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SYNTHESIS, DERIVATIZATION, CHARACTERIZATION AND ANTI-MICROBIAL SCREENING OF AZO COMPOUNDS.

BY

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DECLARATION

I hereby declare that this thesis report is my own work and that the work was undertaken at the Department of Pharmaceutical Chemistry. To the best of my knowledge, it has not been submitted for the award of any degree or certificate from any institution, except where due acknowledgement has been made in the text.

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This work is dedicated to my sister, Ama Anima Addo-Danquah.

ACKNOWLEDGEMENT

ABSTRACT

In our research group, two *azo* dyes were recently demonstrated to have antimicrobial activity. Structural modification of these compounds resulted in seven novel dyes. Five compounds were synthesised by the two-step diazotization-coupling reaction sequence. Employing the Williamson's synthesis and acetylation with acetic anhydride, the ether and acetyl derivatives were synthesised respectively. The synthesised dyes, with good yields, have been characterised with IR, Mass Spec. and ^{1}H NMR, and the melting point as well as retardation factor determined. The Broth Dilution method was used in determining the antimicrobial activity against the organisms, *Staphylococcus aureus, Streptococcus pyrogenes, Salmonella typhi, Pseudomonas aeruginosa and Candida albicans*. The synthesised compounds showed a broad spectrum of activity, with greater gram-negative and antifungal activity. The alkylated products, (E)-1-(4-ethoxynaphthalen-1-yl)-2-(4-nitrophenyl)diazene (KA 008), (E)-4-((4-ethoxynaphthalen-1-yl)diazenyl benzoic acid (KA 009) were the most active of all the compounds synthesised, with an MIC of 35.16μg/ml against *P. aeruginosa* and *S. pyrogenes* for KA 008 and 70.32 μg/ml against *S. typhi* and *P. aeruginosa* for KA 009. The acetylated derivatives showed the least activity against the test organisms. KA 008 also showed comparable activity against the standard Cefuroxime axetil used as the positive control.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SCHEMES

LIST OF TABLES

LIST OF ABREVIATIONS

AMR –   Antimicrobial resistance

Ar –   Aromatic

ATCC –   American Type Culture Collection

CDC –   Center for Disease Control

CD4 –   Cluster Differentiation 4 cell

d –   Doublet

DMAP –   Dimethylaminopyridine

ES –   Electrospray (positive or negative mode)

eq –   equivalent

^{1}H NMR –   Proton Nuclear Magnetic Resonance

HIV –   Human Immunodeficiency Virus

Hz –   Hertz

IR –   Infra-Red Spectroscopy

IUPAC –   International Union of Pure and Applied Chemistry

m –   Multiplet

LCD –   Liquid Crystal Display

M^{+} –   Molecular ion

m/z –   mass-to-charge ratio

MDR –   Multidrug resistance

MDR-TB –   Multidrug-resistant tuberculosis

MHz-   Mega Hertz

MIC –   Minimum inhibitory concentration

mg –   Milligrams

MS –   Mass Spectrometry

NLO –   Nonlinear Optical

nm –   Nanometers

^{o}C –   degrees Celsius

PDT –   Photodynamic Therapy

PEG –   Polyethylene glycol

ppm –   Parts per million

Rf –   Retardation factor

s –   Singlet

TLC –   Thin Layer Chromatography

μg –   Micrograms

μL –   Microliters

δ –   Chemical shift

UV-Vis –   Ultra Violet-Visible Spectroscopy

WHO –   World Health Organisation

CHAPTER ONE

INTRODUCTION

1.1   BACKGROUND OF STUDY

1.1.1   History of antimicrobials

Antimicrobial medicines are agents designed to destroy or inhibit the growth of organisms including bacteria, viruses and parasites. However, in recent times, resistance to commonly administered antimicrobial agents by these organisms is a common occurrence. Organisms that have developed resistance are able to withstand the destruction that otherwise the medicine would have caused. This renders the medicine ineffective and the infections may persist and spread to others (Neu, 1992).

Over the last decade, there has been an increase in the number and proportion of bacterial that has grown resistant to commonly administered anti-bacterial agents, leading to the development of multidrug resistance (MDR) bacteria. This has led to organizations such as the United States' Centre for Disease Control (CDC), the European Centre for Disease Prevention and Control (ECDC) and the World Health Organization (WHO) to label infections caused by MDR bacteria as an emergent global health epidemic (Roca *et al*., 2015).

Responding to the increasing threat posed by antimicrobial resistance (AMR) and MDR, WHO proposed some measures which they believe can help curtail the situation. The measures are;

- bringing all stakeholders together to agree on and work towards a coordinated response;

- strengthening national stewardship and plans to tackle antimicrobial resistance;

- generating policy guidance and providing technical support for Member States;

- actively encouraging innovation, research and development.

The development of fluoroquinolones as antibiotic agents happened about 40 years ago. After fluoroquinolones, there has been limited development of major antibiotic agents. As a result, a gap has been created between the occurrence of new infections and new agents to fight them (WHO, 2014).

1.1.2 Diazo Compounds

Aromatic diazo compounds, containing the functional group Ar–N=N-Ar, form the largest class of dyes in use today. Their extensive conjugation, affording them varying shades of colours, contribute to their use as dyes (Rasheed, 2011).

Interest in diazo compounds, however, is not limited only to their use as dyes, but also their medicinal application. Commercially available medicines containing diazo functionality include; Prontosil, which is used as an antibacterial agent (Wainwright and Kristiansen, 2011) and Balsalazide, used to treat inflammatory bowel disease (Tursi *et al*., 2004) (fig. 1.1).
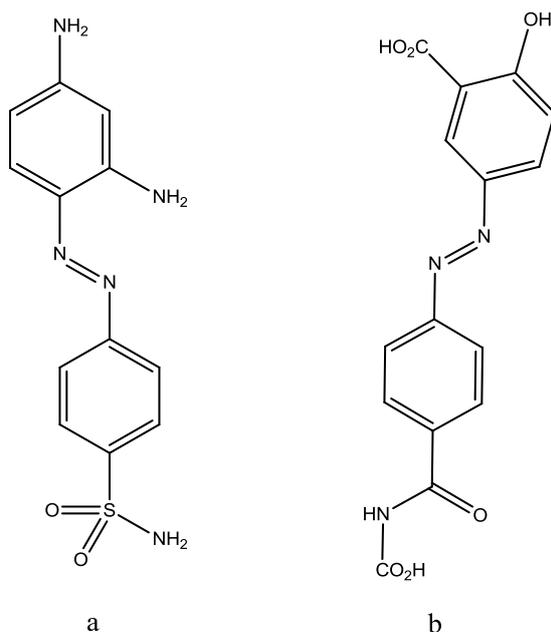
Figure 1.1 Structure of prontosil (a) and balsalazide (b)

Recent research has revealed that some synthetic diazo compounds possess antimicrobial activity (Avci *et al*., 2012, Hayashi *et al*., 2001, Kofie *et al*., 2015, Shridhar *et al*., 2011).

1.2 PROBLEM STATEMENT

In 2014, WHO reported that the problem with antimicrobial resistance is no longer a prediction for the future, but it is now a common occurrence. This is threatening the health provision by making common infections difficult to treat. Treatment for common infections and minor injuries are becoming ineffective and death from such conditions would become common occurrence again. This is leading the world towards a post-antibiotic era. For instance, in several countries, there have been reports of the failure of third generation cephalosporins in treating gonorrhoea. Widespread resistance to fluoroquinolones by *E. coli* as well as resistance to first-line drugs in the treatment of infections by *Staphylococcus aureus* has been reported. The

incident of multidrug-resistant tuberculosis (MDR-TB) is on the increase worldwide (WHO, 2014, Lowy, 2003).

The phenomenon of the development of resistance to antimicrobial drugs by microorganisms is natural. However, the inappropriate use of these drugs has contributed greatly in the resistance process. Another source for the increasing prevalence of resistance is the lack of new antimicrobial agents to tackle new infections. After the fluoroquinolones were discovered, there has not been the development of a major antimicrobial drug in the past few decades. The WHO recommends that the problem with resistance can be addressed and curtailed through the act of collaborative effort between policy makers and researchers, among other recommendations, to develop new vaccines (WHO, 2014, Roca *et al.*, 2015).

In light of these recommendations, seven *azo* compounds were recently synthesised by our research group and their antimicrobial activity determined. Of the compounds synthesised, two demonstrated encouraging antimicrobial potentials (fig 1.2). The two compounds are (E)-4-((4-hydroxynaphthalen-1-yl) diazenyl) benzoic acid, code-named *p*-ABAαN, and (E)-4-((4-nitrophenyl) diazenyl) naphthalen-1-ol, code-named *p*-NAαN.

a   b
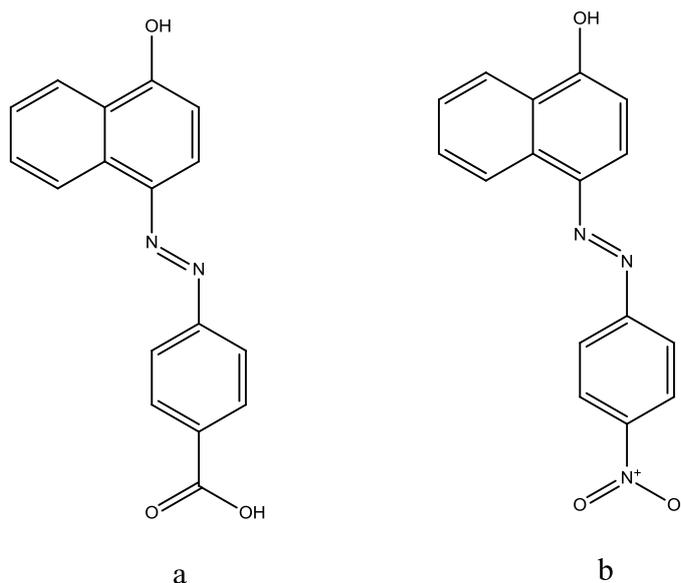
Figure 1.2 Structure of p-ABAαN (a) and p-NAαN (b)

p-ABAαN showed inhibition against the fungus, *Candida albicans*. *p*-NAαN on the other hand showed a broad spectrum antimicrobial activity against all the test organisms used, recording a low minimum inhibitory concentration (MIC) of 0.02mg/ml against *C. albicans* with the highest of 4.06mg/ml against *Mycobacterium smegmatis* (Kofie *et al*., 2015).

Considering the structure of the two compounds, the hydroxyl group on the naphthol, the carboxylic acid group as well as the aromatic ring system provides suitable site for the modification of the structure of the compounds. Therefore the formation of derivatives of these two dyes focusing on the carboxylic, phenol and aromatic ring functionalities will be the basis of this present research.

1.3 OBJECTIVES

1.3.1 General objective

The aim of this research involves the synthesis and derivatization of *azo* compounds, *p*-ABAαN and *p*-NAαN and screening for their antimicrobial properties.

1.3.2 Specific Objectives

- To use readily available reagents to synthesise the *azo* compounds and their derivatives employing the standard diazotization and coupling procedure.

- To use spectroscopic methods − Infra-Red, Proton Nuclear Magnetic Resonance and Mass Spectrometry − to characterize the synthesised compounds.

- To determine the physicochemical parameters of the synthesised compounds including melting point and retardation factor (rf).

- To investigate the *in-vitro* antimicrobial activity of the compounds against the microorganisms, *Staphylococcus aureus, Streptococcus pyrogenes, Salmonella typhi, Pseudomonas aeruginosa* and *Candida albicans.*

CHAPTER TWO

LITERATURE REVIEW

2.1 HISTORY OF ANTIMICROBIAL DRUGS

Introduction of antimicrobial drugs meant an end to the deaths associated with infectious diseases, resulting in improved life of the human race. Such was the advances made in the so-called "golden-era" of antimicrobial drug development that it was believed that total eradication of infectious disease would be achieved in the not so long future.

In the year 1910, Paul Ehrlich successfully synthesised a drug that could treat syphilis through the "magic bullet" idea. This idea was grounded on the observation that, aniline and other synthetic dyes, present at the time, could stain only certain microbes. As a result, Ehrlich embarked on the journey of specifically designing a treatment for the *Treponema pallidum* causing disease. He called the new drug *Salvarsan* (fig 2.1) which became the most widely prescribed drug at the time (Wainwright, 2008).
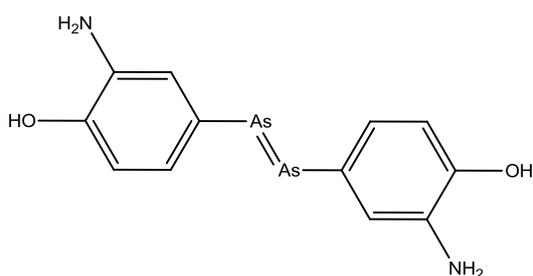
Figure 2.1 Structure of Salvarsan

This was followed by the sulfa drugs, the first of which was synthesised by Mietzsch and Klarer. Gerhard Domagk investigated the antimicrobial activity of the new compound, prontosil. It was revealed later that, prontosil was the precursor of sulphanilamide, (Scheme 2.1) the active part. This kick-started

the development of the sulphonamide group of antibiotics (Wainwright and Kristiansen, 2011).
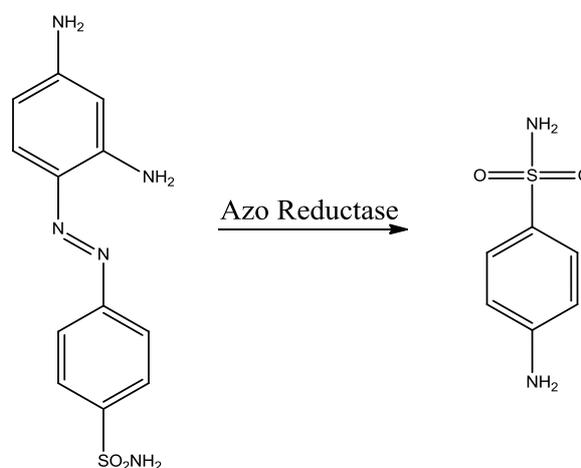
Scheme 2.1 Reduction of Prontosil by azo reductase enzyme

The serendipitous discovery of penicillin by Alexander Fleming in 1928, led to the saving of the lives of many injured soldiers during the Second World War. It was not until the 1940s before the problem of the purification and stabilization of the active compound was addressed and penicillin entered into mass production (Fleming, 1946).With the discovery of these three classes of antimicrobials, the foundation was laid for other researchers to take up the mantle and develop other drugs. The period between the 1950s and 1970s saw the introduction of new classes of antibiotics, from the cephalosporins through to the carbapenems and to the fluoroquinolones (Andriole, 2005, Bo, 2000, Sykes and Bonner, 1985). After this period, a vacuum has been created in terms of the addition of novel class to treat emerging and re-emerging infections. The focus of most research has been the modification of existing antibiotics in recent time (WHO, 2014).

2.2   DYESTUFFS

A dye can be defined as an organic compound used to give colour to a substrate. The mechanism by which the colour is imparted includes physical adsorption, formation of complexes with metals or salts or mechanical retention. Absorption in the visible region (400-800) of the electromagnetic spectrum, gives dyes their colours. Witt proposed that, the presence of a chromophore and an auxochromes are essential for a dye; the chromophore giving the colour while the auxochromes deepens the colour. This theory has been replaced by the modern electronic theory, which states that the excitation of valence π electrons after the absorption of visible light, results in colour (Bafana *et al*., 2011).

The use of dyes dates back to ancient times. Egyptian mummies were wrapped in cloth dyed with *Indigo*. It is believed that, Alexander the Great sprinkled on his army, alizarin (a red dye) to deceive the Persian armies into thinking they were injured. The togas of the Roman Emperors was dyed with *Tyrian purple*, a dye obtained for the Murex snails (Garfield, 2002, Morris and Travis, 1992). The structures for *Indigo*, alizarin and *Tyrian purpl*e are shown in fig. 2.2
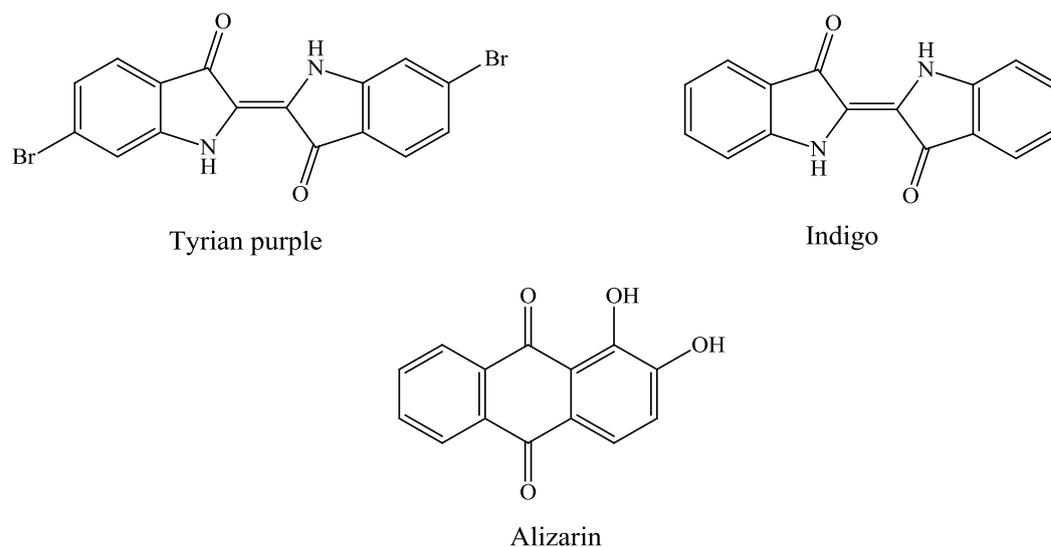
Figure 2.2 Structures of some early dyes

Before the mid-19th century, all the dyes in use were from natural sources, most of which were extracted from vegetables with a few animal sources. The diversity of the colours possessed by these dyes were limited and pushed researchers to develop synthetic dyes with varying colours. With this goal, Woulfe in 1771 developed the yellow coloured picric acid by reacting *Indigo* with nitric acid. The first synthetic dye, *Mauveine* (fig 2.3), however, was developed by William Henry Perkin in 1856. He was trying to synthesise quinine to treat malaria but failed in this quest. However when washing the reaction flask with alcohol, he observed that a purple solution was formed. After this, dozens other synthetic dyes have been developed and are in use to date (Garfield, 2002).
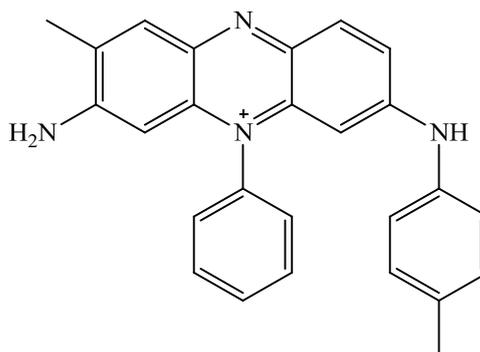
Figure 2.3 Structure of Mauveine

2.3   *AZO* DYES

Azo dyes make up about 70% of all organic dyes in use currently worldwide. The popularity and widespread use of *azo* dyes is as a result of the relative simple method of synthesis, diverse variety of colours, great structural diversity and medium-to-high fastness properties. They find use in the textile, cosmetic, food, medical, printing and paint industries. *Azo* dyes are also used on the high technology areas like electro-optical devices, liquid crystal displays, lasers and ink-jet printers (Natansohn and Rochon, 2002, Yaroshchuk and Reznikov, 2012, Harada *et al*., 2005).

