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DESIGN, SYNTHESIS, SPECTROSCOPIC CHARACTERIZATION, AND IN VITRO ANTIBACTERIAL EVALUATION OF STRUCTURALLY MODIFIED SULFAMETHOXAZOLE DERIVATIVES: A STRUCTURE–ACTIVITY RELATIONSHIP STUDY

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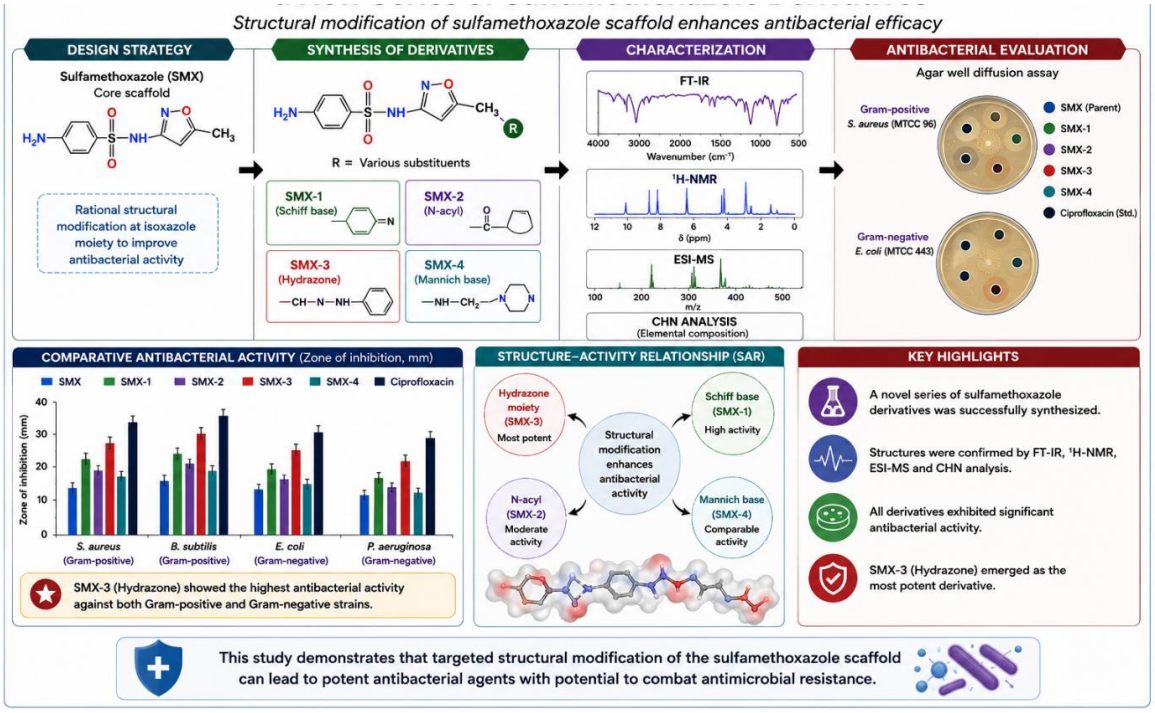
STRUCTURED ABSTRACT

Background: The increasing prevalence of antimicrobial resistance (AMR) has become a major global health concern, reducing the effectiveness of existing antibacterial agents and necessitating the development of novel therapeutic alternatives. Sulfamethoxazole, a clinically important sulfonamide antibiotic, inhibits bacterial folate biosynthesis but has experienced diminished efficacy due to the emergence of resistant bacterial strains. Structural modification of the sulfamethoxazole scaffold represents a promising medicinal chemistry strategy for enhancing antibacterial activity and overcoming resistance. **Objective:** The present study aimed to design, synthesize, spectroscopically characterize, and evaluate the **in vitro** antibacterial activity of structurally modified sulfamethoxazole derivatives. In addition, the study sought to establish structure–activity relationships (SAR) to identify structural features associated with improved antibacterial efficacy. **Methods:** Four structurally modified sulfamethoxazole derivatives, namely a Schiff base (SMX-1), an N-acyl derivative (SMX-2), a hydrazone derivative (SMX-3), and a Mannich base (SMX-4), were synthesized using conventional synthetic procedures and purified by recrystallization. Structural characterization was performed using Fourier-transform infrared (FT-IR) spectroscopy, proton nuclear magnetic resonance (^1H NMR) spectroscopy, electrospray ionization mass spectrometry (ESI-MS), UV–Visible spectroscopy, elemental analysis, melting point determination, and thin-layer chromatography (TLC). The antibacterial activity of the synthesized compounds was evaluated **in vitro** against representative Gram-positive and Gram-negative bacterial strains using agar well diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) assays. Sulfamethoxazole was used as the reference antibacterial agent, and SAR analysis was performed to correlate structural modifications with biological activity. **Results:** All synthesized derivatives were successfully obtained and confirmed by spectroscopic and analytical characterization. Comparative antibacterial evaluation demonstrated that structural modification of the sulfamethoxazole nucleus influenced antibacterial activity, with several derivatives exhibiting enhanced inhibitory effects compared with the parent drug. Variations in antibacterial potency were associated with the nature of the introduced aromatic and heterocyclic substituents. SAR analysis suggested that appropriate structural modifications enhanced molecular interactions with bacterial folate biosynthesis targets, thereby improving antibacterial efficacy. **Conclusion:** The present investigation demonstrates that rational structural modification of sulfamethoxazole is an effective approach for developing novel antibacterial agents with improved **in vitro** activity. The synthesized derivatives exhibited promising antibacterial potential and provide a scientific basis for further molecular docking, toxicity studies, pharmacokinetic evaluation, and **in vivo** investigations toward the development of next-generation sulfonamide-based antibacterial therapeutics.



