

SYNTHESIS, CHARACTERIZATION, AND BIOACTIVITY OF PARA AMINO BENZOIC ACID (PABA) AZO DERIVATIVE

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ABSTRACT

Azo compounds are characterized by the presence of azo group (-N=N-). In this study, the compound named (E)-4-((8-hydroxyquinolin-5-yl) diaziny) benzoic acid was synthesized through a diazo-coupling reaction between Para-aminobenzoic acid and 8-hydroxyquinoline with yield of 85%. The melting point of the compound was found to be greater than 350°C. The prepared compound was purified through a single solvent and two solvent recrystallization methods. The structure of the novel synthesized compound was confirmed through UV-visible, infra-red, and NMR Spectroscopies. The prepared compound was tested for its antifungal activity against *Aspergillus terreus* using the disc diffusion method. Significant antifungal activity was exposed by the novel synthesized compound against the mentioned fungus strain.

Keywords: Azo Compounds, Antifungal activity, UV-visible Spectroscopy, infra-red spectroscopy, NMR spectroscopy. Para-aminobenzoic acid, 8-hydroxyquinoline.

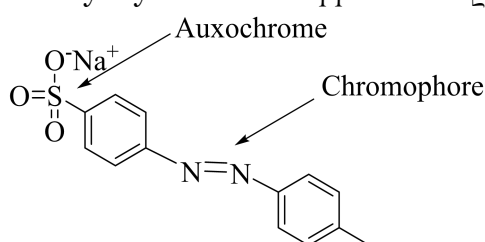
Introduction:

Background

The first azo dye, Prontosil (sulfamidochrysoidine), was synthesized in 1858 by the German scientist Johann Peter Griess, and here is where azo compounds began their start [1]. Although Prontosil is one of the inactive drugs in vitro it demonstrates significant effectiveness in vivo [2]. Through numerous studies and experiments, hundreds of azo compounds have been synthesized throughout the years. More than 7×10^5 tones are synthesized globally according to the most current estimate and more than 2,000 distinct and unique azo dyes are used today [3]. The US Food and Drug Administration (FDA) approved more than 3,000 tons of azo dyes in 1991 [3]. The pharmacological properties of natural azo compounds derived from a variety of sources, including fungi, bacteria, invertebrates, and plants, have been the subject of recent studies and research [4].

Introduction

Azo compounds are the type of synthetic organic compounds having an azo group (—N=N—) in their molecular structure. The azo group, characterized by the nitrogen-nitrogen double bond, gives these compounds their distinctive properties. The atomic groups attached to the nitrogen atoms may be alkyl or aryl, the aryl group are commercially important group due to the benzene ring or its derivatives attached to the azo groups [5]. Azo compounds are usually synthesized by using a simple two-step process called diazo coupling. Normally, most of the azo dyes are produced by diazotizing an aromatic primary amine followed by coupling it with an electron-donating aromatic compound [3]. Auxochromes and chromophores are terms used to describe the nucleophiles and aromatic groups, respectively, in azo dye molecules. These elements come together to form the chromogen, which is a representation of the whole molecular structure of the azo compound. This terminology helps in the classification and understanding of the different parts of azo compounds and their role in dye synthesis and applications [3].



Synthesis

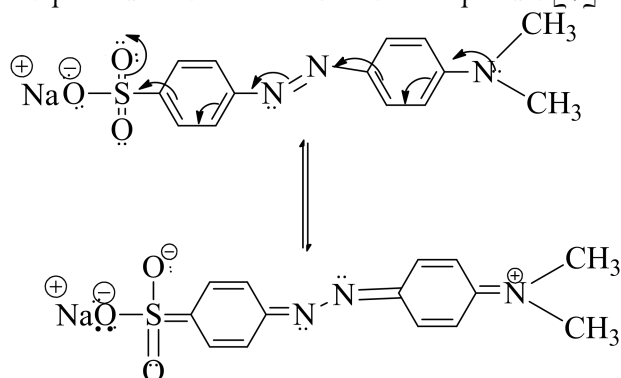
There are several methods used for the synthesis of azo compounds, including azo coupling, oxidation of hydrazine, and reduction of nitro compounds, Mills reaction, and one-spot synthesis. Among these methods, azo coupling is the most commonly used. The azo coupling method for the synthesis of azo compounds is discussed below.

Azo coupling is a widely used method for the synthesis of azo compounds. Azo coupling is two steps reaction [3]. The first step is diazotization. It is the process of converting a primary aromatic amine to diazonium salt. In the first step, the diluted solution of hydrochloric acid (HCl) was added to aromatic amines making the amino group in aromatic amine more reactive. Sodium nitrite (NaNO_2) is reacted with

hydrochloric acid (HCl) to generate nitrous acid (HONO). The protonated aromatic amine reacts with nitrous acid and forms diazonium salt [9]. The reaction is carried out at 0 - 5 °C to prevent the decomposition of the diazonium salt [3]. The second step is coupling, the diazonium salt is then coupled with a coupling agent, an activated aromatic compound. The coupling reaction involves the electrophilic substitution of the diazonium ion with the activated aromatic compound, forming an azo compound [9].

Conjugation

The conjugation of azo compounds refers to the presence of extended π -conjugated systems within the molecule. The conjugation system in azo dyes is due to alternate single and double bonds, which allows the overlap of one p-orbital with another across the sigma bond. As long as each atom in azo dye has an available p-orbital, the system can be considered conjugated. The presence of nitrogen in the ring or groups can also contribute to the conjugation system [6]. The presence of the conjugated system lowers the energy and makes the molecule more stable [6]. The presence of a conjugated π system can also affect the reactivity of azo compounds. The conjugate acids of azo compounds exhibit a highly conjugated structure, characterized by extended π -conjugation. However, conjugation is disrupted when the compound is deprotonated, as a result, the extended π -conjugated system is no longer present in the deprotonated form of the azo compound [7].



Color of Azo Compounds

The color of azo dyes is primarily determined by the presence of azo linkage and their associated chromophores and auxochromes. The azo linkage ($-N=N-$) in the azo compounds contributes to their colors [3]. The chromophore groups are absorbing particular light wavelengths, which results in the color that can be seen. The capacity of azo dyes to maintain their color when exposed to light is considered one of their most important qualities. Azo dyes often have strong light fastness, which makes them appropriate for uses like textiles and printing where color stability is needed. Auxochrome groups enhance the color stability and intensity of dyes, while solubilizing groups increase their solubility in various solvents, facilitating their versatile applications across industries [3]. Azo dyes show a diverse spectrum of colors, ranging from Reds, Oranges, Yellows, and Greens, to Blues and Purples [3].

Classification

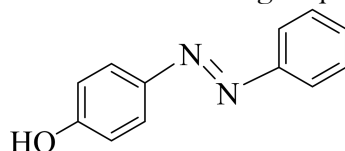
Azo compounds can be classified based on various factors. Two common classification methods for azo compounds are, classification based on alkyl or aryl group attached to the azo group ($-N=N-$) and classification based on the number of azo groups present in the molecule.

Classification based on Aryl or Alkyl groups Attached

Based on the attachment of Aryl or Alkyl groups, the azo compounds are classified into two main classes: Aromatic azo compounds and aliphatic azo compounds.

Aromatic Azo Compounds

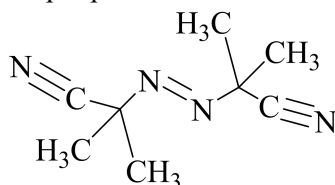
Aromatic azo compounds contain an aromatic ring attached to the azo group. These compounds are usually more stable than the Aliphatic azo compounds because of the aromatic rings attached to both sides of the azo group ($-N=N-$) [8].



Aromatic Azo Compound

Aliphatic Azo Compounds

Aliphatic azo compounds contain aliphatic groups attached to the azo group. These azo compounds are less frequently available than aromatic azo compounds. Aliphatic azo compounds exhibit distinct properties and reactivity [8].