Aniline Yellow and Bismarck Brown (fig 2.4) synthesised by Mene in 1861 and Martius in 1863 respectively, are the first *azo* dyes prepared. Structurally, Bismarck Brown contains two *azo* linkages, making it a *bisazo* dye. The presence of the second *azo* helps to extend the conjugation of the dye hence absorbs visible light of longer wavelength than the Aniline Yellow dye.

Bismarck Brown

Aniline Yellow
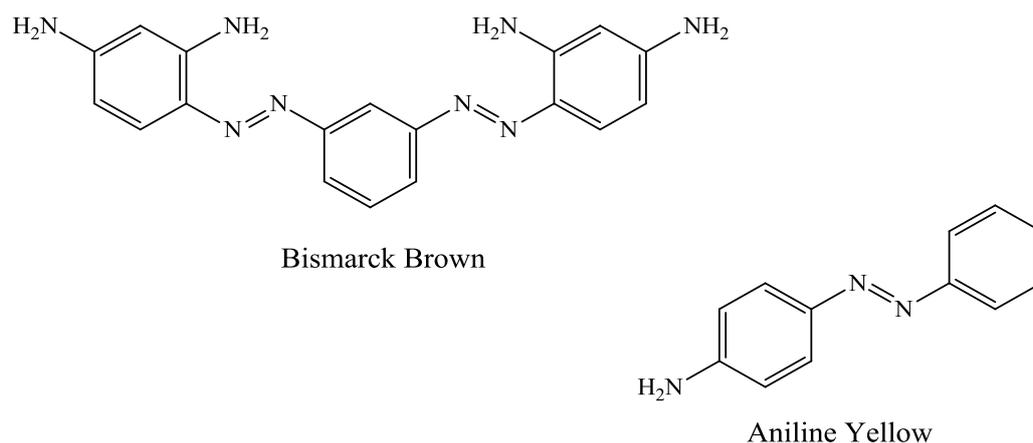
Figure 2.4 Structures of the first synthesised azo dyes

2.4   *AZO* COMPOUNDS

Azo compounds possess the functional group, R-N=N-R′, where R and R′ can either be an alkyl or an aryl group. The International Union of Pure and Applied Chemistry (IUPAC) describes *azo* compounds are derivatives of diazene (diimide), HN=NH, which has both hydrogens substituted by alkyl or aryl groups (Gold *et al*., 1997).

Aliphatic *azo* compounds are mostly colourless, whereas the aromatic counterparts possess variety of colours. The presence of the *azo* bond between the aromatic systems, helps extends the conjugation, leading to a chromophore being formed. Presence of groups such as −OH and −NH_2 on the chromophore will result in the enhancing of the light absorbing property of the chromophore, shifting the wavelength of absorption higher into the visible region (400-800nm) of the electromagnetic spectrum. These groups are termed auxochromes (Solomons, 2012).

12

2.4.1 Origin

In 1858, Peter Griess reported on the synthesis of a new class of compounds. He was initially working on forming the hydroxy derivative of picramic acid. However, after slightly changing the conditions by employing cold alcoholic nitrous acid instead of warm nitrous acid (Scheme 2.2) he observed that the product had properties different from that of the expected product. A similar observation was made when other primary aromatic amines were used in place of picramic acid. He proposed that, the product was formed as a result of two atoms of nitrogen replacing two atoms of hydrogen. Hence, he called this new class of compounds, *"diazo."* Further work on the correct structure of the di*azo* compound formed revealed however that, -NH$_2$ was rather replaced by -N$_2$ (Gordon and Gregory, 2012, Regitz, 2012).
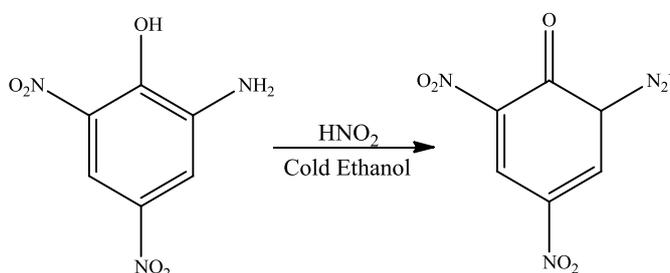
Scheme 2.2 Diazotization of Picramic acid by Griess

2.4.2 General Mechanism

Synthesis of *azo* compounds follows a two-step pathway: diazotization of the primary aryl amine in an acidic medium and coupling with an activated aryl species, such as naphthol, in a basic medium. The diazotization step takes place under acidic conditions at reduced temperatures, and the coupling agent is introduced under basic conditions. The general reaction for this reaction is illustrated in Scheme 2.3.
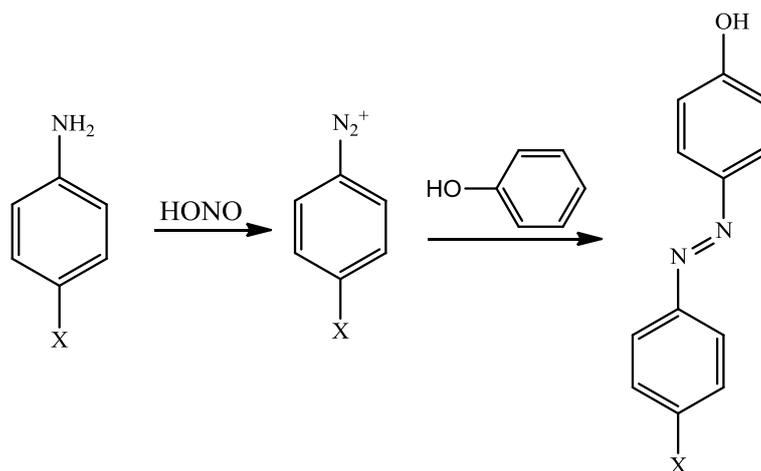
Scheme 2.3 General reaction pathway for the formation of azo dyes

2.5   BIOLOGICAL ACTIVITY OF *AZO* COMPOUNDS

The biological activity of *azo* compounds arises mostly from the particular metabolic pathways, the *in vivo* enzymatic reduction of the *azo* bonds. This leads to the cleavage of the *azo* bond resulting in the releasing of the aryl amine from which the *azo* dye originated. Depending on aryl amine, the resulting product may be more or less toxic than the parent *azo* dye (Chung *et al*., 2008, Pandey *et al*., 2007).

Azo dyes obtained from the benzidine (fig. 2.5), and its derivatives have been shown to possess higher toxicity. Research has demonstrated the carcinogenic activity of benzidine-based compounds (Makena and Chung, 2007, Chung *et al*., 2000).
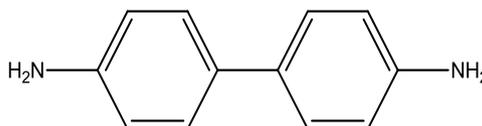
Figure 2.5 Structure of benzidine

The mutagenetic activity of benzidine and its analogues has been reported. For example, the action of intestinal microflorae in humans on the *trisazo* dye

Direct Black 38, fig. 2.6, releases the benzidine moiety (Sponza and Işık, 2005).
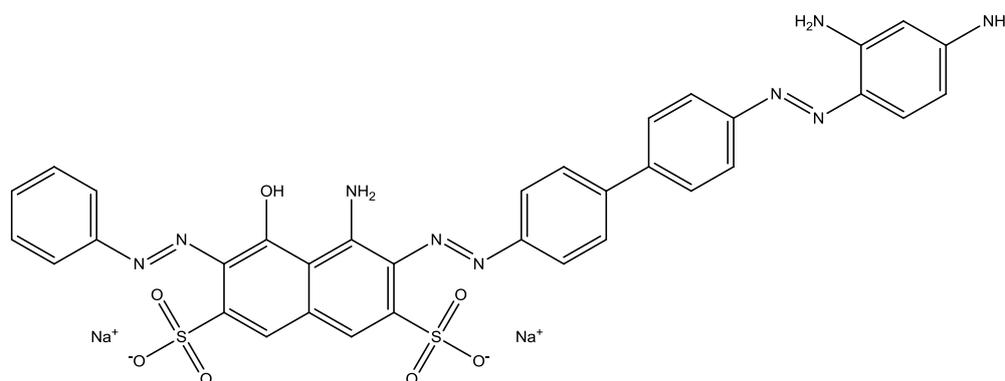
Figure 2.6 Structure of Direct Black 38

In spite of these potential toxicity of *azo* dyes, they also exhibit some encouraging biological activities, conferring on them significant medical use.

Azo dyes and their *in vivo* reduction products play a significant role in the treatment of colon disease such as colitis and irritable bowel syndrome, by acting as specific site delivery drugs. In these drugs, the reduction of the *azo* bond takes place in the colon, making them very site-specific. As a result, drug designers are able to develop target therapy medicines. As example is the *azo* derivative of 5-aminosalicylic acid immobilized on a polyethylene glycol (PEG) matrix, fig. 2.7, which possess anti-inflammatory and cytoprotective activity (Garjani *et al.*, 2004).
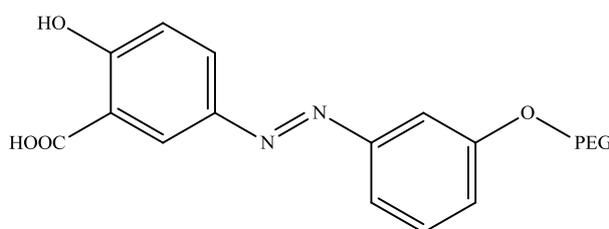
Figure 2.7 Structure of PEG immobilised on a dye

2.5.1 Antiviral Activity

The bis*azo* compound, known and referred to as FP-21399 (fig. 2.8) has demonstrated considerable anti-human immunodeficiency virus (HIV) activity. This drug works by blocking the entry of the virus into the CD4+ cells and also blocks the fusion of infested cells with the healthy ones. The CD4 (cluster differentiation 4) cell is a glycoprotein located on the outer surface of the white blood cells and helps fights infections. After ingestion, FP-21399 is found in much concentrations in the lymph nodes, where a high concentration of HIV and CD4+ are found.

This compound has been demonstrated to be active against all strains of the HIV virus as well as some few resistant strains. Clinical trials have confirmed the anti-HIV activity of the dye (Stellbrink, 2007, Ono *et al.*, 1997).
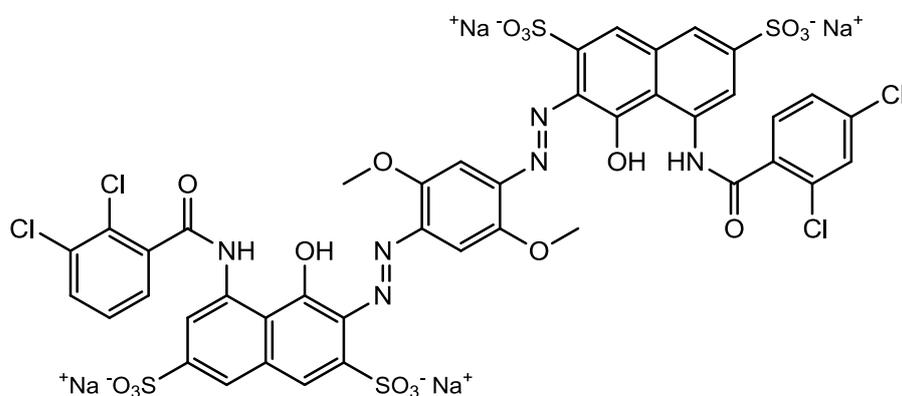
Figure 2.8 Structure of FP-21399

2.5.2 Anti-microbial activity of *azo*-containing Schiff bases

The versatility of *azo* compounds plays a vital role in both fundamental and research applications. Aside their chemical uses, *azo-azo*methine dyes are receiving considerable interest in the plastic, textile and leather sectors.

Azo compounds are able to coordinate with metal ions, the *azo* bond may or may not be involved in the coordination to the ion to form the chelate ring. The extensive use of Schiff base complexes results from the relative stability of such complexes. Metal chelates play vital role in the chemistry of living organisms, a large number of metal-proteins and other metal complexes in biological systems.

Salicyladehyde-derived Schiff bases are polydentate ligands and form coordinates either in the neutral or the deprotonated ionic form. In a study, the antimicrobial potential of such bases was investigated. A number of Schiff ligands were synthesised and complexed with cobalt (II) and copper (IV) (fig. 2.9). The activity of both the ligands and the complexed compounds was determined. It was observed that the metal complexes generally exhibited greater activity against the test organisms than the base ligands. The partial sharing of the charge on the metal ion by the donor atoms leads to an increase in the lipophilicity of the compound, resulting in the increase in the ability of complex to permeate the lipid layer of the cell membrane of the organism. This accounts for the greater activity of the complexes over the ligands (Ispir, 2009).

Ligand
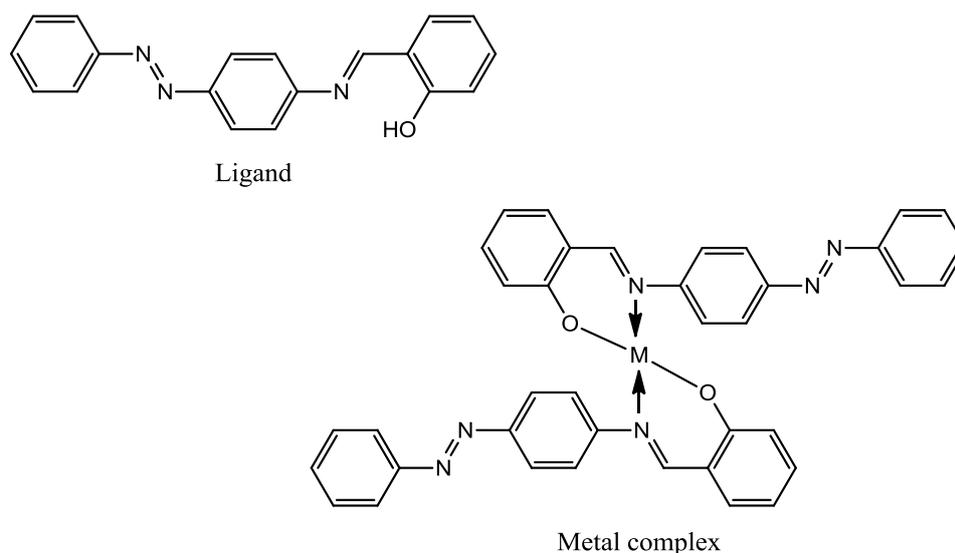
Metal complex

Figure 2.9 Structure of Schiff base ligand and metal complex

From the proposed structure of the complex, the *azo* bond is not involved in the coordination to the metal ion. As the *azo* bond cleaves to release the corresponding amines, the active portion of the dye (Section 2.1), two amines per each molecule of the complex as opposed to one by the ligand, will be released. This obviously explains the increased activity of the complexes – the complexation increasing the rate and ease of transport across cell wall of the organism and the sheer numbers of amines released causing the inhibition.

2.5.3   Anti-microbial activity of *azo*-containing heterocycles

Heterocycle *azo* dyes enjoy a vast range of use as dyestuff in the electronic industries. These applications include colorimetric sensors, nonlinear optical (NLO) devices and liquid crystal display (LCD) used as potential sensitizers for photodynamic therapy (PDT).

In recent times, focus has been shifted to incorporating of *azo* moiety into heterocycle systems. One of such system is 1,3,4-thiadiazole (fig. 2.10) and its derivatives. Antitumor, antibacterial, anti-inflammatory, antimycotic and

antifungal are some of the biological activities exhibited by this nitrogen containing heterocycle. This extensive broad spectrum activity have been attributed to the existence of the toxophoric −N-C-S moiety (Mavrova *et al*., 2009).
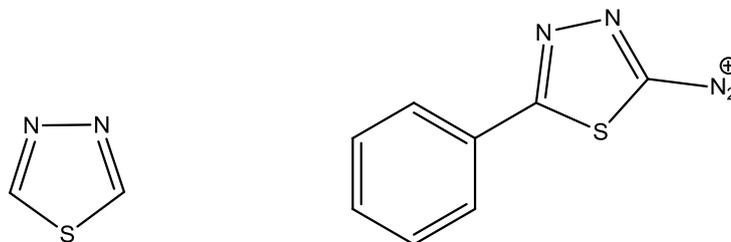
Figure 2.10 Structure of thiadiazole and diazonium salt derivative

Keerthi Kumar *et al* (2013) synthesised and screened for the antimicrobial activity of some *azo* dyes synthesised by the coupling of phenyl amino derivative of 1,3,4-thiadi*azo*le (fig. 2.10) with both heterocyclic and non-heterocyclic aromatic systems. Since all the compounds contain the thiadiazole system, any observed activity may be attributed to the coupling agent. It was observed that the coupling agents with hetero atoms showed a higher activity against the various test organisms than their non-heterocyclic counterparts. For instance, the 8-hydoxyquinoline coupled dye (fig. 2.11) was much active against *S. aureus,* with an MIC of 25μg/ml, with the 2-naphthol dye having an MIC of 200μg/ml against the same organism. Also, an MIC of 50μg/ml and 400 μg/ml was obtained for the amino pyridine and methyl aniline dyes respectively against *P. aeruginosa*. This goes to show that the presence of the hetero atoms in the ring contributes to the observed activity of the dyes. (Keerthi Kumar *et al*., 2013). The presence of the lone pairs of electrons on the hetero atom may have contributed to the observed activity of these compounds. However, there is the need to compare similar structured

heterocyclic compounds to investigate which will offer better activity, which will guide future drug development.
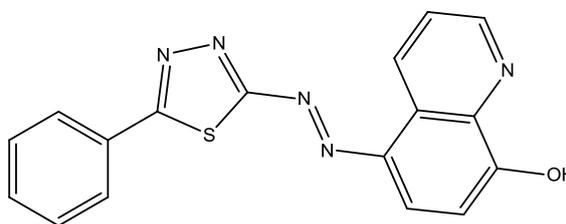
Figure 2.11 Structure of 8-hydroxy quinolone coupled thiazole derivative

Benzothi*azo*le (fig. 2.12) and derivatives have demonstrated variety of biological activity, including antihistamine, antitumor and antibacterial.
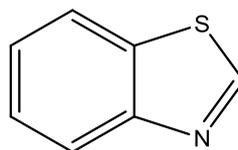
Figure 2.12 Structure of benzothiazole

Kumar *et al*. (2013) also investigated on the activity of various derivatives of phenyl amide and naphthols coupled with the amino benzothi*azo*le. The methoxy derivative of the amide (fig. 2.13) showed a greater activity by inhibiting all test organisms. This can be attributed to the greater non-polar character of the ether functionality over the halogenated and nitro groups. Also the naphthol dyes exhibited a higher activity over the other phenolic counterparts. It can be said therefore that the extra aromatic ring on the naphthol enhances the lipid solubility of the dye (Keerthi Kumar *et al*., 2013)
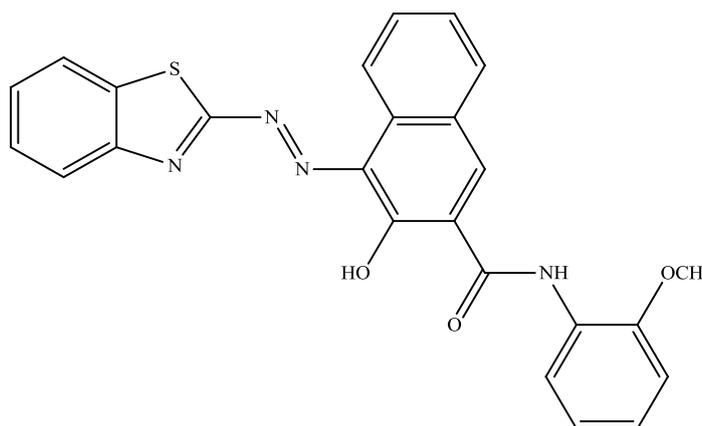
Figure 2.13 Structure of the benzothiazole coupled methoxy amide derivative

Although the methoxy derivative exhibited the greatest activity, careful investigation of the derivatives would reveal that the substitution pattern was not the same. For instance, the nitro derivative has the nitro substituent *para* to the amide bond. It is conceivable that steric hindrances will make the compound shown in fig 2.13 able to withstand modification by cell matter of some organism than if it were a *para* substituted. As a result for a more general and direct comparison to be made, the substituents should have been at the same positions.