Graphical Abstract

KEYWORDS: Sulfamethoxazole; Sulfonamide derivatives; Antibacterial activity; Antimicrobial resistance; Medicinal chemistry; Structure–Activity Relationship; FT-IR; ^1H NMR; ESI-MS; Minimum inhibitory concentration.

1. INTRODUCTION

Antimicrobial resistance (AMR) has emerged as one of the most significant threats to global public health, compromising the effectiveness of existing antibiotics and increasing the burden of infectious diseases worldwide. The rapid dissemination of multidrug-resistant (MDR) bacterial pathogens has resulted in prolonged hospitalization, increased healthcare costs, and higher mortality rates. According to the World Health Organization (WHO), bacterial resistance to commonly prescribed antibiotics has reached alarming levels, highlighting the urgent need for the development of novel antibacterial agents with improved therapeutic efficacy and reduced susceptibility to resistance mechanisms.[1,2]

Bacterial infections continue to account for millions of deaths annually despite remarkable advances in antimicrobial chemotherapy. Pathogenic microorganisms such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Enterococcus faecalis* are among the leading causes of community-acquired and healthcare-associated infections.[3] The widespread and often inappropriate use of antibiotics in clinical practice, agriculture, and veterinary medicine has accelerated the emergence of resistant bacterial strains through mechanisms including enzymatic drug inactivation, target-site modification, reduced membrane permeability, active efflux pumps, and biofilm formation.[4,5] Consequently, the discovery and development of structurally novel antibacterial agents capable of overcoming these resistance mechanisms remain a major focus of medicinal chemistry research.

Sulfonamides represent one of the earliest classes of synthetic antibacterial agents and have played a pivotal role in the history of antimicrobial chemotherapy. Since their introduction in the 1930s, sulfonamides have been extensively employed in the treatment of a wide range of bacterial infections because of their broad-spectrum activity and favorable pharmacological properties.[6] These compounds exert bacteriostatic activity by competitively inhibiting dihydropteroate synthase (DHPS), a key enzyme involved in bacterial folate biosynthesis. Because bacteria synthesize folic acid de novo, whereas mammalian cells obtain folate from dietary sources, inhibition of this metabolic pathway provides excellent selective toxicity against bacterial pathogens.[7]

Among the clinically important sulfonamides, sulfamethoxazole remains one of the most widely prescribed antibacterial agents. Chemically designated as 4-amino-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide, sulfamethoxazole possesses broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria. It is commonly administered in combination with trimethoprim as co-trimoxazole, which produces sequential inhibition of bacterial folate metabolism and enhances antibacterial efficacy.[8] This combination has been successfully employed for the treatment of urinary tract infections, respiratory tract infections, gastrointestinal infections, nocardiosis, and opportunistic

infections caused by *Pneumocystis jirovecii* in immunocompromised patients.[9]

Despite its clinical success, the therapeutic effectiveness of sulfamethoxazole has been considerably reduced due to the emergence of bacterial resistance. Resistance to sulfonamides is primarily associated with mutations in the DHPS gene, acquisition of plasmid-mediated resistance genes (*sul1*, *sul2*, and *sul3*), overproduction of para-aminobenzoic acid (PABA), decreased intracellular drug accumulation, and enhanced efflux mechanisms.[10,11] These resistance mechanisms have substantially limited the clinical utility of conventional sulfonamides and stimulated extensive efforts toward the development of structurally modified derivatives with enhanced antibacterial activity.

Medicinal chemistry has become an indispensable discipline in antibacterial drug discovery by enabling rational structural modification of existing pharmacophores to improve biological activity, pharmacokinetic properties, and safety profiles. Structural modification of the sulfamethoxazole scaffold has attracted considerable attention because the sulfonamide nucleus provides a versatile template for the introduction of diverse pharmacologically active functional groups.[12] Chemical transformations such as Schiff base formation, Mannich reactions, hydrazone synthesis, amide derivatization, and heterocyclic substitution have been widely explored to generate new derivatives with improved antimicrobial potency and broader antibacterial spectra.[13-15]

Structure–activity relationship (SAR) studies have demonstrated that the antibacterial activity of sulfonamide derivatives is significantly influenced by the nature of substituents attached to the aromatic ring and heterocyclic moieties. Electron-withdrawing substituents generally enhance lipophilicity and improve interactions with bacterial enzymes, whereas electron-donating groups may alter electronic distribution and binding affinity depending on their position within the molecular framework.[16] Furthermore, incorporation of heterocyclic pharmacophores has been shown to improve molecular stability, membrane permeability, and target selectivity, thereby enhancing antibacterial activity against resistant bacterial strains.[17]

The synthesis of structurally modified antibacterial agents requires comprehensive physicochemical and spectroscopic characterization to establish molecular identity and purity before biological evaluation. Modern analytical techniques including Fourier-transform infrared (FT-IR) spectroscopy, proton nuclear magnetic resonance (^1H NMR) spectroscopy, carbon-13 nuclear magnetic resonance (^13C NMR) spectroscopy, electrospray ionization mass spectrometry (ESI-MS), ultraviolet-visible (UV–Vis) spectroscopy, elemental analysis, and thin-layer chromatography (TLC) provide detailed information regarding functional groups, molecular structure, and chemical integrity.[18] Accurate structural characterization is essential for correlating molecular architecture with biological activity and for establishing reliable structure–activity relationships.