Aliphatic Azo Compound

Classification based on the number of Azo Groups

Based on a number of azo groups present in the molecular structures of azo compounds, they can be classified into: Mono-azo compounds, Di-azo compounds, and Poly-azo compounds.

Mono-azo compounds are characterized by the presence of only one azo group ($-N=N-$), Di-azo compounds have two azo groups in their molecular structures while Poly-azo compounds have more than 2 azo groups in their molecular structures [3].

Reactions of azo compounds

Reduction

The reduction of azo compounds to aromatic amines is an important reaction in organic chemistry and can be achieved through different methods. One of the most common methods involves using reducing agents such as sodium dithionite or hydrazine [12]. The nitro groups present in the aromatic azo compounds can be reduced through hydrazine hydrate in conjugation with an iron hydroxide catalyst [11]. Hydrazine hydrate is normally used as a strong reducing agent to reduce the aromatic and hetero-aromatic azo compounds [12].

Oxidation

Azo compounds can be oxidized in the presence of oxidizing agents such as potassium permanganate or lead tetraacetate to produce azo radicals or other oxidized products [13]. Applying activated manganese dioxide to oxidize primary aryl amines, produces aromatic azo compounds. Additionally, oxidizing substances including chlorine, bleach, ozone, hydrogen peroxide, the Fenton reagent, and permanganate can be used to react with azo compounds.

Hydrolysis

Hydrolysis is an important reaction of azo compounds that leads to the formation of hydrazine, which serves as an intermediate in the synthesis of various organic compounds [14]. During hydrolysis, the cleavage of the azo group occurs, resulting in the formation of new azo bonds. A study has shown that azo compounds are efficient in t-amino-effect reactions, yielding benzimidazoles [15].

Complex formation with metal ions

Azo compounds can form complexes with metal ions, and these complexes have been studied as catalysts in a variety of chemical processes. The (-N=N-) group of azo compounds acts as a donor site for metal ion coordination. To create a connection, the nitrogen atom in the azo group can contribute a single pair of electrons to an empty orbital on the metal ion. The strength of the coordination between the azo compound and the metal ion is determined by a variety of parameters, including the size of the metal ion and charge, the type of the ligand, and the pH of the solution [25].

Applications

Azo compounds are characterized by the presence of azo group (-N=N-) and have a wide range of applications in various fields due to their coloring and other unique chemical properties. These compounds are significantly used as dyes in textile, printing, cosmetics, and food industries, etc. to impart color to different products. Azo compounds are also used as pH and redox indicators. Besides their coloring properties, they have been reported for their various biomedical applications such as anti-bacterial, anti-inflammatory, anti-cancer, and cellular staining, etc.

General application of azo compounds

Azo compounds are abundantly used in the textile industry to color natural textiles, synthetic textiles, and lather in footwear, fibers, and paints [16]. These compounds are frequently used to dye polyester-based materials because they have great coloring characteristics [17]. Azo compounds are normally used in the printing industry due to their bright colors [17]. Azo dyes highlight their various application in the printing industry such as inject printing, screen printing, and flexography [18]. Among dyeing chemistry today, azo dyes hold the top position in terms of production volume and significantly influence the dye and printing market [3]. These compounds are used in cosmetic industries to provide colors to different cosmetic products such as lipsticks and nail polishes etc. The biomedical application of azo compounds highlights these compounds have been reported as antimicrobial, which makes them useful for cosmetic products [16]. Azo compounds can act as pH indicators by changing color in response to changes in the acidity or alkalinity of a solution. The color change occurs due to the protonation or deprotonation of the azo group in the compound. Azo compounds can also serve as redox indicators,

undergoing color changes in response to changes in the oxidation-reduction potential of a system. The redox reactions can cause the reduction or oxidation of the azo group, leading to color change while some azo compounds can selectively bind to specific metal ions, which causes color changes. These compounds are used as metal ion indicators in analytical chemistry to detect and quantify the presence of specific metal ions in a solution [18].

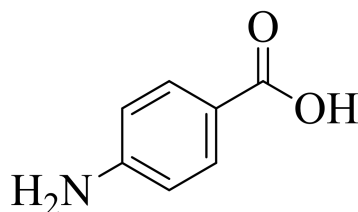
Biomedical application of azo compounds

Azo compounds acquire antimicrobial properties and are used as antifungal, antiviral, and antibacterial agents [10]. It has been proved that azo compounds have antibacterial action against a variety of species, including *Staphylococcus aureus* and *Escherichia coli* [10]. Azo dye features, such as durability and chemical stability, make them appropriate for use as antibacterial agents [4]. They can inhibit the growth and multiplication of bacteria, making them useful in the development of novel anti-infection therapy techniques [19]. The azo compounds have anti-inflammatory effects and are used to treat inflammatory diseases. Azo-reductase (an enzyme used to reduce azo compounds) plays a crucial role in the detoxification of azo dyes, which are used to activate anti-inflammatory azo pro-drugs. Various investigations have been conducted to assess the anti-inflammatory effects of azo compounds. Research, for example, looked at the anti-inflammatory efficacy of NO-releasing furoxan/1,2,4-triazole hybrid derivatives and discovered that compound 5f had potential anti-inflammatory action [22]. Therefore, azo compounds have demonstrated anti-inflammatory activity and can be researched further for their medicinal potential in the treatment of inflammatory diseases. Azo compounds have been used as the foundation for developing drug delivery systems. In polymer chemistry, the reduction of the azo group is commonly used to fabricate drug delivery systems. These drug delivery techniques use the advantages of azo compounds to regulate drug release, enabling prolonged and tailored drug release. Azobenzene has been examined in terms of its structure, production, and drug delivery methods for azo-based polymers [23]. Similar to the utilization of sulfasalazine and other azo compounds for achieving targeted drug release facilitated by the enzymatic action of colonic bacteria's azo reductase, research has explored the potential application of azo compounds for colonic drug delivery [24]. Several azo dyes exhibit cytotoxicity, or the capacity to kill or harm cells [20]. Numerous investigations have noted the anticancer properties of azo compounds due to their capacity to intercalate with RNA and/or DNA as well as the inhibition of several proteins. [20]. Azo dyes also affect glioblastoma cells in vitro and found that at equimolar concentration azo dyes activate more cytotoxic effect than TMZ, which are alkylating agents used as reference drugs [21]. Biomedical applications of azo compounds are still in their early days, but researchers are exploring their potential in drug delivery, cancer chemotherapy, and other pharmacological activities.

Para-aminobenzoic acid (PABA)

Para-aminobenzoic acid which is also known as 4-aminobenzoic acid is an organic compound that is found naturally within the body and also present in different kinds of foods like grains, eggs, milk, meat, etc. [27]. This compound is used as an essential vitamin for the treatment of many human diseases, while in bacteria it is used for the synthesis of folic acid [26]. Para-aminobenzoic acid is used as a

precursor in the synthesis of coenzyme Q6 within *Saccharomyces cerevisiae*. Because of its early adaption as one of the initial Ultraviolet B (UVB), it has received significant adverse publicity [28]. To enhance the bioavailability and facilitate targeted delivery to skin tissues of PABA Liquid-crystal oral formulations were employed. Para-aminobenzoic acid can be used as a precursor in the synthesis of various azo compounds, including its use in the production of Mono-azo dyes as a diazotizing component [27]. It can be utilized in the synthesis of azo-carboxylate ligands, and subsequently employed in the formation of tri organotin (IV) azo-carboxylates. These compounds exhibit antibacterial activities. Following diazotization, PABA was acetylated using acetic anhydride to produce amide derivatives, which were then assessed for their antimicrobial properties [26].

