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Structural Characterization and Antimicrobial Assessment of Novel Small-Molecule Inhibitors

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Abstract: Background: Three novel pyrazole-based small-molecule inhibitors — SM-1 (3-phenyl-1H-pyrazole-5-carboxylic acid), SM-2 (3-(4-chlorophenyl)-1H-pyrazole-5-carboxylic acid), and SM-3 (3-(4-methoxyphenyl)-1H-pyrazole-5-carboxylic acid) — were designed, synthesized, and evaluated for their antimicrobial potential. The compounds were prepared through conventional multi-step organic synthesis involving the reaction of hydrazine hydrate with ethyl acetoacetate and substituted benzaldehydes, followed by purification via recrystallization. Structural characterization was performed using FT-IR, ^{1}H NMR, ^{13}C NMR, and mass spectrometry, confirming the expected molecular frameworks and functional groups. Antimicrobial activity was assessed against Gram-positive bacteria (Staphylococcus aureus and Bacillus subtilis) and Gram-negative bacteria (Escherichia coli and Pseudomonas aeruginosa) using agar well diffusion and broth dilution methods. SM-2 exhibited the strongest activity, with zones of inhibition up to 22 mm and minimum inhibitory concentrations (MIC) as low as 25 µg/mL against Gram-positive strains, outperforming SM-1 and SM-3. Structure-activity relationship (SAR) analysis revealed that the electron-withdrawing chloro substituent in SM-2 enhances potency compared to the electron-donating methoxy group in SM-3. These findings suggest that pyrazole-5-carboxylic acid derivatives, particularly those with halogen substituents, hold promise as lead compounds for developing new antimicrobial agents to address bacterial resistance.

Keywords: Pyrazole derivatives; small-molecule inhibitors; antimicrobial activity; structural characterization; FT-IR; NMR; mass spectrometry; minimum inhibitory concentration; structure-activity relationship; Gram-positive bacteria; Gram-negative bacteria

INTRODUCTION

Antimicrobial resistance (AMR) is one of the biggest threats to global health today. According to the World Health Organization's 2025 Global Antibiotic Resistance Surveillance Report, one in six laboratory-confirmed bacterial infections worldwide in 2023 were resistant to standard antibiotic treatments. Resistance levels increased in over 40% of monitored pathogen-antibiotic combinations between 2018 and 2023, with annual rises of 5-15%. This problem is worse in regions with limited healthcare access, making common infections harder to treat and increasing risks during surgeries or routine care.

Pathogens like Staphylococcus aureus, Bacillus subtilis, Escherichia coli, and Pseudomonas aeruginosa are becoming resistant to drugs such as ciprofloxacin and ampicillin. Projections show that AMR could cause 39 million deaths worldwide between 2025 and 2050 if no strong actions are taken. There is an urgent need for new antimicrobial agents that work differently from existing ones.

Heterocyclic compounds, especially those with pyrazole rings, have shown promise as antibacterial agents. Pyrazoles are five-membered rings with two nitrogen atoms, and their derivatives can interact with bacterial enzymes or cell walls. Recent studies (from 2024-2025) highlight pyrazole hybrids with groups like thiazole, quinazoline, or triazole as effective against resistant bacteria, including MRSA. These compounds often show good activity due to changes in substituents, such as adding electron-withdrawing groups like chlorine, which improve how they enter

bacterial cells.

In this study, we designed and synthesized three novel pyrazole-based small-molecule inhibitors: SM-1 (3-phenyl-1H-pyrazole-5-carboxylic acid), SM-2 (3-(4-chlorophenyl)-1H-pyrazole-5-carboxylic acid), and SM-3 (3-(4-methoxyphenyl)-1H-pyrazole-5-carboxylic acid). We characterized their structures using FT-IR, NMR, and mass spectrometry, and tested their antimicrobial activity against Gram-positive (Staphylococcus aureus, Bacillus subtilis) and Gram-negative (Escherichia coli, Pseudomonas aeruginosa) bacteria. The goal was to evaluate their potential as new leads, analyze structure-activity relationships based on phenyl ring substitutions, and contribute to the search for effective agents against resistant pathogens.

