$C (Lit 266-267 °C). ^{1}H NMR (400 MHz, DMSO-d_6), 7.32–7.30 (2H, m), 7.56–7.52 (1H, m), 7.78 (1H, *J*=8.3 Hz, d), 8.16 (1H, s). ^{13}C NMR (100 MHz, DMSO-d_6,) 116.2, 123.8, 129.2, 131.2, 132.2, 132.5, 152.0, 155.4 ppm.

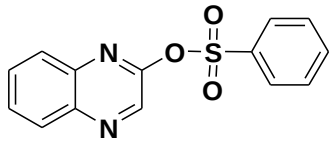
Preparation of 6-nitroquinoxalin-2-ol ***1b***



6-nitroquinoxalin-2-ol

A similar procedure as **1a** was followed, 4-nitro-*o*-phenylenediamine (10.00 g, 0.052 mol) was dissolved in a mixture of acetic acid (5 mL) and methanol (5 mL). The reaction mixture was stirred at room temperature for 10 min. A solution of glyoxylic acid (4.25 g, 0.046 mol) in water (10 mL) was added to the solution, drop-wise for 30 min, while stirring at 0°C, the solution was further stirred for 90 min. After recrystallization from DMF the product 6-nitroquinoxalin-2-ol was obtained as a dark brown solid (10.72 g, 74%); M.p 222–225 °C (Lit 224–226 °C); ^{1}H NMR (400 MHz, DMSO-d_6), 2.92 (1H, s), 7. 99 (1H, *J*= 8.3 Hz, d), 8.08–8.03 (2H, m), 8.34 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$,) 111.4, 117.9, 130.7, 132.9, 135.8, 148.0, 155.0, 156.0 ppm.

Synthesis of 2-benzenesulfonyloxyquinoxaline ***3a***



2-benzenesulfonyloxyquinoxaline

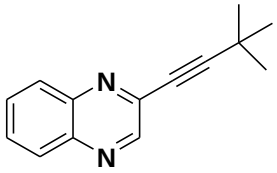
In line with the method reported by Nxumalo [78], in a round bottom flask, quinoxaline-2-ol (1.250 g, 8.5 mmol), 4-dimethylaminopyridine (0.104 g, 0.85 mmol), and benzenesulfonyl chloride (2.18 mL, 17 mmol) were dissolved in dry dichloromethane (DCM) (20 ml), cooled to 0°C and stirred for 5 min. Triethylamine (Et_3N) (3 mL, 22 mmol) was added drop-wise over 5 min. The solution was allowed to stir at room temperature for 1 h and then quenched with 20 mL of saturated aqueous sodium hydrogen carbonate ($NaHCO_3$), two layers were separated and the aqueous layer washed with DCM (2 x 15 mL). The organic layers were combined and dried with sodium sulfate (Na_2SO_4), concentrated and purified on flash silica eluting with DCM to give compound **3a** which was a light brown to yellowish solid (1.140 g, 68%). M.p 89–92 °C (Lit 91 °C). ^{1}H NMR (400 MHz, $CDCl_3$), 7.72 (2H, *J*= 8.2 Hz, t), 7.80–7.72 (3H, m), 7.92 (1H, *J*= 9.1 Hz, d), 8.19 (3H, *J*= 5.0, 1.1 Hz, dd), 8.70 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$), 128.5, 128.6, 129.0, 129.2, 129.8, 131.2, 134.6, 136.5, 139.2, 139.7, 141.31, 150.9 ppm.

General procedure for the Sonogashira ccoupling reaction on 2-benzenesulfonyloxyquinoxaline **3a** and 6-nitro-2-benzenesulfonyloxyquinoxaline **3b**

General procedure of Sonogashira coupling reaction (method 1)

In line with the method reported by Nxumalo [78], in a Schlenk tube or round bottom flask equipped with a stirrer bar, 2- benzenesulfonyloxyquinoxaline **3a** (0.301 g, 1.05 mmol), $PdCl_2(PPh_3)_2$ (38.0 mg, 0.052 mmol, 5 mol%), Et_3N (0.2 mL, 2.1 mmol, 2 eq.) and the ethynyl substrate (1.26 mmol, 1.2 eq.) were dissolved in dry THF (10 mL). The solution was refluxed for 1–24 h under N_2, partitioned between EtOAc/water (20 ml, 3:2) , the layers were separated and the aqueous layer was washed with EtOAc (2 x 15 mL). The organic layers were combined and dried over Na_2SO_4, concentrated and purified on flash silica.

