Original Research Article

Antimicrobial activity and in silico ADME prediction of synthesised 8-hydroxyquinoline *azo* compounds against some ESKAPE human pathogens and *Mycobacterium smegmatis*.

Highlights

- *Azo* compounds have been synthesised using 8-hydroxyquinoline as a coupling agent.
- The compounds have been characterised by ^{1}H-NMR, Ultra-Violet Visible, and Fourier Transform infra-red spectroscopy.
- The compounds show antimicrobial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Proteus mirabilis*.
- The 8-hydroxyquinoline *azo* dyes can be used as structural scaffolds for antimicrobial drug discovery.

Abstract

Antimicrobial resistance has increasingly been a global health concern over the past decades hence the need to synthesise and evaluate the antimicrobial properties of five derivatives of 8-hydroxyquinoline azo compounds (4a–e). The *azo* compounds were synthesised by diazotization and coupling procedures with yields of 60 – 92%. They

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were characterized by melting point determination, Ultra-Violet Visible, and infra-red spectroscopy. High-throughput spot culture growth inhibition (HT-SPOTi) antimicrobial assay was used to evaluate the compounds. From the *in silico* studies, none of the compounds violated Lipinski's rule and therefore have the potential to be developed into an oral drug. They also showed Total Polar Surface Area (TPSA) values < 140 Å^2 (74.91 – 100.98 Å^2) and percentage absorption of 74 – 83 %. They were placed in category III of acute oral drugs. From the high-throughput spot culture growth inhibition antimicrobial assay, all the compounds possessed inhibitory activity against the ESKAPE human pathogens used and *Mycobacterium smegmatis*, with MICs range of 3.9 ≥ 500 µg/mL. Except for 4e which showed liver toxicity, all the compounds demonstrated mutagenic and hepatotoxic tendencies. The modulatory assay of the five *azo* compounds revealed that 4c and 4e modulated the antimicrobial activity of ciprofloxacin against *Pseudomonas aeruginosa* and *Staphylococcus aureus.* 4c and 4e also modulated the antimicrobial activity of rifampicin against *Mycobacterium smegmatis*. Exploiting the ability of 4c and 4e to act by a mode of action revealed that they have biofilm formation inhibitory potential.

Keywords

azo compounds, antimicrobial, biofilm inhibition, resistance modulation, efflux pump.

1.0 Introduction

Reports from the World Health Organisation (WHO) indicate that pathogens that cause infectious diseases, which are the third most notable cause of mortality globally have become increasingly resistant to most antibiotics [1,2].

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Infectious disease load is high in developing countries, just as the emergence of multidrug-resistant (MDR) pathogens due to the irrational use of antimicrobials [3,4]. Although gram-negative bacteria are liable to develop the MDR phenotype, the persistence of resistance is shown to be the same in both gram-positive and gram-negative pathogens [5]. The high prevalence of MDR bacteria such as *Pseudomonas sp.*, *Acinetobacter sp.*, *Staphylococcus sp.,* and *Mycobacterium sp.* are major contributors to nosocomial and community-acquired infections and pose critical challenges in most geographical jurisdictions [6,7]. Fungal pathogens are also shown to develop resistance against routinely prescribed antifungal drugs, such as amphotericin B, fluconazole, and penicillins [8]. This is especially dangerous considering that fungal pathogens such as *Candida* sp. and bacteria are responsible for 75 % of all microbial infections [9]. The situation is worsened by the development of multi- and extensively-drug resistance, like in the case of *Mycobacterium tuberculosis* [10, 11]. *Mycobacterium tuberculosis,* due to its highly impermeable membrane, is gradually becoming resistant to all available antibiotics, including the fluoroquinolones [10, 11]. Laboratory evidence has shown that the presence of efflux pumps and biofilm formation in *Mycobacterium sp*, *Pseudomonas aeruginosa*, *Escherichia coli,* and *Candida albicans* has contributed to the increasing resistance against most antibiotics [11]. It is also a fact that biofilms are responsible for 65 % of all bacterial infections which has contributed to increased antimicrobial resistance [11]. Moreover, most of the concerns in the treatment of nosocomial infections are with the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) [11]. Their infections pose a worrying situation in the health sector since most of them are

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resistant to many antibiotics. Therefore, the understanding of their mechanisms of resistance development would be useful in the development of novel antimicrobial agents [12].