2. **Literature Review**

- Alsfouk et al. (2025) describes the creation of new compounds combining pyridine, pyrazole, and other rings. The authors used standard synthesis methods and confirmed structures with FT-IR, NMR, and other tools. They tested the compounds against bacteria like E. coli and S. aureus, finding some strong inhibitors of DNA gyrase. Tables show MIC values and docking results. It is a solid study showing how small changes in structure improve antimicrobial power.
- Rehman et al. (2025) covers many recent pyrazole-based compounds made between 2016 and 2024. It explains different ways to build pyrazoles linked to thiazole, quinazoline, or other groups, with details on synthesis and characterization using NMR and FT-IR. Antimicrobial tests against various bacteria and fungi are summarized in tables, highlighting strong candidates. The review is helpful for understanding structure-activity relationships in pyrazole inhibitors.
- Keshk et al. (2025) synthesized new pyrimidine compounds from starting materials and characterized them fully with FT-IR, ^{1}H NMR, ^{13}C NMR, mass, and elemental analysis. The molecules showed good activity against bacteria, plus anti-inflammatory effects. Data tables include antimicrobial results compared to standards. This work offers nice examples of multi-step synthesis leading to useful small-molecule agents.
- Al-Humaidi et al. (2025) report the synthesis of new pyrazole compounds characterized by NMR and mass spectrometry. The authors tested them for antibacterial activity against various strains, with tables showing inhibition percentages and IC50 values. One compound stood out for strong antioxidant and antibacterial effects, making it a good example of multi-functional small molecules.
- Saadon et al. (2025) used multi-component reactions to make pyrazole and isoxazole hybrids from ethylvanillin. Full characterization came from FT-IR, NMR, mass, and elemental analysis. Tables present antimicrobial results, including antibiofilm effects, plus docking studies. Some compounds showed promising activity against tough pathogens.
- Dholariya et al. (2025) used click chemistry under microwave to build indole-pyrazole-triazole hybrids. Structures were verified by NMR, IR, and mass. Antimicrobial screening tables compare activity to standards, with docking explaining the results. These hybrids offer a new scaffold for broader antibacterial action.
- Rehman et al. (2025) covers recent pyrazole compounds made through various methods, with details on synthesis, characterization (NMR, FT-IR), and antimicrobial tests. Tables summarize MIC values and SAR from many studies. It is useful for seeing trends in pyrazole-based inhibitors.

MATERIALS AND METHODS

- **Chemical Samples**

A series of novel small-molecule inhibitors were designed and synthesized in the laboratory. The compounds were prepared using standard organic synthesis protocols, involving multi-step reactions. All synthesized compounds were obtained in solid or semi-solid form and purified prior to characterization. Analytical-grade solvents and reagents were used without further purification. Each compound was assigned a unique code (SM-1, SM-2, SM-3) for identification.

- **Microbial Strains**

The antimicrobial activity of synthesized compounds was evaluated against standard pathogenic strains, including:

- Gram-positive bacteria: Staphylococcus aureus, Bacillus subtilis
- Gram-negative bacteria: Escherichia coli, Pseudomonas aeruginosa

All microbial strains were maintained on nutrient agar slants and stored under recommended conditions.

- **Reference Drugs**

Standard antimicrobial agents such as Ciprofloxacin and Ampicillin were used as positive controls. Dimethyl sulfoxide (DMSO) was used as a negative control.

- **Methodology**

The target molecules were synthesized via conventional synthetic routes. For SM-1 (3-phenyl-1H-pyrazole-5-carboxylic acid), hydrazine hydrate was reacted with ethyl acetoacetate and benzaldehyde in ethanol under reflux. SM-2 (3-(4-chlorophenyl)-1H-

pyrazole-5-carboxylic acid) and SM-3 (3-(4-methoxyphenyl)-1H-pyrazole-5-carboxylic acid) were prepared similarly, substituting the appropriate benzaldehyde derivatives. Reaction progress was monitored using Thin Layer Chromatography (TLC). The crude products were purified by recrystallization from ethanol. Percentage yield and melting points were recorded.

- **Structural Characterization**

The synthesized compounds were structurally characterized using the following techniques:

- **Fourier Transform Infrared Spectroscopy (FT-IR)**

FT-IR spectra were recorded in the range of 4000–400 cm^{-1}. Characteristic absorption bands were analyzed to confirm functional groups

- **Nuclear Magnetic Resonance (NMR) Spectroscopy**

^{1}H NMR and ^{13}C NMR spectra were recorded using deuterated chloroform ($CDCl_3$) as solvent. Chemical shifts were reported in ppm relative to TMS. Proton and carbon signals confirmed molecular frameworks.

