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Synthesis and antibacterial activity of 7-substituted 3-bromo-1,4-dimethoxyphenazin-2(10H)-one derivatives

Ch. Sudhakar
Department of Chemistry, SR & BGNR Govt. Arts and Science College (A), Khammam, Telangana, India

Abstract
Phenazinones represent a foundational class of nitrogen-containing heterocyclic redox agents that are highly recognized for their exceptional antibiotic, antifungal, and cellular modulating characteristics. In this study, a novel series of substituted 3-bromo-1,4-dimethoxyphenazin-2(10H)-one derivatives 3(a-d) was successfully synthesized through a direct, base-catalyzed condensation approach. The synthesis involved the reaction of 2,5-dibromo-3,6-dimethoxy-1,4-benzoquinone with various substituted ortho-phenylenediamines in an ethanol medium, using anhydrous sodium acetate as a basic catalyst under reflux conditions for 3 hours. The target phenazinone compounds were isolated in high purity via silica gel column chromatography and characterized systematically. All the newly synthesized derivatives were thoroughly evaluated for their *in vitro* antibacterial efficacy against a selected panel of human pathogens, including four Gram-negative strains (*Escherichia coli, Salmonella paratyphi, and Klebsiella pneumoniae*) and three Gram-positive strains (*Staphylococcus aureus, Micrococcus luteus and Bacillus cereus*) using the agar disc diffusion methodology at multiple concentrations (400, 600, and 800 µg/ml). The biological assays revealed that the derivative carrying a chlorine substitution (3b) exhibited the most potent antibacterial profile, particularly demonstrating a remarkable zone of inhibition of 22–23 mm against *Escherichia coli*, which was highly comparable to the standard antibiotic Streptomycin. Other derivatives, including the unsubstituted (3a), bromo-substituted (3c), and methyl-substituted (3d) analogues, showed moderate to strong broad-spectrum inhibitory potential across both Gram-positive and Gram-negative classes. These findings reveal that the incorporation of halogen groups on the phenazinone core significantly boosts cell membrane penetration and redox-disrupting capabilities, establishing these scaffolds as promising leads for downstream antimicrobial drug development.

Keywords: Phenazinone, heterocyclic synthesis, benzoquinone, antibacterial activity, halo-derivatives, disc diffusion

Introduction

The alarming global rise in multi-drug resistant (MDR) bacterial pathogens has forced the scientific community to aggressively search for novel, structurally diverse chemical entities capable of bypassing existing microbial defense networks. Nitrogen-containing heterocyclic architectures have consistently served as a corner-stone in medicinal chemistry due to their ability to mimic natural biomolecules and interact with essential cellular receptors. Among these specialized structures, phenazines and phenazin-2(10H)-one derivatives have attracted considerable attention as critical redox-active secondary metabolites and core components of numerous advanced antibiotic formulations. Naturally occurring and synthetically derived phenazinones function effectively as intracellular electron shuttles, intercepting electron transport chains and generating highly destructive reactive oxygen species (ROS) that induce oxidative stress and cell death within targeted microbes [1, 2, 3].

Historically, the synthetic exploration of the phenazin-2(10H)-one framework has remained relatively limited due to regioselectivity challenges and low reaction yields associated with classical condensation methods. Early methodologies frequently relied on the oxidative coupling of aniline variants or the rigid Wohl-Aue reaction, which required harsh thermal conditions and offered minimal functional group compatibility. Over the decades, diverse approaches have emerged to synthesize phenazinone networks. For instance, Marcinkeviciene *et al*. introduced an eco-friendly enzymatic strategy using laccase from Coriolopsis byrsina to catalyze the reaction between hydroquinone and ortho-phenylenediamine, achieving phenazin-2(10H)-one synthesis via consecutive Michael addition and carbonyl condensation [3]. Similarly, Gyanendra and colleagues successfully generated highly functionalized acetyl-dimethoxyphenazinones by reacting substituted benzoquinones with specialized ortho-phenylenediamines, demonstrating the versatile reactivity of quinonoid systems [4]. Further chemical variations by Goswami *et al*. demonstrated that tetrahalo-p-benzoquinones could undergo efficient condensation with phenazine-2,3-diamines to afford highly extended trihaloquinoxalino-phenazinone systems under mildly basic conditions[5]. In parallel, the synthesis of fused benzo(a)phenazin-5-one frameworks was successfully pioneered by Rehberg and coworkers through the direct condensation of 2-hydroxy-1,4-naphthoquinone with ortho-phenylenediamines in glacial acetic acid[6]. Berghot later advanced this domain by demonstrating that the intramolecular cyclization of chlorinated anilino-naphthoquinones provides direct access to 6-chlorobenzo(a)phenazin-5(7H)-ones [7]. Additionally, Da Silva *et al*. expanded the library of substituted phenazinones by exploring the condensation reactions of both unsaturated and hydrogenated naphthalene-1,4-diones with aromatic diamines, establishing a clear link between structural rigidity and broad-spectrum therapeutic effects [8].