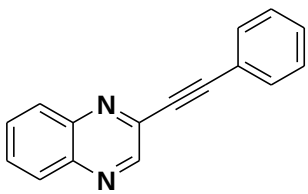
*Synthesis of 2-(3,3-dimethylbutyn-1-yl)quinoxaline **5a***



2-(3,3-dimethylbut-1-ynyl)quinoxaline

Following method 1, 2-benzenesulfonylquinoxaline **3a** (0.302 g, 1.05 mmol) was treated with 3,3-dimethyl-1-butyn-1-yl **4a** (0.155 mL, 1.26 mmol, 1.2 eq). Purification on flash silica, eluting with 1:9 EtOAc/*n*-hexane gave 2-(3,3-dimethyl-1-butyn-1-yl) quinoxaline **5a** as a dark brown solid (0.087 g, 39%). M.p 56–58 °C. ^{1}H NMR (400 MHz, $CDCl_3$), 1.15 (9H, s), 7.55 (2H, *J*= 6.5 Hz, d), 7.78 (2H, *J*= 8.2 Hz, t), 8.70 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$), 51.3, 83.0, 92.2, 92.3, 113.1, 129.2, 130.8, 130.87, 138.9, 141.1, 142.0, 147.0 ppm, *m/z* 211 (M+1).

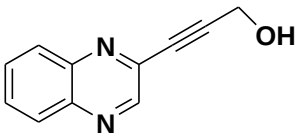
Preparation of 2-(2-phenylethynyl)quinoxaline **5b**



2-(2-phenylethynyl)quinoxaline

In line with method 1, **3a** (0.308 g, 1.34 mmol) was reacted with phenylacetylene **4b** (154 µl, 1.26 mmol, 1.2 eq.). Purification on flash silica and preparative TLC, eluting with 1:9 EtOAc/*n*-hexane gave 2(2-phenylethynyl)quinoxaline **5b** as yellowish solid (0.178 g, 61%). M.p 63–66 °C (Lit 65–66.5 °C). ^{1}H NMR (400 MHz, $CDCl_3$), 7.45 (3H, *J*= 8.3 Hz), d), 7.71–7.69 (2H, q), 7.82–7.78 (2H, m), 8.12 (2H, *J*= 6.5 Hz, d), 8.99 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$), 121.4, 128.6, 129.1, 129.2, 129.2, 129.3, 129.8, 130.6, 130.7, 132.4, 139.14, 139.6, 140.9, 142.2, 147.4 ppm, *m/z* 231 (M+1).

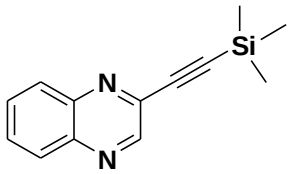
Synthesis of 3-(quinoxaline-3-yl)prop-2-yn-1-ol **5c**



3-(quinoxalin-3-yl)prop-2-yn-1-ol

In line with method 1, **3a** (0.301 g, 1.05 mmol) was treated with propargyl alcohol **4c** (73.3 µL, 1.26 mmol, 1.2 eq.). Recrystallization of the crude product from acetone gave 3-(quinoxalin-2-yl)prop-2-yl-1-ol **5c** as a brown solid (0.124 g, 64%). M.p 140–143 °C (Lit 141–142°C). ^{1}H NMR (400 MHz, $CDCl_3$), 2.20 (1H, s), 3.66 (2H, s), 7.84–7.81 (2H, m), 8.02 (1H, m), 8.19–8.16 (1H, q), 8.74 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$), 29.6, 29.9, 89.7, 128.0, 128.1, 129.0, 129.5, 139.1, 139.6, 140.9, 146.4 ppm, *m/z* 185 (M+1).

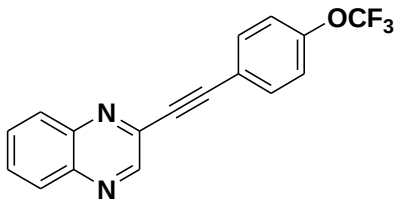
Synthesis of 2-(2-(trimethylsilyl)ethynyl)quinoxaline **5d**



2-(2-(trimethylsilyl)ethynyl)quinoxaline

Following method 1, **3a** (0.303 g, 1.32 mmol) was reacted with ethynyltrimethylsilane (155 µL, 1.26 mmol, 1.2 eq.). Purification on flash silica and prep TLC, eluting with 1:9 EtOAc/*n*-hexane gave 2-(2-(trimethylsilyl)ethynyl)quinoxaline as a brown oily residue (0.173 g, 72%). ^{1}H NMR (400 MHz, $CDCl_3$), 0.15 (9 H, s), 7.55–7.53 (2H, q), 7.87–7.85 (2H, q), 8,70 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$), 100.8, 102.0, 129.6, 130.0, 131.0, 132.0, 139.6, 141.4, 142.4, 147.7 ppm, *m/z* 227 (M+1).