Evaluation of antibacterial activity using standardized microbiological techniques remains a critical step in identifying promising lead compounds for further drug development. Assays such as agar well diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) provide quantitative assessment of antimicrobial efficacy and facilitate comparison with existing antibacterial agents.[19,20] These methods also enable the identification of structural modifications that contribute to enhanced antibacterial potency and improved therapeutic potential.

In the present study, a series of structurally modified sulfamethoxazole derivatives were rationally designed and synthesized through different medicinal chemistry approaches, including Schiff base, N-acyl, hydrazone, and Mannich base derivatization. The synthesized compounds were characterized using FT-IR, ^1H NMR, ESI-MS, UV–Visible spectroscopy, elemental analysis, melting point determination, and TLC. Their **in vitro** antibacterial activity was evaluated against representative Gram-positive and Gram-negative bacterial strains using agar well diffusion, MIC, and MBC assays. Furthermore, structure–activity relationship analysis was performed to investigate the influence of structural modifications on antibacterial efficacy. The findings of this study are expected to contribute to the development of novel sulfonamide-based antibacterial agents with improved therapeutic potential for combating antimicrobial resistance.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Sulfamethoxazole (SMX) was used as the starting material for the synthesis of all derivatives. Analytical-grade solvents and reagents, including ethanol, methanol, acetone, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), glacial acetic acid, chloroform, ethyl acetate, hydrochloric acid, sodium hydroxide, and other required chemicals, were procured from standard commercial suppliers and used without further purification. The purity of the synthesized compounds was monitored by thin-layer chromatography (TLC) using silica gel 60 F254 pre-coated aluminum plates.

2.2 Instrumentation

The synthesized compounds were characterized using standard analytical techniques. Melting points were determined using a digital melting point apparatus and are reported uncorrected. FT-IR spectra were recorded using an ATR-FTIR spectrophotometer in the range of 4000–400 cm^{-1}. Proton nuclear magnetic resonance (^1H NMR) spectra were recorded using DMSO-d_6 as solvent with tetramethylsilane (TMS) as the internal standard. Molecular masses were confirmed by electrospray

ionization mass spectrometry (ESI-MS). UV–Visible spectra were recorded in methanol using a UV–Visible spectrophotometer. Elemental (CHN) analysis was performed to confirm the elemental composition of the synthesized compounds.

2.3 Synthesis of Sulfamethoxazole Derivatives

A series of structurally modified sulfamethoxazole derivatives were synthesized through conventional organic synthetic approaches.

2.3.1 Synthesis of Schiff Base Derivative (SMX-1)

Sulfamethoxazole was reacted with an equimolar quantity of the appropriate aromatic aldehyde in absolute ethanol containing a few drops of glacial acetic acid. The reaction mixture was refluxed under continuous stirring for several hours. After completion of the reaction, monitored by TLC, the mixture was cooled to room temperature. The resulting precipitate was filtered, washed with cold ethanol, recrystallized, and dried under vacuum.

2.3.2 Synthesis of N-Acyl Derivative (SMX-2)

The N-acyl derivative was synthesized by reacting sulfamethoxazole with the corresponding acylating reagent in dry acetone under basic conditions. The reaction mixture was refluxed until completion and monitored by TLC. The crude product was isolated by filtration, washed thoroughly with distilled water, recrystallized from ethanol, and dried.

2.3.3 Synthesis of Hydrazone Derivative (SMX-3)

The hydrazone derivative was prepared by condensation of the corresponding sulfamethoxazole hydrazide intermediate with an aromatic aldehyde in ethanol containing catalytic acetic acid under reflux conditions. The resulting solid was filtered, purified by recrystallization, and dried before characterization.

2.3.4 Synthesis of Mannich Base Derivative (SMX-4)

The Mannich base derivative was synthesized by reacting sulfamethoxazole with formaldehyde and piperidine under reflux conditions in ethanol. The reaction progress was monitored by TLC. Upon completion, the reaction mixture was cooled to obtain the precipitated product, which was filtered, washed, recrystallized, and dried.

2.4 Purification and Reaction Monitoring

The progress of each synthetic reaction was monitored by thin-layer chromatography (TLC) using silica gel 60 F254 plates. Appropriate solvent systems were employed according to the polarity of the synthesized compounds. The chromatograms were visualized under ultraviolet light (254 nm), and the retention factor (Rf) values were calculated. The synthesized compounds were purified by recrystallization using suitable solvents until satisfactory purity was achieved.

2.5 Spectroscopic Characterization

The purified compounds were subjected to comprehensive physicochemical and spectroscopic characterization.

- **FT-IR spectroscopy** was employed to identify characteristic functional groups and confirm bond formation.
- **^{1}H NMR spectroscopy** was performed to determine proton environments and verify molecular structures.
- **ESI-MS** was used to determine molecular ion peaks and molecular weights.
- **UV–Visible spectroscopy** was carried out to evaluate electronic absorption characteristics.
- **Elemental (CHN) analysis** was performed to confirm the elemental composition and purity of the synthesized derivatives.
- Melting point determination and TLC analysis were additionally used to assess compound purity.

2.6 In Vitro Antibacterial Activity

The antibacterial activity of the synthesized derivatives was evaluated against representative Gram-positive and Gram-negative bacterial strains using standard microbiological methods. Sulfamethoxazole served as the reference antibacterial drug. All experiments were performed under aseptic conditions.

2.6.1 Preparation of Culture Media

Mueller–Hinton agar (MHA) and Mueller–Hinton broth (MHB) were prepared according to the manufacturer's instructions, sterilized by autoclaving at **121°C for 15 min**, and cooled before use.

