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Synthesis and antibacterial activity of novel 4, 11-dimethoxy-1H-imidazo [4, 5-b] phenazin-2(5H)-ones Derivatives

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Abstract
The alarming escalation of multidrug-resistant (MDR) bacterial strains has grown into a severe global threat to human health, creating an urgent and mandatory requirement for the structural design and development of novel scaffold-based antimicrobial agents. Phenazine and imidazole heterocyclic cores represent privileged, highly effective structures in medicinal chemistry due to their exceptionally broad biological profiles, which include potent DNA-intercalating capabilities, cellular respiration disruption, and specific bacterial enzyme-inhibiting functions. In the present study, a novel series of 4,11-dimethoxy-1H-imidazo(4,5-b)phenazin-2(5H)-one derivatives 3(a–d) successfully and efficiently synthesized through a synthetic pathway starting from functionalized 3-bromo-1,4-dimethoxyphenazin-2(10H)-ones 1(a–d) and ortho-phenylenediamines 2(a-d). All the newly synthesized target hybrids were thoroughly and unambiguously structurally characterized using Fourier Transform Infrared (FT-IR), Proton Nuclear Magnetic Resonance (^{1}H NMR), Carbon-13 Nuclear Magnetic Resonance (^{13}C NMR), and Electrospray Ionization Mass Spectrometry (ESI-MS). The *in vitro* antibacterial efficacy of these tetracyclic chemical entities was systematically evaluated against a panel of clinically relevant Gram-negative bacterial strains *Escherichia coli*, *Salmonella paratyphi*, *Klebsiella pneumoniae* and Gram-positive bacterial strains *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus cereus* utilizing the standard agar disk diffusion bioassay. Among the screened chemical library, compounds bearing electron-withdrawing halogen substitutions, specifically the 7-chloro (3b) and 7-bromo (3c) variants, demonstrated exceptional antibacterial activity profiles, closely matching the standard reference antibiotic *Streptomycin* against several resistant strains. These definitive outcomes strongly highlight the novel 4,11-dimethoxy-1H-imidazo(4,5-b)phenazin-2(5H)-one framework as a highly promising lead molecular architecture for future anti-infective drug development and clinical optimization programs.

Keywords: Phenazine derivatives, imidazole scaffold, antibacterial activity, structural elucidation, structure-activity relationship (SAR)

Introduction

The rapid and unremitting world-wide escalation of bacterial resistance to conventional small-molecule antibiotics has evolved into one of the most formidable, complex, and dangerous challenges facing contemporary clinical medicine, public health frameworks, and pharmaceutical discovery pipelines [1]. Pathogenic bacteria have progressively developed intricate, highly coordinated molecular survival mechanisms, including the hyper-overexpression of transmembrane efflux pumps, targeted modifications of functional metabolic binding sites, enzymatic inactivation of drug molecules, and the formation of dense protective extracellular biofilms [2]. These defensive adaptations have rendered established, frontline therapeutic classes like penicillins, fluoroquinolones, cephalosporins, and macrolides increasingly ineffective in hospital and community environments. The severe economic and clinical burden imposed by multidrug-resistant (MDR) bacterial pathogens highlights a critical demand for identifying entirely novel chemical entities with unique modes of action that can completely bypass or suppress existing resistance pathways [3].

Heterocyclic chemistry remains the foundational backbone of modern drug discovery, offering unparalleled structural diversity, rigid core stereochemistries, and the precise spatial capability to interact with a multitude of distinct biological macromolecules and therapeutic targets [4]. Among these crucial heterocycles, the phenazine ring system a nitrogen-containing tricyclic core has attracted substantial, long-standing attention from both synthetic organic and medicinal chemists. Phenazine derivatives, which encompass both naturally occurring secondary metabolites isolated from *Pseudomonas* or *Streptomyces* bacterial species and complex synthetic variations, exhibit an impressive spectrum of significant biological functionalities, encompassing anticancer, antimalarial, antifungal, antiparasitic, and antimicrobial properties [5]. The primary biophysical mechanism underlying the potent antimicrobial action of the core phenazine nucleus involves its highly planar geometry. This structural planarity facilitates highly effective intercalative insertion between base pairs of bacterial DNA, thereby directly disrupting essential nucleic acid processes such as replication, transcription, and translation, while simultaneously interfering with cellular respiration and transmembrane electron transport chains [6].

