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Synthesis and antibacterial activity of 2,9-(disubstituted)-6,13-dimethoxy-5,12-dihydroquinoxalino [2,3-*b*] phenazine Derivatives

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Abstract
A novel series of 2,9-(disubstituted)-6,13-dimethoxy-5,12-dihydroquinoxalino [2,3-b] phenazine derivatives 3(a–d) was successfully synthesized with moderate yields (52–61%). The structures of these newly developed nitrogen-fused heterocyclic frameworks were confirmed via FT-IR, ^{1}H-NMR, ^{13}C-NMR, ESI-MS and elemental analysis. All synthesized target molecules were evaluated *in vitro* for their antibacterial efficacy against Gram-positive *Staphylococcus aureus*, *Micrococcus luteus, Bacillus subtilis* and Gram-negative *Escherichia coli*, *Salmonella paratyphi, Klebsiella pneumoniae* bacterial strains using the disk diffusion technique. Among the series, compounds 3b (chloro-substituted) and 3c (bromo-substituted) demonstrated moderate antibacterial activity, producing zones of inhibition (15–19 mm) compared to the commercial standard drug, *Streptomycin*. This study highlights the therapeutic potential of modified phenazine-quinoxaline hybrids as next-generation antibacterial scaffolds.

Keywords: Phenazine derivatives, Quinoxaline, Heterocyclic synthesis, Condensation, Antibacterial screening, Minimal Inhibitory Concentration (MIC)

Introduction

The relentless emergence of multi-drug resistant (MDR) bacterial pathogens poses a severe, immediate threat to global public health infrastructure. Conventional clinical treatments are losing efficacy against critical Gram-positive and Gram-negative strains. Consequently, there is an urgent demand to discover and engineer structurally diverse, novel chemical entities featuring alternative mechanisms of antimicrobial action [1]. Nitrogen-containing fused heterocycles have historically formed the structural backbone of numerous highly effective medicinal designs [2]. Within this class, quinoxaline and phenazine cores are prominent "privileged scaffolds" owing to their exceptional ability to participate in cellular intercalation, redox-cycling, and biochemical enzyme inhibition [3]. Phenazine derivatives exhibit diverse therapeutic properties, acting as anti-cancer, anti-malarial, and anti-tubercular agents [4]. Similarly, quinoxaline hybrids display robust efficacy in disrupting bacterial replication pathways by targeting crucial cellular machinery, such as DNA gyrase [5]. Spurred by these properties, we designed a molecular framework combining both pharmacophores into a single highly conjugated system, 2,9-(disubstituted)-6,13-dimethoxy-5,12-dihydroquinoxalino [2,3-b] phenazine by using precursor 7-Substituted 3-bromo-1,4-dimethoxyphenazin-2(10H)-one. This 7-Substituted 3-bromo-1,4-dimethoxyphenazin-2(10H)-ones was prepared by using known procedure [6]. We hypothesized that placing electronic substituents at the 2,9-positions, combined with electron-donating methoxy groups at the 6,13-positions, would alter the lipophilicity and binding affinity of the molecule [7]. This article details the efficient synthesis, and *in vitro* antibacterial profiling of these novel hybrid derivatives 3(a-d).

Materials and Methods

1. Chemistry and Analytical Instruments

All chemical reagents, solvents, and starting precursors were sourced directly from commercial vendors and utilized without further purification. Melting points were recorded in open capillary tubes using an automated digital melting point apparatus and remain uncorrected. The key structural intermediates, 2,5-dibromo-3, 6-dimethoxy-1,4-benzoquinone, 7-Substituted 3-bromo-1,4-dimethoxyphenazin-2(10H)-ones 1(a-d), and the substituted ortho-phenylenediamine monomers 2(a-d) were verified using standard laboratory specifications. Reaction pathways and product purity were monitored on analytical silica gel TLC plates (Merck, 60 F_{254}) under UV light visualization (254 nm). Analytical characterization was performed by recording FTIR spectra in KBr pellets on a Nexus 470 spectrometer, ^{1}H NMR spectra recorded in DMSO-d_6 solvent on Varian Mercury 400 MHz spectrometer using tetramethylsilane (TMS) as internal standard, ^{13}C NMR spectra recorded on Varian Mercury 75 MHz spectrometer and ESI-Mass spectra obtained on Shimadzu mass spectrometer.