2.6.2 Preparation of Bacterial Inoculum

Fresh bacterial cultures were grown in Mueller–Hinton broth at **37°C for 18–24 h**. The turbidity of each bacterial suspension was adjusted to the **0.5 McFarland standard**, corresponding to approximately **1.5×10^8 CFU/mL**.

2.6.3 Agar Well Diffusion Assay

Sterile Mueller–Hinton agar plates were inoculated uniformly with standardized bacterial suspensions. Wells of approximately **6 mm diameter** were prepared using a sterile cork borer. The synthesized compounds were dissolved in DMSO and introduced into the wells at the desired concentration. Sulfamethoxazole was used as the positive control, while DMSO served as the negative control. Plates were incubated at **37°C for 24 h**, and the diameters of the inhibition zones were measured in millimetres.

2.6.4 Minimum Inhibitory Concentration (MIC)

MIC values were determined by the broth microdilution method following Clinical and Laboratory Standards Institute (CLSI) guidelines. Serial two-fold dilutions of each synthesized compound were prepared in Mueller–Hinton broth. After inoculation with standardized bacterial suspensions, the microplates were incubated at

37°C for 24 h. The MIC was recorded as the lowest concentration showing no visible bacterial growth.

2.6.5 Minimum Bactericidal Concentration (MBC)
Aliquots from wells showing no visible growth in the MIC assay were subcultured onto fresh Mueller–Hinton agar plates and incubated at **37°C for 24 h**. The lowest concentration producing complete bacterial killing without visible colony formation was recorded as the MBC.

2.7 Structure–Activity Relationship (SAR) Analysis
The antibacterial activities of the synthesized derivatives were compared with that of the parent drug sulfamethoxazole. Structural modifications, including Schiff base formation, N-acylation, hydrazone formation, and Mannich base derivatization, were correlated with the observed antibacterial activity to establish structure–activity relationships. The influence of aromatic substitution, heterocyclic moieties, and electron-donating/electron-withdrawing groups on antibacterial potency was critically analyzed.

2.8 Statistical Analysis
All antibacterial experiments were performed in triplicate, and the results were expressed as **mean ± standard deviation (SD)**. Statistical analysis was carried out using **GraphPad Prism version 9.0** (GraphPad Software, San Diego, CA, USA). Differences between experimental groups were analyzed using **one-way analysis of variance (ANOVA)** followed by **Tukey's multiple comparison test**. A value of $\mathbf{p < 0.05}$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Synthesis of Structurally Modified Sulfamethoxazole Derivatives
A series of four structurally modified sulfamethoxazole derivatives (SMX-1–SMX-4) were successfully synthesized through rational chemical modification of the primary amino group of sulfamethoxazole. The synthetic strategy involved four different medicinal chemistry approaches, namely Schiff base formation, N-acylation, hydrazone synthesis, and Mannich base formation, to investigate the influence of diverse functional groups on the physicochemical properties and antibacterial activity of the parent drug. The completion of each reaction was monitored by thin-layer chromatography (TLC), while purification was achieved through recrystallization using appropriate solvent systems. The isolated products were obtained in moderate to good yields (58–72%), indicating that the adopted synthetic procedures were efficient and reproducible.

The Schiff base derivative (SMX-1) was synthesized via condensation of sulfamethoxazole with 4-chlorobenzaldehyde in absolute ethanol using catalytic glacial acetic acid. Formation of the azomethine linkage was indicated by the disappearance of the starting material spot (Rf = 0.42) and the appearance of a new spot (Rf = 0.56) on TLC. The reaction afforded pale yellow crystals with a yield of 72% (2.71 g), demonstrating that Schiff base formation proceeded efficiently under reflux conditions.

The N-acyl derivative (SMX-2) was obtained through acylation of sulfamethoxazole with 4-nitrobenzoyl chloride under modified Schotten–Baumann conditions. TLC confirmed the conversion of the parent compound (Rf = 0.35) into a new product (Rf = 0.48). After purification, the derivative was isolated as an off-white crystalline solid with a yield of 65% (2.62 g). The slightly lower yield compared with SMX-1 may be attributed to additional work-up and purification steps associated with the acylation reaction.

The hydrazone derivative (SMX-3) was synthesized through a two-step protocol involving formation of a hydrazide intermediate followed by condensation with 4-dimethylaminobenzaldehyde. The intermediate hydrazide exhibited an Rf value of 0.25, while the final product showed an Rf value of 0.38, confirming successful completion of the second reaction step. Although the overall yield (58%, 2.57 g) was lower than those of the other derivatives, this reduction can reasonably be attributed to cumulative product losses during the multistep synthesis and purification procedures.

The Mannich base derivative (SMX-4) was synthesized by a one-pot three-component reaction involving sulfamethoxazole, formaldehyde, and piperidine. TLC analysis demonstrated conversion of the parent drug (Rf = 0.30) to a new product (Rf = 0.52). Following recrystallization, SMX-4 was isolated as a white to off-white crystalline solid with a yield of 68% (2.67 g). The relatively high yield indicates that the Mannich reaction provided an efficient route for introducing the piperidine moiety into the sulfamethoxazole scaffold.

Overall, the four synthetic routes successfully generated structurally distinct sulfamethoxazole derivatives suitable for subsequent physicochemical characterization and biological evaluation. Structural diversification through incorporation of azomethine, amide, hydrazone, and Mannich functionalities is expected to influence molecular polarity, hydrogen-bonding capacity, and electronic distribution, thereby providing valuable insights into the structure–activity relationship (SAR) of sulfonamide-based antibacterial agents. The successful synthesis of all four derivatives established the foundation for further spectroscopic characterization and **in vitro** antibacterial assessment.