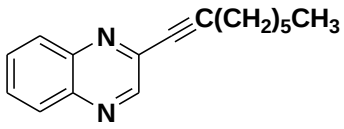
Synthesis of 2-(2-(4-(trifluoromethoxy)phenyl)ethynyl)quinoxaline **5e**



2-(2-(4-(trifluoromethoxy)phenyl)ethynyl)quinoxaline

Following method 1, **3a** (0.304 g, 1.31mmol) was treated with 4-(trifluoromethoxy)phenylacetylene **4e** (0.193 mL, 1.26 mmol, 1.2 eq.). Purification on flash silica, eluting with 1:9 EtOAc/*n*-hexane gave 2-(2-(4-(trifluoromethoxy)phenyl)ethynyl)quinoxaline **5e** as a cream white solid (0.311 g, 92%). M.p 100–103 °C. ^{1}H NMR (400 MHz, $CDCl_3$), 7.31–7.19 (2H, m), 7.47 (2H, *J*= 7.8 Hz, t), 7.76 (2H, *J*= 8.2 Hz, t), 8.07–8.04 (2H, m), 8.92 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$), 88.3, 121.0, 121.2, 121.8, 123.8, 128.9, 129.3, 129.4, 130.6, 131.0, 138.9, 142.2, 147.1, 155.4 ppm, *m/z* 315 (M+1).

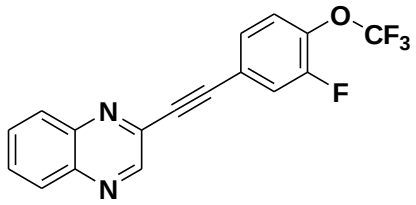
Synthesis of 2-(oct-1-ynyl)quinoxaline **5f**



2-(oct-1-ynyl)quinoxaline

Following method 1, **3a** (0.306 g, 1.32 mmol) was treated with oct-1-yne **4f** (0.186 mL, 1.26 mmol, 1.2 eq.). Purification on flash silica, eluting with 1:9 EtOAc/*n*-hexane gave 2-(oct-1-ynyl)quinoxaline **5f** as a dark brown solid (0.085 g, 33%). M.p 132–135 °C. ^{1}H NMR (400 MHz, $CDCl_3$), 0.91–0.87 (3H, t), 1.32–1.09 (2H, m), 1.49–1.42 (2H, q), 1.62–1.58 (2H, m) 2.49 (2H, *J*= 7.2 Hz, t). ^{13}C NMR (100 MHz, $CDCl_3$), 27.9, 28.7, 29.3, 59.3, 120.8, 123.6, 125.4, 128.7, 129.2, 130.1, 130.9, 138.1, 141.3, 147.8, 148.5 ppm.

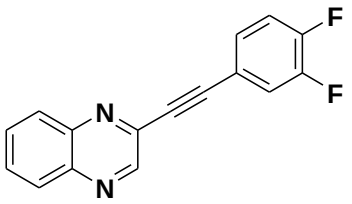
Synthesis of 2-(2-(3-fluoro-4-(trifluoromethoxy)phenyl)ethynyl)quinoxaline **5g**



2-(2-(3-fluoro-4-(trifluoromethoxy)phenyl)ethynyl)quinoxaline

Following method 1, **3a** (0.301 g, 1.26 mmol) was treated with 3-fluoro-4-(trifluoromethoxy)phenylacetylene **4g** (0.201 mL, 1.26 mmol, 1.2 eq.). Purification on flash silica, eluting with 1:9 EtOAc/*n*-hexane gave compound (**5g**) was as a light brown to yellowish solid (0.230 g, 68%). M.p 119–122 °C. ^{1}H NMR (400 MHz, $CDCl_3$), 7.39–7.35 (1H, q), 7.55 (2H, *J*= 8.2 Hz, t), 7.84–7.82 (2H, m), 8,14–8,12 (2H, m), 8,99 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$), 110.7, 111.3, 120.6, 127.8, 128.5, 129.4, 129.5, 129.8, 130.0, 131.6, 132.4, 134.6, 139.1, 146.9 ppm, *m/z* 211 (M+1).

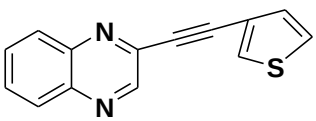
Synthesis of 2-(2-(3,4-difluorophenyl)ethynyl)quinoxaline **5h**



2-(2-(3,4-difluorophenyl)ethynyl)quinoxaline

Following method 1, **3a** (0.303 g, 1.26mmol) was treated with 3,4-difluorophenylacetylene **4h** (0.155 mL, 1.26 mmol, 1.2 eq.). Purification on flash silica, eluting with 1:9 EtOAc/*n*-hexane gave 2-(2-(3,4-difluorophenyl)ethynyl)quinoxaline **5h** as a solid brown (0.167 g, 59%) M.p 164–167 °C. ^{1}H NMR (400 MHz, $CDCl_3$), 7.38–7.33 (2H, m), 7.82–7.75 (3H, m), 8.12 (2H, *J*= 8.2 Hz, t), 8.97 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$), 120.7, 125.9, 129.2, 129.3, 130.1, 130.4, 130.7, 131.6, 140.9, 147.2 ppm, *m/z* 267 (M+1).