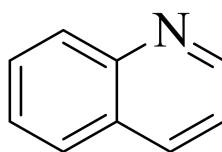


4-aminobenzoic acid

Structure of Para-amino benzoic Acid

Quinoline

It is a heterocyclic organic compound that is composed of a benzene ring fused with a pyridine ring. Because of its wide spectrum of potential biological activities, quinoline has attracted substantial attention in the field of organic synthesis and medicinal chemistry [29]. Quinoline and its derivatives show great potential in various biological processes, particularly in the context of pharmaceutical drug development. Quinoline has been reported as a potent anti-cancer agent and it can be synthesized through various synthetic routes [30]. One of the easy accesses to highly substituted quinoline is metal-free synthesis [31].



quinoline

Structure of Quinoline

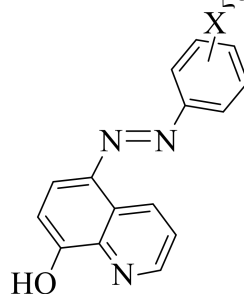
Literature Review

Azo-compounds are an important class of organic compounds that contain a Nitrogen-Nitrogen double bond (-N=N-) as a functional group. These compounds can be synthesized through a diazo-coupling reaction [32]. Azo dyes have a wide range of applications in various fields like textile, fiber, leather, printing, and painting, etc. Several biomedical activities of azo compounds have also been reported such as antibacterial, antifungal, antiviral, and antioxidant etc.[33]. Many chemists have conducted work on versatile biomedical applications like antibacterial, antifungal, anticancer, anti-inflammatory, and antioxidant activities of diverse azo

compounds.

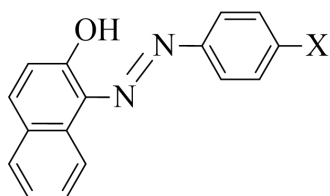
Antibacterial properties of Azo compounds

In a study conducted by C. D. K. Amengor *et al.* (2022) a series (4a-4e) of five azo compounds were synthesized through coupling 8-hydroxyquinoline with diazonium salts of substituted amines. The synthesized azo compounds were characterized using ultra-violet visible, and Infra-red spectroscopy. Moreover, these compounds were assessed for antibacterial activity against human pathogens through a High-throughput spot culture growth inhibition (HT-SPOTi) assay. All the synthesized compounds showed better antibacterial activities [34].



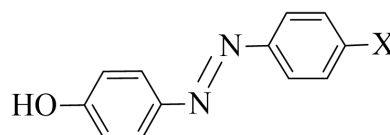
4a-4e

J.K. Adu and co-workers (2020) prepared a series (A1-A4, B1-B4) of 2-naphthalic and phenolic-based azo compounds via diazo-coupling reaction, and their Structures were elucidated using UV-Visible and FT-IR spectroscopy. The antibacterial activity of the synthesized compounds was checked against certain Gram-positive and Gram-negative strains using a high-throughput spot culture growth inhibition assay (HT-SPOTi). The prepared compounds displayed good antibacterial activities [35].



2-naphtholic based azo compounds
(A1-A4)

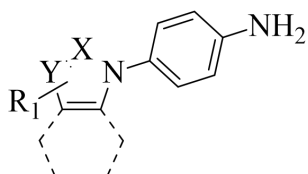
X-SO₂NH₂, Cl, COOH,
COOC₂H₅, NO₂, o-Cl/p-NO₂



phenolic based azo compounds
(B1-B4)

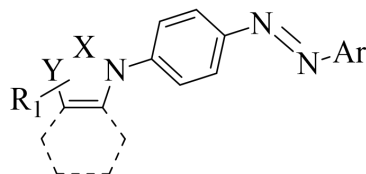
X-COOH, COOC₂H₅, o-Cl/p-NO₂

Following the same procedure of diazo-coupling reaction Z. Ghasemi *et al.* (2017) prepared a series (2a-2p) of azo compounds using 4-(1-azolyl) aniline derivatives and several coupling agents as reactants. Various spectroscopic techniques such as UV-visible, FT-IR, ¹³C NMR, ¹H NMR, and elemental analysis were used to confirm their structures. The compounds were then examined for antibacterial activity against different strains of Gram-negative and Gram-positive bacteria via broth dilution assay using gentamicin as a reference antibiotic. The synthesized compounds displayed good results against the employed bacterial strains [36].



1a-1e

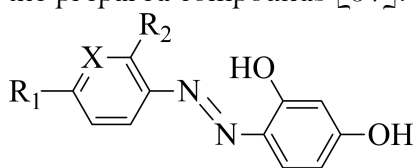
- a: X=CH, Y=N, R1=H
- b: X=CH, Y=N, R1=H
- c: X=N, Y=CH, R1=H
- d: X=N, Y=CH, R1=3-Me
- e: X=N, Y=CH, R1=4-Me



2a-2p

Ar-H: phenol, 2-naphthol,
N,N-dimethylaniline, 8-hydroxyquinoline

Similarly, a series of azo derivatives (TR1-TR9) were designed by Tehreem Tahir (2021) through diazotization of substituted aromatic amines followed by coupling with phenol or naphthol. The compounds were characterized through FT-IR, ^1H NMR, ^{13}C NMR, and mass spectrometry (HRMS) techniques. The compounds were screened for their antibacterial activity against six Gram-positive and Gram-negative bacterial strains via agar disc diffusion method and effective antibacterial results were declared by the prepared compounds [37].



R1= NO₂, OMe, H

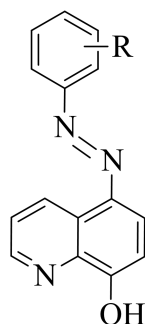
R2= H, Cl

X= N, CH

Azo compounds (TR-1-TR-9)

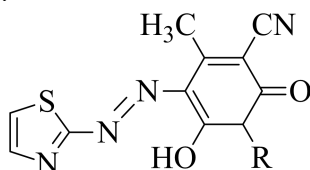
Antifungal properties of Azo compounds

In a similar way P. Vibhute and S. A. Khiste (2019) obtained a series (1-7) of azo compounds by coupling 8-hydroxyquinoline with diazonium salt of substituted anilines and the structures were elucidated using spectroscopic techniques like UV-Visible, ^1H NMR Spectroscopy and elemental analysis. The synthesized compounds were then tested for their antifungal activity using the cup plate diffusion method having potato dextrose agar (PDA) as a medium. Compounds 3, 4, and 7 displayed potent antifungal activity among the synthesized series [38].



(1-7) synthesized azo compounds R: Cl and NO₂

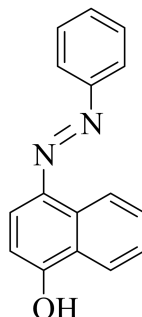
By continuing the diazo coupling reaction between 2-aminothiazole and various pyridone derivatives B.N. Ravi *et al.* (2019) synthesized a series (A1-A3) of azo compounds. Spectroscopic techniques like UV-visible, FT-IR, ¹H NMR, ¹³C NMR, and mass spectrometry were used for structural determination of the compounds. The antifungal activity of these compounds was then assessed against several fungal strains through a Food Poison assay using Potato Dextrose Agar (PDA) as a medium. The novel azo compounds were declared effectively active against the employed fungal strains [39].



Where R= H, CH₃, CH₃CH₂

(A1-A3)

Similarly, 4-phenylazo-1-naphthol, an azo compound was reported by Nimra Tanvir and co-workers (2021) using diazo-coupling reaction between phenyl diazonium salt and α - naphthol. Fourier transform infrared spectroscopy (FT-IR) was used to characterize the compound. The synthesized compound was evaluated for antifungal activity against fungi and declared to be inactive against the exploited fungi [40].

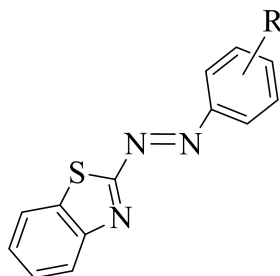


(E)-4-(phenyldiazenyl)naphthalen-1-ol

Antioxidant properties of Azo compounds

By using the classical method of diazo coupling reaction S. Prakash *et al.* (2020) prepared a series (4a-4d) of 2-amino benzothiazole-based azo compounds. The structural confirmation of the novel azo compounds was done via a range of

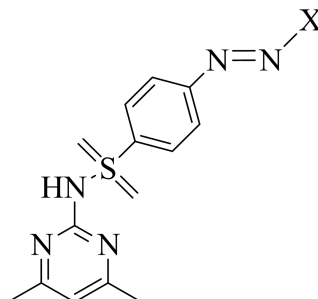
spectroscopic techniques including UV-visible, FT-IR, ^1H NMR, ^{13}C NMR spectroscopy, and LC-(ESI) MS. Furthermore, the antioxidant activity of the novel azo-compounds was evaluated using DPPH radical scavenging activity method and the synthesized compounds shown effective antioxidant activities [41].