However, over the past few years, the global antibiotics pipeline has been running dry, as a result of the surge in resistance development, coupled with a decline in the number of new antibiotics being approved and reaching the market or the patient's bedside [13]. For instance, the production of only two novel classes of antibiotics in the last five decades indicates that it may be a challenging task to produce an adequate set of compounds to boost modern drug discovery programs in the years to come [14]. As a consequence, plans are always needed in advance to design and develop new therapeutic warheads that could either play a role as standalone drugs or adjuvants to existing drug therapy [15].

To this end, *azo* dyes are a subset of suitable candidates to achieve this objective since they are an important class of antimicrobial agents with versatile applications [16, 17]. It has been established that the inclusion of a suitable heterocyclic moiety increases the activity of the azo linkage [18]. Furthermore, the introduction of nitrogen-containing aromatic heterocycles such as quinoline, pyridine, thiadiazole, and triazole, confer significant biological activities including antimicrobial, anticancer, anti-inflammatory, and anti-mycotic to the compound [19]. Their synthetic route follows the diazotization of primary aromatic amines and an electron-rich moiety (coupling reagents) which mimics the principle of molecular hybridization [20, 21] (**Scheme 1**). Also, recent drug discovery strategies involve the development of novel chemical moieties by employing pharmacophore hybridization: a

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process of linking two biologically active groups together by a covalent bond [22, 23]. The selection criteria for molecular hybridization is usually based on their expected or practical pharmacological activities [23]. In the prospects of the hitherto evidence, and for the contribution to the research towards the development of pharmacophore hybrids as potential antimicrobial compounds, authors chose to chemically construct a library of novel molecular hybrids using 8hydroxyquinoline as a scaffold, under suitable experimental reaction conditions, and access the antimicrobial properties.

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2.0 Materials and Methods

2.1 Bacterial strains

Standard reference strains of gram-negative pathogens, including, *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (clinical strain)*, Klebsiella pneumonia* (clinical strain), *Salmonella typhi* (clinical strain), and gram-positive pathogens, including, *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212) were used for the antimicrobial study. *Mycobacterium smegmatis* mc^2 155, an acid-fast mycobacterium, was also used in this study. The test organisms were all obtained from the cell culture laboratory, Department of Pharmacology, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana.

Glycerol stocks of the pathogens stored at -80 °C in a freezer, were removed, thawed, and cultured either on nutrient agar slants for most of the bacteria, or Middlebrook 7H10 agar for *Mycobacterium smegmatis.*

2.2 Culture Media and Reference Antibiotics

Nutrient Agar and Broth, as well as Middlebrook 7H10 Agar and Middlebrook 7H9 Broth, were purchased from Oxoid Limited, (Basingstoke, United Kingdom). Ciprofloxacin and Rifampicin were obtained from Sigma Aldrich ™ (Michigan, USA).

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2.3 Chemicals, Reagents and Instrumentation

The reactants and solvents were all obtained commercially from Fisher Scientific ™ (United

Kingdom). The progress of each synthetic reaction was monitored using thin-layer chromatography (TLC), on a pre-coated silica gel plate (Merck F254) and visualized with UV light (254 nm and 357 nm) or iodine vapour where necessary. The synthesised compounds were purified by recrystallization using suitable solvents. The samples were run neat to obtain their infra-red (IR) spectra, in the range 400 – 4000 cm^{-1} on a Bruker FTIR spectrophotometer (Bruker FTIR 94133). The ultraviolet-visible (UV-Vis) spectra were measured on a UV-Vis spectrophotometer (Jenway, U.K/7315), at 200 – 800 nm with methanol as blank. The melting point of the synthesised compounds was obtained by using one end or open capillary tubes on a Stuart melting point apparatus (England) and are uncorrected.

2.4 Synthesis, and Characterization of Azo Compounds

This study included the design and synthesis of analogues of 8-hydroxyquinoline *azo* compounds, bearing benzene with different substituents ($-NO_2$, -COOH, $-COCH_3$). The *azo* compounds were synthesised as previously described in literature [24]. The primary aromatic amine compounds (2 mmol) were independently reacted with $NaNO_2$ (1 M, 20 mL) in the presence of HCl (2 M, 20 mL, 36 % w/v) at a temperature range of 0 – 5 ^{o}C with the help of an ice bath to achieve diazotization. The clear diazonium solutions were confirmed by the presence of an instantaneous blue color on testing with starch iodide paste or paper. The diazonium compounds were then coupled

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with 8hydroxyquinoline in the presence of NaOH (2 M, 200 mL, 98 %) at a temperature range of 0 – 5 ^{o}C in an ice bath while stirring to achieve coupling. The precipitate of the coupled products was filtered by suction and dried, washed, and recrystallized from hot ethanol to give coloured solids **4a-e,** with yields of 60-92 % (**Figure 1**).