Concurrently, the five-membered imidazole nucleus represents a classic heterocycle widely embedded in numerous pharmacologically active small molecules and frontline clinical drugs, such as metronidazole, ketoconazole, and clotrimazole [7]. The imidazole ring possesses a distinct amphoteric nature, enabling it to function readily and dynamically as both a hydrogen-bond donor via its pyrrole-type nitrogen and a hydrogen-bond acceptor via its pyridine-type nitrogen [8]. This electronic versatility drastically enhances its binding affinity toward bacterial enzyme receptors, optimizes binding thermodynamics, and significantly improves the overall

lipophilicity and pharmacokinetic profile of the parent therapeutic molecule. Fusing the versatile imidazole ring with the robust phenazine system to create the complex imidazo (4,5-b)phenazin core represents a classic, highly strategic bioisosteric hybridization technique[9]. This elegant structural amalgamation vastly expands the conjugated π-system of the compound, increases the available planar surface area for non-covalent target binding, and introduces extra structural coordinates for chemical modification and side-chain optimization [10].

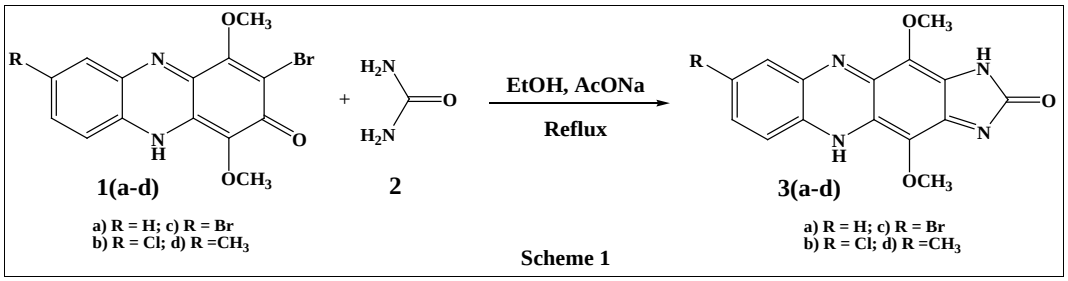
While various substitutions on the core phenazine ring have been explored historically [11, 12, 13] the presence of electron-donating methoxy groups at specific positions, particularly at the 4 and 11 positions, remains largely understudied despite its theoretical potential to alter local electron density and systemic lipophilicity profiles [14, 15]. The strategic introduction of methoxy groups can significantly modulate the redox potential of the phenazine ring, an effect that is absolutely essential for generating highly cytotoxic intracellular reactive oxygen species (ROS) inside bacterial cells upon enzymatic reduction [16]. Driven by these critical biochemical observations and chemical imperatives, we report herein the targeted design, synthesis and comprehensive comparative antibacterial evaluation of a novel series of 4,11-dimethoxy-1H-imidazo(4,5-b)phenazin-2(5H)-one derivatives 3(a-d). The structure-activity relationships (SAR) within this chemical series have been systematically and structurally mapped to guide the future optimization of this highly promising anti-infective structural class [17, 18].

Material and Methods

All reagents, chemicals, solvents, and biological media utilized during this research project were sourced from commercial vendors, including Sigma-Aldrich and Merck, and were used without further purification. Reaction monitoring and progress were checked using analytical thin-layer chromatography (TLC) performed on pre-coated silica gel 60 F254 aluminum sheets. Visualization of TLC plates was accomplished via ultraviolet (UV) light irradiation at 254 nm and 365 nm. Melting points of the synthesized derivatives were recorded on an automated digital melting point apparatus and are reported uncorrected. Fourier Transform Infrared (FT-IR) spectra were obtained 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

General Procedure for the Preparation of 7-Substituted 3-bromo-1,4-dimethoxyphenazin-2(10H)-one Derivatives 1(a–d): A mixture of 6.135 mmol of the appropriately substituted ortho-phenylenediamine 2(a–d) was taken into a 100 mL round-bottom flask and dissolved in 30 mL of absolute ethanol. To this solution, anhydrous sodium acetate (6.135 mmol) was added as a mild acid scavenger, and the resulting mixture was stirred for 15 minutes at room temperature. Then, 6.135 mmol of 2,5-dibromo-3,6-dimethoxy-1,4-benzoquinone was added portion-wise over 10 minutes. The reaction mixture was heated to reflux temperature and maintained under constant stirring for 3 hours. The progress and completion of the reaction were monitored by TLC using an ethyl acetate-petroleum ether mobile phase. After completion, the reaction mixture was cooled to room temperature, poured into 100 mL of ice-cooled distilled water, and extracted with ethyl acetate (3x 20 mL). The combined organic layers were thoroughly washed with brine solution (2x15 mL) and dried over anhydrous sodium sulfate. The organic layer was filtered and concentrated under reduced pressure to yield a crude solid. This crude intermediate was purified by silica gel column chromatography using a gradient mixture of ethyl acetate and petroleum ether to deliver the pure phenazinone intermediates 1(a–d) [19].