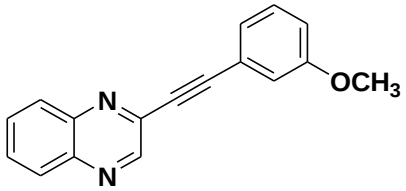
Synthesis of 2-(2-(thiophen-3-yl)ethynyl)quinoxaline **5i**



2-(2-(thiophen-3-yl)ethynyl)quinoxaline

Following method 1, **3a** (0.307 g, 1.32 mmol) was treated with 3-ethynylthiophene **4i** (0.124 mL, 1.26 mmol, 1.2 eq.). Purification on flash silica, eluting with 1:9 EtOAc/*n*-hexane gave 2-(2-(thiophen-3-yl)ethynyl)quinoxaline **5i** as a solid back brown (0.101 g, 42%). M.p 80–84 °C. ^{1}H NMR (400 MHz, $CDCl_3$), 7.40 (2H, *J*= 8.2, 7.0 Hz, td), 7.81–7.77 (3H, m), 8.12–8.10 (2H, m), 8.97 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$), 118.0, 121.2, 129.2, 129.3, 130.7, 131.0, 142.2, 147.1, 149.5, 153.4 ppm, *m/z* 211 (M+1).

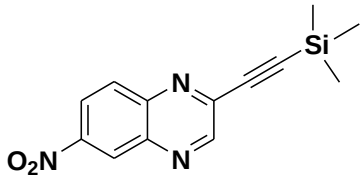
Synthesis of 2-(2(3-methoxyphenylethynyl)ethynyl)quinoxaline **5j**



2-(2-(3-methoxyphenyl)ethynyl)quinoxaline

Following method 1, **3a** (0.300 g, 1.26 mmol) was treated with 3-ethynylanisole **4j** (0.160 mL, 1.26 mmol, 1.2 eq.). Purification on flash silica, eluting with 1:9 EtOAc/*n*-hexane gave 2-(2(3-methoxyphenylethynyl)ethynyl)quinoxaline **5j** as a cream white solid (0.252 g, 91%). M.p 63–66 °C. ^{1}H NMR (400 MHz, $CDCl_3$), 3.80 (3H, s), 7.44 (2H, *J*= 6.7 Hz, d), 7.53 (2H, *J*= 9.1 Hz, d), 7.93–7.91 (2H, m), 8.19 (2H, *J*= 8.4 Hz, d) 8.82 (1H, s). ^{13}C NMR (100 MHz), $CDCl_3$), 55.6, 86.7, 93.7, 116.7, 116.9, 122.4, 124.2, 129.2, 129.3, 129.7, 130.4, 131.4, 139.5, 140.9, 142.2, 147.3, 159.4 ppm, *m/z* 261 (M+1).

Synthesis of 2-(2-trimethysily)ethynyl)-6-nitroquinoxaline **5k**

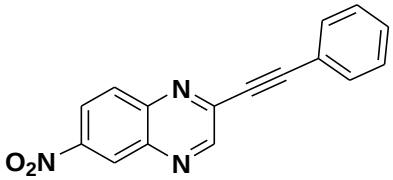


2-(2-(trimethylsilyl)ethynyl)-6-nitroquinoxaline

Following method 1, 6-nitro-2-benzenesulfonyloxyquinoxaline **3b** (0.301 g, 1.10 mmol) was treated with ethynyltrimethylsilane **4a** (155 µL, 1.26 mmol, 1.2 eq.). Purification on flash silica, eluting with 1:9 EtOAc/*n*-hexane gave 2-(2-trimethysily)ethynyl)-6-nitroquinoxaline **5k** as a dark brown solid (0.128 g, 52%). M.p 157–160 °C. ^{1}H NMR (400 MHz, $CDCl_3$), 0.17 (4H, s), 8.07 (1H, *J*= 8.0 Hz, t), 8.40–

8.33 (1H, m), 8.87–8.82 (1H, m). ^{13}C NMR (100 MHz), $CDCl_3$), 29.6, 85.6, 89.56, 110.7, 120.9, 128.7, 129.67, 130.67, 131.32, 136.7, 141.8, 144.48, 150.3 ppm.

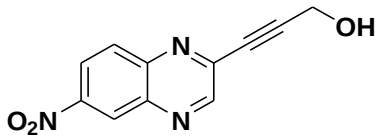
Synthesis of 6-nitro-2-(2-phenylethynyl)quinoxaline **5l**



6-nitro-2-(2-phenylethynyl)quinoxaline

Following method 1, **3b** (0.300 g, 1.10 mmol) was treated with phenylacetylene **4b** (154 µL, 1.26 mmol, 1.2 eq.). Purification on flash silica, eluting with 1:9 EtOAc/*n*-hexane gave 6-nitro-2-(2-phenylethynyl)quinoxaline **5l** as a yellowish solid (0.191 g, 77%). M.p 77–80 °C. ^{1}H NMR (400 MHz, $CDCl_3$), 7.72 (2H, *J*= 8.3 Hz, d), 7.50–7.43 (3H, m), 8.25 (1H, *J*= 8.3 Hz, d), 8.58–8.55 (1H, m), 9.00 (1H, *J*= 8.4 Hz, d), 9.10 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$), 86.6, 91.2, 110.9. 111.6, 120.24, 121.4, 124.6, 127.2, 129.1, 129.8, 130.9, 141.2, 147.7 ppm, *m/z* 276 (M+1).