Figure 1: General scheme for the synthesis of the azo compounds **4a-e**; **4a** (X=*o*-NO_2), **4b** (X= *m*NO_2), **4c** (X=*p*-NO_2), **4d** (X= *p*-$COCH_3$), **4e** (X=*p*-COOH): *o*, *m* and *p* represent ortho, meta, and para substitution respectively.

2.5 In silico Pharmacokinetic and Toxicity studies

Computer-aided analysis of the pharmacokinetics and toxicity parameters of the compounds was performed using admetSAR (http://lmmd.ecust.edu.cn/admetsar2/), an open-access online software, to analyze the Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) properties. The results were further validated with ADMETlab [25], another open-access online software for pharmacokinetic predictions. The parameters for drug-likeness measured included molecular weight, the number of hydrogen bond acceptors (HBA) and donors (HBD), topological polar surface area

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(TPSA), and partition coefficient (cLogP). Absorption was calculated as: Percentage (%) Abs = 109 – (0.345 × TPSA) [26]. Toxicity prediction focused on carcinogenicity, Ames mutagenicity, hepatotoxicity, and acute toxicity dose levels.

2.5 Antimicrobial Activity of Synthesised Azo Compounds

2.5.1 Preparation of the Standard 96-Well Plates

A stock concentration of the compounds was serially diluted using dimethyl sulfoxide (DMSO) in a polymerase chain reaction (PCR) half-skirted 96-well plate to give a concentration range 500 – 0.5 μg/mL. DMSO was used at a final concentration of < 1 %. A volume of 2 μL of the compounds was transferred into their corresponding wells in a standard 96-well plate, and the 200 μL of melted agar dispensed into each well with shaking to mix thoroughly. The plates were left undisturbed to solidify.

The bacterial suspension (2 μL) of 1×10^6 cfu/mL was spotted on each well. The plates were sealed with transparent foil and wrapped with aluminium foil and incubated at a temperature of 37 °C for 18 to 24 h. Ciprofloxacin and Rifampicin were used as reference drugs for bacteria and *Mycobacterium* respectively. Wells with no drugs were included to serve as growth control. The well containing the lowest concentration of a compound for which no growth was visually observed within the incubation period, was determined as the minimum inhibitory concentration (MIC) of that compound against the microorganisms [27].

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2.5.2 Susceptibility of Test Organisms to synthesised compounds

Different concentrations of the *azo* compounds were tested against a panel of Gram-positive bacteria (*Enterococcus faecalis*, and *Staphylococcus aureus*), Gram-negative bacteria (*Proteus mirabilis, Klebsiella pneumoniae, Salmonella typhi*, *Escherichia coli*, and *Pseudomonas aeruginosa*), and an acid-fast bacterium (*Mycobacterium smegmatis*), to determine their MICs using the high-throughput spot culture growth inhibition assay (HT-SPOTi). In the HT-SPOTi assay, molten agar maintained at 55-60 °C was dispensed into 96-well plates to which 2 µL of serially diluted *azo* compounds have been added starting from a stock of 50 mg/mL. The bacterial suspension (2 µL; 1×10^6 cfu/mL) was added to each plate, sealed, and incubated for 18 – 24 h. The lowest concentration at which bacterial growth was completely inhibited by the compound was observed visually, and the MIC was recorded [28, 29].

2.5.3 Resistant Modulatory Assay

To evaluate the effect of the compounds **4c** and **4e** on selected antibiotics, the combination assay of the compounds with ciprofloxacin and/or rifampicin was evaluated using the HT-SPOTi assay in a 96-well microtitre plate as [29, 30] with modifications. The compounds were serially diluted in DMSO to include MIC and sub-MIC concentrations. The checkerboard was constructed by adding 1 µL of each of the dilution concentrations to the corresponding well and 1 µL of the MIC of the standard drug was added. The same was done for the standard drug ciprofloxacin and the MIC's of the compounds added. A volume of 200 µL nutrient agar medium or Middlebrook 7H10 agar medium supplemented with 10 % (*v*/*v*) Oleic acid Albumin Dextrose Catalase (OADC) was dispensed

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into the plates as previously described for the gram-positive and -negative bacteria and *Mycobacterium* respectively. The plates were then spotted with 2 μL of bacteria (~ 1 x 10^6 CFU/mL), sealed, and incubated at 37 °C for 24 h. The Fractional Inhibitory Concentration Indices (FICI) values were calculated using the formula:

$$\text{FICI} = \frac{\text{MIC}_A \text{ in the presence of B}}{\text{MIC}_A} + \frac{\text{MIC}_B \text{in the presence of A}}{\text{MIC}_B}$$