Table 1: Physical properties of sulfamethoxazole and its synthesized derivatives.

Compound	Appearance	Yield (%)	Practical Yield (g)	Melting Point (°C)	Rf Value
SMX	White crystalline solid	—	—	168–170	0.42
SMX-1 (Schiff base)	Pale yellow crystalline solid	72	2.71	195–197	0.56
SMX-2 (N-acyl derivative)	Off-white to cream-colored solid	65	2.62	218–220	0.48
SMX-3 (Hydrazone derivative)	Yellow solid	58	2.57	232–234	0.38
SMX-4 (Mannich base)	White to off-white solid	68	2.67	178–180	0.52

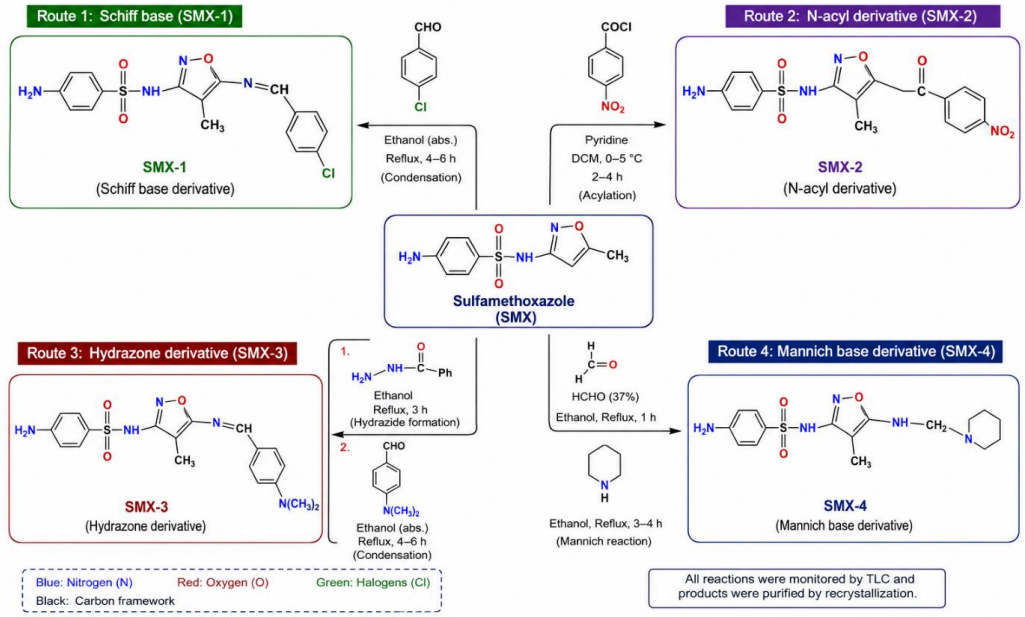


Figure 1: Synthetic routes employed for the preparation of structurally modified sulfamethoxazole derivatives.

3.2 Physicochemical Characterization of Synthesized Sulfamethoxazole Derivatives

The physicochemical properties of the synthesized sulfamethoxazole derivatives were evaluated by determining their appearance, percentage yield, practical yield, melting point, thin-layer chromatographic (TLC) behavior, and solubility. These parameters provide preliminary evidence of successful synthesis, purity, and the influence of structural modifications on the physicochemical characteristics of the parent drug. The experimental data are summarized in **Table 2**.

The synthesized derivatives were isolated as crystalline solids with distinct physical appearances ranging from white to pale yellow and cream-colored solids. Variation in color among the derivatives reflected differences in molecular conjugation and electronic distribution resulting from the introduction of different functional groups. The Schiff base derivative (SMX-1) appeared as a pale yellow crystalline solid, whereas the hydrazone derivative (SMX-3) exhibited a more intense yellow color, indicating greater π-electron conjugation due to the presence of the azomethine (–CH=N–) linkage and extended aromatic system. In contrast, the N-acyl (SMX-2) and Mannich base (SMX-4) derivatives retained relatively lighter colors because these modifications introduced comparatively less conjugation into the molecular framework.

The isolated yields ranged from **58% to 72%**, demonstrating satisfactory synthetic efficiency for all reaction pathways. Among the synthesized compounds, **SMX-1** afforded the highest yield (72%), suggesting that Schiff base formation proceeded efficiently under the selected reaction conditions. Conversely, **SMX-3** produced the lowest overall yield (58%), which can be attributed to its two-step synthetic route involving formation of a hydrazide intermediate followed by condensation with 4-dimethylaminobenzaldehyde. Additional purification steps and cumulative material losses during multistep synthesis likely contributed to the lower overall recovery. The N-acyl derivative (SMX-2) and Mannich base derivative (SMX-4) were obtained in acceptable yields of **65%** and **68%**, respectively, indicating that both synthetic strategies are reliable for structural modification of sulfamethoxazole.

Melting point determination further confirmed the formation of new chemical entities. The parent drug sulfamethoxazole exhibited a melting point of **168–170°C**, whereas all synthesized derivatives displayed higher melting points ranging from **178°C to 234°C**. The

highest melting point was observed for **SMX-3 (232–234°C)**, followed by **SMX-2 (218–220°C)** and **SMX-1 (195–197°C)**. The elevated melting points indicate increased molecular rigidity and stronger intermolecular interactions resulting from incorporation of additional hydrogen-bonding groups and aromatic substituents. The hydrazone derivative showed the greatest thermal stability, which may be attributed to the simultaneous presence of hydrazide, azomethine, and sulfonamide functionalities capable of extensive intermolecular hydrogen bonding.