2.5.4 Anti-microbial activity of naphthol *azo* dyes

Most dyes in use consist of the *azo* and naphthalene moieties. However their antimicrobial activity are less exploited.

Swati *et al.* (2011) studied the effect of substitution on various anilines coupled with 2-naphthol. It was observed that the carboxylic acid and hydroxyl aniline derivatives showed a greater antimicrobial activity. Also, the presence of a chloro group, *ortho* to the *azo* bond contributed nothing to the activity of the dye, the same MIC was obtained against all the test organisms

21

as the unsubstituted anilines. The methoxy derivative demonstrated a greater inhibition against the gram negative organisms used with *E. coli* being the most susceptible with an MIC of 35µg/ml obtained against this organism. The hydroxy derivative was more active against the gram negative *B. subtilis* with an MIC of 35µg/ml (Swati *et al*., 2011).

In a recent study, the substituents effect of some naphthol and phenolic *azo* dyes were investigated. The 1-naphthol moiety was shown to be more active than the 2-naphthol and phenolic systems. As discussed earlier, the extra lipophilicity offered by the naphthol contribute to the higher activity over the phenol. The structures of the most active compounds, *p*-ABAαN and *p*-NaαN, are shown in fig. 1.2 (Sec 1.2)

a   b
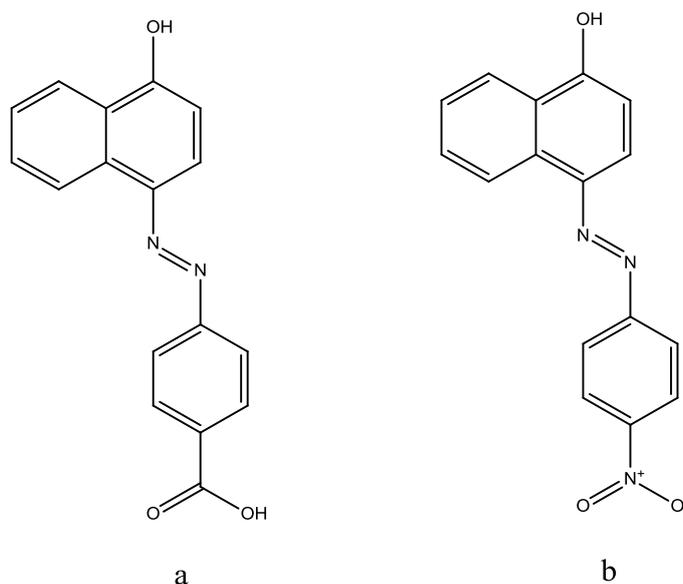
Figure 2.14 Structure of p-ABAαN (a) and p-NAαN (b)

Of the two, *p*-NAαN exhibited greater activity by recording a minimum inhibitory concentration (MIC) of 20µg/ml against *C. albicans* (Kofie *et al*., 2015). The focus of the study was the investigation of the effect of substituents

on the activity. However, from the critical examination of the structures in fig 1.2, it can be observed that the carboxylic acid functional group, the hydroxyl group of the naphthol as well as the aromatic ring provides suitable sites for structural modification. Exploration of the possible modifications with the corresponding activity, therefore, forms the basis for this study.

CHAPTER THREE

MATERIALS AND METHODS

3.1   MATERIALS

3.1.1   Reagents

Table 3.1 Reagents and materials used with their sources

Materials/Reagents	Source
Petroleum ether 40/60	BDH
1-Naphthol	BDH
2-chloro-4-nitroaniline	BDH
p-nitroanile	BDH
4-aminobenzoic acid	BDH
Ethyl-p-aminobenzoate	BDH
Ethyl acetate	BDH
Potassium hydroxide (KOH)	BDH
Ethyl bromide	BDH
Potassium iodide	BDH
Sodium nitrite	VWR Chemicals
Sodium thiosulphate penta hydrate	BDH
Sodium hydroxide	Fisher Chemical
Sodium chloride	BDH
Acetic anhydride	BDH
Hydrochloric acid	BDH
Hexane	Fisher Chemical
Silica gel pre-coated thin layer chromatography (TLC) plates	Alugram Sil G
Ethanol (96%)	BDH
Methanol	BDH

3.1.2 Equipment and Instrument

The Infra-Red (IR) spectra were obtained with a PerkinElmer Spectrum Two Spectrometer in the 4000-400 cm^{-1} range. The ^{1}H NMR spectra were obtained with a Bruker 400 Spectrometer with a frequency of 400 MHz and the MS data were obtained with an Openlynx LCMS Generic Chromatograph. The melting points were determined by the open capillary method with a Stuart Digital SMP10 melting point apparatus. A reflux system with a circulator chiller and water bath from Buchi were also used. All structures were sketched with the aid of ChemDraw Ultra 12.0 software.

3.2 METHODS

3.2.1 General procedure for the formation of the diazonium salt
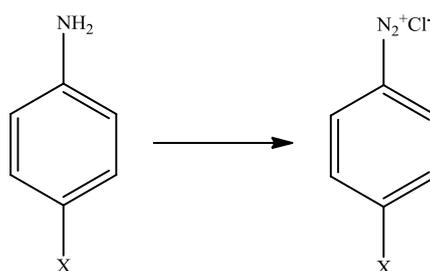
In a 250ml volumetric flask, primary aromatic amine (1 eq) was dissolved with a mixture of 15ml of 2M hydrochloric acid (HCl) and 5ml of water. The resulting solution was immersed in an ice bath and the temperature maintained below 5°C. 50ml of 1M sodium nitrite (NaNO$_2$) solution was added gradually to this solution with constant stirring whiles keeping the temperature below 5°C. Any precipitate formed was filtered off and discarded. The salt was covered and kept in the ice bath.

3.2.2 General procedure for the coupling reaction
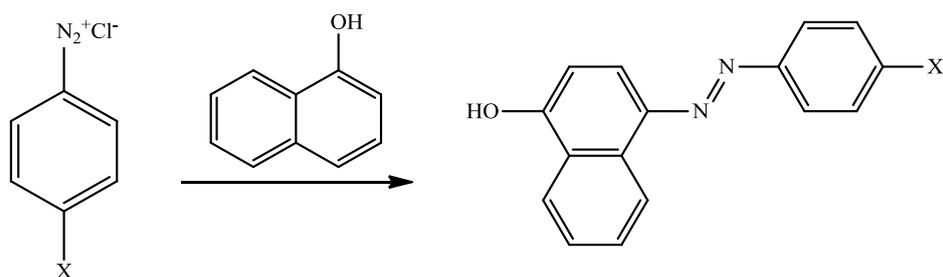
In a 250ml beaker, 1 eq of 1-naphthol was dissolved in 50ml of 3M sodium hydroxide (NaOH), cooled to below 5°C by immersing in an ice bath with stirring. The cold diazonium solution was slowly added to the naphthol solution while stirring, ensuring that the temperature is always below 5°C. Coloured crystals of the azo compound developed. After the addition of all the diazonium salt, the mixture was left in the ice bath for about 30 minutes with occasional stirring. The mixture was suction filtered and the solid crude crystals dried. The crude was purified by either recrystallization from appropriate solvent or column chromatography and the pure compounds obtained and allowed to dry.

(E)-4-((4-hydroxynaphthalen-1-yl)diazenyl)benzoic acid
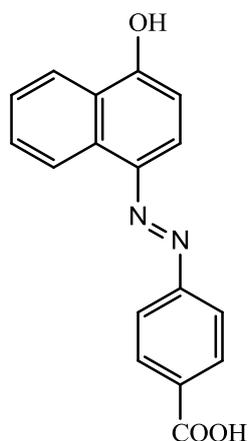
The product was obtained in (2.44g, 83.7%) as a dark brown solid.

Rf.: EtOAc: MeOH, 8:2 (0.64)

mp 287-289ºC, Lit 288-290ºC

IR (V_{max} cm^{-1}): 3373.4, 1681.98, 1497.55, 1402.99, 1159.22, 1243.61.

^{1}H NMR (400MHz, DMSO-d$_6$) δ ppm: 6.90 (1H, d, J=16Hz, Ar-H$_a$), 8.1 (1H, d, J=16Hz, Ar-H$_b$) 8.78 (2H, d, J=8.0Hz, Ar-H$_c$), 8.23 (2H, d, J=8.0Hz, Ar-H$_d$), 8.17 (1H, d, J=8Hz, Ar-H$_e$), 7.86 (1H, d, J=8Hz Ar-H$_f$) 7.97 (1H, m, Ar-H$_g$), 7.61 (1H, m, Ar-H$_h$).

m/z (ES): 293 (M$^+$ + H), 246.9 (M$^-$ - CO$_2$H), 135.0 (M$^+$ - C$_{10}$H$_7$NO)

(E)-ethyl-4-((4-hydroxynaphthalen-1-yl)diazenyl)benzoate
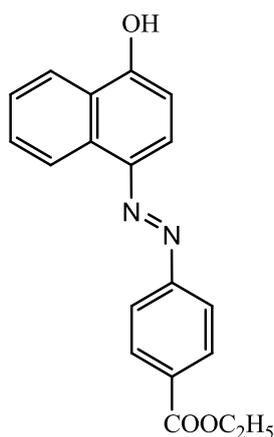
The product was obtained in (2.395g, 74.8%) as a dark brown solid.

R$_f$.: EtOAc: MeOH, 8:2 (0.57)

Mt pt. 245-247

IR (V_{max} cm^{-1}): 3376.82, 1380.25, 1169.33

^{1}H NMR: (400MHz, DMSO-d$_6$, δ ppm): 1.23 (3H, t, J=4.8 Hz, **CH$_3$**), 4.1 (2H, q, J=4.4 Hz, **CH$_2$**), 6.15 (1H, d, J= 6.8Hz, Ar-H$_c$), 7.60 (1H, d, J= 6.8Hz, Ar-H$_d$), 8.72 (2H, d, J=6Hz, Ar-H$_e$), 7.90 (2H, d, J =6Hz, Ar-H$_f$), 8.15 (1H, d,

J=3.6Hz, Ar-H$_g$), 8.04 (1H, d, J=4.0Hz, Ar-H$_h$), 7.45 (1H, m, Ar-H$_i$), 7.19 (1H, m, Ar-H$_J$)

m/z (ES): 293 (M$^+$ - CH$_2$CH$_3$), 275.5 (M$^-$ - C$_2$H$_5$O +1), 247.0 (M$^-$-COOC$_2$H$_5$+1)

(E)-4-((2-chloro-4-nitrophenyl)diazenyl)naphthalen-1-ol
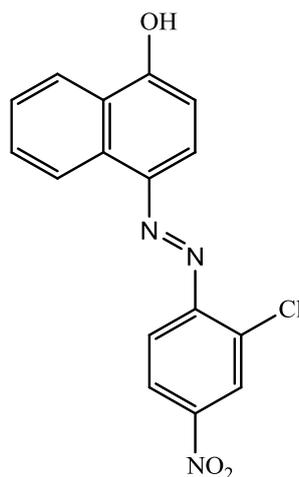
The product was obtained in (3.09g, 94.5%) as a dark reddish solid.

Rf.: EtOAc: MeOH, 8:2 (0.62)

Mp: 183-185°C

IR (V$_{max}$ cm^{-1}): 3378.14, 1637.35, 1584.75, 1538.05, 1512.76, 1406.79, 1276.23, 1168.6

^{1}H NMR: (400MHz, DMSO, δ ppm): 6.40 (1H, d, J=4Hz, Ar-H$_a$), 7.98 (1H, d, J=4Hz, Ar-H$_b$), 8.60 (1H, d, J=4.4Hz, Ar-H$_c$), 8.19 (1H, s, Ar-H$_d$), 8.41 (1H, d, J=4.8Hz, Ar-H$_e$), 7.40-7.30 (1H, m, Ar-H$_f$), 8.12-8.00 (1H, m, Ar-H$_g$), 7.65-7.50 (2H, m, Ar-H$_h$)

m/z (ES): 325.9 (M$^-$), 281.1 (M$^-$ - 46), 183.9 (M$^-$ - 142), 168.9 (M$^-$ - 157)

(E)-4-((4-nitrophenyl)diazenyl)naphthalen-1-ol
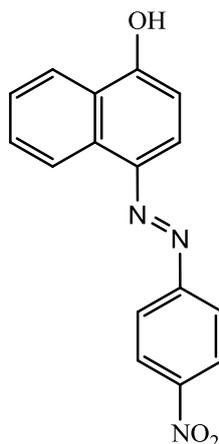
The product was obtained as a dark brown solid in (2.61g, 88.9%)

Rf: EtOAc: MeOH, 8:2 (0.68)

Mp. 124-126ºC, lit 123-125ºC

IR (V_{max} cm^{-1}): 3367.1, 1594.29, 1502.28, 1258.74, 1167.5.

^{1}H NMR: (400MHz, DMSO, δ ppm): 6.82 (1H, d, J=8Hz Ar-H$_a$), 8.09 (1H, d, J=8Hz, Ar-H$_b$), 8.53 (2H, d, J=7.2Hz, Ar-H$_c$), 8.33 (2H, d, J=7.2Hz, Ar-H$_d$), 8.46 (1H, d, J=5.6Hz, Ar-H$_e$), 7.83 (1H, m, Ar-H$_f$), 7.66 (1H, m, Ar-H$_g$), 7.76 (1H, d, J=5.6Hz, Ar-H$_h$).

m/z (ES): 292.0 (M$^-$, 100%) 137.0 (M$^-$ - C$_6$H$_4$N$_2$O$_2$+1), 144.8 (M$^-$- C$_{10}$H$_8$O)

(E)-4-((4-chlorophenyl)diazenyl)naphthalen-1-ol
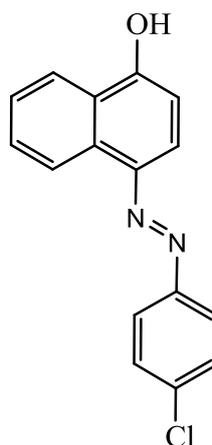
The product was obtained in (2.69, 95.4%) as a dark brown solid.

Rf.: EtOAc: MeOH, 8:2 (0.65)

IR (V_{max} cm^{-1}): 3300, 1596.69, 1479.22, 1404.11, 1272.28, 1155.61.

^{1}H NMR: (400MHz, DMSO, δ ppm): 6. 98 (1H, d, *J*= 12Hz Ar-**H$_a$**), 7.85 (1H, d, *J*=12Hz, Ar-**H$_b$**), 7.66 (2H, d, *J*=10Hz, Ar-**H$_c$**), 8.64 (2H, d, *J*=10Hz, Ar-**H$_d$**), 7.52-7.48 (2H, m, Ar-**H$_e$**), 8.02-7.97 (2H, m, Ar-**H$_f$**)

m/z (ES): 282.9 (M$^+$, 100%), 298.8 (M$^-$ + H$_2$O), 138.9 (M$^-$ - C$_{10}$H$_7$O)

3.2.3 Procedure for formation of the acetylated products
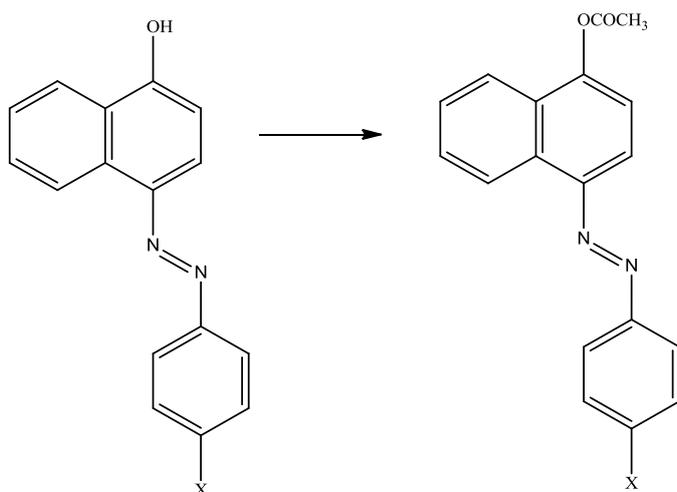
In a 150ml round bottom flask, 5ml of 25% NaOH was added to the *azo* compound (1 eq) and stirred for 15 minutes at room temperature. To the resulting solution, acetic anhydride (1 eq) was added and stirred for a further 1 hour at room temperature. The solid crude was filtered off and washed with cold water. The pure crystals was obtained after recrystallization from ethanol and ethyl acetate.

(E)-4-((4-nitrophenyl)diazenyl)naphthalene-1-yl acetate
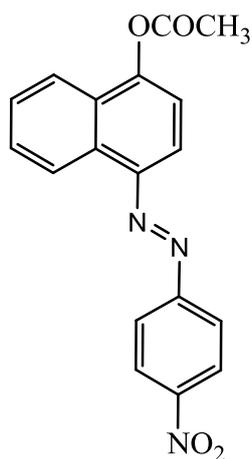
The product was obtained as a dark brown solid in (250mg, 74.6%)

Rf.: Pet: EtOAc, 6:4 (0.61)

Mp. 147-150°C

IR (V$_{max}$ cm^{-1}): 3267.51, 1628.23, 1594.61, 1547.6, 1502.56, 1259.51.

(E)-4-((4-acetoxynaphthalen-1-yl)diazenyl benzoic acid
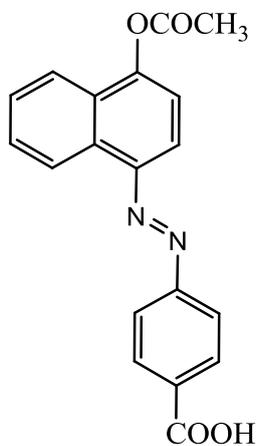
The product was obtained as a dark brown solid in (238mg, 71.3%).