The combining effect of the compounds in with ciprofloxacin or rifampicin against the pathogens was interpreted as follows: **Synergy, FICI $\leq$ 0.5; Indifference, 0.5 $<$ FICI $>$ 4.0; and antagonism, FICI $\geq$ 4.0.**

2.5.4 Biofilm Growth and Biofilm Inhibition Assay

A colorimetric microplate-based assay modified from Cheng *et al* [31], was used to screen the most active compounds (**4c** and **4e**) for biofilm inhibitory activity against *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Mycobacterium smegmatis. Pseudomonas aeruginosa* and *Staphylococcus aureus* were cultured on Mueller Hinton agar and sub-cultured in Tryptic Soy Broth (TSB), while Middlebrook 7H10 agar media supplemented with 10 % OADC was used to culture *M. smegmatis* and subsequently sub-cultured in TSB. An overnight culture of the micro-organisms was inoculated in 5 mL TSB and incubated at 37˚C for 24h with shaking at 120 rpm. The cultures were diluted in TSB (supplemented with additional glucose to a final concentration of 1 %) to give ~ 1x 10^6 cfu/mL (determined by OD_{600}). Two-fold serial dilutions of the compounds were done with TSB to give sub-inhibitory concentrations. An amount of 10 μL of the compounds was transferred into a 96-well tissue cultured

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plate and mixed with TSB supplemented with glucose to give a final concentration of 1/2 MIC, 1/4 MIC, 1/8 MIC, and 1/16 MIC. The wells containing only the innocula were included to serve as growth control whiles Ciprofloxacin and Rifampicin (for *M. smegmatis*) served as drug control. Sterile TSB was added as an additional control to ensure that the media remained sterile during the experiment. The plates were incubated at 37 °C for 24 h without shaking to allow cell attachment and biofilm development. Following incubation, the planktonic cells were gently aspirated, and the wells were washed with phosphate buffer saline (PBS, *pH* 7.2) twice. After rinsing, the biofilm was fixed by incubating for 20 min at 37 °C and staining with 200 µL of 0.1 % (w/v) crystal violet for 10 minutes at room temperature. The excess stain was removed by washing with distilled water and left to air dry. Subsequently, the stain was solubilized with 125 µL of 95 % ethanol for 15 minutes. The optical density of each well was measured at 600 nm (OD_{600}) using an automated plate reader (Biotek Synergy H1 Hybrid MultiMode Reader: 271230). The bioassay was performed in triplicate for validation. The results were expressed as percentage inhibition:

$$Percentage\ Biofilm\ Inhibition\ (\%) = \left(\frac{Control\ OD_{600} - Test\ OD_{600}}{Control\ OD_{600}}\right) \times 100\%$$

2.6 Statistical Analysis

The data from the study were analyzed using GraphPad Prism (Version 8.01, GraphPad Software Inc., USA). The data were described using descriptive statistics and tested inferentially using One-Way analysis of variance (ANOVA) with the Neuman-Keuls post-test. The levels of significance were set at $p < 0.05$ in all scenarios.

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3.0 RESULTS AND DISCUSSION

3.1 Characterization of synthesised Azo Compounds

The synthesis of the library of azo dyes **(4a-e)** (**Table 1; Figure 1**) was performed according to methods in the literature. The compounds with NO_2, $COCH_3$ and COOH substituents were chosen because of their potential antimicrobial activities in studies as reported by works from [32] and [33].The compounds were obtained in good yields and high purity confirmed by TLC, melting point, and spectroscopic methods. The treatment of 8-hydroxyquinoline (3) with various primary aromatic amines (**1a-e**) resulted in the formation of reactive diazonium intermediates (**2a-e**) which were coupled with 8-hydroxyquinoline (3) to produce *azo* quinolin-8-ol derivatives. The compounds, **4a-e**, known and reported in literature were confirmed based on IR, UV-Vis, and ^{1}H NMR spectroscopic data [34]. For instance, the IR spectrum of **4c** showed vibrational bands for OH and N=N at 3088 and 1447 cm^{-1} respectively. The UV-Vis absorption spectrum of 370 nm confirmed the presence of extended chromophores after molecular hybridization. The sharp uncorrected melting points confirmed the purity of the compounds. The visible colours of the compounds were due to the presence of extended conjugation, which increases the wavelength of absorption. However, the different colours suggested the presence of varying substituents and their positions on the chromophore system (**Table 1**). This was confirmed by their UV-Vis absorption spectra (**Figure SM1**) and their respective electron transitions (**Table SM1**) [34].