Recent literature highlights the ongoing modification of the phenazinone core to achieve better pharmacological profiles. Zsila *et al*. utilized Friedel-Crafts alkylation techniques to introduce methyl functionalities onto aminophenazinones, evaluating their specific binding interactions with transport proteins and nucleic acids [9]. Meanwhile, Conda-Sheridan and co-workers successfully

synthesized methoxy-substituted phenazine analogues from ortho-benzoquinones, highlighting their potent chemopreventive, antioxidant, and anti-inflammatory roles [10]. Modern advancements continue to optimize these scaffolds; recent work by researchers in 2026 focused on developing regioselective synthetic pathways using (3+3) annulations of enaminones and halogenated nitrobenzenes to build customized phenazin-2(10H)-ones with enhanced cytotoxic selectivity against specialized carcinoma cell lines. Given that these core structures are widely documented for their broad-spectrum of antibiotic, anti-oomycete, and antitumor profiles, the development of novel halogenated and alkoxy-substituted phenazinone variations holds immense therapeutic potential [11]. Phenazinone based nitrogen heterocycles show significant therapeutic promise. Structural and analytical profiling reveals their multi-target biological efficacy, highlighting potent antimicrobial, antioxidant, and anticancer properties [12]. Mechanistically, these frameworks function as transmembrane ion transporters that disrupt cellular pH and induce cytotoxicity in cancer cells [13]. Recent synthetic advancements utilize regioselective condensation pathways to couple functionalized ortho-phenylenediamines with substituted quinonoid matrices [14]. Additionally, metal-free, PIDA-mediated one-pot synthesis of phenazin-1-ones from Schiff bases allows functional diversification via glycosylation and N-alkylation, yielding diverse, biologically valuable compound libraries [15].

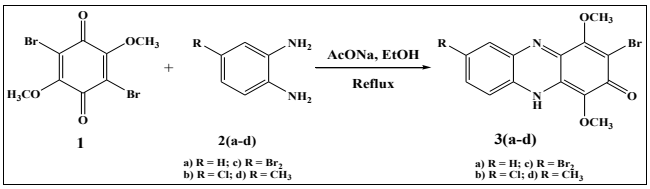
Motivated by these ongoing structural advancements and the urgent clinical necessity for novel antibiotics, the present work details a highly streamlined chemical synthesis of a novel series of 3-bromo-1,4-dimethoxyphenazin-2(10H)-one derivatives 3(a-d) utilizing a highly reactive 2,5-dibromo-3,6-dimethoxy-1,4-benzoquinone building block[16], followed by an in-depth comparative investigation of their antibacterial properties against challenging clinical isolates.

Material and Methods

All chemical reagents, starting precursors, and analytical-grade solvents were obtained from commercial suppliers and used without further purification. The key structural intermediate, 2,5-dibromo-3,6-dimethoxy-1,4-benzoquinone (1), and the substituted ortho-phenylenediamine monomers 2(a-d) were verified using standard laboratory specifications. Reaction monitoring was regularly conducted via thin-layer chromatography (TLC) utilizing aluminum sheets pre-coated with silica gel 60 F254, with visualization achieved under ultraviolet (UV) light at 254 nm and 365 nm. Purification of the crude solid products was carried out via silica gel column chromatography using optimized gradient mixtures of analytical-grade ethyl acetate and petroleum ether.