Rf.: Pet: EtOAc, 6:4 (0.54)

Mp. 250-252°C

IR (V$_{max}$ cm^{-1}): 3359.22, 1738.21, 1497.55, 1402.99, 1159.22, 1243.61

3.2.4 Procedure for the formation of the alkylated products
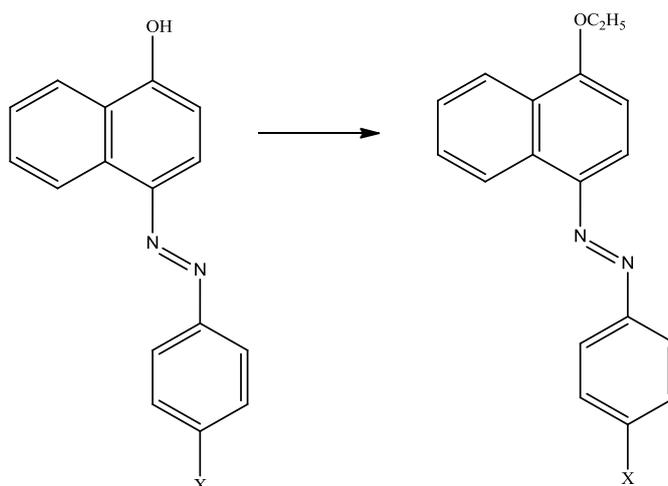
In a 150 ml round bottom flask, 10 ml of methanol was added to the *azo* dye (1 eq). 5 ml of 25% KOH was added and stirred at room temperature for 15 minutes. To this solution, ethyl bromide (2 eq) and potassium iodide (1.5 eq of ethyl bromide) was added and stirred for two hours at 90-95°C. The mixture was then transferred into a separatory funnel and shaken with 25 ml of 1M sodium thiosulphate solution. The organic layer was then extracted with three 15 ml portions of ethyl acetate, washed with saturated sodium chloride solution and dried. The crude was purified by recrystallization from ethanol (70%).

(E)-1-(4-ethoxynaphthalen-1-yl)-2-(4-nitrophenyl)diazene
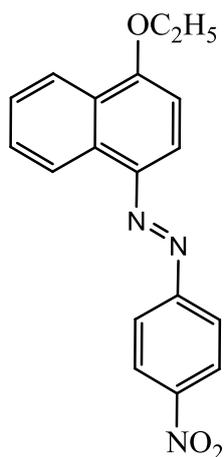
Using the above procedure, the product was obtained in (252mg, 78.5%) as a

dark solid.

Rf.: Hexane: EtOAc, 9:1 (0.71)

Mp. 121-123°C

IR (V_{max} cm^{-1}): 1486.9, 1254.26, 1210.15, 1147.19.

(E)-4-((4-ethoxynaphthalen-1-yl)diazenyl benzoic acid
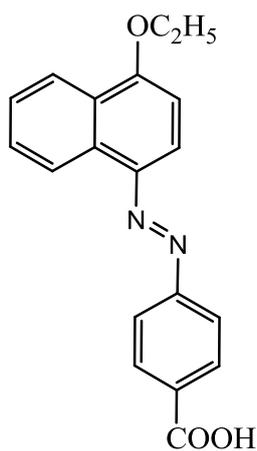
The product was obtained as dark brown solid in (252mg, 72.7%)

Rf.: Hexane: EtOAc, 9:1 (0.65)

Mp. 102-104°C

IR (V_{max} cm^{-1}): 3359.22, 1738.21, 1497.55, 1402.99, 1159.22, 1263.25

3.2.5 Antimicrobial Assay

Various concentrations of the synthesised compounds (5000, 2500, 1250, 625, 312.5, 156.25, 78.13, 39.07µg/mL) were prepared by serial dilution using methanol: water (50:50) as diluting solvent.

Using a micropipette, 100µL of the sterile nutrient broth was transferred into each well of the micro titre plate. 90µL of each of the test solution was added to the broth in the wells and labelled appropriately. 10µL of each of the test microorganism was then dispensed into the appropriate well and the plate covered and incubated at 37°C for 18-24 hours. After the inoculation, the resultant concentration of the test compounds in each well were 2250, 1125, 562.50, 281.25, 140.63, 70.32, 35.16 and 17.58 µg/mL. The results were recorded and the Minimum Inhibitory Concentration (MIC) determined for each test compound.

CHAPTER FOUR

RESULTS

4.1 SYNTHESIS

Table 4.1 Synthesised azo compounds with their physical data

Compound	Code	Mol. Weight	% Yield	Melting pt, ºC	R$_f$
	KA 001	292.29	83.7	287-289ºC (EtOAc:EtOH)	0.64 (EtOAc:MeOH)
	KA 002	320.34	74.8	245-247	0.57 (EtOAc:MeOH)
	KA 003	327.74	94.5	183-185 (CH$_3$Cl)	0.62 (EtOAc:MeOH)
	KA 004	293.28	88.9	124-126 (EtOAc)	0.68 (EtOAc:MeOH)
	KA 005	282.72	95.4	151-154 (EtOH)	0.65 (EtOAc:MeOH)
	KA 006	335.31	74.6	147-150 (EtOAc:EtOH)	0.61 (Pet:EtOAc)
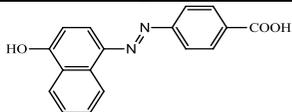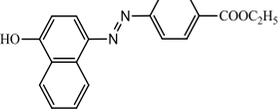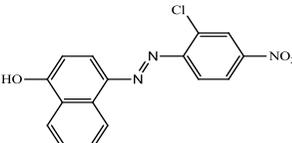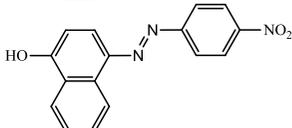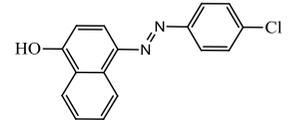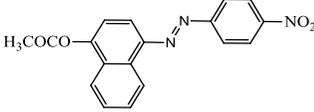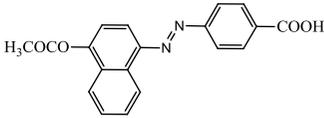
Structure	Code	MW	% Yield	Melting point (°C)	R_f
H₃COCO—naphthyl—N=N—C₆H₄—COOH	KA 007	334.33	71.3	250-252 (EtOAc:EtOH)	0.54 (Pet:EtOAc)
C₂H₅O—naphthyl—N=N—C₆H₄—NO₂	KA 008	321.33	78.5	121-123 (Hexane:EtOAc)	0.71 (Hexane:EtOAc)
C₂H₅O—naphthyl—N=N—C₆H₄—COOH	KA 009	320.34	72.7	102-104 (Hexane:EtOAc)	0.65 (Hexane:EtOAc)
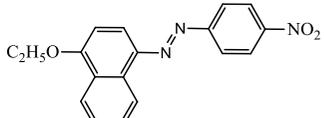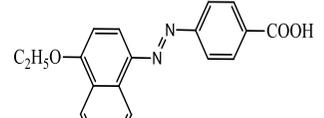
From Table 4.1, all the synthesised dyes were purified by recrystallization from their respective solvents, except KA 002 which was column chromatographed on silica gel. The solvents underneath the melting point values represent the solvents from which recrystallization was done.

4.2 ANTIMICROBIAL ASSAY

Table 4.2 Minimum Inhibition Concentrations (MIC) of the synthesised dyes

	MIC (µg/ml)				
Organisms / Compounds	*S. aureus*	*S. pyrogenes*	*S. typhi*	*P. aeruginosa*	*C. albicans*
KA 001	562.5	562.5	1125	281.5	**140.13**
KA 002	**70.32**	281.5	281.5	562.5	281.5
KA 003	140.63	140.63	**70.32**	140.63	**70.32**
KA 004	281.5	140.63	140.63	281.25	**70.32**
KA 005	1125	562.5	562.5	**140.63**	281.50
KA 006	1125	281.5	281.5	**140.63**	281.50
KA 007	**281.5**	140.63	1125	562.50	562.50
KA 008	70.32	**35.16**	70.32	**35.16**	70.32
KA 009	140.63	140.63	**70.32**	**70.32**	140.63
Cefuroxime axetil	70.32	**35.16**	**35.16**	**35.16**	70.32

All the synthesised compounds demonstrated encouraging antimicrobial activity, with the alkylated derivatives being the most active compounds. Acetylation results in the formation of a less active compound. Replacing the nitro group in KA 004 with a chloro group leads to a reduction in the activity.

CHAPTER FIVE

DISCUSSIONS, CONCLUSION AND RECOMMENDATIONS

5.1   DISCUSSION

5.1.1   Synthesis

Azo compounds have been shown to possess a variety of biological activity including antimicrobial, antiviral, and antifungal among others. The first report of the synthesis of an *azo* compound happened in 1856. However, the derivatization of these compounds is largely unexplored. Recently in our research group, a number of *azo* compounds were synthesised and two of these dyes exhibited encouraging antimicrobial activity, fig. 5.1 (Sec 1.2).
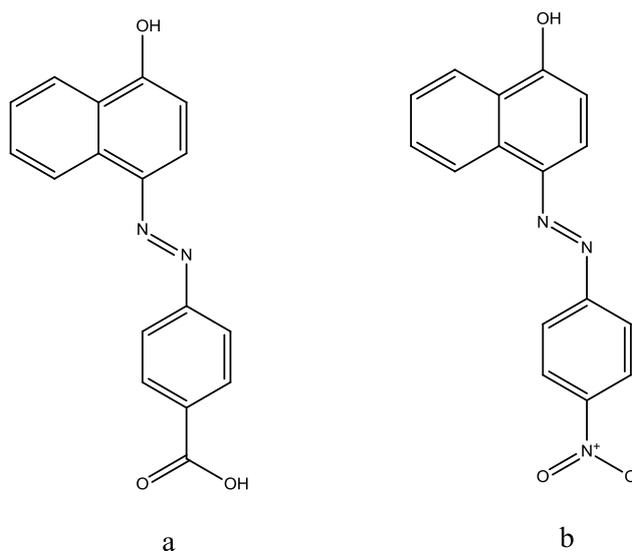
Figure 5.1 Structure of p-ABAαN (a) and p-NAαN (b)

As a medicinal chemist, considering the structure of the two compounds, the hydroxyl group on the naphthol provide a suitable site for structural modifications, such as the formation of an acetylated and alkylated derivatives of the dyes. Also, the presence of the carboxylic acid in *p*-ABAαN makes it favourable for the esterification of this compound. The substitution with a

halogen on the aromatic ring of *p*-NAαN is also a possible structural transformation to be explored.

The Directly Coupled Dyes

By employing the direct and well known route, the coupling between the di*azo*nium salt and naphthol under carefully controlled conditions of temperature and pH, five *azo* compounds were successfully synthesised and in very good yield. The general scheme for this process is shown in scheme 5.1 (section 2.4.2)
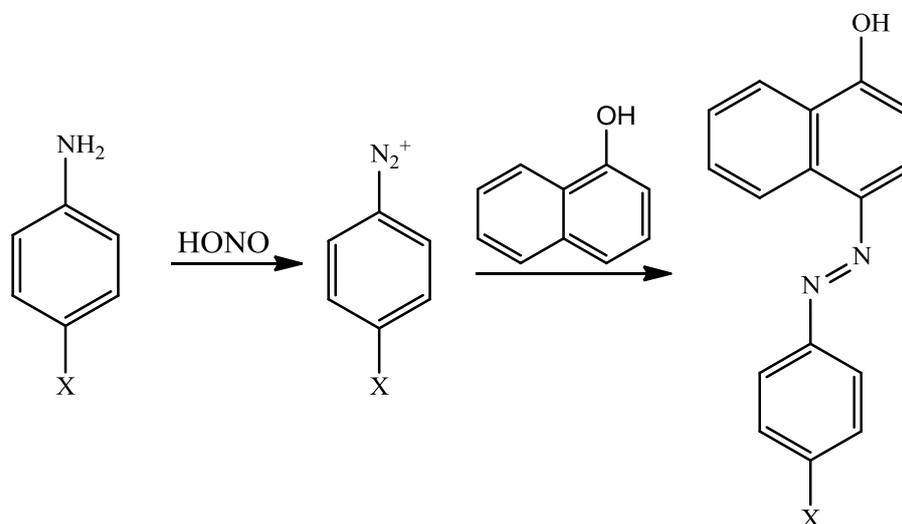
Scheme 5.1 General reaction pathway for the formation of azo dyes

The formation of the diazonium salt was detected by the instantaneous formation of a blue-black colour when a drop of the solution was tested with a potassium starch-iodide paper. The blue colour formed gave an indication of the presence of excess nitrous acid.

The Acetylated Products

The acetylation products were obtained by the general scheme (Sec. 3.2.3). This method for the acetylation was used in this work, owing to the readily availability of the reagents as well as the time taken to run the whole

40

experiment. From literature, a non-solvent method with a very high yield requires the use of a specialised catalyst, polystyrene-supported dimethylaminopyridine (DMAP). Also this procedure requires a maximum of 69 hours to produce the product. Coupled with the unavailability of the catalyst, the procedure used in this work requires stirring at room temperature to obtain the desired products.
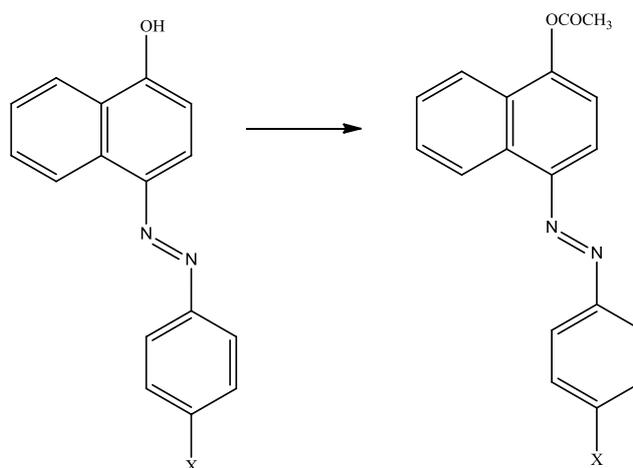
Scheme 5.2 General scheme for the synthesis of the acetylated products

The Alkylated Products

The alkylated products were synthesised *via* the Williamson's synthesis procedure, the synthesis of an ether from an alcohol and alkyl halide, scheme 4.2. From our preliminary work, it was observed that when sodium hydroxide (NaOH) was used in the deprotonation of the hydroxyl of the naphthol, after reaction with the alkyl halide, a product with a lower rf than the starting material resulted. The implication of this was that the product formed was more polar than the starting material, which should not be the case since the ether product is expected to be less polar than the alcoholic starting material. To explain this observation, it can be said that the ether product formed was again hydrolysed by any excess NaOH, which is a much stronger base, present

in the reaction mixture, leading to the formation of the deprotonated naphthol product, which is more polar than the starting naphthol material, as shown in appendix J. As a result, a milder base, potassium hydroxide (KOH) was used.

Also, potassium iodide was added to the reaction mixture to convert the ethyl bromide to a more reactive ethyl iodide *in situ.* This conversion is referred to as the Finkelstein reaction (Caron, 2011). This conversion leads to the formation of molecular iodine which is non-polar. In order to easily separate the formed iodine from the desired product, sodium thiosulphate was added to convert the non-polar iodine into a more polar iodide form and extracted into the aqueous phase.
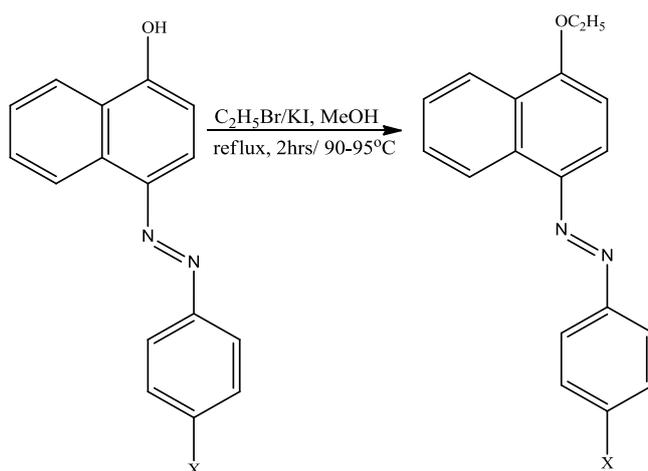
Scheme 5.3 General scheme for the synthesis of the alkylated products

5.1.2 Spectral Analysis

Directly Coupled Compounds

The Infra-Red (IR) region of the electromagnetic spectrum covers the range 4000 – 400 cm^{-1}. Interaction between the molecules and light of this wavelength provides important information about the functional groups present in the molecule.

From the IR spectrum for compound KA 001 (appendix A1), some major functional groups can be identified by their absorption bands. For instance, the free hydroxyl group the carboxylic acid functionality (-COOH) is indicated by the presence of a broad band peaking at 3373.4 cm^{-1} which slopes into the – CH stretching vibrational region at ~3000 cm^{-1}. The absorption at 1681.98 cm^{-1} indicates the presence of a carbonyl (-C=O) of the carboxylic acid. The vibrational frequency for the *azo* bond (-N=N-) can be observed at 1497.55 cm^{-1}. Vibrational frequencies at 1243.61 cm^{-1} and 1159.22 cm^{-1} represent the – C-O vibrations for the carboxylic acid and naphthol respectively. The C-O of the acid will vibrate at a slightly higher frequency as the alternating of the proton on the two oxygen atoms introduces a slight double bond character into this bond. At this point, it is important to state that this results are consistent with that obtained by Kofie *et al.* (2015).

Mass Spectrometry (MS) differs from the other spectroscopic methods, in that whiles the other methods (IR, UV-Vis and NMR) studies the interaction between the molecules with electromagnetic radiations, in MS, interaction between the molecules with high energy electrons is of interest. This provides useful data about the molecular weight of the molecule. Electrospray

ionization is used in this work because fragmentation is reduced hence the possibility of obtaining the molecular ion peak.

From the MS data for the synthesised compounds, it is important to state that generally, they can be ionised in both the positive and negative modes, producing similar peak intensities in both modes. This is not far-fetched, given that the hydroxyl group on the naphthol can be protonated and deprotonated easily.

Examining the spectrum for KA 001, appendix A2, intense signals are obtained in the positive ionization mode (MS ES+ : 293, 1.4 x 10^6) and negative mode (MS ES- : 291 x 10^6). In the negative mode, an intense peak is observed at m/z of 291.0 which represents an $[M-H]^-$ peak. The molecular weight for this compound is predicted as 292, hence the loss of a proton will result in the formation of a fragment, approximately having a molecular weight of 291. A peak at m/z ratio of 292.9 in the positive mode further, representing the $[M+H]^+$ ion confirms the molecular weight of the compound. The molecular ion peaks in both modes are shown in fig 5.2.

