IJEAST

INTERNATIONAL JOURNAL
OF ENGINEERING APPLIED SCIENCE
AND TECHNOLOGY

VOLUME : 5 ISSUE : 3 Print / Issue Publication Date: 15-Sep-2020

ISSN : 2455-2143

DOI : 10.33564/IJEAST.2020.v05i03.005

Indexed In

Google SCHOLAR OPEN ACCESS doi Crossref

WWW.IJEAST.COM

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International Journal of Engineering Applied Sciences and Technology, 2020
Vol. 5, Issue 3, ISSN No. 2455-2143, Pages 26-32
Published Online July 2020 in IJEAST (http://www.ijeast.com)

ANTIMICROBIAL ANALYSIS AND CHARACTERIZATION OF PHENAZINEAZO-2-NAPHTHOL SYNTHESIZED FROM PHENAZINE-1-CARBOXYLIC ACID ISOLATED FROM *PSEUDOMONAS AERUGINOSA*

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AbstracT – The containment of microbial infections and diseases in our environment have prompted scientists all over the world to search for improved antibiotics to be used for such treatments, as some already existing antibiotics have become toxic to the infected hosts as well as resistant to the pathogens. Phenazines are para diazine with annular two nitrogen atoms at 1,4 position joined to two phenyl group in a side by side pattern. The derivatives of the compound(s) are primarily produced from microorganisms and can also be synthesized, example of this is phenazine-1-carboxylic acid (PCA). In the present study, phenazine-1-carboxylic acid biosynthesized from *Pseudomonas aeruginosa* isolated from soil was subsequently used to synthesize phenazineazo-2-naphthol following amidation with ammonium carbonate and Hoffmann degradation reactions to reduce the compound to phenazineamine under reflux condition of 50 °C before diazotization and coupling with 2-naphthol. The results obtained from the UV-vis, IR and NMR spectra were used to elucidate the important peaks in the compound. The pathogenic organisms (*Staphylococcus aureus*, *Escherichia coli* and *Aspergillus fumigatus*) were tested against 50 µg/mL, 100 µg/mL, 150 µg/mL, 200 µg/mL and raw sample concentrations of the compound as well as 30 µg/mL Augmentin (Au, control drug) and 30 µg/mL Oflocitoxin (OFX, control drug) to determine the inhibition activities. In summary, the compound was active against *Staphylococcus aureus* at concentrations of 150 µg/mL and above, then against *Escherichia coli* at concentrations of 100 µg/mL and above. and against *Aspergillus fumigatus* at concentrations of 200 µg/mL and above whilst the 30 µg/mL Augmentin and Oflocitoxin also tested against the organisms were more active against the bacteria, although the Augmentin was less active (almost inactive) to the fungus (*Aspergillus fumigatus*) than the Oflocitoxin which was totally inactive to it.

Keywords – Phenazineamine, 2-naphthol, Phenazine, Phenazineazo-2-naphthol, Antimicrobial.

I. INTRODUCTION

Natural phenazines can be produced from *Pseudomonas aeruginosa* and several other gram-negative and gram-positive bacteria such as *Streptomyces* and *Bacillus* [1-5]. Phenazine compound secreted by *Pseudomonas aeruginosa* are largely found in phenazine-1-carboxylic acid and this can be synthetically constructed [6]. The toxicity problems caused by some synthetic compounds or dyes to the environment are part of the reasons scientists consider natural alternative which has proven wide applications, more especially from production through microbial sources [7]. Coloured organic compound with diverse applications, synthesized through diazotization and azo coupling reactions is known as azo dye [8-9]. Most azo coloured (dyes) compounds possess resistance to oxidizing agents, non-toxic, non-basic and slightly acidic [10], and azo-naphthol compounds or dyes possess interesting colours on substrates, not limited to their use in dyeing of fabrics,

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Published Online July 2020 in IJEAST (http://www.ijeast.com)

food colorants, pharmaceutical agents and other purposes [11-14].