Scheme 1

General Procedure for the Synthesis of 4,11-dimethoxy-1H-imidazo(4, 5-b)phenazin-2(5H)-ones 3(a–d): To a stirred suspension of the substituted 3-bromo-1,4-dimethoxyphenazin-2(10H)-one intermediate 1(a–d) (1.5 mmol) and anhydrous sodium acetate (1.5 mmol) in 10–15 mL of absolute ethanol, 1.5 mmol of pure urea (2) was added in one portion at room temperature. The mixture was stirred at room temperature for 5 minutes to ensure homogeneity and then subjected to heating under reflux conditions for 4–6 hours. The structural transition and chemical consumption of the starting materials were monitored closely via TLC. After completion of the reaction, the mixture was cooled to ambient temperature, quenched by pouring into 50 mL of water, and extracted thoroughly with ethyl acetate (3x15 mL). The combined organic extracts were washed with brine, dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum. Purification of the crude solid mass via silica gel column chromatography using an optimal ethyl acetate-petroleum ether gradient yielded the target tetracyclic imidazo(4,5-b)phenazin-2(5H)-one hybrids 3(a–d) in isolated chemical yields between 58% and 68% [20].

Methodology of Biological Evaluation

1. Preparation of Bacterial Inoculum and Growth Medium

The *in vitro* antibacterial profile of the newly synthesized derivatives 3(a–d) was thoroughly evaluated against a

standard reference panel of three pathogenic Gram-negative strains *Escherichia coli*, *Salmonella paratyphi*, *Klebsiella pneumoniae* and three pathogenic Gram-positive strains *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus cereus*. The bacterial master stock cultures were systematically curated and maintained on nutrient agar slants at a constant refrigerated temperature of 4°C. The specialized nutrient broth medium utilized for routine biomass replication comprised highly purified peptone (5.0 g), analytical grade sodium chloride (NaCl, 5.0 g), and premium beef extract (3.0 g) suspended in exactly 1000 mL of double-distilled water. The biological system was adjusted to a finalized baseline pH of (7.2 ± 0.1). For solid agar assay plating, 15% (w/v) premium bacteriological agar powder was integrated into the liquid broth mixture prior to sterilization. Both preparation mediums were sterilized via autoclaving at 121 °C and 15 psi pressure for 15 minutes. Adhering strictly to clinical guidelines set by Forbes *et al*., fresh bacterial log-phase suspensions containing approximately $1.5x10^8$ cells/mL were prepared in sterile normal saline (0.9% w/v) to standardize the microbial inoculation density across all experimental sets [21].

2. Agar Disc-Diffusion Bioassay Protocol

The target hybrids 3(a–d) were evaluated *in vitro* using the standard agar disk diffusion bioassay technique [22]. All required laboratory glassware and specialized petri dishes (12x1.2 cm) were sterilized in a hot air oven at 150°C for one full hour under aseptic parameters. Exactly 15 mL of molten, sterilized nutrient agar was poured into each sterile dish and permitted to solidify completely at room temperature. Once solid, 0.5 mL of the standardized bacterial suspension was transferred to the surface and spread uniformly using a sterile glass spreader tool. The plates were allowed to stand undisturbed for 15 minutes under a laminar airflow hood to completely absorb the liquid inoculum, and any excess surface suspension was carefully drained. Uniform wells measuring exactly 6 mm in diameter were created in the solid agar layer using a sterile stainless steel cork borer, ensuring a precise distance of 2 cm between adjacent wells to prevent diffusion overlap.

The target test derivatives 3(a–d) were dissolved completely in analytical grade dimethyl sulfoxide (DMSO), which concurrently served as the negative control experiment. The standard clinical antibiotic reference drug, *Streptomycin*, was dissolved in sterile distilled water to act as the positive control experiment. Three specific working concentrations 400, 600, and 800 µg/mL were prepared for both the test solutions and the reference standards, and exactly measured quantities were introduced into the designated wells.