Chemical Formula: $C_{17}H_{13}N_2O_3{}^+$
Exact Mass: 293.09
Molecular Weight: 293.30

a

Chemical Formula: $C_{17}H_{11}N_2O_3{}^-$
Exact Mass: 291.08
Molecular Weight: 291.28

b
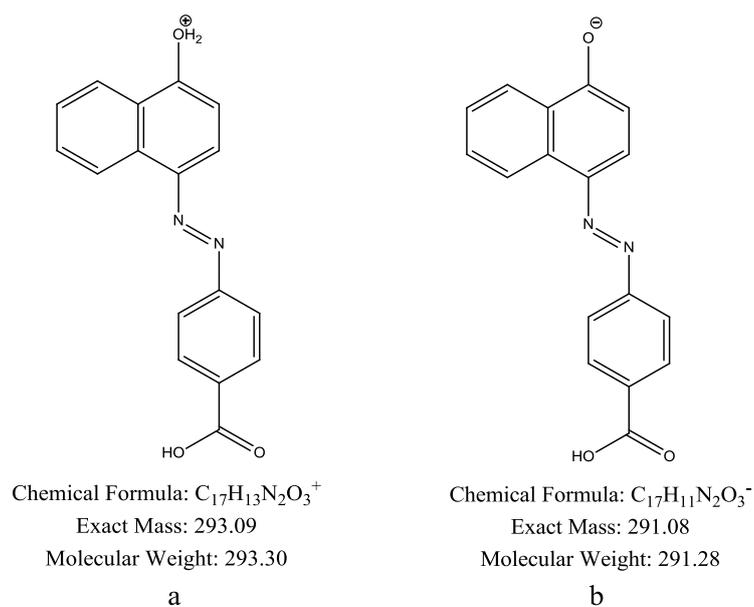
Figure 5.2 Molecular ion in a) positive and b) negative ionization modes

Again, a very intense peak is observed in the positive mode at m/z of 126.0 corresponding to the fragmentation of the naphthalene moiety (fig 5.2). A peak is observed at m/z of 128, which can be attributed to the $[X+2]^+$ peak. This is not far-fetched, since the uptake of 2 protons will result in the formation of the neutral naphthalene molecule. This fragment as a matter of fact is present in the spectra of all the other compounds as well. In addition, a peak corresponding to the loss of the carboxylic acid unit (-COOH) is observed at m/z of 246.9. The obtained fragment is shown in fig 5.3.

Chemical Formula: $C_{10}H_6^{2\bullet}$
Exact Mass: 126.05
Molecular Weight: 126.15
a

Chemical Formula: $C_{16}H_{10}N_2O^{\bullet-}$
Exact Mass: 246.08
Molecular Weight: 246.26
b
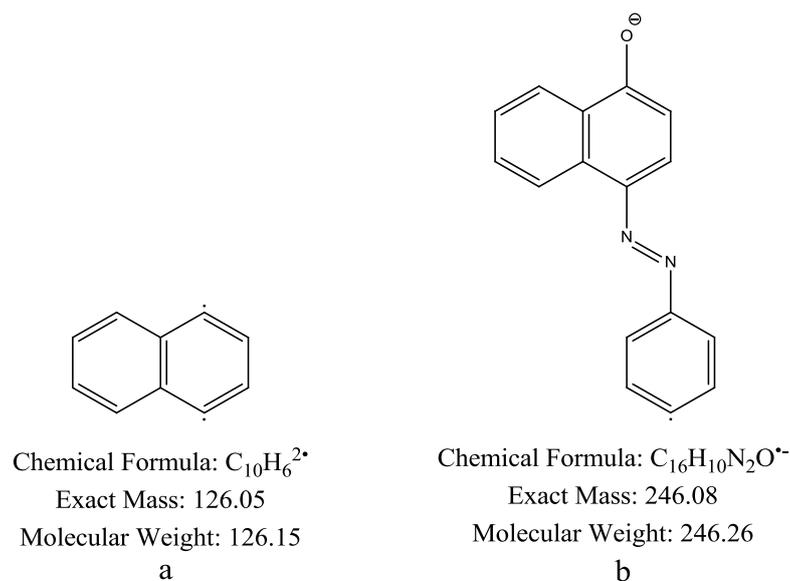
Figure 5.3 Fragment (ion) with m/z of 126.0 (a) and 246 (b)

For a chemist to understand the properties of a compound, it starts by being able to know the structure of the said compound. In recent years, X-ray crystallography has emerged as the tool of choice for determining the complete molecular structure of pure crystalline compounds. NMR spectroscopy follows by aiding the chemist with a direct and general tool for the elucidating the structures of both pure compounds and mixtures, either in solid or liquid forms. In NMR, the magnetic property of the nuclei of the atoms in the compound is harnessed to give information about the chemical shift and splitting patterns to provide useful information about the structure of the compound.

From the ^{1}H NMR spectrum for the compound, appendix A3, a signal at a chemical shift of about 12 was expected for the carboxylic acid proton (-**COOH**). However, this signal is absent, which can be attributed to the fact that looking at the spectrum, it cover from 0 – 9.5 ppm. Also, the naphthol –**OH**, can be seen to have been superimposed by the signal due to water. That

notwithstanding, two doublet signals at chemical shift 6.9 and 8.1 both having integration for 1H are assigned to the protons at C-3 and C-4 respectively. This assignment results from the fact that C-4 proton is closer to the much more electronegative –OH group hence will experience a greater deshielding effect hence it appearing further downfield than the C-3 proton. Two doublet signals with integration for 2H at 8.78 and 8.23 are attributed to the protons at C-1 and C-2 respectively. In this case too, the proximity of the C-1 proton to the deshielding –COOH group shift it further downfield in the spectrum. Again, two doublets are 8.17 and 7.86 both with integration for 1H are assigned protons at C-5 and C-8 respectively. Here also the C-5 proton can be seen to be closer to a deshielding hydroxyl group, hence the assignment. Finally, there are two multiplet signals resulting from the C-6 and C-7 protons at 7.87-7.85 and 7.67-7.64 respectively.

For the compound KA 002, from the IR spectrum (appendix B1), a broad band at 3376.82 cm^{-1} signifies the absorption vibrations of the naphthol –OH. There is a –C=O absorption at about 1720 cm^{-1} which gives an indication of the presence of an ester functional group. When compared with that of KA 001, the carbonyl for the ester vibrates at a higher wavenumber than that of the carboxylic acid. This results from the fact that in the carboxylic acid, the hydrogen atom alternates between the two oxygen atoms. This leads to the introduction of a somewhat single bond character in its carbonyl functionality, reducing the energy required to cause vibrations in this molecule.

To compliment this observation, the MS for the compound was obtained, appendix B2. The molecular weight for this compound is predicted to be 320.

A molecular ion peak occurring at m/z of 321 in the positive mode and 319 in the negative mode was expected. However this is not observed, and can be attributed to the fact that ester linkages are generally weak bonds and breaks relatively easily. This fact can be ascertained by the presence of an intense peak at m/z ratio of 293.0 resulting from the cleavage of the ethyl ($-C_2H_5$) group, an [M-X]$^+$ ion, resulting in the formation of the carboxylate ion (fig 5.4). A similar fragment with an m/z of 291.0 corresponding to the [X-H]$^-$ in the negative mode.
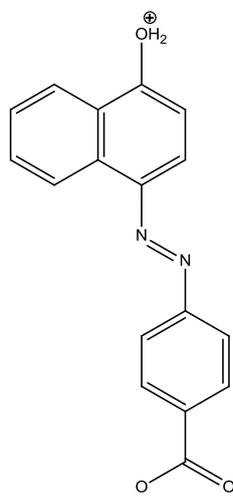
Chemical Formula: $C_{17}H_{12}N_2O_3^{\bullet+}$
Exact Mass: 292.08
Molecular Weight: 292.29

Figure 5.4 Fragment (ion) with m/z of 292.3

There is a peak at m/z of 247.0 which can be attributed to the [M-COOC$_2$H$_5$+1]$^-$ fragment. A peak at m/z of 177.2 can be attributed to the fragment obtained after the cleavage of the naphthol system. These two fragments are shown in fig. 5.5.

Chemical Formula: $C_{16}H_{10}N_2O^{\bullet-}$
Exact Mass: 246.08
Molecular Weight: 246.26

a

Chemical Formula: $C_9H_9N_2O_2^{\bullet}$
Exact Mass: 177.07
Molecular Weight: 177.18

b
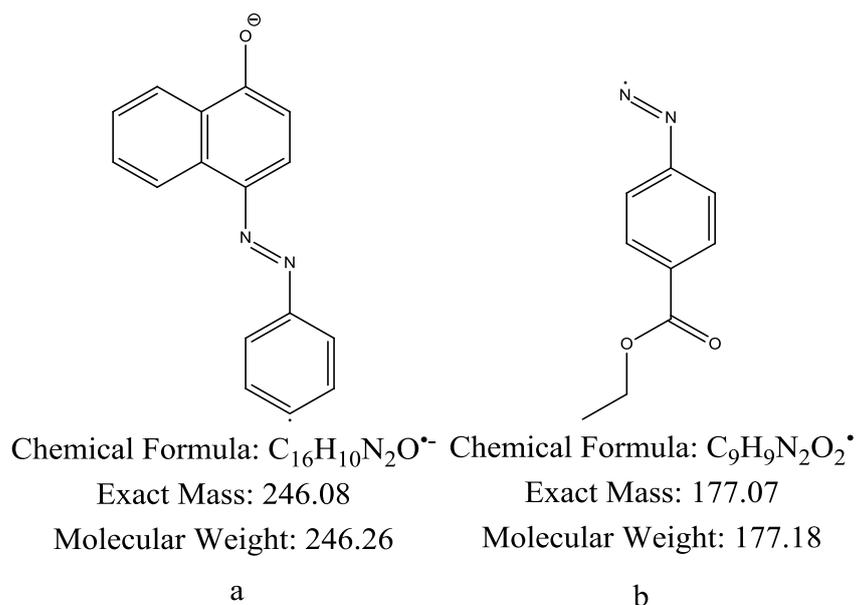
Figure 5.5 Fragment (ion) with m/z of (a) 246.3 and (b) 177.2

Investigation of the ^{1}H NMR spectrum for this compound, appendix B3, revealed the presence of a triplet signal, with an integration for 3H, at the chemical shift of 1.23 resulting from the protons on the methyl group (C-1). A quartet signal due to the methylene (-CH$_2$-) protons, is also observed at chemical shift 4.1 with an integration for 2H. The quartet signal arising from the splitting experienced by the neighbouring protons on the methyl group on C-1. Also a chemical shift of 6.15 and 7.60 corresponds to the signals of the protons at C-3 and C-4 respectively, both integrated for 1H. The strongly deshielding effect, results in the downfield shift of the C-4 signal, as a result of the close proximity to the hydroxyl group. Two doublets, with integration for 2H each, corresponding to the C-5 and C-6 protons appear at 7.90 and 8.72 respectively. Again, here too, the ester functional group causes the downfield shift of the C-6 protons. In addition, two doublets corresponding to the C-7 and C-8 protons are observed at 8.15 and 8.04. The signal due to the proton at C-7 appears downfield due to its closeness to the strong deshielding hydroxyl

group. As expected, there are multiplet signals at shifts 7.45 and 7.19 for the C-9 and C-10 protons respectively. The multiplet signal is not far-fetched since each proton is coupled to two other protons.

The IR spectrum for compound KA 003 can be located at appendix C1. From this spectrum, a broad band at 3378.14 indicates the presence of the naphthol – OH group. A peak at $1584.57cm^{-1}$ indicates the presence of the nitro ($-NO_2$) functional group, with the –C-N vibrations appearing at the 1242.36 cm^{-1}. Strong bands at 894.74 and 832.43 cm^{-1}, representing –C-Cl vibrations, give indication of the presence of the chloro group in the compound.

Analysis of the MS data of the compound (KA 003), appendix C2, provided the following observations on the structure for the compound. There is a peak at m/z of 327.9 represented the molecular ion peak, $[M+H]^+$, since the molecular weight for the compound is predicted to be 327, hence a gain of a proton in the positive mode will lead to the formation of the molecular ion. There is another peak at m/z of 329.9 with a percentage abundance of almost a third that at 327.9. This gives an indication of the presence of an isotopic atom, the chlorine atom. The chlorine atom exist naturally as the isotopes ^{35}Cl and ^{37}Cl in the abundance ratio of 75:25 (^{35}Cl: ^{37}Cl). This observation is repeated in the negative mode as well.

The cleavage of the nitro group results in the formation of a fragment (fig 5.6) with m/z of 280.0. Another at m/z 281.1 can be attributed to the formation of an $[X+1]^-$ for this particular fragment.
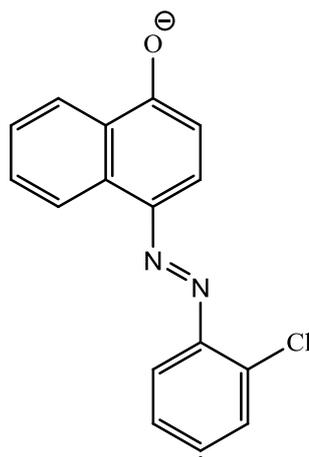
Chemical Formula: $C_{16}H_9ClN_2O^{\bullet-}$

Exact Mass: 280.04

Figure 5.6 Fragment with m/z of 280.0

In addition, at m/z of 168.9, a peak that can be associated with an [X-H]⁻ fragment resulting from the cleavage of the *azo* bond. The loss of the naphthol moiety will result in the fragment with an m/z of 183.9. These fragments are shown in fig 5.7.
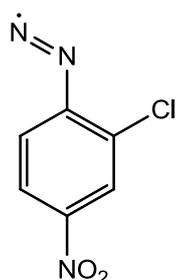
Chemical Formula: $C_6H_3ClN_3O_2^{\bullet}$

Exact Mass: 183.99
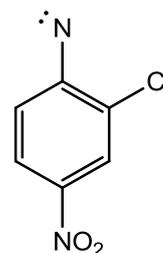
Chemical Formula: $C_6H_3ClN_2O_2^{2\bullet}$

Exact Mass: 169.99

Figure 5.7 Fragment (ion) with m/z of (a) 183.9 and (b) 169.9

To confirm all this observations, the ¹H NMR spectrum was obtained, appendix C3. From the spectrum, a singlet is observed at the chemical shift of 8.19, with integration for 1H, which is as a result of the proton at C-2. The

other two protons at C-5 and C-6 are observed as doublets at chemical shifts 8.60 and 8.41 respectively. The C-6 experiences strong deshielding by the nitro group, hence the downfield shift. The spectrum can be seen at appendix C3.

From the IR spectrum for KA 004, located at appendix D1, vibrational peaks obtained at 1547.51 cm^{-1} signifies the presence of the $-NO_2$ functional group accompanied by the $-C-N$ vibration at 1258.74 cm^{-1}.

The MS data for this compound can be located at appendix D2. The molecular weight for this compound is calculated as 293. In the positive mode therefore, the peak at m/z of 293.9 can be attributed to the $[M+H]^+$. In the negative mode, an $[M-H]^-$ fragment is indicated at the m/z of 292.0 with an $[M-H+1]^-$ peak at 293.0.

An $[X+1]^+$ fragment, corresponding to the ion due to the naphthol moiety is observed at m/z of 144.8 in the positive mode. Also, the cleavage at the *azo* bond will result in a fragment with m/z of 137.0. These two fragments are shown in fig 5.8.
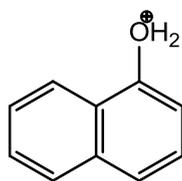
Chemical Formula: $C_{10}H_8O^{•+}$
Exact Mass: 144.06
Molecular Weight: 144.17
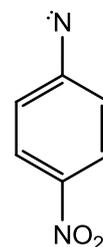
Chemical Formula: $C_6H_4N_2O_2^{2•}$
Exact Mass: 136.03
Molecular Weight: 136.11

Figure 5.8 Fragment (ion) with m/z of 144.8 (a) and 137.0 (b)

From the ^{1}H NMR spectrum, appendix D3, it can be observed that the singlet that was obtained in the spectrum for KA 003 is absent in this particular case. This further confirms the absence of the chloro group. Also, there are two doublets each with an integration for 2H at chemical shifts 8.53 and 8.33 for the protons C-2 and C-3 respectively. Again, here, the $-NO_2$ causes the downfield shifting of the C-2 protons.

From the IR spectrum for compound KA 005, appendix E1, a peak at 824.05 cm^{-1} in the fingerprint region, is as a result of the vibrations of a C-Cl bond. This indicates the presence of the chloro group in this compound.

To compliment this observation, the MS data was obtained, appendix E2. The calculated molecular weight for the compound is 282, hence peaks at m/z of 281.0 and 282.9 in the negative and positive modes respectively can be said to the molecular ion peaks each mode. The isotopic chlorine group is shown by the appearance of peaks with about a third the abundance of the molecular ion at 283.0 and 284.9 in the positive and negative modes respectively. The molecular ions in both modes are shown in fig 5.9.
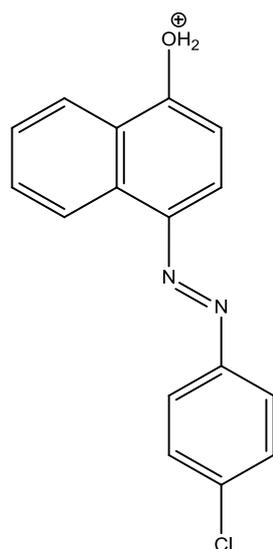
Chemical Formula: $C_{16}H_{12}ClN_2O^+$
Exact Mass: 283.06
Molecular Weight: 283.73

a
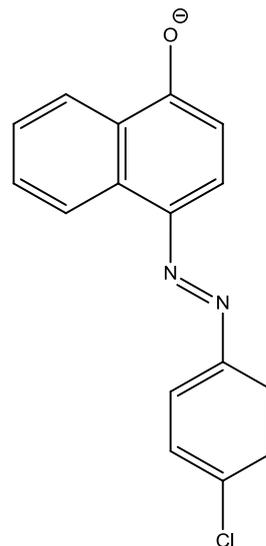
Chemical Formula: $C_{16}H_{10}ClN_2O^-$
Exact Mass: 281.05
Molecular Weight: 281.72

b

Figure 5.9 Molecular ion formed in positive (a) and negative (b) modes

Also in the negative mode, there is a peak at m/z of 298.8 which is attributed to a peak resulting from an $[M+H_2O]^-$ fragment. In addition, the peak at m/z of 171.3 is an $[X+1]^-$ can be linked to the fragment formed after the cleavage of the chloro benzene. Also the cleavage of the naphthol leads to the generation of a fragment with m/z of 138.9. These fragments are shown in fig 5.10.
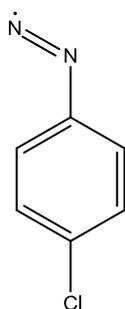
Chemical Formula: $C_6H_4ClN_2^{\bullet}$
Exact Mass: 139.01

a
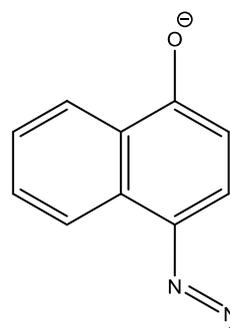
Chemical Formula: $C_{10}H_6N_2O^{\bullet-}$
Exact Mass: 170.05

b

Figure. 5.10 Fragments with m/z of 139.0 (a) and 170.0 (b)

The ^{1}H NMR for this compound is very similar to that obtained for the KA 004. This is not far-fetched, seeing that the chloro group in KA 005 is at exactly the same position, *para*, as the nitro group in KA 004.

The Acetylated Products

For compound KA 006, the IR spectrum was obtained as shown in appendix F. From the spectrum, there is an absorption band at 1772.65 cm^{-1} representing the presence of a carbonyl of the acetyl group introduced. It is important to note that, the broad band at about 3200 cm^{-1} that was obtained for the starting compound, KA 004, is absent in this case.