Most phenazine compounds especially the naturally occurring ones have antibiotic properties and have been referred to be "phenazine antibiotics", because of their primary function as bacterial warfare agents used in microbial competition [15]. Phenazine-1-carboxylic acid biosynthesized from chorismate by genes constituting the redundant phzA1-G1 and phzA2-G2 operons (phenazine enzyme) is known as "Tubermycin B" because it has strong antibiotic activity against *Mycobacterium tuberculosis* [5, 16, 4] and has displayed an array of interesting and therapeutic biological importance [17] whilst phenazine-5,10-dioxide (PDO) synthesized from phenazine also possesses strong antibiotic activity against some strains of microorganisms [6]. Since azo-naphthol compounds, for example 1-(1-phenylazo)-2-naphthol, have antibiotic activity against some strains of microorganisms [18], and phenazine compound combined with azophenol from both natural and artificial routes also possesses some antibiotic properties [19], therefore phenazine compound combined with azo-2-naphthol from both natural and artificial reaction routes could as well have antibiotic activity against both bacteria and fungi.

II. MATERIAL AND METHODS

The chemical reagents purchased were of analytical grade and were used without further purification. The equipment used are centrifuge (Health Med. England. 80-2), Petri dishes, incubator, melting point apparatus (microscopic), autoclave (Desco), Meta-lab water bath (MSI 17B), pH meter (Thermo scientific PHS-3C), thermocool refrigerator (HTF-259H), magnetic stirrer (constant temp. HY-3D), orbital shaker/vibrator (mechanical HY-4), weighing balance (Electric FA2004), UV-Visible spectrophotometer (Metro UV-5800PC), FT-IR spectrometer (Agilent Happ-Genzel) and NMR spectrometer (Agilent-NMR-vnmrs 400 and Bruker Avance III HD 500).

1. Collection and preparation of soil sample.

Following the previous research by Onunkwo and Okerulu [19], the soil sample was collected randomly after digging 100 cm with a washed and sterilized container from the waste disposal channel of A. C Drug Company Limited, Thinkers Corner Enugu, Enugu State (swampy area channeled to a flowing stream). The soil sample was serially diluted following Ten-fold dilution method by addition of the soil sample (1 g) into a 100 mL volumetric flask and then made up to the mark with de-ionized water (10^{-1}). Other dilutions (10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) were prepared by serially diluting 1 mL to the remaining four test-tubes containing 9 mL of de-ionized water.

2. Isolation of *Pseudomonas aeruginosa*

Following the manufacturers guide and the previous work by Onunkwo and Okerulu [19], Cetrimide agar (selective medium for *P. aeruginosa*) was prepared and sterilized in an autoclave at 121 °C for 15 mins and left to cool on a sterilized and microorganism-free environment to about 40 – 60 °C. The prepared media (20 mL) was poured into each of the petri dishes containing 1 mL of different dilutions of the contaminated soil samples and the whole agar plates was incubated for 24 h at 37 °C. After 24 h morphologically the bacterial colony developed on the agar plates. A pure culture of the isolate was prepared by taking streaks from the agar plates on a fresh Cetrimide agar media of the isolate in a bijou bottles, which were incubated at 37 °C for 24 h and stocked.

3. Characterization of the *P. aeruginosa*

Isolates were characterized and identified using conventional microbiological procedures such as colony and culture morphology, gram staining reactions and biochemical tests such as catalase test, oxidase test, indole test, hydrogen sulphide test, motility test and sugar fermentation test as described below [19-20]:

I. Colony and culture morphology

The colony and morphology of *Pseudomonas aeruginosa* was identified by physical observations. A creamy raised colony and smooth edge was observed.

II. Gram staining

A drop of sterile normal saline was placed on a clean slide and the isolate was collected using an inoculating loop. The isolate was emulsified on the slide containing the normal saline forming a thin smear. It was left to dry and then heat fixed using a Bunsen burner flame. The slides were placed on a staining rack. Crystal violet was applied on it for 60 secs, drained off and washed with water. Lugol's iodine was applied and allowed to act for 1 min. It was rinsed off with water and acetone alcohol applied until no colour appeared to flow from the preparation for 30 secs, before washing with water. There was decolorization

of the organism colony when viewed under the microscope, showing a gram negative organism.