R= Isobutyl, Methoxyl, OH

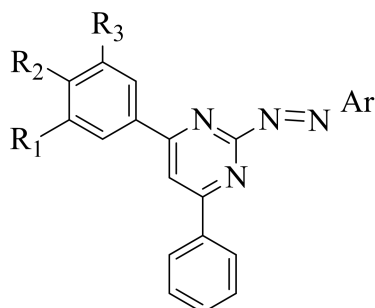
novel azo compounds (4a-4d)

Similarly, Vinodkumar and J. Keshavayya (2019) synthesized a series (S1-S3) of heterocyclic amine-based azo compounds through a classical diazo-coupling reaction. The structures of the synthesized compounds were then identified via multiple spectroscopic techniques such as UV-visible, FT-IR, ^1H NMR spectroscopy, and mass spectrometry. Moreover, the compounds were subjected to their antioxidant activity using a DPPH radical scavenging assay, and potent antioxidant activities were shown by the series [42].



(S₁-S₃) X= N, N-dimethyl aniline derivatives

Aziz Unnisa *et al.* (2020) reported a library (5a-5l) of 6-aryl substituted pyrimidine azo dyes through a diazo-coupling reaction between phenyl pyrimidine 2-amine with different aromatic amines. Moreover, the structural elucidation of the compounds was done using IR, ^1H NMR, ^{13}C NMR spectroscopy, and Mass spectrophotometry. The compounds were then tested for their antioxidant activity using a DPPH assay. The novel compounds displayed enhanced antioxidant activities [43].

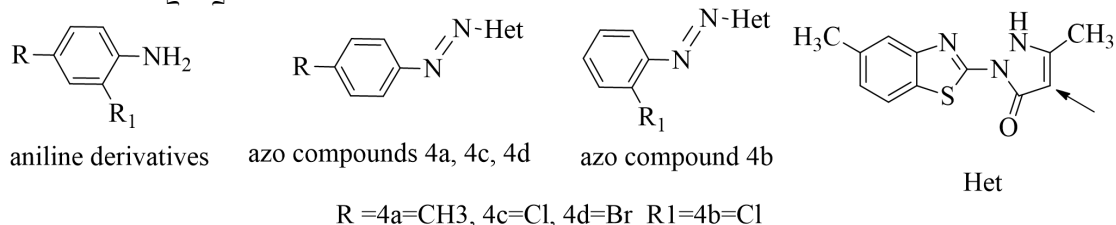


R1 and R3:H,OCH3 R2: NO2,Cl,OCH3

5a-5l
Azo-dyes

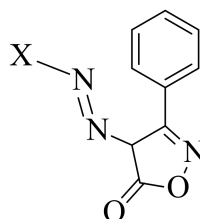
Anticancer properties of Azo compounds

A series (4a-4d) of four different heterocyclic azo compounds was reported by Maliyappa M. R *et al.* (2019) through a diazo coupling reaction between aniline derivatives and a coupling agent. Several spectroscopic techniques like UV-visible, ^1H NMR spectroscopy, and Vibrational and liquid chromatography-mass spectrometry were used for structural elucidation. The anticancer activity of the compounds was studied against human cancer cells using MTT assay. The results declared potent anticancer activities of the synthesized compounds against human cancer cells [44].



Anti-inflammatory properties of Azo compounds

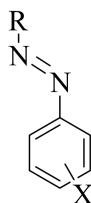
Manjunatha B *et al.* (2021) prepared a series (Io1-Io4) of novel azo dyes by coupling isoxazolone-thiazole with various diazonium salts of heterocyclic amines. Moreover, the structures were elucidated using various spectroscopic techniques like FT-IR, ^1H NMR, ^{13}C NMR, and HRMS. The anti-inflammatory activity of the prepared compounds was then studied against two matrix metalloproteinases by gel electrophoresis method. The result displayed the remarkable anti-inflammatory activity of the series [45].



isoxazolone-thiazole based azo dyes (Io1-Io4)

X= Heterocyclic amines

C. N. D. Attoh *et al.* (2022) prepared a library of 10 aryl-substituted azo-dyes (1-10) by diazotization of various aromatic amines followed by coupling with a number of coupling agents. The novel compounds were characterized through UV-visible, FT-IR, ^1H NMR, ^{13}C NMR spectroscopy, and Mass spectrometry. Further, the in vitro anti-inflammatory activity of the compounds was checked using egg albumin denaturing bioassay. The result demonstrated that quinol and naphthol-based azo compounds had the best anti-inflammatory activities [46].



R= Beta-naphthol, quinol, catechol

Methodology

Materials and Methods

Chemicals and Reagents Used for the Synthesis of Azo Compounds

Various chemicals, reagents, and solvents have been used. The information about the chemicals and reagents is displayed in Tables 3.1 and 3.2.

Table 3.1 Chemicals used

Material	Chemical formula	Store
4-aminobenzoic acid	$\text{C}_7\text{H}_7\text{NO}_2$	Haq chemical
Sulfanilic acid	$\text{C}_6\text{H}_7\text{NO}_3\text{S}$	Haq chemical
8-hydroxyquinoline	$\text{C}_9\text{H}_7\text{NO}$	Haq chemical

Table 3.2 Reagents and Solvents Used

Material	Chemical formula	Store
Hydrochloric acid	HCl	Haq chemical
Sodium nitrite	NaNO_2	Haq chemical
Sodium hydroxide	NaOH	Haq chemical
Acetone	$(\text{CH}_3)_2\text{CO}$	Haq chemical
Ethyl acetate	$\text{C}_4\text{H}_8\text{O}_2$	Haq chemical
n-hexane	C_6H_{14}	Haq chemical
Ethanol	$\text{C}_2\text{H}_6\text{O}$	Haq chemical
Methanol	CH_3OH	Haq chemical
Dichloromethane	CH_2Cl_2	Haq chemical
Dimethylformamide	$\text{C}_3\text{H}_7\text{NO}$	Haq chemical

Synthesis of 4-aminobenzoic Acid Derivative

The 4-aminobenzoic acid azo derivative was synthesized through a two-step reaction involving diazotization and coupling reaction. The reaction was carried out under controlled temperature conditions using an ice bath and stirring to maintain a temperature range of $0-5^\circ\text{C}$. The Author Miroslav Krátký, Klára Konečná, and Jarmila Vinšová [52].

Step 1: Diazotization Reaction

4-aminobenzoic acid solution (4mmol, 0.55g) in acetone (10ml) and distilled water (30ml) was treated with Sodium nitrite (12mmol, 0.83g) in 10ml distilled water and HCl (2ml). Gradually add HCL and sodium nitrite to the 4-aminobenzoic acid solution while stirring on the magnetic stirrer in an ice bath for 20-30 minutes. The temperature was kept under 0-5 °C diazonium salt was formed [47].

Step 2: Coupling Reaction

The diazonium salt was further coupled with the coupling agent 8-hydroxyquinoline (2mmol, 0.29g) in 4ml acetone. The NaOH solution (2mmol, 0.08g) in 10 mL distilled water was prepared and added to the diazonium salt while stirring. The entire reaction was carried out in an ice bath to maintain the temperature between (0-5 °C). The reaction was then stirred for 1 hour to obtain the final product. The orange color product was formed [47].

The crystalline product was filtered and washed with cold acetone and hot water. The obtained compound was then identified by chromatography and purified by recrystallization methods [48].