From the IR spectrum of KA 007, appendix G, two signals can be observed at 1764.59 and 1657.15 cm^{-1} which represents the vibrations due to the two carbonyls groups present, the carboxylic acid and acetyl group. As explained earlier, the carbonyls of ester linkages (in this case the acetyl group) with higher frequencies as compared with the carboxylic acid. Therefore, the signal at 1764.59 cm^{-1} represent that of the acetyl group and that at 1657.15 cm^{-1} is that for the acid.

The Alkylated Products

From the IR spectrum, appendix H, the signal due to the –OH vibration at ~3200cm^{-1} is not present. This signifies that the alcohol functional group has been converted into a new one, that is, the ether which is indicated by the appearance of a peak at 1210.15 cm^{-1}.

Furthermore, for the compound KA 009, appendix I, the characteristic broad band stretching over 3550 – 3200 cm^{-1} representing the naphthol –OH cannot be observed. However the vibration of the –OH of the carboxylic acid is

recorded at 2966.78 cm^{-1}. A new absorption band is also recorded at 1247.23 cm^{-1} representing the C-O-C vibrations of the ether bond formed.

5.1.3 Antimicrobial Assay

The minimum inhibitory concentration (MIC) of an antimicrobial agent can be defined as the lowest concentration of the agent needed to cause an inhibition of growth of a microorganism. The current technique of choice employed in the determination the MIC of antimicrobial agents is the broth dilution method. This method has gained grounds over the agar well diffusion because in this method, the issues of the diffusion of the test compound through the growth media is eliminated (Wiegand *et al*., 2008).

In the dilution method, the series of the test sample contained in a nutrient broth are placed in wells on a micro-titre plate in the order of decreasing concentration, prepared through serial dilution. The microorganisms are inoculated in the wells and incubated for a period of time, mostly for 18 − 24 hours. After the incubation period, the wells are examined and the lowest concentration of the test sample that shows no microbial growth is recorded as the MIC of the sample. It is important to note that, sometimes there is the need to add a colour reagent, a tetr*azo*lium salt, which stains the cell matter of the organism, to aid the determination of the MIC.

In this experiment, sterility and growth controls, and Cefuroxime Axetil were used as negative and positive controls respectively. In the sterility control, the organisms were inoculated with the diluting solvents without the test compounds. This gives an indication as to whether or not the solvents contribute to the inhibition. For the growth control, inoculation of the test compound and solvent without the organisms. This helps to check

contamination. Cefuroxime is a commercially available antibiotic with a general broad spectrum activity, and comparing the MIC of the synthesised dyes with this drug will provide some preliminary efficacy of dyes. The determinations were performed in duplicated in order to ensure that the results obtained were reproducible. Two gram positive organisms [(*Staphylococcus aureus* (ATCC 25923) *and Streptococcus pyrogenes* (clinical strain)], two gram negative organisms [*Salmonella typhi* (clinical strain)*, Pseudomonas aeruginosa* (ATCC 4853)] and a fungus [*Candida albicans* (clinical strain)] were used in the assay. The choice of these organisms was to aid give an indication as to whether the synthesised compounds would exhibit a broad or narrow spectrum of activity.

The results obtained from the assay are summarised in table 4.2

From the results obtained, the compounds KA 001 and KA 004 showed greater activity against the fungus, *C. albicans*, recording MIC of 70.32 µg/ml and 140.63 µg/ml respectively. This is consistent with the results obtained from the work of Kofie *et al.* From that work however, an MIC of 20µg/ml was obtained for the compound KA 004 (referred to as *p*-NAαN in their work) (Kofie *et al.*, 2015). It is important to note that an ATCC 9027 strain of the organism was used in their study whereas a clinical strain sourced from the Komfo Anokye Teaching Hospital was used in this work. This may have accounted for the differences in the activities obtained.

Compound KA 002, the carboxylic acid of KA 001 is replaced by an ethyl ester group. This transformation resulted in a general increase in the activity. The ester group is less polar than the carboxylic acid, as a result, the lipophilic

character of KA 002 is improved. Hence it is able to easily move across the cell wall of the organism, to cause inhibition or death of the organism.

Also, there is the introduction of a chloro group *ortho* to the *azo* linkage of KA 004 which leads to the compound KA 003. In a study to investigate the effect of substituent and substitution pattern of the decolourization of *azo* dyes by Hsuech *et al*., it was observed that the presence of an electron-withdrawing group *ortho* to the *azo* linkage will lead to a reduced rate of decolourization of the dye by microbes, as compared with an isomer with the substituent at the *para* position (Hsueh and Chen, 2008, Hsueh *et al*., 2009). This observation is consistent with the results obtained from this work, in which KA 003 showed a general improvement in activity over KA 004. To explain this, steric hindrance may play a crucial role in this observed activity. Some microorganisms such as the penicillin-resistant organisms produce certain protective enzymes that adds certain groups which binds to specific positions on the antimicrobial agents which ends up inactivating the agent (Silver, 2011). In KA 003 however, binding at the chloro group will result in considerable steric problems due to the presence of the *azo* bond in the close proximity. As a result the structural integrity of the compound may be said to be intact at the site of action to cause inhibition.

In addition, the activity of KA 005 was lower than KA 004. Hsuetch *et al*. demonstrated that when an electron-withdrawing group is present in a *para* position to the *azo* linkage in a dye, it is much more susceptible to microbial decolouration (Hsueh and Chen, 2008, Hsueh *et al*., 2009). The chloro group is more electronegative than the nitro group as a result will exhibit a higher electron-withdrawing effect, hence the susceptibility to microbial degradation

will be higher than that of the nitro dye. This explains the reduced activity of KA 005 compared with KA 004.

Furthermore, from the microbial testing of the alkylated products, KA 008 and KA 009, it was revealed that presence of the ether group contributed significantly in improving the activity of these compounds. These results are not far-fetched, considering that the ether compound is less polar than the base phenolic compound. This reduced polarity increases the lipophilicity of these compounds therefore making them easy to transport across the cell wall of the microorganism to cause death or inhibition of growth.

This same improvement in the activity was expected for the acetylated products, KA 006 and KA 007, since they are also more non-polar. However, from the study, a rather decrease in activity was obtained instead. The acetylated products are about 42 units heavier than their base compound. This extra weight may have contributed in slowing down the rate of diffusion of the compounds across the cell walls of the organisms to cause inhibition, hence the lower MIC.

Finally, the commercially available antibiotic, cefuroxime axetil, was used as the positive control in this work. The most active synthesised compound, KA 008, recorded some comparable activities against some of the organisms used as the control drug, 35.16 μg/ml against *S. pyrogenes* and *P. aeruginosa* for both KA 008 and the control. This goes to show that this compound has great potential to be used as an antimicrobial agent.

In spite of this great promise, before any claims can be made, there is the need for *in vivo* and toxicity studies to ascertain the efficacy and safety of this compound in living cells.

5.2   CONCLUSION

In summary, nine *azo* compounds have been successfully synthesised, and in good yields. Using the standard direct di*azo*tization-coupling procedure, five compounds were synthesised. The Williamson's ether synthesis as well as the acetylation with acetic anhydride were also used to synthesize the alkylated and acetylated products respectively. The compounds have been partially characterised.

All the synthesised dyes showed antimicrobial activity, generally showing more activity against the gram negative and fungus.

Generally, the introduction of electron-withdrawing groups *ortho* to the *azo* linkage, results in an increase in the activity of the compound through the introduction of steric issues, as seen in the activity of KA 003. Also, alkylation of the hydroxyl of the naphthol leads to the improvement in the lipid solubility profile of the products hence an increase in the activity of the alkylated derivatives. The structures of the most active compounds are shown in fig. 5.11.
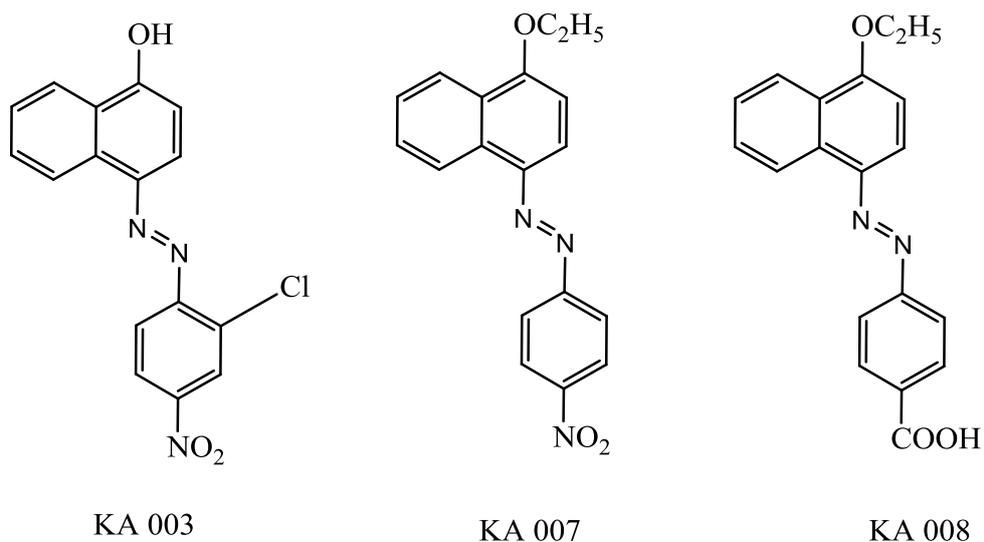
Figure 5.11 Structures of the most active synthesised dyes

Also, the most active compound, KA 008, showed comparable activities with the standard Cefuroxime axetil used, indicating it's potential to be used as an antimicrobial agent.

5.3   RECOMMENDATION

- Further structural modification by the increasing of the carbon chain in the ether and ester derivatives can be explored. This will help to show whether or not increasing the carbon chain of these derivatives would improve activity.

- Toxicity studies should be conducted to ascertain the safety profile of the active compound. Compound KA 008 showed comparable activity as the standard cefuroxime, hence if should be developed as an antimicrobial agent, its safety should be assured.

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# APPENDICES

Appendix A; Analytical Data for KA 001

(1) IR Spectrum

(2) Mass Spec

Openlynx ~~OA3 LCMS Generic Chromatography.~~ Page 2
File:OA3_160108_012
Vial:3:2
Submitter:

ID:KA1
Date:08-Jan-2016

Description:
Time:12:23:55
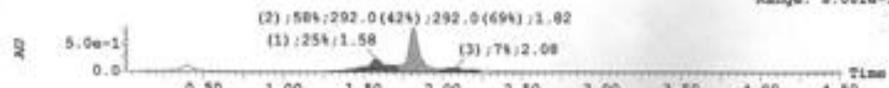
Printed: Fri Jan 08 12:43:38 2016

2:(Time: 1.83) Combine (49:53-(43:44+59:62))

1:MS ES+
4.1e+005

100
126.0
292.9
294.0
209.9
441.4
583.0 607.2
200.0   400.0   600.0   800.0   1000.0
m/z
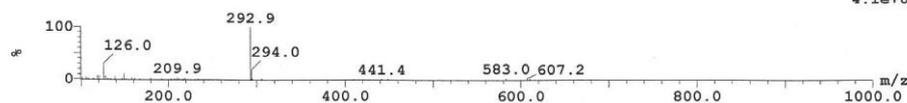
2:(Time: 1.81) Combine (48:52-(43:44+58:61))

2:MS ES-
4.3e+005

100
291.0
292.0
135.0   246.9
439.0
581.0 604.9
200.0   400.0   600.0   800.0   1000.0
m/z
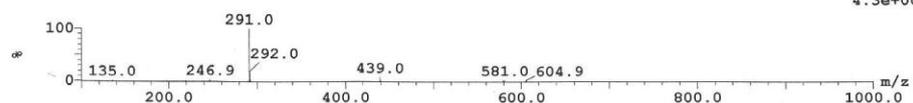
3:(Time: 2.09) Combine (56:60-(50:51+64:67))

1:MS ES+
9.5e+004

100
173.9
445.9
102.0   195.9
329.8   434.9   447.0 508.9
632.1 673.3
891.1   931.3
200.0   400.0   600.0   800.0   1000.0
m/z
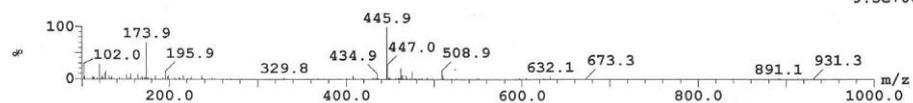
4:(Time: 2.19) Combine (58:62-(54:56+66:69))

1:MS ES+
2.8e+005

100
126.0
173.9   213.9   293.0   430.8
445.9   508.9   632.1
200.0   400.0   600.0   800.0   1000.0
m/z
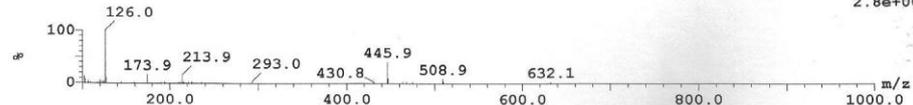
5:(Time: 2.26) Combine (60:64-(57:58+74:77))

1:MS ES+
4.5e+005

100
126.0
128.0 213.9   302.1
445.9 508.9   632.1
200.0   400.0   600.0   800.0   1000.0
m/z

69
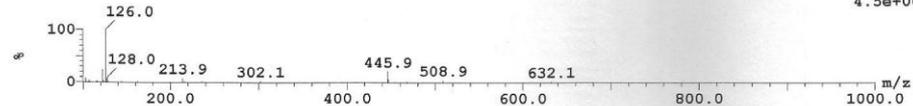
(3) ^{1}H NMR
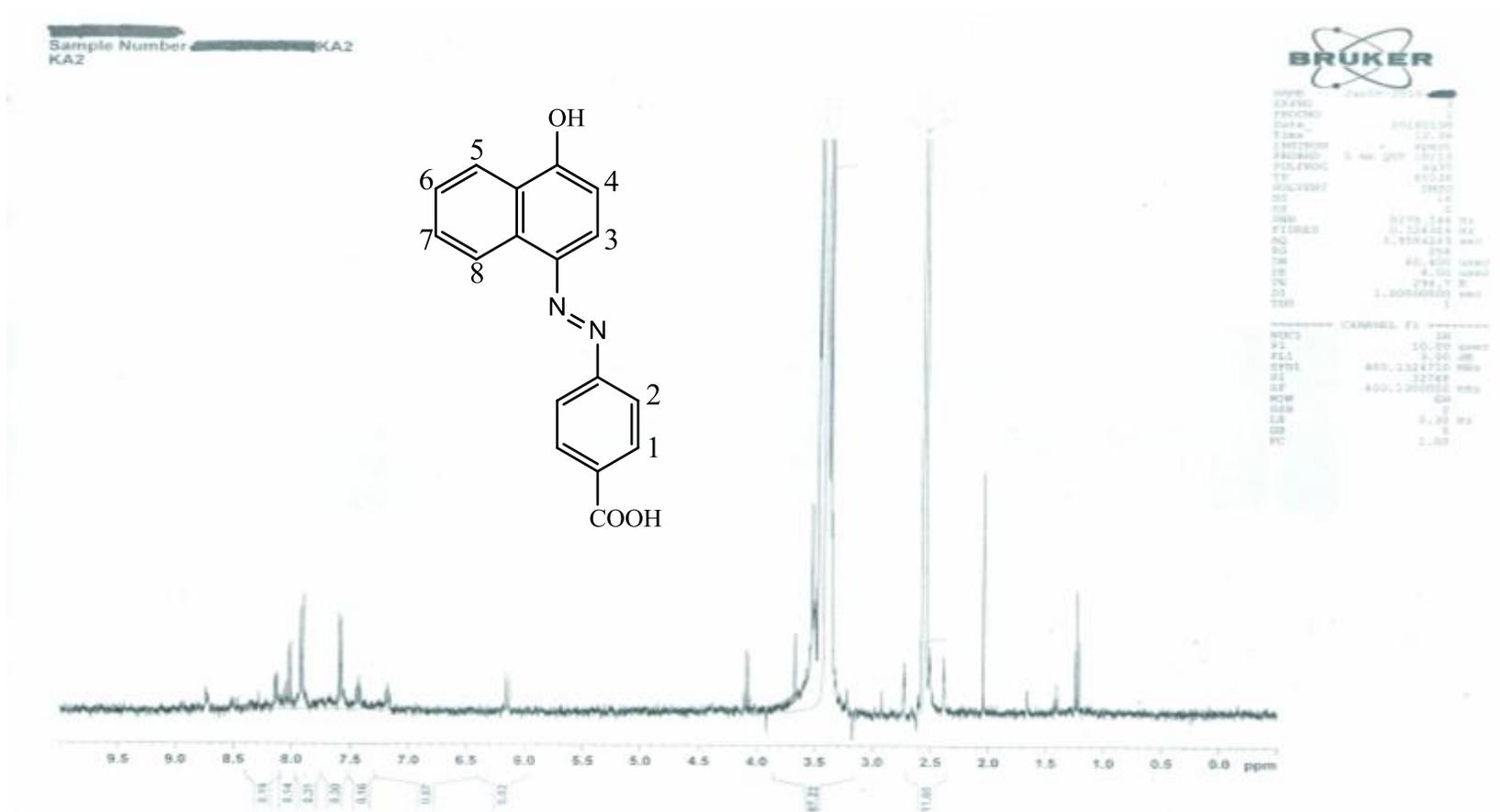
Appendix B; Analytical Data for KA 002

(1) IR Spectrum
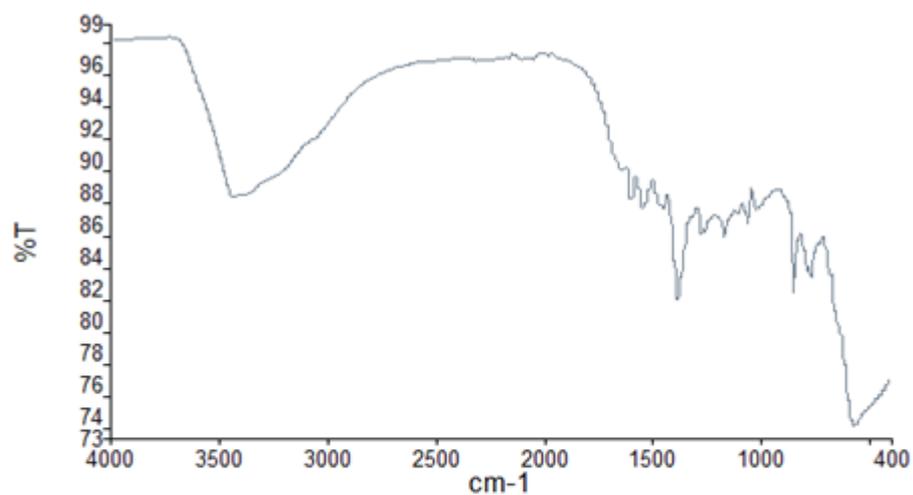
Name	Description
——Kwaku Addo-Danquah	KA 002

Peak Table

Peak	X	Y	Peak	X	Y	Peak	X	Y	Peak	X	Y
1	3376.82	88.73	2	1380.25	82	3	1169.33	85.97	4	846.66	82.46
5	762.54	83.5	6	558.91	74.05						