III. Indole test

Peptone water (1.5 g) was dissolved in 100 mL distilled water, autoclaved for 15 mins at 121 °C, then cooled and inoculated with the test organism and incubated. After 24 h of incubation, 0.5 mL Kovac's indole reagent was added and shaking to observe for a colour change. There was appearance of a red ring at the surface of the medium which indicated a positive test.

IV. Oxidase test

A piece of filter paper was placed in a sterile dish and was flooded with oxidase reagent (tetramethylparaphenylenediamine dihydrochloride) and the test organism smeared across the impregnated paper. A positive result is indicated by a deep purple after 30 secs.

V. Catalase test

A loopful of isolates was individually emulsified on a clean slide with a drop of sterile distilled water. A drop of hydrogen peroxide was added. A positive result was observed indicated by a bursting bubble which gives rise to an effervescence.

VI. Motility test

A single straight stab was made at the centre of the test tubes containing the semi-solid medium about half the depth of the medium. The medium was then incubated at room temperature for 24 hrs. A positive result was observed for motility test as detected by the migration of the organism from the stab line and diffusion into the medium causing turbidity and rendering the medium opaque.

VII. Sugar Fermentation test

The sugar fermentation tests were carried out using 1 % (w/v) of the sugars in normal peptone water containing a drop of bromothymol blue indicator. The solutions were dispensed in test tubes with inverted Durham tubes for the collection of gas. The contents in the test tubes were sterilized at 121 °C for 15 mins and allowed to cool before inoculation. The test organisms were inoculated into the test tubes and incubated for 24 h at room temperature.
A positive result was noted by a change in colour from green to yellow for acid production, while gas production was indicated by displacement of the medium in the Durham tubes. Before incubation of the media, absence of gas bubbles in the Durham tubes were confirmed. An uninoculated sterile medium served as a control. The procedure was carried out using glucose, lactose and sorbitol.

VIII. Hydrogen Sulphide test

Nutrient broth was prepared and poured into test tubes before sterilization at 121°C for 15 mins. The test organisms were aseptically inoculated into the medium after cooling using lead acetate paper. This was incubated at 37 °C for 24 h and then examined for a black colour which indicates a positive result.

4. Production of Phenazine-1-carboxylic acid (PCA) from the isolated *Pseudomonas aeruginosa* –

[6, 19].

The *Pseudomonas aeruginosa* was streaked on Luria-Bertani (LB) agar plates and incubated at room temperature for 24 hrs. A single colony of *P. aeruginosa* on a LB agar plate was transferred into 100 mL of modified King's A broth (KA): bactopeptone (15.0 g), sodium chloride (13.0 g), glycerol (9.0 mL) and potassium sulphate (1.0 g) were all added to 1,000 mL distilled water; and incubated at 29 - 30 °C with an orbital shaker (200 rpm) for 24 hrs. For increasing PCA production, the starter culture was transferred into an Erlenmeyer flask (1,500 mL) containing fresh modified KA medium with 1:50 bacterial dilutions and incubated for 48 h under the same conditions as described above. An Amberlite XAD-16 resin column was used for the PCA isolation by eluting this column with 70 % (v/v) acetonitrile in distilled water.

I. Purification of the PCA

The purification of the compound was achieved in two steps: firstly, the pH of the crude phenazine solution was adjusted to 2.5 and residues removed by centrifugation at 3,500 rpm for 15 mins. Secondly, this solution was separated by a liquid-liquid extraction with dichloromethane. The extracted phenazine was then purified on a silica gel column, equilibrated with dichloromethane. The optimum solvent system for the silica gel column was 90 % (v/v) dichloromethane in ethyl acetate. The yellow crystals formed after concentration, were dried, weighed and the yield, as well as the melting point determined. The procedure was repeated to obtain more yields.