Chemical Reaction

The chemical reaction is given in Figure 3.1

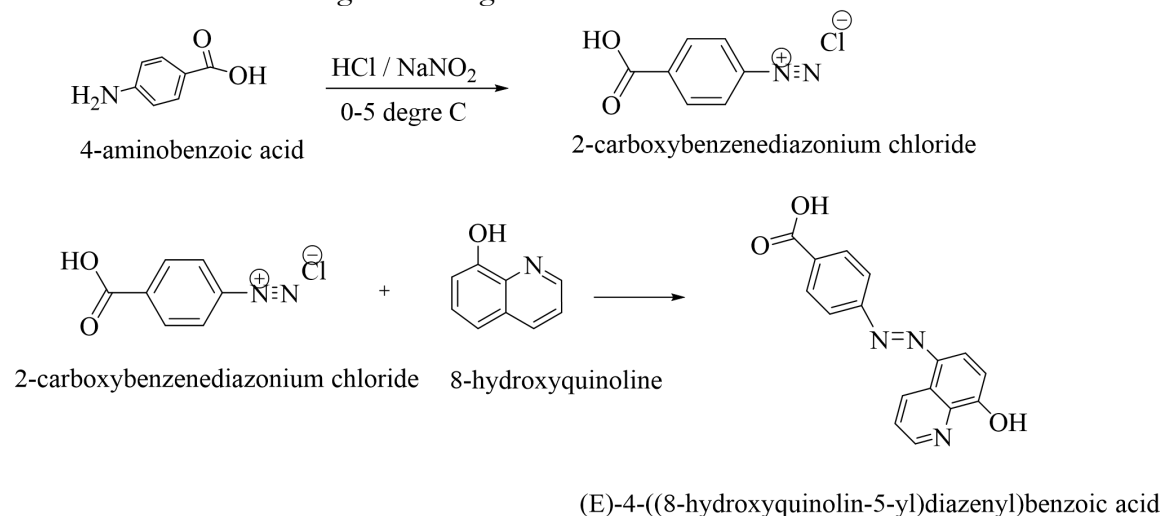


Figure 3.1 chemical reaction

Instrumentation and Techniques

Melting Point

The determination of the melting point of the obtained compound was done using a melting point apparatus. The sample is heated in a capillary tube until it melts. The temperature at which the sample was melted was above 300°C [49].

Thin-layer Chromatographic

Thin-layer chromatography technique was used to observe the completion of the reaction and purity of the prepared azo compound. 35% (3.5mL) n-hexane and 65% (6.5mL) ethyl acetate is mixed to prepare the solvent system for thin-layer chromatography. 0.019g of the synthesized compound was dissolved in 1 mL of DMF (Dimethylformamide) to prepare the sample. The sample is then spotted on a

TLC plate and placed in the solvent system to move the sample. After, 5-10 minutes intervals of time the movement was observed under ultraviolet (UV) light [48].

Recrystallization

Recrystallization is a purification technique used to remove impurities from organic compounds which are solid at room temperature. Two basic types of recrystallization processes are used to purify the synthesized compound: Single Solvent Recrystallization and Two Solvent Recrystallization.

Single Solvent Recrystallization:

The single solvent recrystallization of the synthesized azo compound was done by dissolving the impure sample in a minimum amount of hot solvent (methanol) and then being allowed to cool. As the solution cools, the azo compound recrystallizes into pure form. The crystals were then filtered through vacuum filtration. The appropriate solvent for recrystallization should be one that fully dissolves the sample at boiling so that slow, selective crystallization can occur as the solution cools [50].

Two Solvent Recrystallization:

Two-solvent recrystallization is a purification technique that involves the use of a mixture of two solvents (dimethylformamide and chloroform). The two solvents must be miscible with each other. The choice of solvents depends upon their different solubility properties. One solvent should have high solubility for the compound at elevated temperatures (dimethylformamide) and the other should have low solubility for the compound at low temperatures (chloroform).

UV-visible Spectroscopy:

A qualitative and quantitative technique that measures the amount of visible or UV light absorbed by the chemical substance. The structure of the synthesized compound was characterized through UV-visible spectroscopy. Generally, the UV-visible spectrometer was used to identify conjugation in organic compounds. Light intensity that passes through the sample in comparison with a blank or reference sample is detected by the spectrometer. A blank sample is the solvent in which the compound is dissolved. DMF is used as a blank sample or reference solvent for our compound [51].

Infrared Spectroscopy

Infrared spectra of the compound were determined using FT-IR spectrophotometer (4000 to 500 cm^{-1}) by using KBr disk.

NMR Spectroscopy

The Proton NMR data of synthesized quinoline derivative was determined at 500 MHz spectrometer. TMS was used as an internal reference and the chemical shifts are given in δ scale (ppm).

Antifungal activity of synthesized derivative

The antifungal activity of the synthesized 4-aminobenzoic acid azo derivative was tested in vitro using the disc diffusion method, with minor modifications to the procedure described by Marinescu et al. [53]. RPMI 1640 agar supplemented with 2% glucose was prepared as the growth medium to provide suitable conditions for the test organism.

A 24-hour cultured plate of *Aspergillus terreus* was used to prepare the inoculum. Colonies were suspended in 0.85% saline solution, and the turbidity was standardized to 0.5 McFarland (corresponding to $1 \times 10^6 - 5 \times 10^6$ cells/mL). A

sterile cotton swab was dipped into this suspension, excess liquid removed by pressing against the tube wall, and the agar surface inoculated by streaking three times, rotating the plate $\sim 60^\circ$ after each streak to ensure uniform distribution.

Wells were created on the agar surface, and 50 μL of the test compound solution (1 mg/mL) was added to each well. Plates were incubated at 35°C for 24 h and 48 h to observe inhibition at different time points, considering the growth characteristics of *Aspergillus terreus*.

Following incubation, zones of inhibition were measured as the diameter (in whole millimetres) where fungal growth was completely absent. This measurement served as the indicator of antifungal activity and allowed comparison of the compound's effectiveness. Fluconazole (1 mg/mL, 50 μL per well) was used as the positive control for reference comparison. The entire procedure was carried out with standardized steps to ensure consistent and reliable results, providing a clear evaluation of the synthesized azo derivative's antifungal potential relative to the established antifungal agent.

Results and Discussion

Synthesis

The desired compound (E)-4-((8-hydroxyquinolin-5-yl) diaziny) benzoic acid was prepared through a diazo-coupling reaction between Para Amino Benzoic Acid and 8-hydroxyquinoline according to the previously reported diazo-coupling reaction by H. U. R. Shah *et al.* [54]. The diazo-coupling reaction is a type of electrophilic aromatic substitution reaction in which the diazonium salt is an electrophile and the coupling agent is a nucleophile. In this process, the diazonium salt coupled with the activated aromatic ring of the coupling agent leads to the formation of azo compounds [32]. The whole reaction was carried out at $0-5^\circ\text{C}$ temperature.

This temperature was maintained throughout the reaction because the intermediate (diazonium salt) produced in the diazo-coupling reaction is sensitive to temperature and decomposes at slightly higher temperatures [55]. Upon the dropwise addition of the basic solution of 2mmol of 8-hydroxyquinoline to the diazonium salt of Para Amino Benzoic Acid, the color change to radish brown was observed which identifies the formation of precipitates. The reaction pH was eventually adjusted to 3-5 to enhance the product yield of the reaction. The product was then washed with cold acetone to remove impurities. Vacuum filtration was used to collect the crystals and dry them at room temperature. The reaction progress was monitored using TLC.

The diazotization step of the diazo-coupling reaction is conducted in concentrated aqueous hydrochloric acid (HCl) solution to produce the nitrous acid (HONO) required for diazonium salt formation. Using highly concentrated acidic or alkaline reagents disrupts the pH equilibrium and leads to product degradation. Therefore, to maintain the pH neutral, the coupling agent is dissolved in a basic solution [56]. The azo compounds are thermally very unstable, it has been reported that during processing, storage, and transportation of these compounds at high temperatures cause degradation [57].