Experimental Section

The general procedure for the synthesis of 3-bromo-1,4-dimethoxyphenazin-2(10H)-one derivatives 3(a-d) was systematically executed as follows: A precise amount of the substituted ortho-phenylenediamine derivative 2(a-d) (6.135 mmol) was charged into a 100 ml round-bottom flask containing 30 ml of absolute ethanol. Anhydrous sodium acetate (6.135 mmol) was added to the mixture, and the resulting solution was allowed to stir efficiently at room temperature for 15 minutes to ensure uniform deprotonation and activation. Subsequently, an equimolar quantity of 2,5-dibromo-3,6-dimethoxy-1,4-benzoquinone (1) (6.135 mmol) was added portion-wise over several minutes to control the initial exothermic profile of the condensation. The reaction mixture was then brought to a steady reflux temperature and maintained under constant stirring for exactly 3 hours, during which the progress and complete consumption of starting materials were rigorously monitored via TLC. Upon full completion, the reaction matrix was permitted to cool naturally to room temperature, poured slowly into 100 ml of ice-cold distilled water, and subsequently extracted with ethyl acetate (3 x 20 ml). The combined organic fractions were isolated, washed thoroughly with saturated brine solution, and dried over a layer of anhydrous sodium sulfate. Evaporation of the volatile solvent under reduced pressure yielded a crude brown solid mass, which was further purified via silica gel column chromatography using variable ratios of ethyl acetate and petroleum ether to afford the pure target derivatives 3(a-d).



Methodology of Biological Evaluation

To establish a clear understanding of the antimicrobial profile of these newly synthesized phenazinones, an *in vitro* antibacterial assay was conducted using the standard agar disc diffusion methodology [17]. The pathogen screening panel was strategically chosen to include a diverse array of human pathogens, bridging both Gram-negative and Gram-positive classifications. The tested Gram-negative strains comprised *Escherichia coli, Salmonella paratyphi, and Klebsiella pneumoniae,* while the Gram-positive cohort was represented by *Staphylococcus aureus, Micrococcus luteus, and Bacillus cereus*.

The experimental setup involved preparing uniform bacterial inocula suspended in sterile normal saline to an approximate concentration of 1.5×10^8 cells/ml. These suspensions were evenly distributed across sterilized nutrient agar plates. Standardized wells (cups) were formed in the solidified medium using a sterile cork borer [18]. The test derivatives 3(a–d) were prepared as solutions dissolved in dimethyl sulfoxide (DMSO), which simultaneously served as the negative vehicle control to rule out any independent solvent-induced toxicity. The evaluation was executed in duplicate across three specific dosing concentrations: 400 µg/ml (Dose A), 600 µg/ml (Dose B), and 800 µg/ml (Dose C). Streptomycin was integrated into the study as the baseline positive reference antibiotic. Following an incubation phase at 37°C for 18 hours, the relative antibacterial potency was systematically quantified by measuring the diameter of the zones of inhibition (DIZ) in millimeters.

Performance Analysis of Phenazinone Derivatives

The experimental findings documented in the study reveal that the newly synthesized core modifications exhibit prominent, broad-spectrum antimicrobial properties that are driven forward in a distinct, dose-dependent manner. Compound 3b, which features a highly electronegative

chlorine substitution on the aromatic core, emerged as the most potent antimicrobial lead developed within this study. It demonstrated an exceptional zone of inhibition measuring 22 mm at the lowest dose (400 µg/ml) and escalating to 23 mm at higher concentrations against the Gram-negative pathogen *Escherichia coli*. This performance is highly remarkable

Because it closely challenges the inhibition zones generated by the commercial standard *Streptomycin* (28–32 mm). Beyond its exceptional performance against *E. coli*, compound 3b also maintained stable, broad-spectrum inhibitory properties against *Salmonella paratyphi* (14–17 mm), *Klebsiella pneumoniae* (15–16 mm), and *Staphylococcus aureus* (15–16 mm).

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	16	16	16	19	19	19	15	16	16	14	15	16	-	-	10	15	16	16
2	3b	22	23	23	14	17	17	15	16	16	15	16	16	15	15	15	-	10	10
3	3c	16	16	17	15	17	19	14	15	15	16	16	17	15	15	16	18	19	20
4	3d	17	17	18	14	16	16	14	14	15	17	21	21	16	16	16	16	17	17
5	S treptomycin	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.

The unsubstituted parent framework, compound 3a, displayed a highly consistent and well-rounded performance profile across multiple bacterial strains. It was particularly effective against *Salmonella paratyphi*, generating a stable zone of inhibition of 19 mm across all tested concentrations, while showing solid inhibition against *Escherichia coli* (16 mm) and *Klebsiella pneumoniae* (15–16 mm). This widespread baseline activity validates the choice of the phenazinone core as a therapeutic foundation.

In contrast, the bromo-substituted derivative, compound 3c, showed highly targeted selectivity toward Gram-positive bacteria. It produced an exceptional zone of inhibition measuring 18–20 mm against Bacillus cereus, an effect that directly rivals the 28–30 mm zone displayed by *Streptomycin*. Compound 3c also exerted steady, mid-tier broad-spectrum control against *Salmonella paratyphi* (15–19 mm), *Klebsiella pneumoniae* (14–15 mm), and *Staphylococcus aureus* (16–17 mm). Lastly, the methyl-substituted analogue, compound 3d, showed strong action against Gram-positive strains. It generated an excellent zone of inhibition against *Staphylococcus aureus*, starting at 17 mm and reaching 21 mm at higher doses, alongside consistent action against *Bacillus cereus* (16–17 mm). However, its efficacy against Gram-negative paths remained strictly moderate.