5. Synthesis of phenazineazo-2-naphthol 5 from PCA isolated from *P. aeruginosa*

The phenazineazo-1-naphthol (PCA-1N) **5** synthesis from the phenazine-1-carboxylic acid (PCA) isolated from *P. aeruginosa* is as follows (shown in scheme I):

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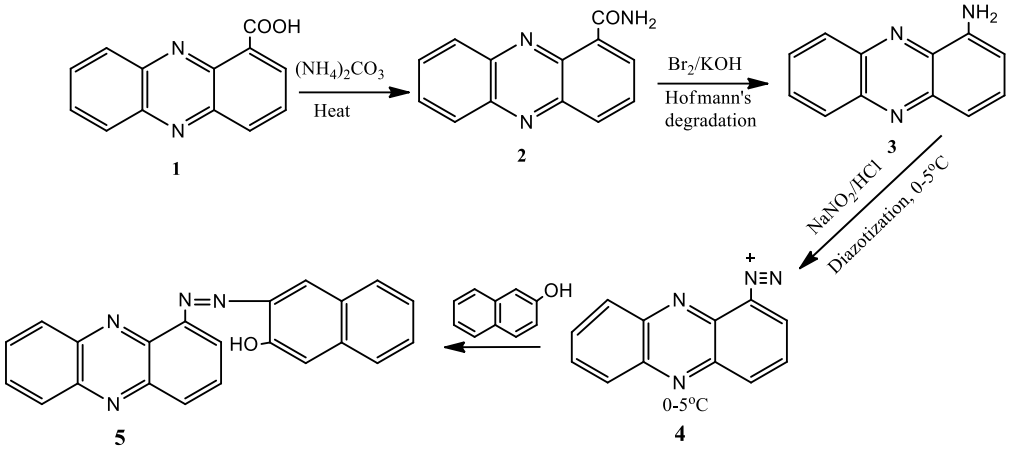

Scheme I: Synthesis of phenazineazo-2-naphthol compound from the PCA

Phenazine-1-carboxylic acid **1** (0.05 g) was refluxed with 1 gram of ammonium carbonate, $(NH_4)_2CO_3$, for 4 h at 50 °C. A mixture of solution of bromine (8.5 mL) and KOH (30 %, 3.8 mL) was added, it was heated for 4 h at 50 °C to degrade the phenazine amide **2** formed (amidation) to produce phenazine amine **3** which was filtered and dried. The procedure was repeated to obtain more of the phenazine amine. The phenazine amine **3** (0.019 g) was mixed with a solution of sodium nitrite (1 g) precooled to 0 - 5 °C and concentrated hydrochloric acid (5 mL) to form the phenazonium ion **4** in an ice bath at 0 - 5 °C. A solution of 1 % 2-naphthol was separately prepared and allowed to cool at 0 - 5 °C for 10 mins. The cooled 1% 2-naphthol solution was added to the phenazonium solution with stirring at 0 - 5 °C. The whole mixture was allowed to stand for 5 mins for complete reaction and the reddish yellow phenazineazo-2-naphthol **5** crystal formed was recovered through suction filtration. It was recrystallized using ethanol, dried in a desiccator and the weight and melting point determined.

5. Determination of the zone of Inhibition and Minimum Inhibitory Concentration Estimation (disks method) – [18-19].

By using Whatman filter paper No. 1, Discs of 5 mm in diameter were produced by using a paper borer. After that, the prepared disks were put in suitable containers. Then, the discs were subjected to autoclaving in order to sterilize the disks (adjusting the conditions of autoclave to 121°C for 15 mins) and left to cool. Later on, the discs were allowed to suck up the sample filtrate at 50 µg/mL, 100 µg/mL, 150 µg/mL, 200 µg/mL, raw and 30 µg/mL each of control antibiotics [Augmentin (Au) and Oflocitoxin (OFX) drug discs] concentrations, maintained for later assay. The produced discs (each one) have the ability to absorb about 0.01 mL of the sample concentrations. The discs with concentrations were place on the prepared plates inoculated with *Staphylococcus aureus*, *Aspergillus fumigatus* and *Escherichia coli* and incubated for 24 hrs. The zone of inhibition was observed, measured in millimeter and the minimum inhibitory concentration estimated.