The synthesized compound was completely soluble in DMF (Dimethylformamide) and DMSO (Dimethyl Sulfoxide) solvents, partially soluble in Ethyl acetate, Acetone, Ethanol, Methanol, and Benzene while completely insoluble in Water, n-hexane, DCM (Dichloromethane) and chloroform. The melting point of the synthesized

compound was noted above 350 °C.

Briefly, the desired azo compound was synthesized successfully using a diazo-coupling reaction in a good yield. Some of the physical properties of the synthesized compound are listed in Table 4.1.

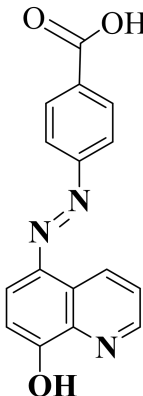
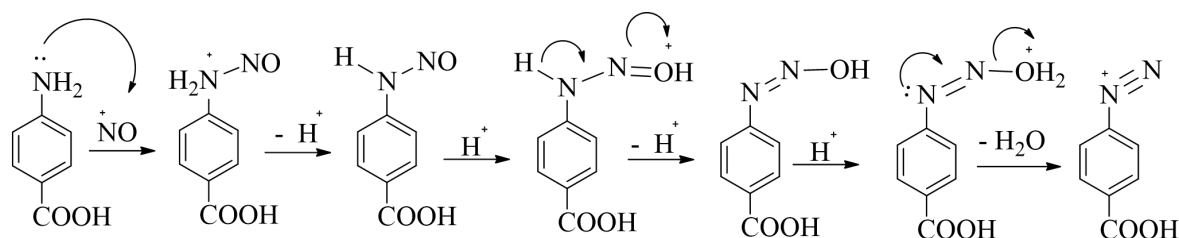
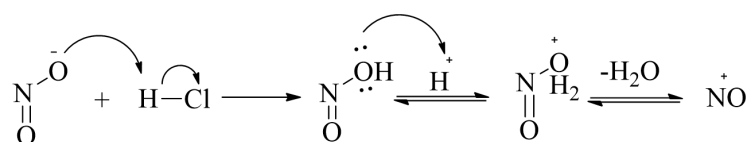
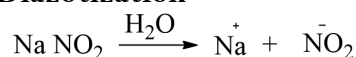
Name	Molecular Formula	Molecular Mass (g/mol)	Structure	Yield	Melting point	Solubility	Color
(E)-4-((8-hydroxyquinolin-5-yl) diaziny) benzoic acid	C ₁₆ H ₁₁ N ₃ O ₃	293.28		85%	Above 350°C	DMF/DMSO	Redish Brown

Table 4.1. Physical Properties of Synthesized Azo Compound

General Mechanism of Synthesized Azo Compound

The general mechanism of the synthesized compound is sketched in Figure 4.1.

Diazotization



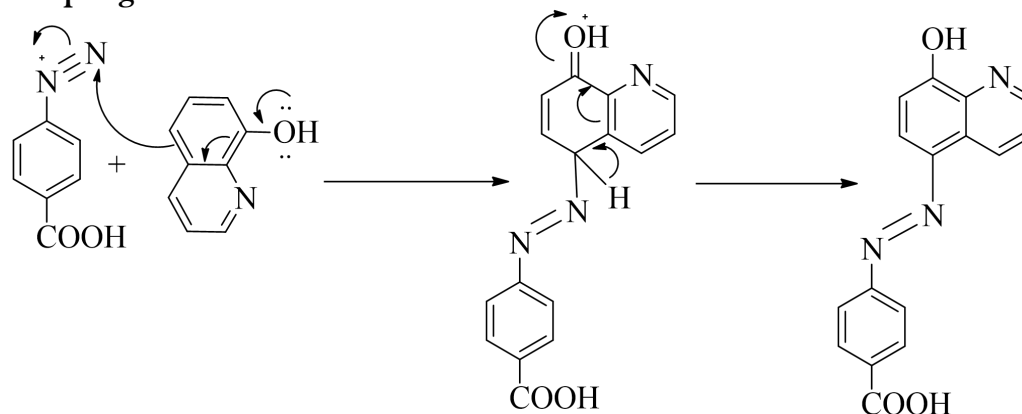
Coupling

Figure 4.1. Stepwise Mechanism of Diazo-coupling Reaction

Purification

Following the synthesis of the desired compound the TLC (Thin-Layer Chromatography) was carried out to check the purity of the synthesized compound. The sample solution and the mobile phase (solvent system) for TLC were prepared by the previously documented procedure by J. Silver (with slight changes) [58]. The sample solution was prepared by weighing 2mg of the synthesized compound and dissolved in 1 mL of DMF (Dimethylformamide) solvent. The mobile phase (solvent system) for TLC was prepared by combining 3.5mL of n-hexane (constituting 35% of the mixture) and 6.5mL of Ethyl acetate (constituting 65% of the mixture) within a beaker. The beaker was covered with aluminum foil to facilitate the equilibrium. A small spot of the prepared sample solution was then applied onto the baseline of the TLC plate and placed in the solvent system. After 5-10 minutes intervals of time, the migration of the chromatographic spot was visually observed on the TLC plate under the UV lamp. The prepared compound was then processed for purification using two types of recrystallization techniques: Single Solvent Recrystallization and Double Solvent Recrystallization.

Single Solvent Recrystallization

In single-solvent recrystallization, only one solvent is used to purify the impure sample. Solvent selection is one of the most challenging aspects of performing the recrystallization process. A solvent in which the compound of interest is partially soluble at room temperature and completely soluble at elevated temperature should be chosen. Such solvent will dissolve the target compound at high temperatures and recrystallize upon cooling. Several factors should concern such as chemical reactivity, molecular bonding interaction, and degree of solubility of solute in the solvent. Poor selection of the solvent may lead to poor quality and quantity of the recrystallized product after the recrystallization process [59]. Considering all the factors methanol was chosen as a solvent for the recrystallization process of the synthesized compound. The sample was dissolved in hot methanol solvent and then allowed to cool at room temperature to form crystals. The purified crystals were then collected using vacuum filtration.

Double Solvent Recrystallization

Following the single solvent recrystallization, double solvent recrystallization was

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performed. For the double solvent recrystallization, two solvents should be chosen: Solvent 1 and Solvent 2. The sample should be completely soluble in Solvent 1 at high temperatures and insoluble in Solvent 2 at low temperatures. For the double solvent recrystallization of the synthesized compound two solvents were chosen: DMF (Dimethylformamide) as Solvent 1 and Chloroform as Solvent 2. The sample was dissolved in hot Solvent 1 DMF (Dimethylformamide) and allowed the solution to cool at room temperature. The cooled solution was then followed by the dropwise addition of Solvent 2 (Chloroform) till the formation of a cloudy appearance in the mixture. Hot Solvent 1 (DMF) was added in a small amount again to clarify the solution and then allowed the solution to cool and crystallize at room temperature. The crystals were collected using vacuum filtration. TLC was employed to confirm the purification status of the prepared compound. The purification status of the recrystallized product is given in Figure 4.2.

The recrystallization process can be influenced by the use of less or more quantity of solvents and can reduce purification efficiency. Using a small amount of solvent can result in incomplete dissolution of solute even at high temperatures. Similarly, in an excess amount of solvent, the solute remains in dissolved form even upon cooling.