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Table 1: Physical properties of the azo compounds.

Compound	R	% yield	Melting points (°C)	Colour
4a	*o*-nitro	70	218-221	Orange
4b	*m*-nitro	78	224-227	Orange
4c	*p*-nitro	85	130-132	Dark brown
4d	*p*-$COCH_3$	60	214-217	Brick red
4e	*p*-COOH	92	290-293	Dark brown

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The template below represents the structural scaffold for the synthesis showing the varying substituents **(Figure 2).**

Figure 2: Scaffold design for 8-hydroxyquinoline *azo* compounds **4a-**

3.1.1 5-(2-nitrophenyl) diazenylquinolin-8-ol (4a)

According to the general procedure, *o*-nitroaniline (1.04 g) was reacted with $NaNO_2$ (1 M, 20 mL,) in the presence of HCl (2 M, 20 mL, 36%) to afford the *title compound* (70 %) as an orange solid after purification (R_f 0.70; EtOAc: MeOH (9:1); Mpt: 218-221 ^oC; UV-Vis/ nm: 245 and 405; IR; v_{max}/cm^{-1} :3217 (O-H), 1453 and 1433 (N=N), 1340 (C-N stretch)

3.1.2 5-(3-nitrophenyl) diazenylquinolin-8-ol (4b)

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According to the general procedure, *m*-nitroaniline (1.04 g) was reacted with $NaNO_2$ (1 M, 20 mL,) in the presence of HCl (2 M, 20 mL, 36 %) to afford the *title compound* (78 %) as an orange solid after purification (R_f 0.73; EtOAc: MeOH (9:1); Mpt: 224-227 ^oC; UV-Vis/ nm: 245 and 405, IR: ν_{max}/cm^{-1}:3078 (O-H), 1426 and 1404 (N=N), 1348 (C-N stretch).

3.1.3 5-(4-nitrophenyl) diazenyl) quinolin-8-ol (4c)

According to the general procedure, *p*-nitroaniline (1.04 g) was reacted with $NaNO_2$ (1 M, 20 mL) in the presence of HCl (2 M, 20 mL, 36 %) to afford the *title compound* (85 %) as a dark brown solid after purification (R_f 0.76; EtOAc: MeOH (9:1); Mpt: 130-132 ^oC; UV-Vis/ nm: 255, 275 and 370; IR: ν_{max}/cm^{-1}: 3088 (O-H), 1506 and 1447 (N=N), 1232 (C-N stretch).

3.1.4 1-(4-(8-hydroxyquinolin-5-yl) diazenyl) phenyl) ethan-1-one (4d)

According to the general procedure, *p*-aminoacetophenone (1.04 g) was reacted with $NaNO_2$ (1 M, 20 mL,) in the presence of HCl (2 M, 20 mL, 36 %) to afford the *title compound* (60 %) as a brick red solid after purification (R_f 0.65; EtOAc: MeOH (9:1); Mpt: 214-217 ^oC; UV-Vis/ nm: 250 and 440, IR ν_{max}/cm^{-1}: 3080 (O-H), 1554 (N=N), 1357 (C-N stretch).

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3.1.5 4-(8-hydroxyquinolin-5-diazenyl) benzoic acid (4e)

According to the general procedure, *p*-aminobenzoic acid (1.04 g) was reacted with $NaNO_2$ (1 M, 20 mL,) in the presence of HCl (2 M, 20 mL, 36 %) to afford the *title compound* (92 %) as a dark brown solid after purification (R_f 0.80; EtOAc: MeOH (9:1); Mpt: 290-295 ^oC; UV-Vis/ nm: 250 and 410, IR ν_{max}/cm^{-1}: 3066 (O-H), 1581 and 1545 (N=N), 1372 (C-N stretch).

3.2 In Silico Pharmacokinetic and Toxicity Studies

The determination of the pharmacokinetic and toxicity properties of compounds is of great importance in the drug development process, as it has been shown that approximately 40 % of drug candidates fail at the clinical trial stages due to poor absorption, distribution, metabolism, and excretion (ADME) profiles [35]. Therefore, assessing the pharmacokinetics and toxicity properties of molecules in the early stages of drug discovery considerably reduces the risk of late-stage attrition by detecting early problematic properties and focusing on lead optimization efforts to enhancing the desired ones. With this in mind, *in silico* analysis of the 8-hydroxyquinoline *azo* dyes was performed for the evaluation of their ADME properties. The outcomes from the *in-silico* modelling of the synthesised compounds are summarized in **Table 2** and **Table SM3**.