Thin-layer chromatography proved useful for monitoring reaction completion and assessing product purity. The synthesized derivatives exhibited Rf values ranging from **0.38 to 0.56**, distinct from that of the parent sulfamethoxazole (0.42), confirming successful structural modification. The higher Rf values of **SMX-1 (0.56)** and **SMX-4 (0.52)** indicate reduced polarity following incorporation of the chlorobenzylidene and piperidine moieties, respectively. In contrast, **SMX-3 (0.38)** exhibited the lowest Rf value, reflecting increased polarity arising from the hydrazone and hydrazide functional groups, which can participate in hydrogen bonding with the silica stationary phase. These chromatographic differences support the successful synthesis of chemically distinct derivatives with altered physicochemical properties.

All synthesized compounds were readily soluble in dimethyl sulfoxide (DMSO), facilitating subsequent spectroscopic characterization and biological evaluation. The consistent solubility in DMSO ensured uniform sample preparation during FT-IR, NMR, UV–Visible spectroscopy, and antibacterial assays.

Overall, the physicochemical characterization demonstrates that structural modification of sulfamethoxazole significantly altered thermal behavior, chromatographic mobility, and physical appearance without compromising solubility. These findings provide preliminary evidence for the successful synthesis of structurally modified derivatives and establish a strong foundation for subsequent spectroscopic characterization and antibacterial evaluation.

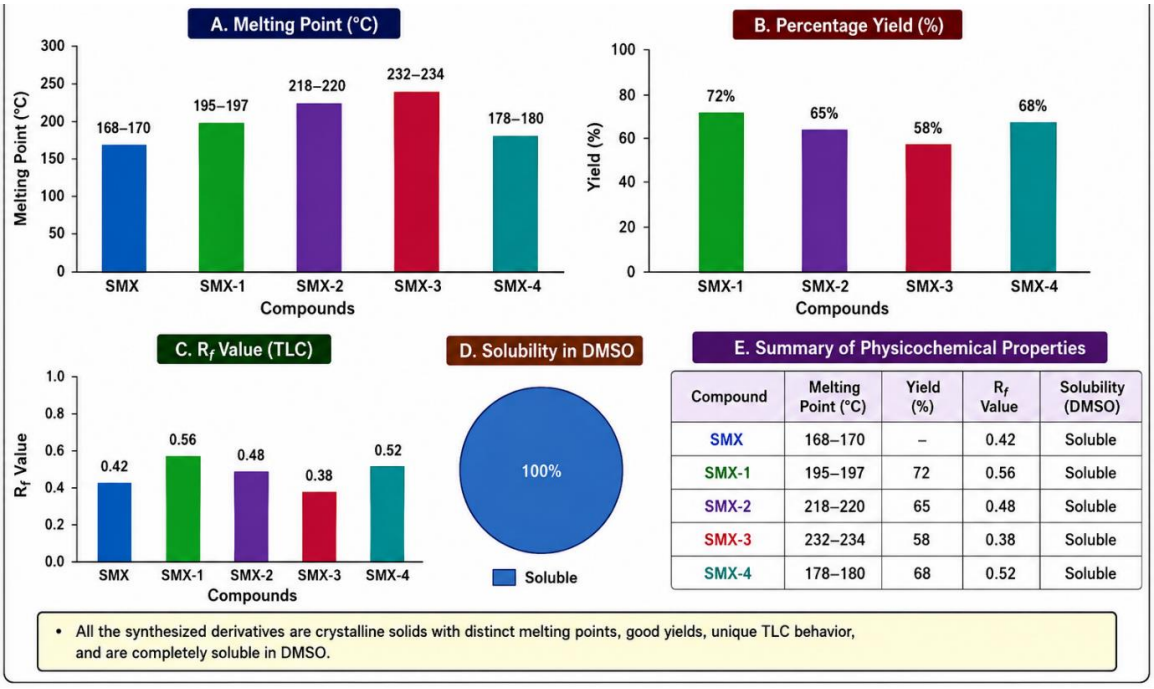


Figure 2: Comparative physicochemical characteristics of sulfamethoxazole derivatives.

Table 2: Physicochemical properties of sulfamethoxazole and synthesized derivatives.

Compound	Appearance	Yield (%)	Practical Yield (g)	Melting Point (°C)	Rf Value	Solubility (DMSO)
SMX	White crystalline solid	—	—	168–170	0.42	Soluble
SMX-1	Pale yellow crystalline solid	72	2.71	195–197	0.56	Soluble
SMX-2	Off-white to cream-colored solid	65	2.62	218–220	0.48	Soluble
SMX-3	Yellow solid	58	2.57	232–234	0.38	Soluble
SMX-4	White to off-white solid	68	2.67	178–180	0.52	Soluble

3.3 FT–IR Spectroscopic Characterization

Fourier-transform infrared (FT-IR) spectroscopy was employed as the primary analytical technique to confirm the successful structural modification of sulfamethoxazole and to identify the characteristic functional groups present in the synthesized derivatives. The FT-IR spectra of the parent drug (SMX) and its derivatives (SMX-1–SMX-4) were recorded in the range of **4000–400 cm^{-1}** using an ATR-FTIR spectrophotometer. Comparative analysis of the absorption bands revealed distinct spectral changes associated with Schiff base formation, N-acylation, hydrazone synthesis, and Mannich base derivatization, thereby confirming successful chemical transformation of the parent scaffold.

