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SYNTHESIS, CHARACTERIZATION AND ANTIMICROBIAL ANALYSIS OF PHENAZINEAZOPHENOL FROM PHENAZINE-1-CARBOXYLIC ACID ISOLATED FROM *PSEUDOMONAS AERUGINOSA*

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AbstracT - Disease causing microorganisms have become a threat to the other living things in the environment and this has prompted researchers to search for antibiotics to be used in treatment of ill-health conditions caused to their hosts. 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 this compounds are primarily produced from microorganisms and can also be synthesized, example of this phenazine-1-carboxylic acid (PCA). In the present study, phenazine-1-carboxylic acid was biosynthesized from *Pseudomonas aeruginosa* isolated from soil. Subsequently, phenazineazophenol was synthesized from the produced phenazine-1-carboxylic acid following conversion with Ammonium carbonate (amidation) and Hoffmann degradation reactions to reduce the compound to phenazineamine under reflux condition of 50 °C before diazotization and coupling with phenol. The results obtained from the UV-vis, IR and NMR spectra were at a reasonable extent able to elucidate the important peaks in the compound. The *Staphylococcus aureus* was inhibited at 0.1 mm, 15 mm, 13 mm and 0.4 mm marks by the concentrations of 200 µg/mL, 30 µg/mL control drugs [Augmentin (Au) and Oflocitoxin (OFX)] and raw sample of the compound respectively, while *Escherichia coli* was inhibited at 0.2 mm, 1.2 mm, 21 mm, 19 mm and 3.1 mm marks against the concentrations of 150 µg/mL, 200 µg/mL, 30 µg/mL Augmentin (Au, control drug), 30 µg/mL Oflocitoxin (OFX, control drug) and raw sample of the compound; *Aspergillus fumigatus* proven resistant across all prepared concentrations and raw sample of the compound, including the 30 µg/mL control drugs. The minimum inhibitory concentrations of the compound was estimated at 200 µg/mL concentration.

Keywords – Phenazineamine, Phenol, Phenazine, Phenazineazophenol, Antimicrobial.

I. INTRODUCTION

Natural phenazines are coloured secondary metabolites primarily containing heterocyclic nitrogens isolated from Pseudomonas, Streptomyces, and a few other genera from soil or marine habitats [1] and phenazine compound secreted by Pseudomonas aeruginosa are largely found as phenazine-1-carboxylic acid [2-3]. The toxicity problems caused by some synthetic dyes to the environment have created interest towards natural dyes or compounds with proven wide applications, more especially from microbial sources [4]. Coloured organic compound, for example azo dyes are used to impart colours to substrates and can have other applications [5]. The synthesis of azo coloured compounds or dyes involves diazotization and azo coupling reactions; diazotization is a strong chemical reaction to produce diazonium salts (electrophile) and azo coupling involves the electrophile attack on the available nucleophile group (electron withdrawing groups – example is hydroxyl group such as phenol) at ice temperatures [6]. Most azo coloured or dyes compounds possess resistance to

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oxidizing agent, non-toxicity, non-basicity and slight acidity properties [7].

Phenazine-1- carboxylic acid, the precursor of all other phenazines produced by *P. aeruginosa*, is (a brightly yellow coloured compound) biosynthesized from chorismate by genes constituting the redundant phzA1-G1 and phzA2-G2 operons (phenazine enzyme), each of which encodes a full set of functional phenazine-1-carboxylic acid biosynthetic enzymes [1]. This phenazine natural product possess great antiparasitic or antibiotic activities against some strains of bacteria and fungi alike, has also displayed an array of interesting and therapeutically relevant biological activities thus, has been nicknamed as Tubermycin B because of its antibiotic activity against *Mycobacterium tuberculosis* [2-3,8-9] and all these make it important for a modified nature of this natural phenazine compound to be studied and analysis carried out at different bases. On that note, since azo coloured compounds or dyes have displayed a wide use in today's industries (60-70% of the market) for many uses such as dyeing of fabrics, food colorants, pharmaceutical agents [10-13], antimicrobial compounds [10], etc., therefore phenazineazo compounds such as phenazineazophenol (synthesized from phenazine-1-carboxylic acid) is proposed to possess antibiotic activity against some strains of microorganisms.