(2) Mass Spec

Openlynx ▓▓▓▓▓▓ OA3 LCMS Generic Chromatography. ▓▓▓▓▓▓ Page 3
File:OA3_160108_013   ID:KA2   Description:
Vial:3:3   Date:08-Jan-2016   Time:12:30:43
Submitter:

Printed: Fri Jan 08 12:43:38 2016

1: MS ES+ :321   3.3e+004

(5) ;100%;320.0(3%) ;3.79

2: MS ES- :319   5.2e+004

(4)
47%
3.18
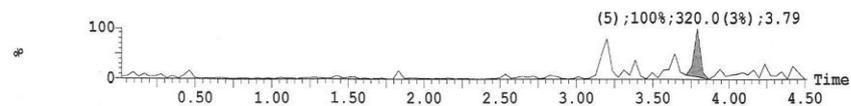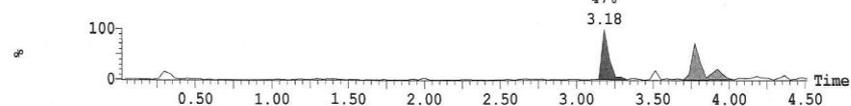
3: UV Detector: TIC   8.517e+1
   Range: 8.516e+1

0.35   (1)   (3)
            67%   27%
            1.80   2.09
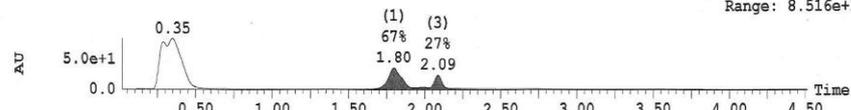
Peak Number	Mass Found	Time	Area %Total
1		1.80	67.24
2		1.99	5.38
3		2.09	27.38

3: UV Detector: 252_256   2.226
                                                         Range: 2.226

            (1)   (3)
            70%   22%
            1.80   2.09
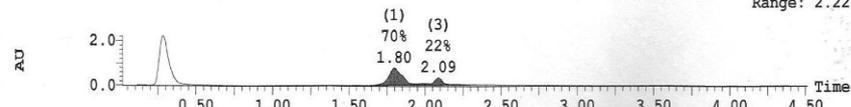
Peak Number	Mass Found	Time	Area %Total
1		1.80	70.38
2		1.99	7.18
3		2.09	22.44

1:(Time: 1.80) Combine (48:52-(39:40+58:61))   1:MS ES+
                                                          6.0e+005

293.0
126.0 198.9 294.0
                        565.1582.9
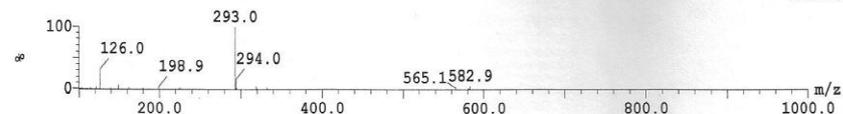
1:(Time: 1.80) Combine (47:51-(38:40+58:60))   2:MS ES-
                                                          8.6e+005
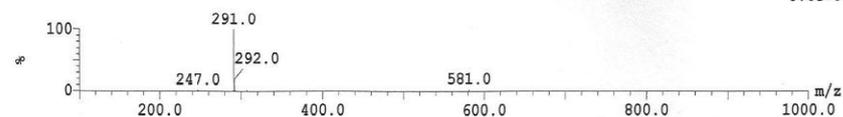
291.0
292.0
247.0   581.0

72

Openlynx ████████████ _OA3 LCMS Generic Chromatography: ████████████████████ Page 4
File:OA3_160108_013   ID:KA2   Description:
Vial:3:3   Date:08-Jan-2016   Time:12:30:43
Submitter:

Printed: Fri Jan 08 12:43:38 2016

2:(Time: 1.99) Combine (53:57-(49:50+61:63))   1:MS ES+
                                                                 2.6e+005
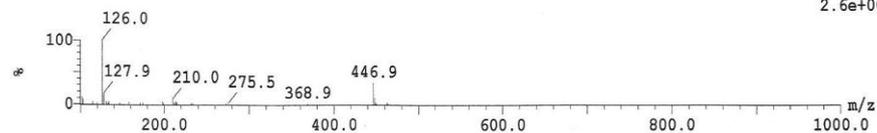

3:(Time: 2.09) Combine (56:60-(51:53+70:73))   1:MS ES+
                                                                 3.1e+005
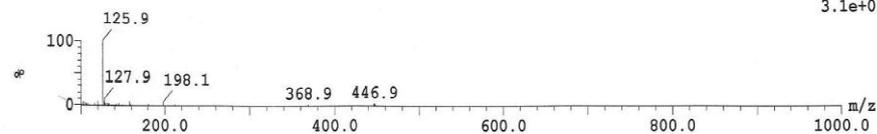

3:(Time: 2.09) Combine (55:59-(51:52+70:73))   2:MS ES-
                                                                 1.7e+005
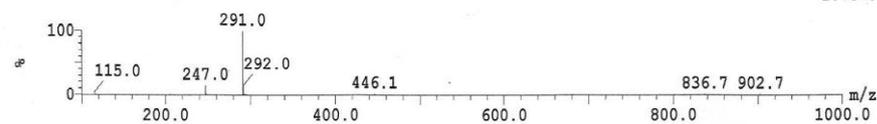
5:(Time: 3.79) Combine (102:106-(97:98+110:113))   1:MS ES+
                                                                 2.6e+005
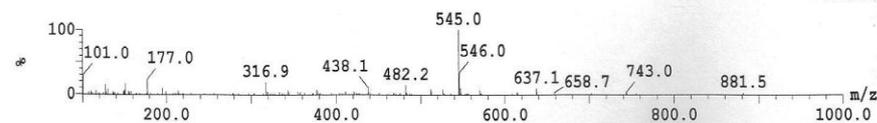
(3) ^{1}H NMR
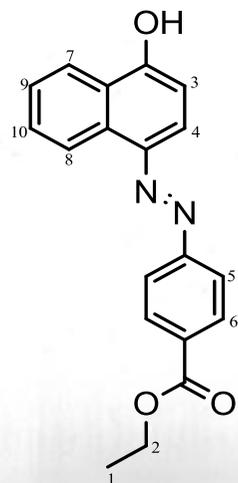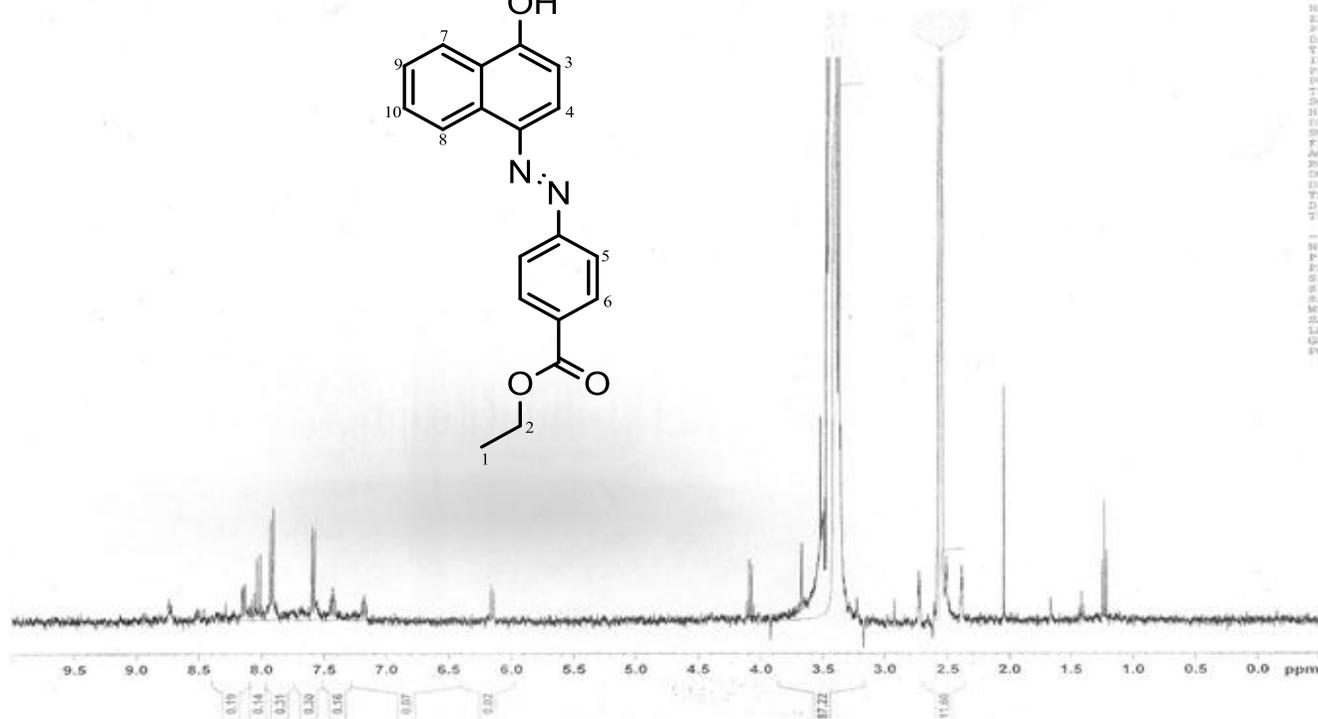
Appendix C; Analytical Data for KA 003

(1) IR Spectrum
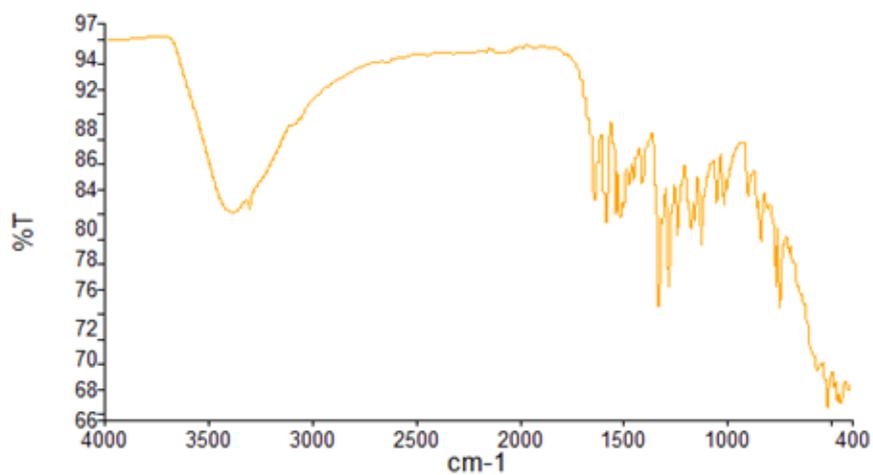
| Name	Description
—Kwaku Addo-Danquah	KA 003

Peak Table

| Peak	X	Y	Peak	X	Y	Peak	X	Y	Peak	X	Y
1	3378.14	82.36	2	1637.35	83.37	3	1584.75	81.33	4	1538.05	82.05
5	1512.76	81.74	6	1406.79	84.6	7	1329.22	74.51	8	1302.43	81.2
9	1276.23	76.2	10	1242.36	80.29	11	1168.6	80.83	12	1118.77	79.56
13	1047.37	83.07	14	1011.07	82.94	15	894.74	83.57	16	832.43	79.84
17	761.67	76.17	18	742.77	74.46	19	514.81	66.32	20	447.63	66.62

(2) Mass Spec

1: MS ES+ :328   3.7e+006

(2);100%;327.0(50%);327.0(58%);2.98

2: MS ES- :326   5.9e+006

(2);88%;327.0(50%);327.0(58%);3.03

(6);12%;327.0(50%);3.77

3: UV Detector: TIC   7.491e+1
                                                         Range: 7.491e+1

0.36

(2);68%;327.0(50%);327.0(58%);2.99

Peak Number	Mass Found	Time	Area %Total
1		1.98	3.41
2	327.00, 327.00	2.99	67.77
3		3.27	9.08
4		3.42	10.23
6	327.00	3.75	9.51

3: UV Detector: 252_256   9.133e-1
                                                         Range: 9.133e-1

(2);73%;327.0(50%);327.0(58%);2.99

Peak Number	Mass Found	Time	Area %Total
2	327.00, 327.00	2.99	72.67
3		3.27	9.61
4		3.40	7.22
5		3.51	4.49
6	327.00	3.75	6.01

2:(Time: 2.98) Combine (80:84-(74:75+100:103))   1:MS ES+
                                                                   1.6e+006

126.0   327.9
     183.9   329.9

76

1: MS ES+ :328   3.7e+006

100   (2):100%;327.0(50%);327.0(58%);2.98

0
       0.50   1.00   1.50   2.00   2.50   3.00   3.50   4.00   4.50   Time

2: MS ES- :326   5.9e+006

100   (2):88%;327.0(50%);327.0(58%);3.03
                                     (6):12%;327.0(50%);3.77
0
       0.50   1.00   1.50   2.00   2.50   3.00   3.50   4.00   4.50   Time

3: UV Detector: TIC   7.491e+1
                                                              Range: 7.491e+1
     0.36
5.0e+1   (2):69%;327.0(50%);327.0(58%);2.99

0.0
       0.50   1.00   1.50   2.00   2.50   3.00   3.50   4.00   4.50   Time

Peak Number	Mass Found	Time	Area %Total
1		1.98	3.41
2	327.00, 327.00	2.99	87.77
3		3.27	9.08
4		3.42	10.23
6	327.00	3.75	9.91

3: UV Detector: 252_256   9.133e-1
   Range: 9.133e-1

5.0e-1   (2):73%;327.0(50%);327.0(58%);2.99

0.0
       0.50   1.00   1.50   2.00   2.50   3.00   3.50   4.00   4.50   Time

Peak Number	Mass Found	Time	Area %Total
2	327.00, 327.00	2.99	72.67
3		3.27	9.61
4		3.40	7.22
5		3.51	4.49
6	327.00	3.75	6.01

2:(Time: 2.98) Combine (80:84-(74:75+100:103))   1:MS ES+
                                                                       1.6e+006

100   126.0   327.9
               329.9
       183.9
0
       200.0   400.0   600.0   800.0   1000.0   m/z

(3) ^{1}H NMR

Appendix D; Analytical Data for KA 004

(1) IR Spectrum
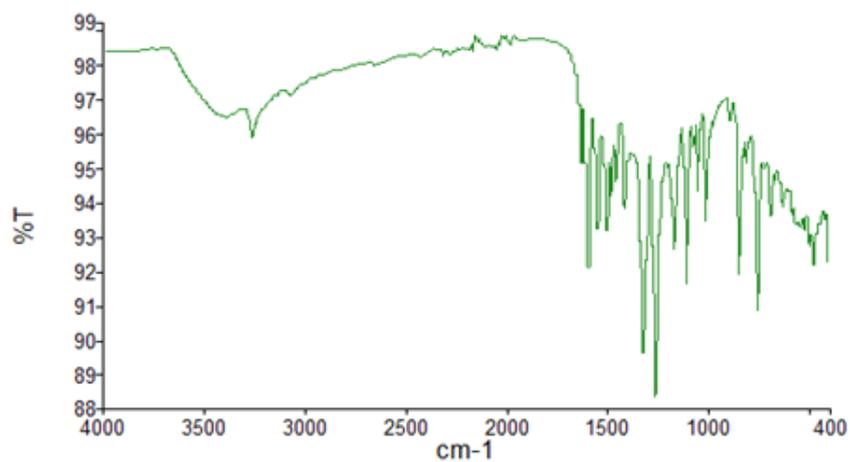
| Name	Description
—Kwaku Addo-Danquah	KA 004

Peak Table

| Peak	X	Y	Peak	X	Y	Peak	X	Y	Peak	X	Y
1	3267.1	95.99	2	1594.29	92.1	3	1547.51	93.27	4	1502.28	93.24
5	1319.45	89.59	6	1258.74	88.3	7	1167.5	92.69	8	1107.99	91.68
9	1046.99	94.39	10	1013.34	93.48	11	847.59	91.95	12	750.48	90.83

79

(2) Mass Spec

1: MS ES+ :294   7.5e+005

(1);100%;293.0(28%);293.0(81%);3.02

2: MS ES- :292   6.4e+006

(1);100%;293.0(28%);293.0(81%);3.03

3: UV Detector: TIC   6.822e+1
                                                            Range: 6.822e+1

0.41

(1);86%;293.0(28%);293.0(81%);2.99
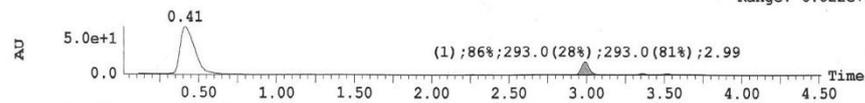
Peak Number	Mass Found	Time	Area %Total
1	293.00, 293.00	2.99	86.35
2		3.36	6.45
3	293.00	3.52	7.20

3: UV Detector: 252_256   4.947e-1
                                                            Range: 4.947e-1

(1);91%;293.0(28%);293.0(81%);2.99

(2);5%;3.36
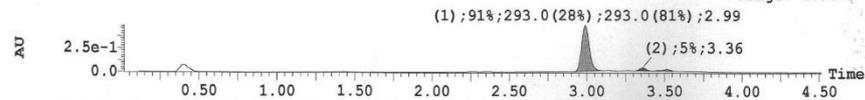
Peak Number	Mass Found	Time	Area %Total
1	293.00, 293.00	2.99	90.59
2		3.36	5.35
3	293.00	3.52	4.06

1:(Time: 3.02) Combine (81:85-(75:76+99:102))   1:MS ES+
                                                         8.5e+005

126.0
144.8 196.3 293.9 295.0
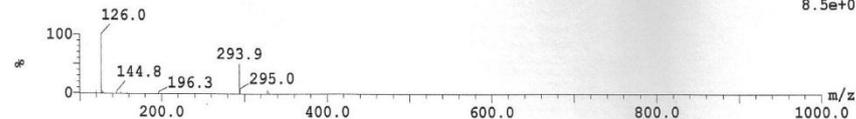
1:(Time: 3.03) Combine (81:85-(74:75+96:99))   2:MS ES-
                                                         2.9e+006

292.0
212.0 293.0

80

Openlynx_____OA3 LCMS Generic Chromatography_____ Page 2
File:OA3_160108_015   ID:KA4   Description:
Vial:3:5   Date:08-Jan-2016   Time:12:43:31
Submitter:
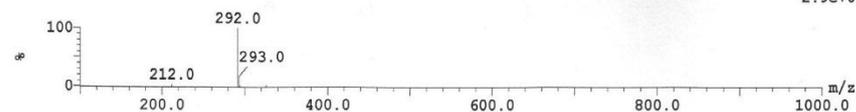
Printed: Fri Jan 08 13:03:10 2016