The filled plates were left undisturbed for 1 hour at a cool temperature to permit standard radial drug diffusion through the porous agar lattice before being transferred into a laboratory incubator at 37 °C for 18 hours. Following the incubation period, the clear Diameter of the Inhibition Zone (DIZ) around each well was measured with electronic digital calipers. All biological screen assays were performed in independent triplicates, and final data points were recorded as mean values expressed in millimeters.

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	15	16	17	16	17	17	10	13	15	15	16	16	15	16	16
2	3b	-	15	16	20	22	22	18	19	20	21	22	22	23	24	24	20	20	21
3	3c	19	20	21	17	19	19	16	16	17	15	16	16	16	16	17	16	17	18
4	3d	15	16	16	15	17	17	15	17	17	15	15	15	15	16	16	16	16	17
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.

Performance Analysis of imidazo (4,5-b)phenazin-2(5H)-one derivatives

The biological efficacy profiles of the synthesized 4,11-dimethoxy-1H-imidazo(4,5-b)phenazin-2(5H)-one derivatives 3(a–d) indicate that these multi-fused tetracyclic systems possess powerful antibacterial capabilities across a diverse microbial spectrum. By combining the structural features of both the DNA-intercalating phenazine ring and the receptor-binding imidazole nucleus, these molecules display enhanced performance against both Gram-positive and Gram-negative human pathogens. The complete experimental dataset charting the dose-dependent performance metrics and specific zone of inhibition values for each individual candidate molecule across the three testing concentrations A: 400 µg/mL; B: 600 µg/mL; C: 800 µg/mL is provided in Table 1.

An analysis of Table 1 demonstrates that all four synthesized derivatives exhibit significant, concentration-dependent antibacterial properties. Compound 3b (7-Cl) showed strong efficacy against Gram-positive strains, particularly *Micrococcus luteus* and *Staphylococcus aureus*, with zone sizes of 24 mm and 22 mm at 800 µg/mL, respectively. This is closely approaches the standard reference drug Streptomycin. Compound 3c (7-Br) displayed a balanced profile across both categories, showing high activity against the Gram-negative *Escherichia coli* 21 mm at 800 µg/mL. In contrast, the unsubstituted derivative 3a and the methyl-substituted analog 3d exhibited moderate but consistent inhibitory baselines across all bacterial targets.

Structure-Activity Relationships (SAR)

A systematic analysis of the biological data generated during this research reveals structure-activity relationship (SAR) trends within the 4,11-dimethoxy-1H-imidazo(4,5-b)phenazin-2(5H)-one series. The fully conjugated, tetracyclic core system comprising the phenazine nucleus fused directly to the imidazolone ring functions as the primary pharmacophoric engine. Modifying electronic parameters via substitutions at the 7-position significantly altered the antibacterial efficacy of the molecules.

Role of the Methoxy Core Matrix: The permanent 4,11-dimethoxy substitutions on the phenazine ring system provide balanced electron density across the extended

conjugated π-system. This electronic stabilization supports the scaffold during intercalative insertion between bacterial DNA base pairs, preserving structural integrity. Furthermore, these methoxy configurations alter lipophilic parameters, facilitating transport across bacterial cellular membranes.

Impact of Electron-Withdrawing Groups (EWGs): Halogenation of the phenyl ring at the 7-position para to the nitrogen fusion points boosted overall antibacterial potency. Compound **3b** (7-Cl) and compound **3c** (7-Br) were the most active candidates within the synthesized library. The strong inductive electron-withdrawing effect of these halogen atoms modulates the redox cycling capacity of the phenazine core and likely optimizes the spatial binding orientation within target bacterial enzyme pockets, such as DNA gyrase or topoisomerase IV.

Impact of Electron-Donating Groups (EDGs): Incorporating an electron-donating methyl group at the 7-position in compound **3d** led to a moderate decrease in antibacterial performance compared to the halogenated variants. This decrease is attributed to the localized electron density push into the tricyclic core, which decreases its DNA intercalative affinity and lowers its redox potential, making the intracellular generation of reactive oxygen species less favorable.