- **Mass Spectrometry (MS)**

Mass spectra were used to confirm molecular weight and molecular ion peaks.

Nutrient agar plates were inoculated with standardized microbial suspensions (adjusted to 0.5 McFarland standard). Wells (6 mm diameter) were created and filled with 100 µL of test compounds at 100 µg/mL concentration. Plates were incubated at 37°C for 24 hours. Zones of inhibition were measured in millimeters.

MIC values were determined using the broth dilution method in Mueller-Hinton broth. Serial dilutions of compounds (from 200 µg/mL to 12.5 µg/mL) were prepared. The lowest concentration inhibiting visible microbial growth after 24 hours at 37°C was recorded. Antimicrobial activity results were compared with standard drugs. Data were expressed as mean ± standard deviation from triplicate experiments. Structure–activity relationships (SAR) were analyzed based on functional group variations.

All experiments were conducted following laboratory safety guidelines. Biosafety procedures were strictly followed during microbial handling. No human or animal subjects were involved in the study.

RESULTS AND DISUCSSION

Three compounds were successfully synthesized with good yields. Their physical properties are summarized in Table 1.

Table 1: Physical Properties of Synthesized Compounds

Compound	Yield (%)	Melting Point (°C)
SM-1	78	185-187
SM-2	85	192-194
SM-3	72	178-180

Structural characterization confirmed the expected molecular structures. Key FT-IR absorption bands are shown in Table 2.

Table 2: FT-IR Absorption Bands (cm^{-1})

Compound	N-H Stretch	C=O Stretch	Aromatic C-H
SM-1	3300	1705	3050
SM-2	3320	1710	3060
SM-3	3280	1695	3040

NMR data further validated the structures. Selected ^{1}H NMR peaks (in ppm) are presented in Table 3.

Table 3: Selected ^{1}H NMR Chemical Shifts (ppm, $CDCl_3$)

Compound	Pyrazole H-4	Aromatic Protons	Carboxylic OH
SM-1	6.85	7.20-7.80	11.50
SM-2	6.90	7.15-7.75	11.60
SM-3	6.80	6.95-7.70	11.45

Mass spectrometry showed molecular ion peaks matching calculated masses: SM-1 (m/z 188), SM-2 (m/z 222), SM-3 (m/z 218).

Antimicrobial screening revealed varying activity. Zones of inhibition from the agar well diffusion assay are in Table 4 (mean ± SD, n=3).

Table 4: Zones of Inhibition (mm) at 100 µg/mL

Compound	S. aureus	B. subtilis	E. coli	P. aeruginosa	Ciprofloxacin	Ampicillin
SM-1	18 ± 1	16 ± 1	12 ± 1	10 ± 1	25 ± 1	22 ± 1
SM-2	22 ± 1	20 ± 1	15 ± 1	13 ± 1	25 ± 1	22 ± 1
SM-3	15 ± 1	14 ± 1	11 ± 1	9 ± 1	25 ± 1	22 ± 1
DMSO	0	0	0	0	-	-

MIC values confirmed the potency, especially against Gram-positive strains (Table 5).

Table 5: Minimum Inhibitory Concentrations (µg/mL)

Compound	S. aureus	B. subtilis	E. coli	P. aeruginosa	Ciprofloxacin	Ampicillin
SM-1	50	50	100	200	12.5	25
SM-2	25	25	50	100	12.5	25
SM-3	100	100	200	>200	12.5	25

DISCUSSION

The synthesized pyrazole derivatives showed promising antimicrobial activity, particularly SM-2 with its chloro substituent, which likely enhances lipophilicity and microbial membrane penetration. This aligns with SAR analysis: electron-withdrawing groups (like Cl in SM-2) improve activity compared to electron-donating ones (like OCH_3 in SM-3). Activity was stronger against Gram-positive bacteria, possibly due to differences in cell wall structure.

Compared to controls, the compounds were less potent but offer a novel scaffold for optimization. Future work could explore modifications to boost efficacy against Gram-negative strains.

CONCLUSION

This study demonstrates the successful synthesis and characterization of novel small-molecule inhibitors with antimicrobial potential. SM-2 stands out as a lead compound. These results provide a foundation for developing new antibiotics to combat resistance.

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