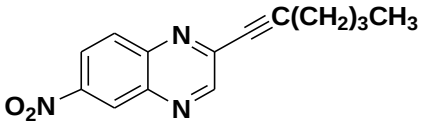
Synthesis of 3-(6-nitroquinoxalin-2-yl)prop-2-yl-1-ol **5m**



3-(6-nitroquinoxalin-2-yl)prop-2-yn-1-ol

Following method 1, **3b** (0.300 g, 1.10 mmol) was treated with propargyl alcohol **4c** (73.3 µL, 1.26 mmol, 1.2 eq.). Purification on flash silica, eluting with 1:9 EtOAc/*n*-hexane gave 3-(6-nitroquinoxalin-2-yl)prop-2-yl-1-ol **5m** as a bark brown solid (0.129 g, 62%). M.p 187–190 °C. ^{1}H NMR (400 MHz, $CDCl_3$), 2.79 (1H, s), 3.39 (2H, s), 7.61–7.52 (1H, *J*= 7.2 Hz, t), 7.81–7.78 (2H, *J*= 6.7 Hz, d), 7.99 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$), 31.1, 89.6. 92.4, 116.0, 120.1, 129.3, 130.3, 131.9, 133.2, 148.5 ppm.

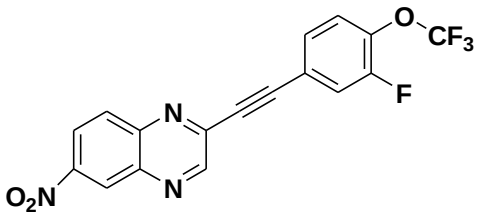
Synthesis of 2-(hex-1-ynyl)-6-nitroquinoxaline **5n**



2-(hex-1-ynyl)-6-nitroquinoxaline

Following method 1, **3b** (0.302 g, 1.10 mmol) was treated with hex-1-yne **4k** (0.145 mL, 1.26 mmol, 1.2 eq.). Purification on flash silica, eluting with 1:9 EtOAc/*n*-hexane gave 2-(hex-1-ynyl)-6-nitroquinoxaline **5n** as a brown solid (0.098 g, 42%). M.p 120–124 °C. ^{1}H NMR (400 MHz, $CDCl_3$), 0.94 (3H, *J*= 7.0 Hz, t), 1.18 (2H, m), 1.48 (2H, *J*= 7.0 Hz, d), 1.66 (2H, 3H, *J*= 7.3 Hz, t), 8.16–8.10 (1H, m), 8.48–8.41 (1H, m), 8.90–8.86 (1H, m), 9.00 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$), 43.0, 52.0, 25.2, 52.5, 88.7, 90.0, 112.2, 123.2, 127.1, 127.6, 128.5, 134.3, 140.1.147.9 ppm.

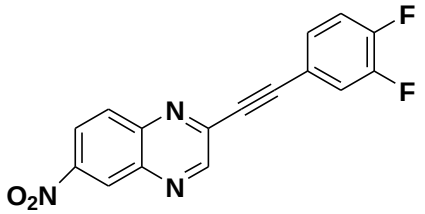
Synthesis of 2-(2-(3,4-(trifluoromethoxy)phenyl)ethynyl)-6-nitroquinoxaline **5o**



2-(2-(3-fluoro-4-(trifluoromethoxy)phenyl)ethynyl)-6-nitroquinoxaline

Following method 1, **3b** (0.300 g, 1.10 mmol) was treated with 3-fluoro-4-(trifluoromethoxy)phenylacetylene **4g** (0.201 mL, 1.26 mmol, 1.2 eq.). Purification on flash silica, eluting with 1:9 EtOAc/*n*-hexane gave 2-(2-(3,4-(trifluoromethoxy)phenyl)ethynyl)-6-nitroquinoxaline **5o** as a light brown solid (0.150 g, 44%). M.p 95–97 °C. ^{1}H NMR (400 MHz, $CDCl_3$), 7.36–7.35 (1H, m), 7.90 (1H, *J*= 7.7 Hz, d), 7.83 (1H, s), 8.26 (1H, *J*= 7.7 Hz, d), 8.51 (1H, *J*= 8.8 Hz, d), 8.98 (1H, *J*= 8.5 Hz, d), 9.07 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$), 120.1, 120.9, 129.1, 129.3, 129.3, 129.8, 130.6, 130.8, 134.0, 139.2, 140.1, 142.2, 147.2 ppm.