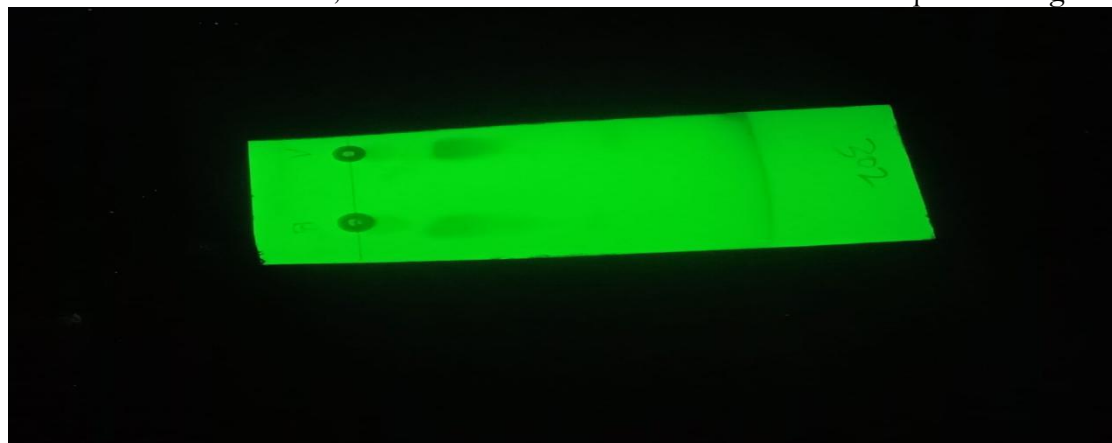


Figure 4.2. Purification Status Before and After Purification

Structural Elucidation

Structural elucidation is the process used to determine the chemical structure of an unknown organic compound. Several techniques can be used to elucidate the structure of an unknown compound like NMR spectroscopy, UV-visible spectroscopy, IR spectroscopy, and Mass spectrometry, etc. The structure of the synthesized compound was elucidated using UV-visible spectroscopy, IR spectroscopy, and NMR spectroscopy. These techniques determine the presence of double, conjugation, functional groups and details about protons present in a compound to elucidate structure completely.

UV-visible Spectrum

Azo compounds are colored compounds due to the presence of the chromophore ($-N=N-$) group and two possible transitions: $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transition. The $\pi \rightarrow \pi^*$ transition of an azo compound is responsible for shorter wavelengths while the $n \rightarrow \pi^*$ transition is responsible for longer wavelengths [60]. The auxochrome (carboxylic group) in the prepared compound shows a bathochromic effect because of which the peak is shifted towards a high wavelength. The UV-visible spectra of the synthesized

compound were recorded in DMF solvent at (0.05 M) dilution between 200-800nm wavelengths. Prominent peaks were recorded at desired regions (400-490nm) and determined the presence of the azo group (-N=N-) of the synthesized compound. The λ max of the synthesized compound is given in Table 4.2. and the UV-visible spectrum graph for the synthesized azo compound is sketched in Figure 4.3.

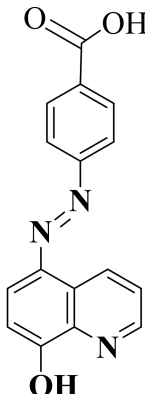
Synthesized Compound	Molar Concentration (M)	λ max (nm)	Solvent
	0.05 M	400-490	DMF

Table 4.2. λ max(nm) at (0.05M) Using DMF Solvent

Wavelength VS Absorbance

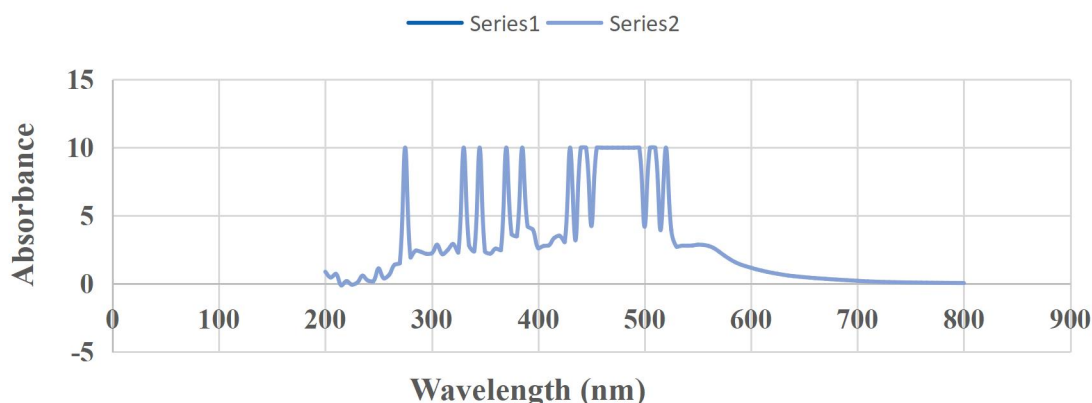


Figure 4.3. UV-Visible Spectra of Synthesized Azo Compound at (0.05 M).
FT-IR Spectral Analysis

The structure of the synthesized 4-aminobenzoic acid azo derivative was successfully confirmed by Fourier Transform Infrared (FT-IR) spectroscopy employing the KBr pellet technique. The recorded FT-IR spectrum exhibited several diagnostic absorption bands that are fully consistent with the anticipated molecular structure. A prominent N-H stretching vibration characteristic of the primary amine group (-NH₂) was clearly observed at 3391 cm⁻¹. In the higher wavenumber region, O-H stretching bands originating from the hydroxyl groups appeared between 3258 and 3280 cm⁻¹, with distinct peaks at 3258, 3280, and 3279 cm⁻¹; these signals likely

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include some contribution from N–H stretching due to intramolecular hydrogen bonding interactions of the type O–H $\cdots$ N, which is commonly encountered in such molecular systems.

At lower frequencies, a notably high-wavenumber C–N stretching vibration was detected at 1641 cm⁻¹, this shift to higher frequency being attributable to strong conjugation effects involving the azo linkage. Aromatic C=C stretching modes of the conjugated azo dye framework were identified at 1573 cm⁻¹. The quinoline ring contributed a well-defined C=N stretching vibration at 1516 cm⁻¹, while the characteristic –N=N– stretching band of the azo group appeared at 1493 cm⁻¹, confirming the presence of the central azo bridge. Furthermore, multiple C–O stretching vibrations associated with the amino and hydroxyl substituents attached to the quinoline heterocycle were observed in the fingerprint region, specifically within the ranges 1250–1210 cm⁻¹, 1240–1190 cm⁻¹, and 1190–1125 cm⁻¹.

Collectively, these spectral assignments are in excellent agreement with the expected functional groups present in the target molecule—namely the azo linkage, quinoline core, primary amine, and hydroxyl moieties—and correspond closely to literature-reported values for structurally related azo-quinoline derivatives. Thus, the FT-IR data provide strong evidence for the successful synthesis and structural integrity of the compound.