2:(Time: 3.36) Combine (90:94-(86:87+98:101))   1:MS ES+
   3.1e+004
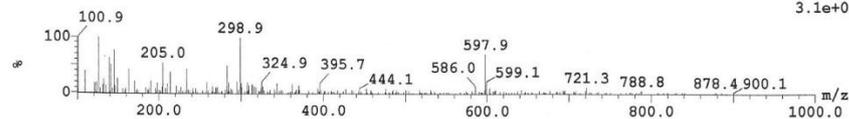
2:(Time: 3.36) Combine (89:94-(85:87+98:101))   2:MS ES-
                                                                       3.5e+005
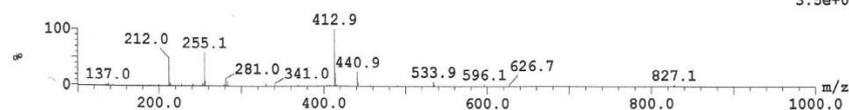
3:(Time: 3.52) Combine (94:98-(89:90+104:107))   1:MS ES+
                                                                       2.6e+005
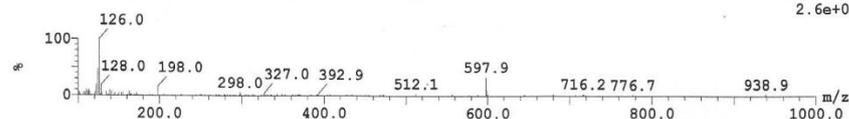

3:(Time: 3.52) Combine (94:98-(88:90+103:106))   2:MS ES-
                                                                       2.2e+005

81

(3) ^{1}H NMR
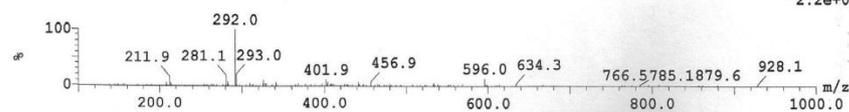
Appendix E; Analytical Data for KA 005

(1) IR Spectrum
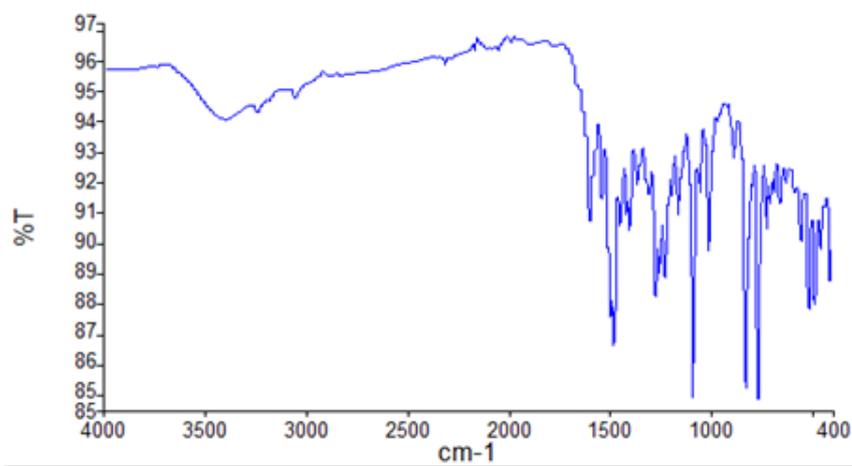
Name	Description
—Kwaku Addo-Danquah	KA 005

Peak Table

Peak	X	Y	Peak	X	Y	Peak	X	Y	Peak	X	Y
1	1596.69	90.82	2	1479.22	86.63	3	1404.11	90.43	4	1272.28	88.25
5	1155.61	90.97	6	1088.97	84.93	7	1008.34	89.79	8	824.05	85.15
9	763.76	84.82	10	727.4	90.5	11	511.48	87.8	12	484.51	87.95

(2) Mass Spec

Openlynx OA3 LCMS Generic Chromatography. Page 3
File:OA3_160108_016   ID:KA5   Description:
Vial:3:6   Date:08-Jan-2016   Time:12:50:21
Submitter:

Printed: Fri Jan 08 13:03:10 2016

1: MS ES+ :283   3.5e+006

(4);100%;282.0(44%);282.0(60%);3.39

2: MS ES- :281   8.0e+006

(4);100%;282.0(44%);282.0(60%);3.40
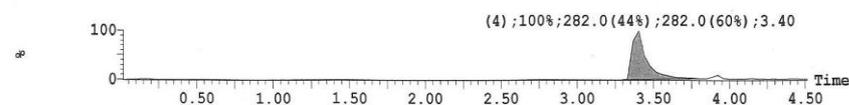
3: UV Detector: TIC   (4)   7.345e+1
                                                       61%   Range: 7.345e+1
                                                     282.0(44%)
                                                     282.0(60%)
                                                       3.38
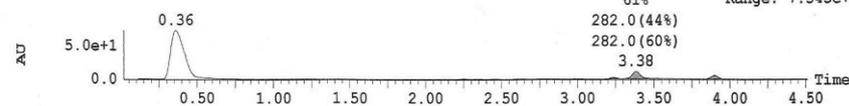
| Peak Number	Mass Found	Time	Area %Total
1		2.25	1.65
2		3.00	2.43
3		3.24	11.93
4	282.00, 282.00	3.38	61.03
6	282.00	3.90	22.97

3: UV Detector: 252_256   2.869e-1
                                                              Range: 2.869e-1

(4);63%;282.0(44%);282.0(60%);3.38
(3);13%;3.24

| Peak Number	Mass Found	Time	Area %Total
2		3.00	3.42
3		3.24	13.32
4	282.00, 282.00	3.38	62.97
5		3.81	3.40
6	282.00	3.90	16.88

2:(Time: 3.00) Combine (80:84-(76:77+89:92))   1:MS ES+
                                                           3.4e+005

126.0
128.0 198.1 294.0 327.9   456.8

84

Openlynx ███████ ___OA3 LCMS Generic Chromatography.█████████████ Page 4
File:OA3_160108_016   ID:KA5   Description:
Vial:3:6   Date:08-Jan-2016   Time:12:50:21
Submitter:
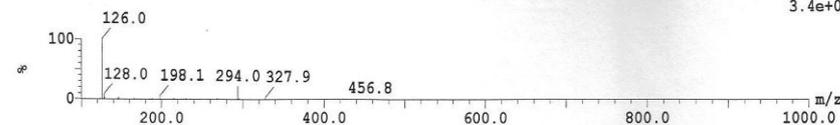
Printed: Fri Jan 08 13:03:10 2016

2:(Time: 3.00) Combine (80:84-(75:77+88:91))   2:MS ES-
                                                     3.5e+005
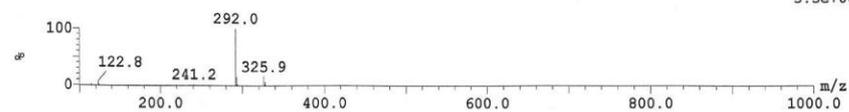
3:(Time: 3.24) Combine (87:91-(82:83+95:97))   1:MS ES+
                                                     1.4e+005
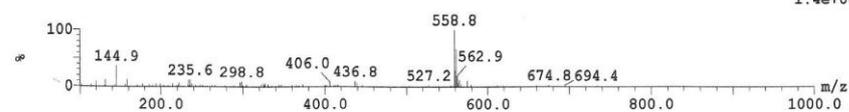
4:(Time: 3.39) Combine (91:95-(84:85+109:112))   1:MS ES+
                                                     1.5e+006
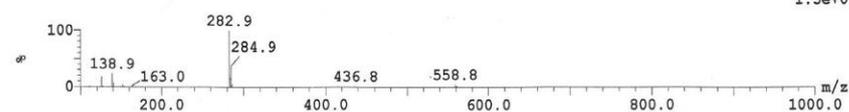
4:(Time: 3.40) Combine (91:95-(85:86+108:111))   2:MS ES-
                                                     4.2e+006
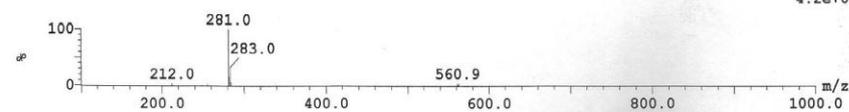
6:(Time: 3.90) Combine (104:108-(99:101+113:116))   2:MS ES-
                                                     1.4e+005
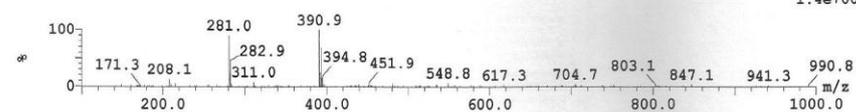
(3) ^{1}H NMR
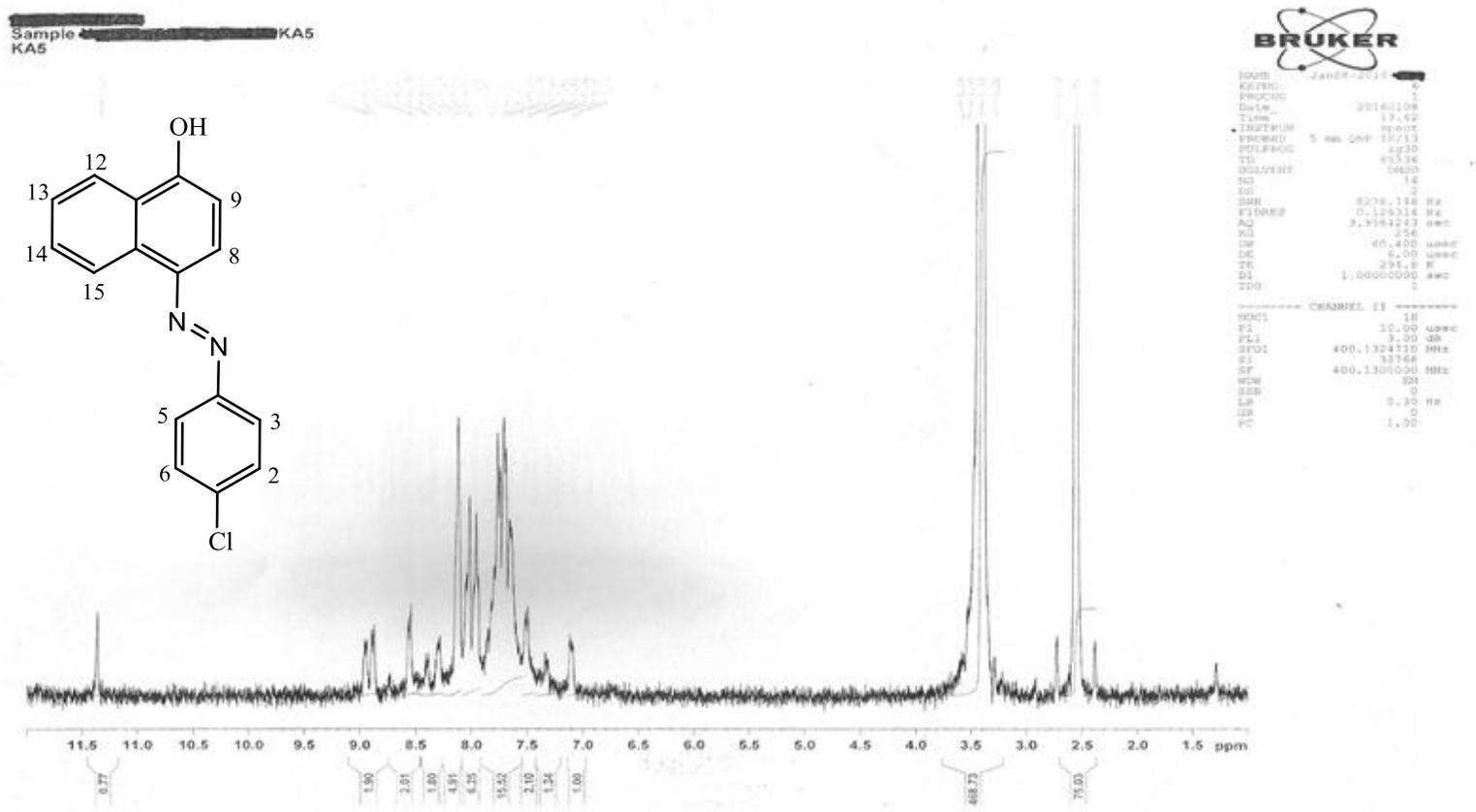
Appendix F; Analytical Data for KA 006

(1) IR Spectrum

Spectrum Graph

| Name	Description
—Kwaku Addo-Danquah	KA 006

Peak Table

Peak	X	Y	Peak	X	Y	Peak	X	Y	Peak	X	Y
1	2966.78	94.78	2	1772.65	87.53	3	1685.15	88.63	4	1577.14	87.17
5	1518.84	90.34	6	1481.54	88.22	7	1395.43	88.7	8	1378.23	89.95
9	1312.63	89.1	10	1281.89	89.91	11	1247.23	85.87	12	1143.7	90.85
13	1119.69	90.41	14	1075.97	92.66	15	1020.36	93.77	16	847.43	90.58
17	732.67	88.83	18	695.21	89.59	19	655.54	87.7	20	554.83	83.04
21	522.79	83.85	22	470.72	83.68	23	436.86	85.31			

Appendix G; Analytical Data for KA 007

(1) IR Spectrum

Spectrum Graph
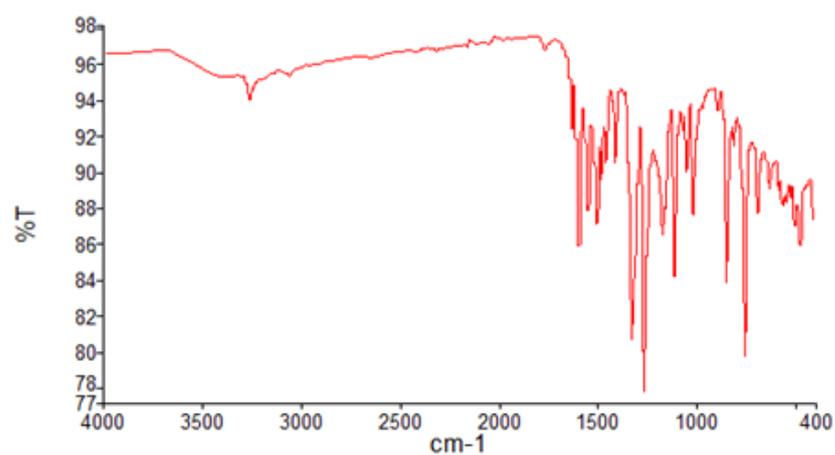
Name	Description
—Kwaku Addo-Danquah	KA 007

Peak Table

Peak	X	Y	Peak	X	Y	Peak	X	Y	Peak	X	Y
1	3267.51	94.19	2	1628.26	92.44	3	1594.61	85.86	4	1547.6	87.9
5	1502.56	87.14	6	1479.77	89.65	7	1457.81	90.58	8	1412.14	90.59
9	1320.05	80.67	10	1259.51	77.69	11	1167.67	86.55	12	1107.96	84.17
13	1047.12	90.04	14	1013.34	87.64	15	847.64	83.81	16	750.48	79.69
17	686.77	87.7	18	468.39	85.84						

Appendix H; Analytical Data for KA 008

(1) IR Spectrum
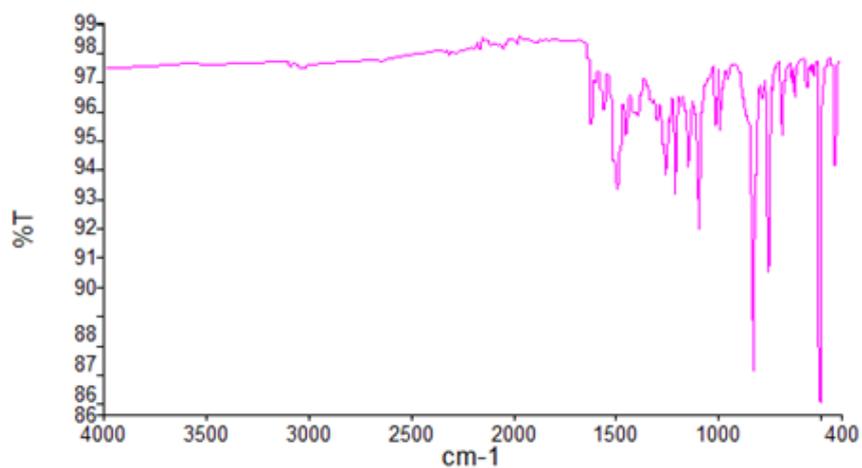
| Name	Description
—Kwaku Addo-Danquah	KA 008

Peak Table

Peak	X	Y	Peak	X	Y	Peak	X	Y	Peak	X	Y
1	1486.9	93.42	2	1254.26	93.9	3	1210.15	93.26	4	1147.19	94.19
5	1088.63	91.99	6	985.48	95.42	7	820.78	87.08	8	749.16	90.56
9	679.7	95.27	10	496.26	85.98	11	423.15	94.24			

Appendix I; Analytical Data for KA 009

(1) IR Spectrum

Spectrum Graph

Name	Description
—Kwaku Addo-Danquah	KA 006

Peak Table

Peak	X	Y	Peak	X	Y	Peak	X	Y	Peak	X	Y
1	2966.78	94.78	2	1772.65	87.53	3	1685.15	88.63	4	1577.14	87.17
5	1518.84	90.34	6	1481.54	88.22	7	1395.43	88.7	8	1378.23	89.95
9	1312.63	89.1	10	1281.89	89.91	11	1247.23	85.87	12	1143.7	90.85
13	1119.69	90.41	14	1075.97	92.66	15	1020.36	93.77	16	847.43	90.58
17	732.67	88.83	18	695.21	89.59	19	655.54	87.7	20	554.83	83.04
21	522.79	83.85	22	470.72	83.68	23	436.86	85.31			

Appendix J: TLC profile for the alkylation using NaOH
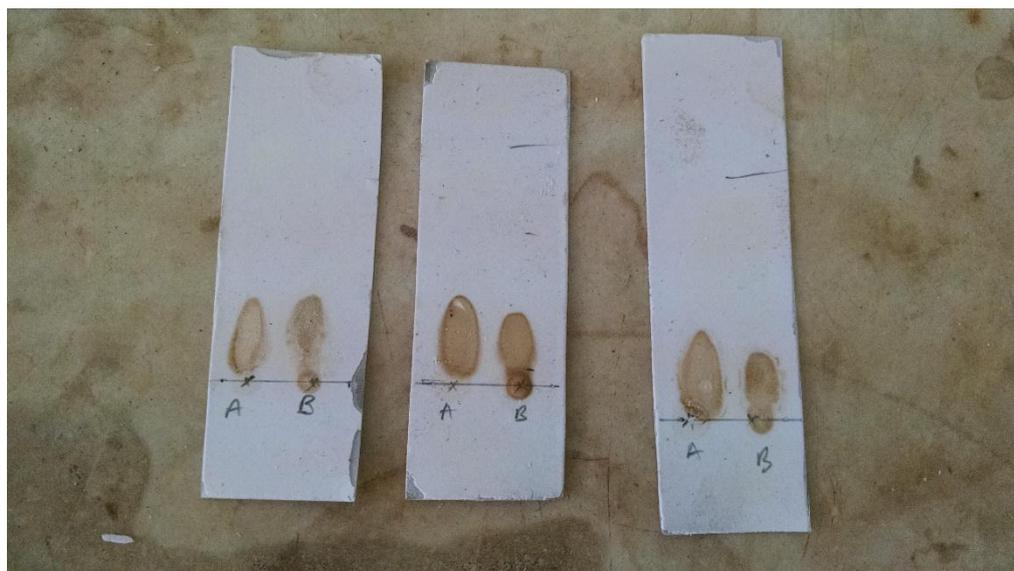
A represents the spot for the starting material and B represents that of the

reaction mixture. The TLC was developed after times, 0, 30 and 60 mins.