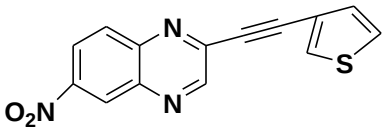
Synthesis of 2-(2-(3,4-difluorophenyl)ethynyl)-6-nitroquinoxaline **5p**



2-(2-(3,4-difluorophenyl)ethynyl)-6-nitroquinoxaline

Following method 1, **3b** (0.302 g, 1.10 mmol) was treated with 3,4-difluorophenylacetylene **4h** (0.155 mL, 1.26 mmol, 1.2 eq.). Purification on flash silica, eluting with 1:9 EtOAc/*n*-hexane gave 2-(2-(3,4-difluorophenyl)ethynyl)-6-nitroquinoxaline **5p** isolated as light brown solid (0.142 g, 50%). M.p 117–119 °C. ^{1}H NMR (400 MHz, $CDCl_3$), 6.97–7.20 (1H, *J*= 7.3 Hz t), 7.33–7.30 (1H, m), 7.41 (1H, *J*= 7.2 Hz, t), 7.64–7.58 (1H, m), 7.89 (1H, *J*= 8.8 Hz, d), 8.08–8.06 (1H, m), 9.09 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$), 121.0, 127.4, 128.5, 129.3, 129.7, 129.9, 130.6, 133.1, 141.9, 147.7 ppm.

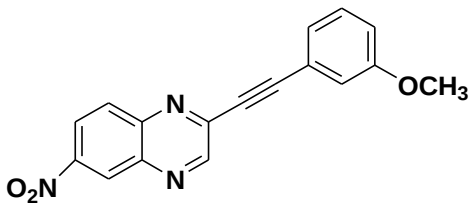
Synthesis of 6-nitro-2-(2-(thiophen-3-yl)ethyl)quinoxaline **5q**



6-nitro-2-(2-(thiophen-3-yl)ethynyl)quinoxaline

Following method 1, **3b** (0.302 g, 1.10 mmol) was treated with 3-ethynylthiophene **4i** (0.124 mL, 1.26 mmol, 1.2 eq.). Purification on flash silica, eluting with 1:9 EtOAc/*n*-hexane gave 6-nitro-2(2-(thiophen-3-yl)ethyl)quinoxaline **5q** as a light brown solid (0.082 g, 32%). M.p 84–88 °C. ^{1}H NMR (400 MHz, $CDCl_3$), 7.53–7.43 (2H, m), 7.82–7.80 (2H, m), 8.13–8.10 (2H, m) 8.97 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$), 111.0, 121.4, 127.7, 129.1, 129.3, 131.0, 132.2, 144.3, 145.0, 148.1 ppm.

Synthesis of 2-(2-(3-methyphenyl)ethynyl)-6-nitroquinoxaline **5r**

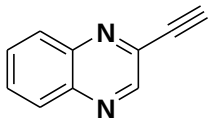


2-(2-(3-methoxyphenyl)ethynyl)-6-nitroquinoxaline

Following method 1, **3b** (0.300 g, 1.10 mmol) was treated with 3-ethynylanisole **4j** (0.160 mL, 1.26 mmol, 1.2 eq.). Purification on flash silica, eluting with 1:9 EtOAc/*n*-hexane gave 2-(2-(3-methyphenyl)ethynyl)-6-nitroquinoxaline **5r** as a light brown solid (0.138 g, 50%). M.p 119–121 °C. ^{1}H NMR (400 MHz, $CDCl_3$), 3.85 (3H, s), 7.01 (2H, *J*= 7.2 Hz, d), 7.32–7.30 (2H, m), 7.81–7.77 (2H, m), 8.12–8.10 (2H, m), 8.99

(1H, s). ^{13}C NMR (100 MHz), $CDCl_3$), 42.6, 112.1, 120.1, 121.7, 127.3, 129.0, 129.4, 129.7, 129.9, 131.5, 142.6, 145.4 ppm, *m/z* 211 (M+1).

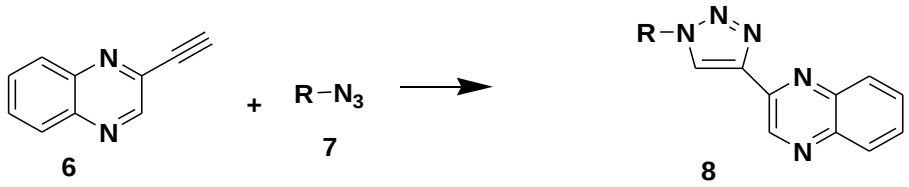
Synthesis of 2-ethynylquinoxaline ***6***