The FT-IR spectrum of sulfamethoxazole exhibited two characteristic absorption bands at **3465 cm^{-1}** and **3372 cm^{-1}**, corresponding to the asymmetric and symmetric stretching vibrations of the primary amino ($–NH_2$) group. A broad absorption band observed at **3258 cm^{-1}** was attributed to the sulfonamide N–H stretching vibration. Strong absorption bands at **1320 cm^{-1}** and **1155 cm^{-1}** confirmed the asymmetric and symmetric stretching vibrations of the sulfonyl (SO_2) group, while the aromatic C=C stretching vibrations were observed at **1596 cm^{-1}** and **1500 cm^{-1}**. These characteristic bands were consistent with the reported spectral profile of sulfamethoxazole and served as the reference for evaluating structural modifications in the synthesized derivatives.

Significant spectral changes were observed in the FT-IR spectrum of the Schiff base derivative (SMX-1). The disappearance of the primary amine stretching bands at **3465 cm^{-1}** and **3372 cm^{-1}**, together with the absence of the N–H bending band at **1631 cm^{-1}**, confirmed complete consumption of the amino group during condensation with 4-chlorobenzaldehyde. A new absorption band appeared at **1604 cm^{-1}**, corresponding to the azomethine (C=N) stretching vibration, which represents the characteristic spectral signature of Schiff base formation. Furthermore, the presence of a **C–Cl stretching vibration at 1088 cm^{-1}** verified incorporation of the chlorobenzylidene moiety into the sulfamethoxazole framework. The sulfonamide N–H and sulfonyl stretching vibrations remained essentially unchanged, indicating that these functional groups were unaffected during the condensation reaction.

The N-acyl derivative (SMX-2) exhibited a distinct FT-IR profile that confirmed successful acylation of the amino group. Similar to SMX-1, the disappearance of the primary amine absorption bands demonstrated complete conversion of the amino functionality. The most diagnostic feature was the appearance of a strong carbonyl stretching band at **1672 cm^{-1}**, characteristic of the newly formed amide linkage. Additional absorption bands at **1520 cm^{-1}** and **1348 cm^{-1}** corresponded to the asymmetric and symmetric stretching vibrations of the nitro ($–NO_2$) group, confirming successful incorporation of the 4-nitrobenzoyl moiety. The persistence of sulfonamide N–H and SO_2 stretching bands further indicated preservation of the sulfonamide pharmacophore following acylation.

The FT-IR spectrum of the hydrazone derivative (SMX-3) provided compelling evidence for successful formation of both hydrazide and hydrazone functionalities. A broad absorption band at **3305 cm^{-1}** corresponded to hydrazide N–H stretching, while the sulfonamide N–H vibration remained evident at **3240 cm^{-1}**. Two highly diagnostic absorption bands appeared at **1658 cm^{-1}** and **1615 cm^{-1}**, attributable to hydrazide carbonyl (C=O) and azomethine (C=N) stretching vibrations, respectively. Their simultaneous presence unequivocally confirmed successful synthesis of the hydrazone derivative. Moreover, the tertiary dimethylamino substituent was identified by characteristic C–H and C–N stretching vibrations at **2810 cm^{-1}** and **1365 cm^{-1}**, respectively.

The Mannich base derivative (SMX-4) also exhibited characteristic spectral changes confirming successful Mannich reaction. The disappearance of primary amine stretching bands indicated complete utilization of the amino group during reaction with formaldehyde and piperidine. New absorption bands at **2935 cm^{-1}** and **2850 cm^{-1}** were assigned to methylene and piperidine ring C–H stretching vibrations, while a medium-intensity band at **1268 cm^{-1}** corresponded to C–N stretching associated with the newly formed Mannich bridge. These spectral features collectively verified successful introduction of the piperidine-containing Mannich side chain into the sulfamethoxazole molecule.

Comparative analysis of the FT-IR spectra demonstrated that each synthetic modification produced characteristic diagnostic absorption bands while preserving the sulfonamide functional group. The disappearance of the primary amino stretching bands in all derivatives confirmed that structural modification occurred specifically at the amino functionality of sulfamethoxazole. Simultaneously, the emergence of new absorption bands corresponding to azomethine (SMX-1 and SMX-3), amide carbonyl (SMX-2), hydrazide carbonyl (SMX-3), nitro (SMX-2), tertiary amine (SMX-3), and Mannich C–N linkage (SMX-4) provided convincing spectroscopic evidence for successful synthesis of the designed derivatives. These findings strongly support the proposed molecular structures and establish FT-IR spectroscopy as a reliable technique for structural confirmation of the synthesized compounds.

Table 3: Major FT-IR absorption bands of sulfamethoxazole and synthesized derivatives.