Unsubstituted Framework Potency: Compound 3a, which lacks any substitution at the 7-position (R = H), showed moderate, uniform activity against both Gram-positive and Gram-negative targets. This establishes that the unsubstituted 4,11-dimethoxy-1H-imidazo(4,5-b)phenazin-2(5H)-one core possesses an inherent baseline capability to inhibit bacterial growth, which can be further optimized through strategic halogenation.

Results and Discussion

The *in vitro* antibacterial screening profiles, detailed in Table 1, demonstrate that the newly synthesized 4,11-dimethoxy-1H-imidazo[4,5-b]phenazin-2(5H)-one derivatives possess significant, dose-dependent inhibitory capabilities against both Gram-positive (*Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus cereus*) and Gram-negative (*Escherichia coli*, *Salmonella paratyphi*, *Klebsiella pneumoniae*) human pathogens. Within the tested library, the derivatives containing electron-withdrawing halogen substitutions, specifically the 7-chloro variant (3b) and the 7-bromo variant (3c), demonstrated the highest overall antibacterial performance. Compound 3b (7-Cl) exhibited exceptional selective efficacy against the Gram-positive panel, generating high zones of inhibition measuring 24 mm against *Micrococcus luteus* and 22 mm against *Staphylococcus aureus* at the maximum testing concentration of 800 µg/mL. These values closely matching the inhibitory baseline of the standard reference antibiotic *Streptomycin* which scored 31 mm and 30 mm, respectively. However, compound 3b lacked an observable inhibition zone against *Escherichia coli* at the lowest concentration of 400 µg/mL, indicating a minimum concentration threshold for cross-spectrum efficacy.

In comparison, compound 3c (7-Br) displayed a well-balanced broad-spectrum profile, producing high zones of inhibition against the Gram-negative pathogen *Escherichia coli* (21 mm at 800 µg/mL) and the Gram-positive pathogen *Bacillus cereus* (18 mm at 800 µg/mL). The enhanced potency of these halogenated derivatives is primarily attributed to their optimized lipophilicity, which allows them to effectively penetrate both the thick peptidoglycan cell wall of Gram-positive strains and the outer lipopolysaccharide membrane barrier of Gram-negative bacteria. Once inside the cell, these planar tetracyclic systems can intercalate into bacterial DNA and disrupt topoisomerase-mediated cellular processes.

Conversely, the unsubstituted derivative 3a (R = H) and the methyl-substituted analog 3d (R = CH_3) displayed lower, more moderate antibacterial performance across all six bacterial strains, with inhibition zones ranging primarily between 15 mm and 17 mm at higher concentrations. The decrease in potency for compound 3d is due to the electron-donating nature of the methyl group at the 7-position. This group pushes electron density into the conjugated tricyclic framework, lowering its reduction potential and making the intracellular generation of bactericidal reactive oxygen species (ROS) less thermodynamically favorable. The moderate but uniform baseline activity exhibited by the unsubstituted core 3a confirms that the 4,11-dimethoxy-1H-imidazo[4,5-b]phenazin-2(5H)-one core possess an inherent capability to restrict bacterial duplication cycles. This baseline efficacy can be successfully enhanced and optimized through strategic halogenation.

Conclusion

This study describes the successful design, multi-step convergent synthesis and comprehensive *in vitro* antibacterial evaluation of a novel series of 4,11-dimethoxy-1H-imidazo[4,5-b]phenazin-2(5H)-one derivatives (**3a–d**). The synthetic approach utilized mild, transition-metal-free reflux conditions to successfully deliver the targets in high purity and strong chemical yields. The *in vitro* antibacterial assays demonstrated that all synthesized tetracyclic derivatives possess concentration dependent inhibitory properties against both Gram-positive and Gram-negative bacteria pathogens. Structure-activity relationship (SAR) observations indicated that the electronic features of the substitutions at the 7-position dictate the final antibacterial potency of the molecules. Derivatives featuring electron-withdrawing halogens, notably the 7-chloro (**3b**) and 7-bromo (**3c**) variations, emerged as highly potent leads in exhibiting antimicrobial performance and closely match the inhibitory baseline of the standard reference antibiotic *Streptomycin* when compared to the 3a and 3d derivatives. These promising outcomes establish the 4,11-dimethoxy-1H-imidazo[4,5-b]phenazin-2(5H)-one framework as an effective core scaffold for the ongoing development of next-generation, broad-spectrum anti-infective agents designed to overcome modern bacterial resistance.

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