2-ethynylquinoxaline

Following method reported by Montserrat (2001) [83], to a suspension of 2-(trimethylsilylethynyl)quinoxaline **5d** (3,00 g, 13.2 mmol), in dry methanol (34 mL) at room temperature was added potassium carbonate (K_2CO_3) (182 mg, 1.32 mmol) under N_2 and the mixture stirred for 1 h. The MeOH was evaporated under reduced pressure and the residue dissolved in DCM, the solution washed with H_2O, dried, and evaporated under reduced pressure, to give a brown solid material. The compound was purified by column chromatography over silica gel eluting with DCM giving 2-ethynylquinoxaline **6** (1.5 g, 76%) as a black brown solid material. M.p 94–97 °C (Lit 95–96 °C). ^{1}H NMR (400 MHz, $CDCl_3$), 3.10 (1H, s), 7.69–765 (2H, m), 8.32–8.29 (2H, m), 9.01 (1H, s). ^{13}C NMR (100 MHz), $CDCl_3$), 84.5, 89.6, 120.4, 128.7, 139.5, 130.0, 139.3, 144.7, 144.9, 149.5 ppm.

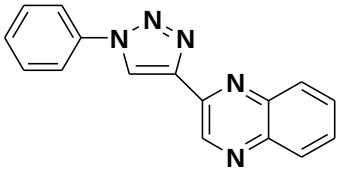
3.5 Preparation of 1,3-dipolar cycloaddition across the alkyne compounds



6 **7** **8**

Following method reported by Kinfe and Belay (2013) [88], to a round bottom flask equipped with stirrer bar, containing 2-ethylquinoxaline **6** (102 mg, 0.649 mmol) in DMF:water (35 mL, 4:1), was added azidobeneze solution (130 µL, 0.649 mmol), $CuSO_4.H_2O$ (162 mg, 0.649 mmol) dissolved in 1ml of H_2O and ascorbic acid (114 mg, 0.649 mmol) and the reaction mixture were stirred at 80 °C for 12 h. After completion of the reaction, the mixture were cool and quenched with EtAOc/H_2O (3:1). The mixture was transferred into a separating funnel and extracted with EtAOc (30 ml x4). The organic layers were combined and dried over anhydrous Na_2SO_4. The resulting crude product was purified by column chromatography.

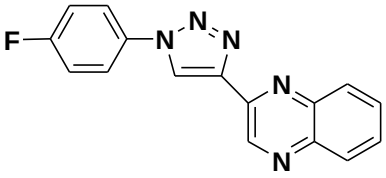
Synthesis of 2-(1-phenyl)-1H-1,2,3-triazol-4-yl)quinoxaline (**8a**)



2-(1-phenyl-1*H*-1,2,3-triazol-4-yl)quinoxaline

To a round bottom flask equipped with stirrer bar, containing 2-ethylquinoxaline **6** (102 mg, 0.649 mmol) in DMF:water (35 mL, 4:1) was added azidobeneze solution **7a** (130 µL, 0.649 mmol), $CuSO_4.H_2O$ (162 mg, 0.649 mmol) was dissolved in 1ml of H_2O and ascorbic acid (114 mg, 0.649 mmol).After workup, the resulting crude product was purified by column chromatography to give 2-(1-phenyl-1*H*-1,2,3-triazol-4-yl)quinoxaline **8a**, 0.126g, 71%. M.p 152–155 °C. ^{1}H NMR (400 MHz, $CDCl_3$), 7.53 (1H, *J*= 7.2 Hz, t), 7.62 (2H, *J*= 7.3 Hz, t), 7.81–7.77 (2H, m), 7.89 (2H, *J*= 8.3 Hz, d), 8.10 (1H, *J*= 9.1 Hz, d), 8.17–8.15 (1H, s), 8.83 (1H, s), 9.83 (1H, s). ^{13}C NMR (100 MHz), $CDCl_3$), 114.1, 120.7, 121.5, 129.1, 129.3, 129.5, 129.9, 130.0, 130.5, 136.8, 139.3, 142.1, 142.2, 143.4, 144.9, 147.1 ppm.

Synthesis of 2-(2-(1-(4-fluorophenyl)-1H-1,2,3-triazol-4-yl)quinoxaline (**8b**)



2-(1-(4-fluorophenyl)-1*H*-1,2,3-triazol-4-yl)quinoxaline

To a round bottom flask equipped with stirrer bar, containing 2-ethylquinoxaline **6** (102 mg, 0.649 mmol) in DMF:water (35 mL, 4:1) was added 4-flouro-azidobeneze solution **7b** (131 µL, 0.649 mmol), $CuSO_4.H_2O$ (162 mg, 0.649 mmol) dissolved in 1ml of H_2O and ascorbic acid (114 mg, 0.649 mmol). Compound 2-(2-(1-(4-fluorophenyl)-1H-1,2,3-triazol-4-yl)quinoxaline **8b** as a light brown solid (0.127 g, 67%). M.p 166–170 °C. ^{1}H NMR (400 MHz, $CDCl_3$), 7.32 (2H, *J*= 7.3 Hz, t), 7.82–7.78 (2H, m), 7.88–7.84 (2H, q), 8.10–8.08 (1H, m), 8.20 (1H, *J*= 7.2 Hz, t), 8.78 (1H,

s), 9.82 (1H, s). ^{13}C NMR (100 MHz), 120.4, 121.5, 129.0, 129.3, 129.5, 129.8, 129.9, 130.5, 136.7, 142.1, 142.2, 143.4, 144.9, 147.1 ppm.