III. EXPERIMENTAL RESULTS

Table 1: Characterization of the isolate (*Pseudomonas aeruginosa*)

											Sugar fermentation		
Isolate	**Diameter (mm)**	Colony and Morphology	Shape	**Gram Staining**	**Catalase**	**Motility**	**Oxidase**	**Indole**	**Hydrogen Sulphide**	**Glucose**	**Lactose**	**Sorbitol**
PA	1-4	Creamy raised colony, smooth edge	Long rod	-	+	+	+	+	+	Acid	Acid	Acid

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Published Online July 2020 in IJEAST (http://www.ijeast.com)

From Table 1, the results the colony and morphology of *Pseudomonas aeruginosa* was confirmed to be creamy and smooth edged, long rod shaped. It was also observed that the bacterium tested negative to gram staining and positive to catalase, motility, indole and hydrogen sulphide biochemical analyses. The bacterium was able to utilize and ferment glucose, lactose and sorbitol releasing acidic gases in the presence of the sugars. The results obtained above were in agreement with Bergey *et al*. [21].

Phenazineazo-2-naphthol with brownish-yellow colour was found to have a melting point of 122.8 °C at 44.9 % yield higher than the brightly yellow coloured natural phenazine-1-carboxylic acid (PCA) – where it was modified from in reference to Onunkwo and Okerulu [19].

The UV-visible data of phenazineazo-2-naphthol was obtained at 496 nm assumes a reflection of a reddish yellow colour of the compound conjugated system. The wavelength scan obtained depicts the colours of the compound in reference to Donald *et al*. [22]. The Infra-red data against KBr showed the functional group O-H (broad 3507.4 cm^{-1}), C-N (s1360.5 cm^{-1}, 1267.3 cm^{-1}), C-C (s1144.3 – 849.8 cm^{-1}), C=C (s1525 cm^{-1}), C=N (s1625.1 – 1572.9 cm^{-1}), C-H (s790.2-682.1 cm^{-1}), N=N (s1438.8 cm^{-1}). The HNMR data using $CDCl_3$ of δ 7.000 - 8.917 corresponding to aromatic protons and the C13NMR data showed slight 76.78 ppm (R-OH), 125.06 - 153.47 ppm (benzene, C-N, C=N aromatic) of the compound with reference to the work of Donald *et al*. [22], Onunkwo and Okerulu [19], Onunkwo and Ejikeme [18] and Arijit *et al*. [11].

Table 2: The effect of different dilutions of the samples against pathogenic organisms

Organisms	Phenazineazo-2-naphthol (µg/mL)					Control		MIC
	50	**100**	**150**	**200**	**Raw**	**Au**	**OFX**	
Staph. Sp	R	R	1.2	5	7	13	14	150
E. coli	R	0.9	3	7	15	17	29	100
Aspergillus. sp	R	R	R	2	4	0.19	R	200