Structure-Activity Relationships (SAR)

A deep look at these results highlights crucial structure-activity relationships governed by the chemical groups appended to the phenazinone structure. The inclusion of highly electronegative halogen atoms, such as chlorine in 3b and bromine in 3c, clearly enhances the overall antibacterial performance compared to alkyl or unsubstituted modifications. From a biophysical perspective, these halogen groups significantly augment the lipophilicity of the molecular scaffold. Increased lipophilicity facilitates superior penetration through the complex lipid bilayers of bacterial cell membranes. Once inside the cellular environment, the electron-withdrawing nature of these halogens facilitates better coordination with cellular targets, amplifying the compound's capacity to intercept electron transport flows and catalyze internal oxidative damage.

Results and Discussions

The synthetic sequence proceeded smoothly, providing the target 3-bromo-1,4-dimethoxyphenazin-2(10H)-one derivatives 3(a-d) via a highly efficient heteroannulation cascade involving double nucleophilic attacks by the aryl amine groups on the carbonyl and halo-substituted carbons of the benzoquinone. The *in vitro* antibacterial profiles of the synthesized molecules were thoroughly characterized against a diverse panel of bacterial pathogens using the agar disc diffusion protocol. The diameter of the zone of inhibition (DIZ) was measured precisely in millimeters at three distinct dosing concentrations: 400 µg/ml (A), 600 µg/ml (B), and 800 µg/ml (C), with the reference standard antibiotic Streptomycin serving as the positive baseline control.

The experimental bioassay results demonstrated that all four phenazinone derivatives possess significant broad-spectrum antibacterial activity, which increased in a strictly dose-dependent manner. Notably, compound 3b (bearing a chlorine substitution) emerged as the most powerful antimicrobial agent in this study. It exhibited a remarkable zone of inhibition measuring 22 mm at 400 µg/ml and reaching up to 23 mm at higher concentrations against the Gram-negative pathogen *Escherichia coli.* This performance was highly competitive with the standard *Streptomycin* control, which recorded zones of 28–32 mm. Compound 3b also maintained steady, moderate-to-strong inhibitory effects across *Salmonella paratyphi* (14–17 mm), *Klebsiella pneumoniae* (15–16 mm), and *Staphylococcus aureus* (15–16 mm).

The unsubstituted derivative 3a displayed well-rounded, consistent efficacy against *Salmonella paratyphi* (19 mm), *Escherichia coli* (16 mm), and *Klebsiella pneumoniae* (15–16 mm), highlighting the core therapeutic utility of the phenazinone framework. The bromo-substituted derivative 3c showed a unique and highly pronounced selectivity towards the Gram-positive strain Bacillus cereus, generating an exceptional zone of inhibition of 18–20 mm, which closely approached the performance of *Streptomycin* (28–30 mm). Meanwhile, the methyl-substituted analogue 3d displayed strong, broad-spectrum growth inhibition against *Staphylococcus aureus* (17–21 mm) and *Bacillus cereus* (16–17 mm), while demonstrating moderate action against the tested Gram-negative pathogens.

This comprehensive comparative analysis indicates that inserting highly electronegative halogen atoms such as chlorine or bromine onto the phenazinone core significantly amplifies lipophilicity, thereby enhancing cellular membrane penetration and increasing the compound's capacity to disrupt internal microbial redox balances. These compelling results clearly establish these newly synthesized bromo-dimethoxyphenazinones as excellent molecular scaffolds for designing next-generation antibacterial agents targeting resistant bacterial populations.

Conclusion

In conclusion, the systematic *in vitro* biological screening successfully highlighted the robust, broad-spectrum antibacterial potential of these molecules against dangerous Gram-positive and Gram-negative bacterial strains. The biological evaluation confirms that modifying 3-bromo-1,4-dimethoxyphenazin-2(10H)-one scaffolds with halogenated functional groups is a highly viable synthetic approach to create advanced antimicrobial options. The outstanding broad-spectrum potency of compound 3b and the targeted Gram-positive efficiency of compound 3c mark these molecules as exceptional starting points for future optimization programs aimed at addressing antibiotic resistance.

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