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Table 2: In silico physicochemical and pharmacokinetic parameters of synthesised compounds

Physicochemical parameters

Compound	**Molecular weight**	**cLogP**	**H-bond Donor**	**H-bond Acceptor**	**TPSA (140 Å^2)**	**Rotatable Bonds**	% ABS
4a	294.27	4.26	1	6	100.98	3	74.16
4b	294.27	4.26	1	6	100.98	3	74.16

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4c	294.27	4.26	1	6	100.98	3	74.16
4d	291.31	4.56	1	5	74.91	3	83.16
4e	293.28	4.05	2	5	95.14	3	76.18

cLog P: Calculated lipophilicity, H-bond Donor: Number of hydrogen bond donors, H-bond Acceptor: Number of hydrogen bond acceptors, TPSA: Polar surface area (Å^2), Violation: Number of violations from Lipinski's rule of five, %ABS: Absorption percentage.

The compounds demonstrated good absorption; with the most active compound(s) recording 74.16% (4c) and 76.18 % (4e) absorption (**Table 2**). According to Lipinski's rule-of-5, orally active drugs should show no more than one violation of the following four criteria: molecular weight ≤ 500, logP (lipophilicity) ≤ 5, number of hydrogen bond donors ≤ 5, and number of hydrogen bond acceptors ≤10 [36]. None of the compounds violated Lipinski's rule and therefore have the potential to be developed into oral pharmaceutical drugs. TPSA was used to predict the cell permeability and transport properties of compounds and those with TPSA ≤ 140 Å^2 were predicted to possess good oral bioavailability (**Table 2**). The compounds displayed TPSA values ranging between 74.91 to

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100.98 Å^2, indicating they had good cell permeabilities and therefore good oral bioavailabilities. The synthesised compounds were predicted to be mildly toxic exhibiting no carcinogenicity but were hepatotoxic and mutagenic (**Table SM3**). They also showed no tendencies to interfere with the cardiac conduction system and were placed in category III of acute oral drugs with LD_{50} between 500 – 5 000 mg/kg. Further optimization could therefore focus on improving their toxicity profiles.

3.3 Antimicrobial Activity of Synthesised Azo Compounds

3.3.1 Susceptibility of Test Organisms to synthesised compounds

A panel of eight different pathogens (clinical strains) was used in the antimicrobial activity study of which five were gram-negative bacteria, two were gram-positive bacteria, and an acid-fast bacterium. The MICs of the compounds following the HT-SPOTi assay are shown in **Table 3**. Seven of the pathogens showed susceptibility to the test compounds with only *Salmonella typhi* showing complete resistance to all the five test agents. Among the pathogens tested, *Mycobacterium* was the most resistant, with little susceptibility to the synthesised compounds employed. *Pseudomonas aeruginosa* and *Staphylococcus aureus* possessed least resistance towards the test agents, followed by *Proteus mirabilis*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterococcus faecalis*. This is also seen in the bacteriostatic activity of phenylazo-diaminopyridine hydrochloride against *Staphylococcus aureus* and *Escherichia coli* [36].

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Table 3: Antimicrobial activity of the synthesised azo compounds

	Pseudomonas aeruginosa	*Staphylococcus aureus*	*Proteus mirabilis*	*Escherichia Coli*	*Klebsiella pneumoniae*	*Salmonella typhi*	*Enterococcus faecalis*	*M. smegmatis*
4a	7.8	3.9	15.6	62.5	500	>500	>500	>500

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4b	15.6	15.6	15.6	62.5	250	>500	>500	>500
4c	125	15.6	15.6	62.5	250	>500	>500	500
4d	31.2	62.5	31.2	125	>500	>500	>500	>500
4e	7.8	3.9	7.8	125	500	>500	500	500
CPR	0.9	0.07	≤ 0.5	0.15	≤ 0.5	15.6	≤ 0.5	

Minimum inhibitory concentration

s (μg/mL)

Key: CPR-Ciprofloxacin; RPN: Rifampicin; NA: not applicable

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3.3.2 Structure-Activity Relationship (SAR) effect on antimicrobial activity of test compounds

Among the substituents on the primary aromatic amine, it was observed that both the nitro and carboxylic groups recorded the highest antimicrobial activity, followed by the ketone group. The study also revealed both the nitro and carboxylic substituents to be active against *Mycobacterium smegmatis* mc^2 155. The structural moieties of the test agents responsible for the antimicrobial activity could be attributed to the varying substituents on the primary aromatic amine as well as their positions on the aromatic ring; be it ortho, meta, or para to the *azo* linkage.