3.6 Antimicrobial activity against *M tuberculosis*

3.6.1 Broth microdilution method

The antimicrobial activity against *M tuberculosis* was performed at the University of Cape Town, drug discovery and development centre (H3-D), following broth microdilution method. The broth microdilution method [89,90] allows a range of antibiotic concentrations to be tested on a single 96-well microtitre plate in order to determine the minimum inhibitory concentration (MIC). Briefly, a 10 ml culture of a mutant *Mycobacterium tuberculosis* (H37RvMA) [91] strain constitutively expressing recombinant green fluorescent protein (GFP) off a plasmid integrated at the *attB* locus is grown to an OD_{600} of 0.6–0.7. The H37RvMA::*gfp* culture is then diluted 1:100 in GAST/Fe medium. In a 96-well microtitre plate, 50 µl of GAST/Fe medium is added to all wells from Rows 2–12. The compounds to be tested are added to Row 1 in duplicate, at a final concentration of 640 µM (stocks are made up to a concentration of 12.8 mM in DMSO, and diluted to 640 µM in GAST/Fe medium). A two-fold serial dilution is prepared, by transferring 50 µl of the liquid in Row 1 to Row 2 and aspirating to mix. 50 µl of the liquid in Row 2 is then transferred to Row 3 and aspirated, and so on. This procedure is repeated until Row 12 is reached, from which 50 µl of the liquid is discarded so as to bring the final volume in all wells to 50 µl. Finally, 50 µl of the 1:100 diluted *M tuberculosis* cultures is added to all wells in Rows 2-12. Cells are not added to Row 1, as this serves as a contamination control. Controls include media only, 5% DMSO, Rifampicin and Kanamycin. The microtitre plate is stored in a secondary container and incubated at 37 °C with humidifier to prevent evaporation of the liquid. The lowest concentration of drug that inhibits growth of more than 90% of the bacterial population is considered to be the MIC_{90} and MIC_{99}. MIC_{90} and MIC_{99} values are scored by quantitative fluorescence using a Fluostar Optima microplate reader (BMG Labtech) at 7-days and 14-days post inoculation, and digital images captured and stored.

3.7 Anti-cancer activity of quinoxaline derivative against A549 cancer cells

3.7.1 Cell viability assay

The anti-cancer activity against A549 human lung cells was done at the University of Limpopo, Biochemistry Department. According to method performed by Boshielo (2014) [91], to determine the cytotoxicity of compounds on lung A549 cancer and myoblast C2C12 cells, the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay was used. The cells were seeded in a 96-well plate, treated with various concentrations of compounds (0, 10, 20, 30, 40 and 50 µM) and incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h. After the incubation, 50 µL of MTT solution (5 mg/ml) (Sigma) was added to each well and the plate was incubated further for 4 h. The cell culture medium was then replaced with 100 µL of DMSO to dissolve the oxidised MTT. The blue crystals of the oxidized MTT (formazan) were quantified by spectrophotometry at 560 nm using the GloMax®-Multi+ Detection System (Promega, Madison, USA). Percentage of survival was plotted using the.

Formula:

$$\%\text{Cell viability} = \left(\frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated control}} \right) \text{X } 100$$

3.7.2 Wound-healing assay

According to method performed by Boshielo (2014) [91], the effect of compounds on the ability of the A549 cells to migrate was assessed using the wound healing assay. Cells were plated into 6-well culture plates for overnight in order to form monolayers. Wounds were created by scratching the monolayer using a 200 µL micro-pipette-tip. Cell debris was removed by rinsing the scratched monolayer with hepes-buffered saline. The scratched monolayers were photographed using an inverted light microscope before being exposed to 0,5 and10 µM of compounds for 6 and 24 hours. The cells were stained with 0.1% (w/v) of crystal violet solution for 30 min, washed with tap water, air-dried and photographed using an inverted light microscope (Nikon, Japan) to monitor wound closure.

3.7.3 Cell adhesion assay

According to method performed by Boshielo (2014) [91], in order to determine the effect of compounds on A459 cell adhesion, the cell attachment assay was employed. A549 cells (1 x 10^5 cells/ml) were exposed to 0, 5 and 10 µM of compounds for 3 h. Cells were then seeded in 12-well plates and allowed to adhere at 37 °C in a humidified atmosphere of 5% CO_2 for 6 and 24 h. After washing with hepes-buffered saline to remove non-adherent cells, the adherent cells were stained with 0.1% crystal violet for 30 min, washed with tap water and air-dried. The stained cells were photographed using an inverted microscope (Nikon, Japan).

Chapter 4

Chapter 4

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