Experimental Section

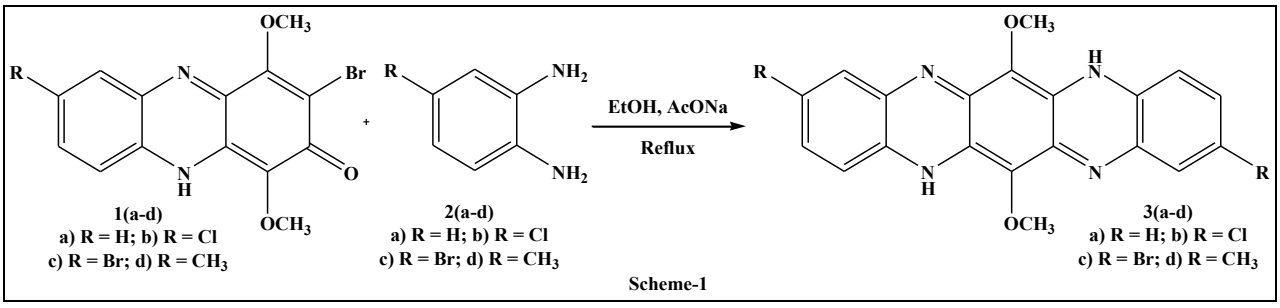
1. General Procedure for the Preparation of 7-Substituted 3-bromo-1,4-dimethoxyphenazin-2(10H)-one Derivatives 1(a–d)

To a stirred solution of substituted *ortho*-phenylenediamine 2(a–d) (6.135 mmol) in absolute ethanol (30 mL) within a 100 mL round-bottom flask, anhydrous sodium acetate (6.135 mmol) was added. After stirring at room temperature for 15 minutes, an equimolar amount of 2,5-dibromo-3,6-dimethoxy-1,4-benzoquinone (6.135 mmol) was introduced in small portions over a few minutes. The reaction matrix was then heated to reflux and maintained under constant

stirring for 3 hours, monitoring progress by TLC. Following full consumption of the starting materials, the mixture was cooled to room temperature, quenched with 100 mL of ice-cold distilled water, and extracted using ethyl acetate (3×20 mL). The combined organic extracts were washed with brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The resulting brown crude solid was purified by silica gel column chromatography eluting with variable ratios of ethyl acetate and petroleum ether to yield the pure target compounds [6] 1(a–d).

2. General Procedure for the Synthesis of Target Substituted 6,13-Dimethoxy-5,12-Dihydroquinoxalino (2,3-b) Phenazines 3(a-d)

The target functionalized multi-fused heterocyclic compounds were synthesized by reacting the prepared intermediates 7-Substituted 3-bromo-1,4-dimethoxyphenazin-2(10H)-ones 1(a-d) with an additional equivalent of substituted 1,2-diaminobenzenes. A precise amount of the appropriate substituted ortho-phenylenediamine 2(a-d) (1.5 mmol) was placed into a 50 mL round-bottom flask and dissolved in 15 mL of absolute ethanol. Anhydrous sodium acetate (1.5 mmol) was added to the solution, and the resulting mixture was stirred continuously at room temperature for 15 minutes to ensure proper activation of the amino groups. To this well-stirred mixture, an equimolar quantity (1.5 mmol) of the previously synthesized 7-Substituted 3-bromo-1,4-dimethoxyphenazin-2(10H)-one derivative 1(a-d) was added portion-wise over 10 minutes. The reaction vessel was then equipped with a reflux condenser and heated under steady reflux conditions for a duration ranging between 5 to 8 hours. The kinetic progress of the cyclization reaction was carefully tracked by executing periodic TLC assays. On complete conversion, the reaction mixture was allowed to cool to ambient temperature and subsequently poured into 50 mL of chilled distilled water. The resulting mixture was extracted three times with ethyl acetate (3x20 mL). The organic layers were pooled, washed with a saturated brine solution, and dried over anhydrous sodium sulfate. The dried organic phase was filtered and concentrated under reduced pressure to yield the crude product. Final purification was successfully achieved via silica gel column chromatography using custom-tailored gradient mixtures of ethyl acetate and petroleum ether, successfully generating the pure target substituted 6,13-dimethoxy-5,12-dihydroquinoxalino(2,3-b) phenazines [8] 3(a-d).