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), UV-Visible spectrophotometer (Metro UV-5800PC), autoclave (Desco), Meta-lab water bath (MSI 17B), pH meter (Thermo scientific PHS-3C), magnetic stirrer (constant temp. HY-3D), orbital shaker/vibrator (mechanical HY-4), thermocool refrigerator (HTF-259H), weighing balance (Electric FA2004), FT-IR spectrometer (Agilent Happ-Genzel), NMR spectrometer (Agilent-NMR-vnmrs 400 and Bruker Avance III HD 500).

1. Collection and preparation of soil sample.

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, 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.

3.1. 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.

3.2. 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.

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3.3. 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.

3.4. 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.

3.5. 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.

3.6. 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.

3.7. 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.

3.8. 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 [14].

4. Production of Phenazine-1-carboxylic acid (PCA) from the isolated *Pseudomonas aeruginosa* – [2].

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.

4.1. 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 phenazineazophenol 5 from PCA isolated from *P. aeruginosa*

The phenazineazophenol (PCA-PH) **5** synthesis from the phenazine-1-carboxylic acid (PCA) already

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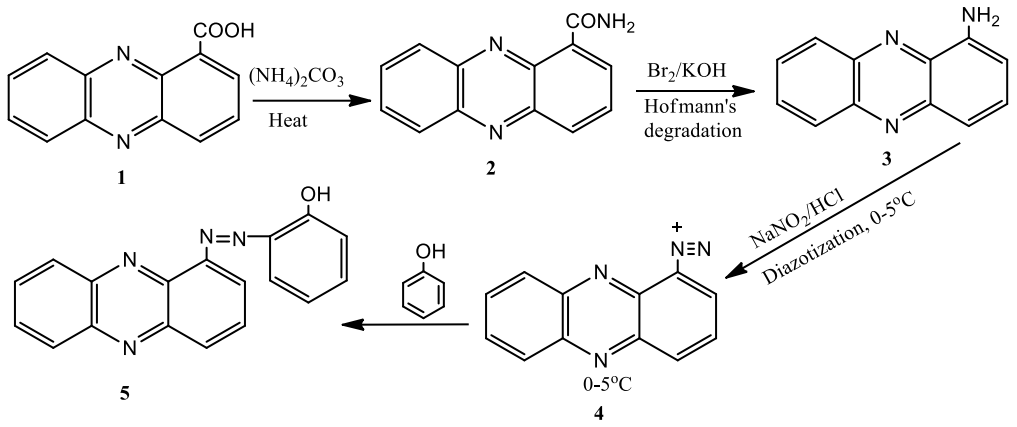
isolated from *P. aeruginosa* is as follows (shown in Figure/scheme 1):



Figure/scheme 1: Synthesis of phenazineazophenol 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 % phenol was separately prepared and allowed to cool at 0 - 5 °C for 10 mins. The cooled 1% phenol 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 greenish yellow phenazineazophenol **5** crystal formed was recovered through suction filtration. It was recrystallized using ethanol, dried in a desiccator with the weight and melting point determined.

6. Determination of the zone of Inhibition and Minimum Inhibitory Concentration Estimation (disks method) – [10].

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 50µg/mL, 100µg/mL, 150µg/mL, 200µg/mL, raw and 30µg/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 Isolate (*Pseudomonas aeruginosa*)

Isolate	Diameter (mm)	Colony and Morphology	Shape	Gram Staining	Catalase	Motility	Oxidase	Indole	Hydrogen Sulphide	Sugar fermentation		
										Glucose	Lactose	Sorbitol

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PA	1-4	Creamy colony, edge	raised smooth	Long rod	-	+	+	+	+	+	Acid	Acid	Acid

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's documentation [15].

Phenazineazophenol (PCA-PH) with yellowish-green colour was found to have a melting point of 330 °C at 44.5 % yield higher than the brightly yellow coloured natural phenazine-1-carboxylic acid (PCA) – where it is modified from in reference with Nansathit and his colleagues [2].

The UV-visible data of phenazineazophenol against ethanol showed max. at 400nm of yellowish-green colour, indicating a conjugated and delocalized n – π^* (non-bonding to pie star) electron transition of the compound. The Infra-red data against KBr showed the functional group C=N (s1640), C-C (s894.6 – 650.0), C=C (s1.438.9 – 1360.5), C=N (s1293.4, 1233.7), O-H (3652.8), C-C (s1185.3 – 827) and N=N (s1490.9). The HNMR data using $CDCl_3$ of δ 7.282 - 7.301 corresponding to aromatic protons and the C13NMR data showed slight 78.90 (R-OH), 125-160 (benzene, C-N, C=N aromatic) of the compound with reference with the works of Donald [16] and Onunkwo and Ejikeme [10].