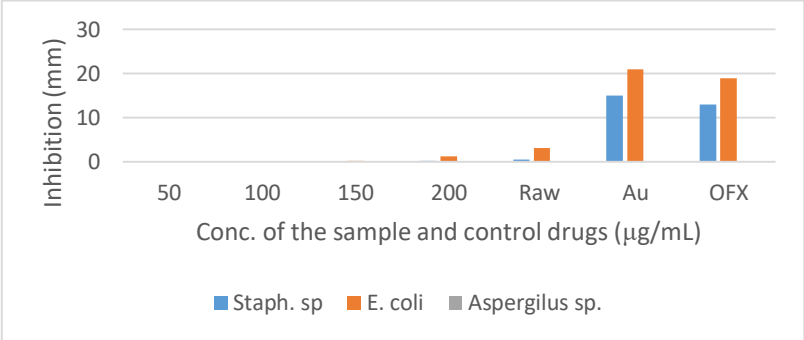

Fig. I: Antimicrobial analysis of phenazineazo-2-naphthol

From the Table 2 and Fig. I, The pathogenic organisms (*Staphylococcus aureus*, *Escherichia coli* and *Aspergillus fumigatus*) were tested against 50 µg/mL, 100 µg/mL, 150 µg/mL, 200 µg/mL and raw sample concentrations of the compound as well as 30 µg/mL Augmentin (Au, control drug) and 30 µg/mL Oflocitoxin (OFX, control drug) to determine the inhibitory activities. The *Staphylococcus aureus* was inhibited from 150 µg/mL concentration of the compound at 1.2 mm and above, and the inhibitions of the 30 µg/mL Augmentin and 30 µg/mL Oflocitoxin occurred at 13 mm and 14 mm respectively; *Escherichia coli* was inhibited from 100 µg/mL concentration of the compound at 0.9 mm and above, and the inhibitions of the 30 µg/mL Augmentin and 30 µg/mL Oflocitoxin occurred at 17 mm and 29 mm respectively while *Aspergillus fumigatus* was inhibited from 200 µg/mL concentration of the compound at 2.0 mm and above, and the inhibition of the 30 µg/mL Augmentin occurred at 0.19 mm, but it resisted the 30 µg/mL Oflocitoxin concentration from the antimicrobial analysis carried out. In summary, the estimated minimum inhibitory concentration of the compound against *Staphylococcus aureus*, *Escherichia coli* and *Aspergillus fumigatus* are at 150 µg/mL, 100 µg/mL and 200 µg/mL respectively. The

compound was active against *Staphylococcus aureus* at concentration of 150 μg/mL and above, then against *Escherichia coli* at concentration of 100 μg/mL and above and against *Aspergillus fumigatus* at concentration of 200 μg/mL and above whilst the 30 μg/mL Augmentin and Oflocitoxin also tested against the organisms were more active against the bacteria, although the Augmentin was more active to the fungus (*Aspergillus fumigatus*) when compared to the Oflocitoxin which was totally inactive to it and this may be as a result of the less concentrations used or on the increased adaptive resistance of the fungus. The results differs slightly from the work of Onunkwo and Okerulu [19] – where the fungus (*Aspergillus fumigatus*) was resistant across all the concentrations of the drugs tested, and differs more widely from the work of Onunkwo and Ejikeme [18], where the fungus (*Aspergillus fumigatus*) was resistant across all the concentrations of the drugs (phenazineazophenol and control drugs – Augmentin and Oflocitoxin) tested; also in the same work, the bacteria (*Staphylococcus aureus* and *Escherichia coli*) resisted 1-(1-phenylazo)-2-naphthol concentrations of 50 μg/mL, 100 μg/mL, and 150 μg/mL tested.

IV. CONCLUSION

The reddish-yellow phenazineazo-2-naphthol which was synthesized from a biosynthesized phenazine-1-carboxylic acid through amidation, Hoffmann degradation, diazotization and azo coupling reactions possesses antimicrobial property against the bacteria and fungus strains tested. Although each of the control drugs also used possess greater inhibitory activity against the bacteria when compared to this compound but one of the control drugs was totally inactive and the other was partially active to the fungus strain. This effect observed in the control drug may be due to the concentration used. In summary, the compound phenazineazo-2-naphthol can be used as antibacterial and antifungal drug to treat infections as it possesses antimicrobial property.

V. ACKNOWLEDGEMENTS

This work acknowledges Tshuwane University of Technology, Acadia South Africa in the person of Prof. Okonkwo O.J. for his assistance in the IR and NMR analysis, and also the Vetech Research Centre and Laboratory, Institute of Management and Technology (IMT), Enugu, in the person of Victoria Mbah, as well as the staff of Godfrey Okoye University Chemical and biochemical Sciences laboratories, Enugu, Nigeria for their assistances in the experimental and analytical aspects of this research work.

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