Scheme-1

3. Methodology of Biological Evaluation

a. Preparation of Bacterial Inoculum and Growth Medium

The *in vitro* antibacterial activity of synthesized derivatives 3(a–d) was evaluated against three Gram-negative (*Escherichia coli, Salmonella paratyphi, Klebsiella pneumoniae*) and three Gram-positive (*Staphylococcus aureus, Micrococcus luteus, Bacillus cereus*) human pathogens. Stock cultures were maintained on nutrient agar slants at 4°C. The nutrient broth medium comprised peptone (5.0 g), NaCl (5.0 g), and beef extract (3.0 g) in 1000 mL distilled water (pH 7.2 ± 0.1). For the solid medium, 15% (w/v) agar powder was added to the broth. Both media were sterilized via autoclaving at 121 °C and 15 psi for 15 minutes. Following clinical guidelines by Forbes *et al*., fresh bacterial suspensions containing approximately 1.5x108 bacteria cells/ml were prepared in sterile normal saline to standardize bacterial density [9].

b. Agar Disc-Diffusion Bioassay Protocol

The freshly synthesized target hybrids 3(a–d) were evaluated *in vitro* using the standard agar disk diffusion assay [9,10]. Glassware and petri dishes (12x1.2 cm) were sterilized in a hot air oven at 150 °C for one hour under aseptic conditions. Molten nutrient agar (15 mL) was poured into each dish and solidified at room temperature. Then, 0.5 mL of the bacterial suspension was spread uniformly across the agar surface using a sterile glass spreader. The plates stood for 15 minutes to absorb the inoculum, and excess liquid was drained. Uniform 6-mm wells were made in the agar using a sterile cork borer, spaced 2 cm apart. Test derivatives 3(a–d) were dissolved in pure DMSO (negative control), while the standard reference antibiotic, Streptomycin, was dissolved in sterile distilled water. Three concentrations (400, 600, and 800 µg/mL) were prepared and introduced into designated wells. Plates were left for one hour to allow radial diffusion before incubation at 37°C for 18 hours. The Diameter of the Inhibition Zone (DIZ) was measured in millimeters using a digital caliper. Assays were conducted in independent triplicates, and results were recorded as mean values.

4. Performance Analysis of Dihydroquinoxalino(2,3-b) Phenazine Derivatives

The performance profile of the newly synthesized 2,9-(disubstituted)-6,13-dimethoxy-5,12-dihydroquinoxalino(2,3-b) phenazine derivatives was critically analyzed by comparing their measured diameters of zones of inhibition against both Gram-negative and Gram-positive bacteria classes across the three established dosing thresholds. The collected empirical bioactivity dataset is presented in Table 1.

Table 1: Anti-bacterial activity of the compounds 3(a-d)

S. No	Compound	Zone of inhibition (in mm)																	
		E. coli			*Salmonella paratyphi*			*Klebsiella pneumoniae*			*Staphylococcu s aureus*			*Micrococcus luteus*			*Bacillus cereus*		
		A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
1	3a	-	-	10	10	12	13	-	16	16	10	10	10	10	12	13	12	14	15
2	3b	17	18	18	15	16	16	15	16	16	16	17	17	17	18	18	15	15	16
3	3c	16	17	17	15	15	16	15	17	17	16	17	17	17	18	19	15	16	16
4	3d	15	15	15	15	17	17	16	16	17	15	16	17	15	16	16	-	10	12
5	Streptomycin	28	30	32	27	28	29	29	29	30	29	30	30	28	30	31	28	28	30
Test solution and standard solution; A: 400 µg/ml; B: 600 µg/ml; C: 800 µg/ml.																			

An examination of the data reveals that the synthesized compounds exhibit distinct, highly localized performance characteristics dependent on the nature of the chemical functionalization at the 2,9-positions. The unsubstituted derivative 3a displayed the weakest overall antibacterial profile. It failed to show any inhibitory action against *Escherichia coli* and *Klebsiella pneumoniae* at lower and intermediate doses (400 and 600 µg/mL), requiring a maximum concentration of 800 µg/mL to produce a minimal zone of 10 mm and 16 mm, respectively.