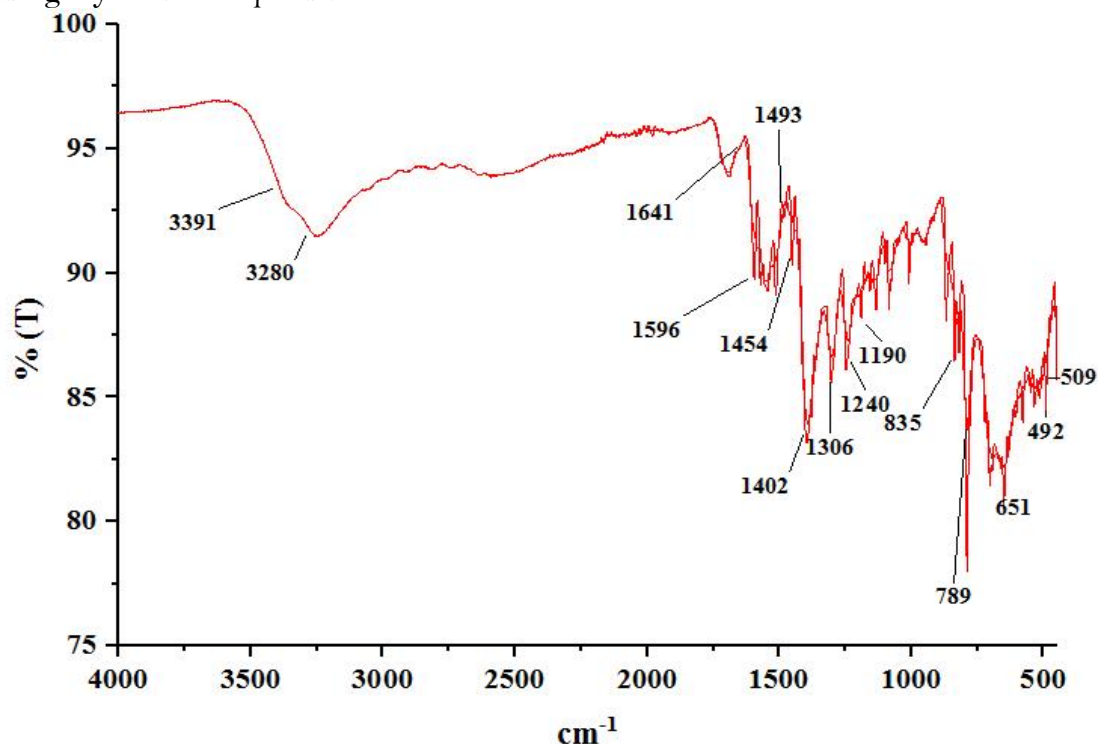
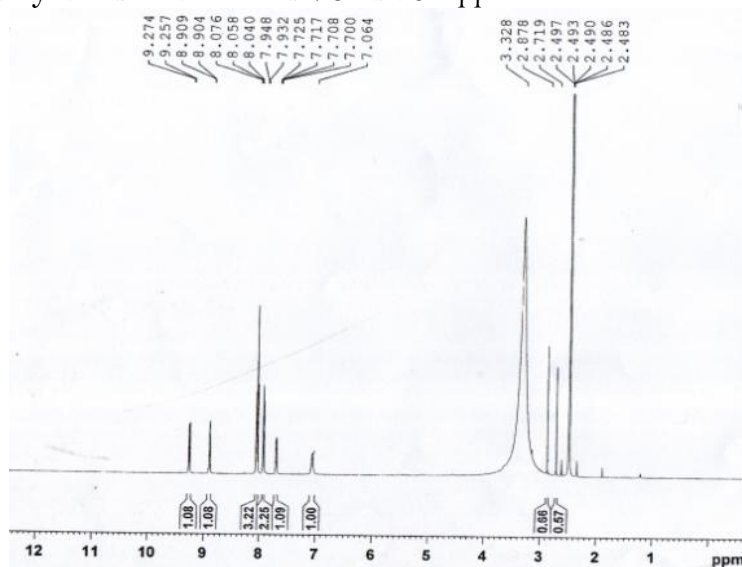


Fig. 4.3 *Infrared spectrum of benzoic acid derivative*

¹H NMR spectroscopy

The ¹H NMR spectrum displayed the following characteristic proton signals. Solvent resonances from DMSO-d₆ appeared at 2.5 ppm and 3.3 ppm. The aromatic protons of the benzene ring directly attached to the azo group (–N=N–) were significantly deshielded and resonated in the downfield region between 9.0 and 10.0

ppm, consistent with the strong electron-withdrawing effect of the azo linkage. Protons on the phenolic ring of the quinoline moiety gave signals in the range 7.2–7.9 ppm, while those associated with the pyridine ring (fused to the phenolic ring) appeared slightly downfield between 7.9 and 8.1 ppm.



A broad singlet corresponding to the phenolic hydroxyl group (O-H) was observed at approximately 7.0 ppm. The phenolic ring exhibited a characteristic doublet at around 7.9 ppm with a coupling constant of 8 Hz, indicative of ortho coupling. In contrast, the benzene ring protons appeared as two distinct doublets (reflecting two sets of non-equivalent protons) in the deshielded region of 8.9–9.2 ppm. This pronounced downfield shift for the benzene ring protons is attributed to the presence of the strongly electron-withdrawing carboxylic acid group (-COOH), which deshields the ortho and para positions relative to the substituent. Additionally, triplet signals in the narrow range 8.04–8.07 ppm were assigned to the protons of the pyridine ring. Notably, no distinct singlet for the carboxylic acid proton (-COOH) was observed at the expected position around 12.71 ppm; this absence is likely due to rapid intermolecular hydrogen bonding and exchange interactions between the carboxylic acid proton, the phenolic hydroxyl group, and the DMSO solvent, which broadens and shifts the signal beyond clear detection or merges it into the baseline. These ^1H NMR assignments are fully consistent with the proposed molecular structure, confirming the presence of the azo linkage, quinoline core, phenolic hydroxyl, benzene ring protons influenced by electron-withdrawing groups, and pyridine ring protons. The spectral data align with typical chemical shift ranges reported for analogous azo-quinoline derivatives containing aromatic and hetero aromatic systems.

Evaluation of Anti-Fungal Activity

The antifungal efficacy of the novel 4-aminobenzoic acid azo derivative was assessed against *Aspergillus terreus* using the disk diffusion method. The synthesized compound demonstrated considerable inhibitory activity against the tested fungal strain, as evidenced by the formation of clear zones of inhibition. These findings

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were benchmarked against the standard antifungal agent Fluconazole, which served as the positive reference control.

The inhibition zone diameters (measured in millimetres) for the test compound and Fluconazole are summarized in the accompanying table. Zone measurements represent the mean values obtained from replicate experiments, recorded to the nearest whole millimetre after incubation at 35°C for 24 h and 48 h, respectively.

The observed zones indicate that the novel azo derivative exhibited significant antifungal activity relative to Fluconazole under the experimental conditions employed. No inhibition was observed in solvent-only controls, confirming that the observed effects were attributable to the test compound itself.

Strain used	Compound vs. Fluconazole
<i>Aspergillus terreus</i> (4)	5mm, 20mm

Table 4.3. Minimum Inhibition Concentration of the Synthesized Compound

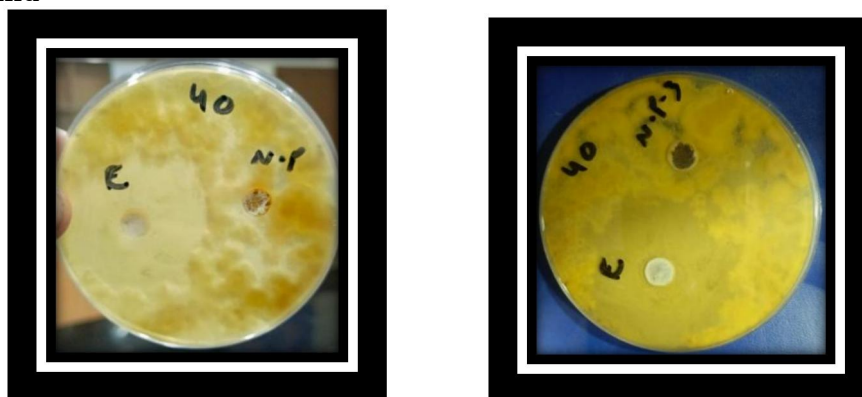


Figure 4.4. Inhibition Zone of Synthesized Compound against Mentioned Fungal Strain

Conclusion

In this study, the targeted compound (E)-4-((8-hydroxyquinolin-5-yl) diaziny) benzoic acid was successfully synthesized through a diazo-coupling reaction between Para Amino Benzoic Acid and 8-hydroxyquinoline. The prepared compound was obtained in a good yield (85%). The resultant compound was found to be insoluble in water and some other common organic solvents like n-hexane, DCM (Dichloromethane), and chloroform. The melting point of the compound was determined to be notably above 350 °C. Through the recrystallization method, the prepared compound was purified effectively.

The structure of the synthesized compound has been elucidated via UV-visible, IR, and NMR spectroscopies. The prominent peaks observed at a range of 400-490 nm wavelength confirm the presence of the azo group (-N=N-).

Furthermore, the compound under consideration was examined for its antifungal activity against a fungal strain: *Aspergillus terreus* using disc diffusion assay. The result displayed potent anti-fungal activity against the mentioned fungal strain.

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dispersed azo dyes coupling with 5-methyl-2-(6-methyl-1, 3-benzothiazol-2-yl)-2, 4-dihydro-3H-pyrazol-3-one. The study also included computational and biological evaluations. The article was published in the Journal of Molecular Structure and can be cited as follows:

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