3.3.3 Antimicrobial Resistance Modulation Studies (Combination Assay)

Based on the results obtained from the susceptibility studies, an antimicrobial resistance modulatory activity was carried out using the combination assay for the two most active test compounds (4c and 4e) against the two most susceptible pathogens, *Staphylococcus aureus,* and *Pseudomonas aeruginosa*, as well as *Mycobacterium smegmatis,* the most resistant pathogen, to investigate their synergistic or antagonistic effects. The results of the modulatory studies are shown in **Table SM4** and **Table SM2**. It was observed that 4c and 4e modulated the antimicrobial activity of ciprofloxacin against *Pseudomonas aeruginosa* and *Staphylococcus aureus.* The compounds, 4c and 4e, also modulated the antimicrobial activity of rifampicin against *Mycobacterium smegmatis* (**Table SM2**). The fractional inhibitory concentration index (FICI) of the activity of the test agents were calculated as indicated above (**Table SM3**). A compound is said to be synergistic with the reference drug if its FICI is ≤ 0.5,

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indifference with no interaction if FICI is between 0.5 and 4.0 and antagonistic with the reference drug if FICI is above 4.0. As shown in the FICI test results (**Table SM2**), combination of 4c with ciprofloxacin was synergistic against *Pseudomonas aeruginosa*. Combination of 4e with ciprofloxacin was antagonistic against *Staphylococcus aureus*. Both 4c and 4e combination with rifampicin were indifferent against *Mycobacterium smegmatis*, but with high synergistic potential rather than antagonism. Again, combination of 4c with ciprofloxacin against *Staphylococcus aureus* and combination of 4e with ciprofloxacin against *Pseudomonas aeruginosa* were also indifferent with high synergistic potential rather than antagonism (**Table SM2**).

3.3.4 Biofilm Inhibition Activity

Microorganisms may develop resistance against antimicrobial agents through the formation of biofilms. Biofilms contribute a significant percentage to microbial infections globally. Therefore, test agents in antimicrobial drug discovery and development may be expected to work to prevent antimicrobial resistance by reducing or inhibiting the formation of these biofilms by the microorganisms [38].

The compounds, 4e and 4c were assessed for their ability to reduce or inhibit formation of bacteria biofilms of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Mycobacterium smegmatis* at 24 h using the sub-inhibitory concentrations 3.9 – 0.49 μg/mL, 1.95 – 0.24 μg/mL, and 250 – 31.25 μg/mL respectively (which corresponded to 1/4, 1/8 and 1/16 MIC by 4c, and 1/2 and 1/16 MIC by 4c) for both compounds. The study showed that both

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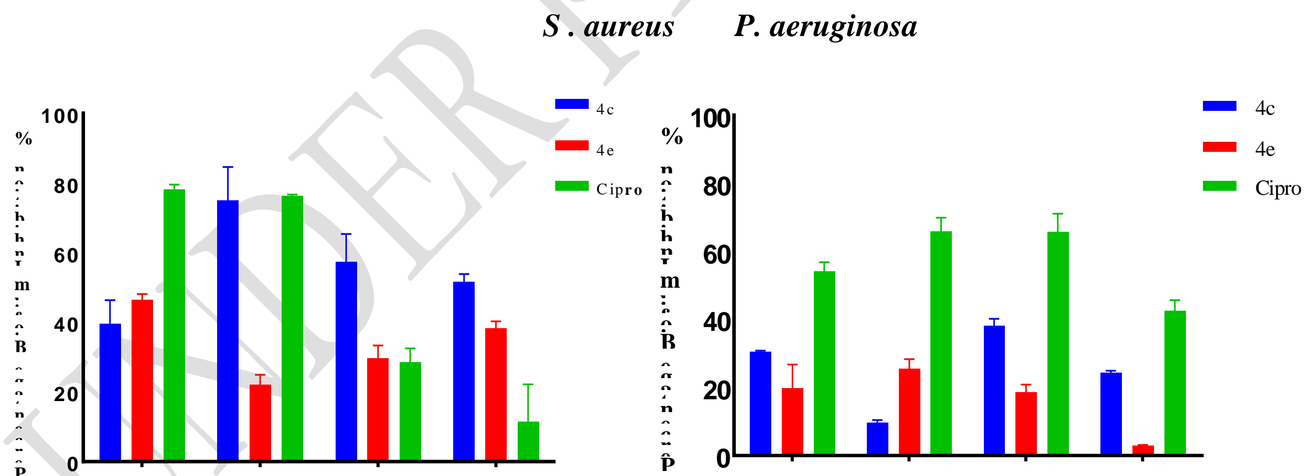
compounds demonstrated biofilm inhibitory effects against the test microorganisms (**Figure 3**). All the concentrations of 4e demonstrated biofilm inhibition against *M. smegmatis* mc^2 155, whiles an inhibitory activity was recorded for 4c at 1/8 and 1/16 MIC. Biofilm inhibition activities of 4e were recorded at 1/4, 1/8 and 1/16 MIC, while that of 4c were at 1/2, 1/4 and 1/18 MIC respectively against *P. aeruginosa* (**Figure 3**). The results further indicated that the antibiofilm activities of 4c and 4e against all the test microorganisms were not concentration dependent, as increasing concentrations of both compounds did not result in an increase in anti-biofilm effect (**Figure 3**). This shows that the compounds 4c and 4e showed biofilm inhibitory potential at the different concentrations used. The antibacterial activity of 4c and 4e as demonstrated in this study suggests their potential use as scaffolds for further structure optimisation and expansion of the library to improve the antimicrobial profile.