In sharp contrast, the introduction of halogen atoms onto the framework resulted in an immediate enhancement of potency. The dichloro-substituted analog 3b and dibromo-substituted analog 3c exhibited high performance across all bacterial lines, even at the lowest administered dose of 400 µg/mL. For instance, compound 3b achieved an inhibition zone of 17 mm against *E. coli* at 400 µg/mL, which expanded to 18 mm at 800 µg/mL. Similarly, compound 3c excelled against *Micrococcus luteus*, yielding a notable zone of 19 mm at the 800 µg/mL concentration threshold.

The dimethyl-functionalized derivative 3d demonstrated intermediate performance, showing reliable activity against Gram-negative pathogens but exhibiting a drop in efficacy against the Gram-positive rod *Bacillus cereus*, where it was completely inactive at 400 µg/mL and scored a minimal 10 mm zone at 600 µg/mL. Overall, the performance analysis highlights that the synthesized derivatives operate via dose-dependent pathways, with halogenation being the primary driver of high antimicrobial performance.

5. Structure-Activity Relationships (SAR)

The structural modifications performed on the outer benzenoid rings of the 6,13-dimethoxy-5,12-dihydroquinoxalino(2,3-b) phenazine core provide a solid foundation for evaluating Structure-Activity Relationships (SAR). The core tricyclic phenazine unit combined with the quinoxaline moiety yields a highly extended, planar polycyclic system. This flat, aromatic architecture facilitates electronic delocalization and promotes cellular uptake via passive membrane diffusion. The SAR trends observed in this study can be broken down into three main structural factors.

a. Influence of Halogenation (Electronegativity vs. Lipophilicity)

The attachment of chlorine 3b and bromine 3c at the 2,9-positions dramatically increased antibacterial activity compared to the unsubstituted core 3a. Halogen atoms are known to act as electron-withdrawing groups via induction (-I effect), while simultaneously providing electron density through resonance (+R effect). More importantly, halogens significantly enhance the lipophilicity of the entire molecule. This increased lipophilicity facilitates the breakdown of the hydrophobic lipid bilayers that constitute the cell membranes of both Gram-positive and Gram-negative bacteria. The chloro derivative 3b slightly outperformed the bromo derivative 3c against *E. coli*, likely due to the smaller atomic radius and higher electronegativity of chlorine, which may allow for tighter binding interactions within bacterial enzyme active sites.

b. Impact of Electron-Donating Alkyl Substitution

Compound 3d features methyl groups at the 2,9-positions, which act as electron-donating groups via hyperconjugation and inductive mechanisms (+I effect). While compound 3d displayed decent activity against *Salmonella paratyphi* (17 mm at 800 µg/mL) and *Klebsiella pneumoniae* (17 mm at 800 µg/mL), its performance against *Bacillus cereus* was poor. This indicates that increasing electron density on the peripheral rings without a corresponding strong lipophilic or electronegative effect reduces the compound's ability to interfere with critical physiological processes in certain bacterial strains.

c. Role of the Central Methoxy and Dihydro Diimine System

The presence of the fixed 6,13-dimethoxy groups on the central phenazine ring maintains a baseline level of electron distribution and structural stabilization across the series. Additionally, the secondary amine nitrogen atoms (-NH) at the 5,12-positions act as crucial hydrogen-bond donors. These sites can form strong intermolecular hydrogen bonds with the phosphate head groups of bacterial phospholipids or the amino acid residues of bacterial transmembrane proteins, securing the molecule at its site of action.

Results and Discussion

1. Antibacterial Screening Analysis

The freshly synthesized target hybrids 3(a–d) were evaluated *in vitro* using the standard agar disk diffusion assay. Each compound was prepared at concentrations of 400 µg/ml, 600 µg/ml and 800 µg/ml by dissolving in sterile Dimethylsulfoxide (DMSO). Standard inhibition zones were carefully measured after 24 hours of incubation at 37°C. Streptomycin was run concurrently as the positive standard drug control, while pure DMSO served as the negative control. Structure-activity relationship (SAR) tracking indicates that the biological efficacy of this chemical series depends significantly on the electronic profile of the functional groups located at the 2,9-positions. Compounds containing electron-withdrawing groups, such as 3b (chloro) and 3c (bromo), showed higher antibacterial potency against both bacterial classes than compounds with electron-donating groups 3d. This elevated potency is likely due to

increased lipophilicity, which enhances the molecule's ability to cross lipid-rich bacterial cell walls and bind tightly to intracellular targets.

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