Table 2: The effect of different dilutions of the sample and control drugs against pathogenic isolate

Organisms	**phenazineazophenol (µg/mL)**					**Control drugs**		**MIC**
	50	**100**	**150**	**200**	**Raw**	**Au**	**OFX**	
Staph. Sp	R	R	R	0.1	0.4	15	13	200
E. coli	R	R	0.2	1.2	3.1	21	19	150
Aspergillus. sp	R	R	R	R	R	R	R	-

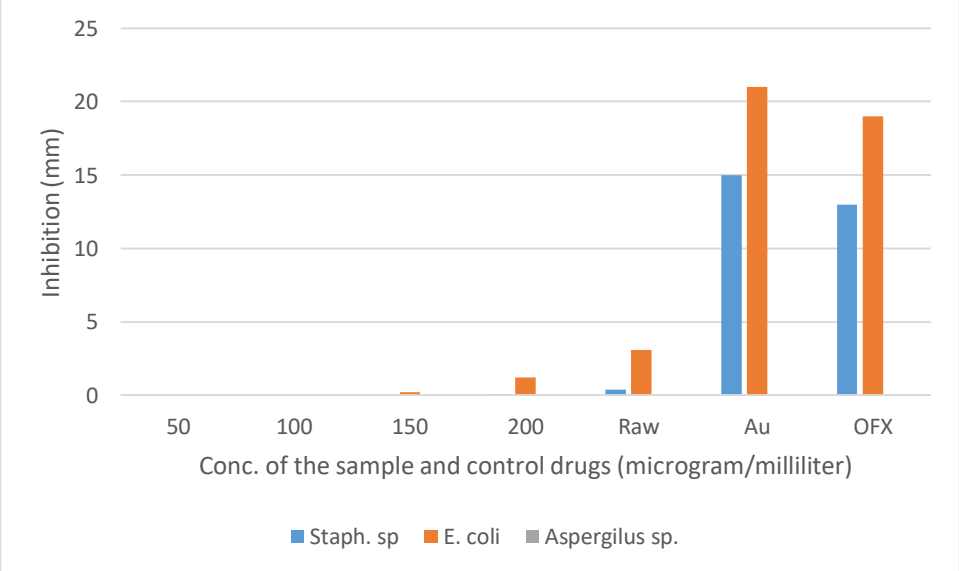

Figure 2: Antimicrobial analysis of phenazineazophenol

From the Table 2 and Figure 2, the organism (*Aspergillus sp.*) was resistant on all the concentrations (50 µg/mL, 100 µg/mL, 150 µg/mL, 200 µg/mL and raw) of compound and the minimum inhibitory concentration is zero. The organism was also resistant against the control drug Augmentin (Au) and Oflocitoxin (OFX) at 30 µg/mL concentrations.

The organism (*E. coli*) was resistant against 50 µg/mL and 100 µg/mL concentrations of the compound but 150 µg/mL, 200 µg/mL and raw concentrations of the compound were able to inhibit the organism at 0.2 mm, 1.2 mm and 3.1 mm respectively. The minimum inhibitory concentration is 200 µg/mL while the control drugs Augmentin (Au) and Oflocitoxin (OFX) at 30 µg/mL concentrations have 21 mm and 19 mm inhibitions respectively.

The organism (*Staphylococcus. sp*) was resistant (R) against 50 µg/mL, 100 µg/mL and 150 µg/mL concentrations of the compound but 200 µg/mL and raw concentrations of the compound were able to inhibit the organism at 0.1mm and 0.4 mm respectively. The minimum inhibitory concentration is 200 µg/mL while the control drugs Augmentin and Oflocitoxin at 30 µg/mL concentrations have 15 mm and 13 mm inhibitions respectively. The results differs lightly from the work of Onunkwo and Ejikeme in 2020 [10].

IV. CONCLUSION

The yellowish green phenazineazophenol compound possess antimicrobial property against *Staphylococcus aereus* and *E. coli* at some certain concentrations, lower in action than the control drugs (Augmentin and Oflocitoxin) but was resisted by *Aspergillus sp.* at all concentrations; thus, can be used as antibiotics for drug treatment of certain bacterial related illnesses and infections.

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