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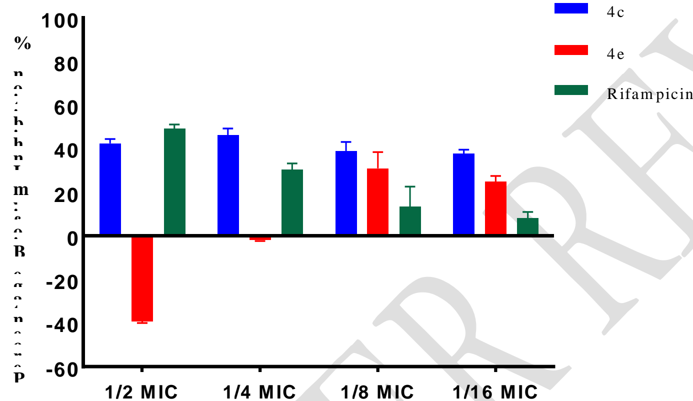
1/2 M IC 1/4 M IC 1/8 M IC 1/16 M IC 1/2 MIC 1/4 MIC 1/8 MIC 1/16 MIC

C on c(µ g/L) Conc(µg/L)

(a) (b)

M. smegmatis mc^2 155



Conc (µg/mL)

(c)

Figure 3. Percentage biofilm inhibition caused by concentrations of 4c and 4e [1/2 MIC: 1.9500 µg/mL, 1/4 MIC: 0.975 µg/mL, 1/8 MIC: 0.4875 µg/mL, and 1/16 MIC: 0.2438 µg/mL] against the biofilm forming **(a)** *S. aureus* and **(b)** *P. aeruginosa*. Ciprofloxacin was employed as the reference drug at 1/2 MIC: 0.0780 µg/mL, 1/4 MIC: 0.0390 µg/mL, 1/8 MIC: 0.0195 µg/mL and 1/16 MIC: 0.00975 µg/mL. **(c)** *M. smegmatis* mc^2 155: 4e and 4c [1/2 MIC: 250 µg/mL, 1/4 MIC: 125 µg/mL, 1/8 MIC: 62.5 µg/mL, and 1/16 MIC: 31.25 µg/mL] against the biofilm

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forming *M. smegmatis* mc^2 155. Rifampicin was employed as the reference drug at 1/2 MIC: 3.9063 µg/mL, 1/4 MIC: 1.9531 µg/mL, 1/8 MIC: 0.9766 µg/mL and 1/16 MIC: 0.4883 µg/mL.

4.0 Conclusions

A library of five (5) azo compounds were synthesised through the diazotisation and coupling pathways. 5-(4-nitrophenyl) diazenyl) quinolin-8-ol (4c) and 4-(8-hydroxyquinolin-5-diazenyl) benzoic acid (4e) exhibited better antibacterial and antimycobacterial activities against *Mycobacterium tuberculosis* and two ESKAPE organisms (*Staphylococcus aureus*, and *Pseudomonas aeruginosa*) respectively. Compounds 4c and 4e also exhibited antimicrobial resistance modulatory effects. The resistant modulatory activity and biofilm inhibition potential of these compounds may suggest their potential use as antibacterial agents after further drug development.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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