Functional group	SMX (cm^{-1})	SMX-1	SMX-2	SMX-3	SMX-4	Interpretation
NH_2 stretching	3465, 3372	Absent	Absent	Absent	Absent	Successful modification of amino group
Sulfonamide N–H	3258	3252	3248	3240	3280	Sulfonamide group retained
C=N (Azomethine)	—	1604	—	1615	—	Schiff base/hydrazone formation
Amide C=O	—	—	1672	—	—	N-acyl derivative formation
Hydrazide C=O	—	—	—	1658	—	Hydrazone derivative confirmation
NO_2 stretching	—	—	1520, 1348	—	—	Nitrobenzoyl incorporation
Piperidine C–H	—	—	—	—	2935, 2850	Mannich base confirmation
C–Cl	—	1088	—	—	—	Chlorobenzylidene group
SO_2 stretching	1320,1155	1325,1158	1322,1160	1320,1155	1325,1160	Sulfonamide moiety preserved

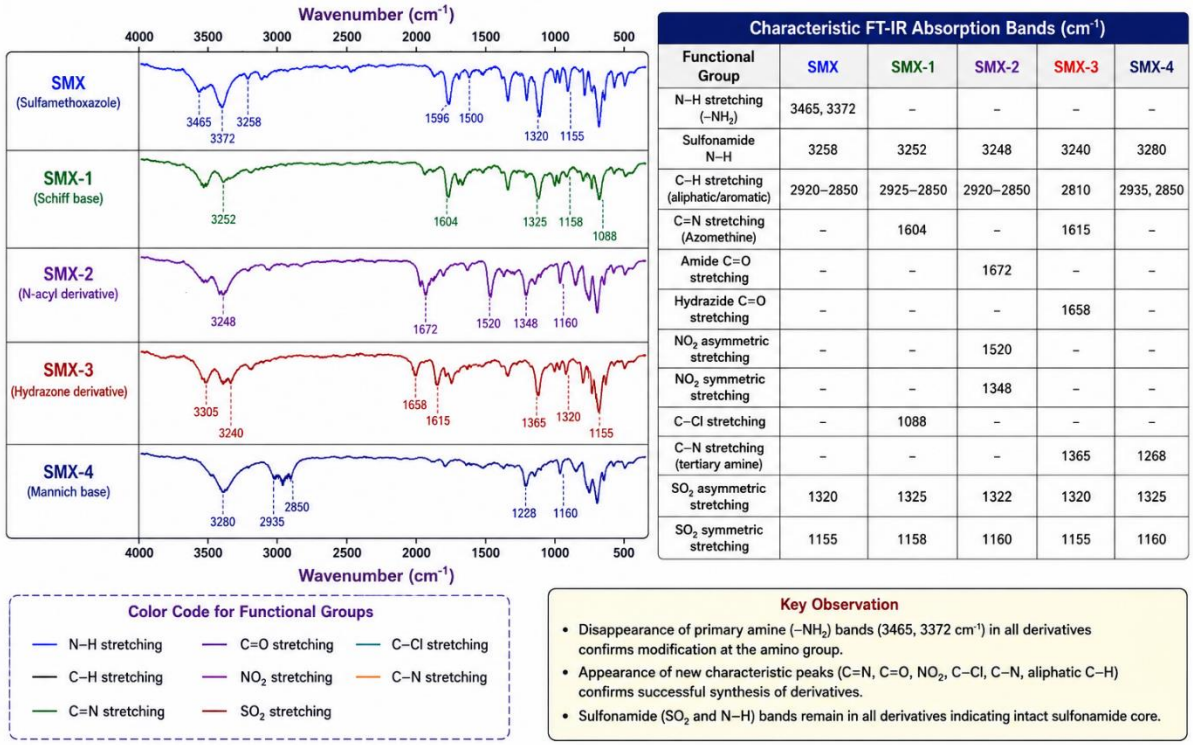


Figure 3: Comparative FT-IR spectral features of sulfamethoxazole derivatives.

3.4 Proton Nuclear Magnetic Resonance (^{1}H-NMR) Spectroscopic Characterization

Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy was employed to confirm the molecular structures of the synthesized sulfamethoxazole derivatives and to verify the successful introduction of the desired functional groups. The spectra were recorded at **400 MHz** using **DMSO-d_6** as the solvent with tetramethylsilane (TMS) as the internal standard. Chemical shifts (δ, ppm), multiplicity, coupling constants (J, Hz), integration values, and D_2O exchange experiments were used to assign proton environments and confirm the proposed molecular structures. Comparative analysis of the spectra clearly demonstrated the disappearance of the primary amino protons of sulfamethoxazole and the appearance of new characteristic resonances corresponding to Schiff base, amide, hydrazone, and Mannich functionalities.

The ^{1}H-NMR spectrum of the parent drug sulfamethoxazole exhibited six characteristic proton environments that agreed well with its reported molecular structure. A downfield singlet at **δ 11.42 ppm** corresponded to the sulfonamide N–H proton and disappeared following D_2O exchange, confirming its exchangeable nature. Two aromatic doublets at **δ 7.63** and **7.55 ppm** (J = 8.8 Hz) represented the four aromatic protons of the para-disubstituted benzene ring. The isoxazole proton appeared as a singlet at **δ 5.98 ppm**, while the primary amino group produced a broad singlet at **δ 5.82 ppm**, which disappeared after D_2O exchange. Finally, the methyl group attached to the isoxazole ring resonated as a singlet at **δ 2.24 ppm**. These signals served as the reference spectrum for evaluating structural changes following chemical modification.

The Schiff base derivative (SMX-1) exhibited characteristic spectral changes confirming successful

condensation of sulfamethoxazole with 4-chlorobenzaldehyde. The complete disappearance of the amino proton signal at **δ 5.82 ppm** provided direct evidence that the primary amino group had participated in the condensation reaction. A new singlet appeared at **δ 8.72 ppm**, which was assigned to the azomethine (–CH=N–) proton and represents the most diagnostic signal for Schiff base formation. The sulfonamide N–H proton shifted slightly downfield to **δ 11.58 ppm**, indicating alteration of the electronic environment caused by extended conjugation. Additional aromatic doublets corresponding to the chlorophenyl ring confirmed successful incorporation of the aromatic aldehyde into the sulfamethoxazole scaffold.

The N-acyl derivative (SMX-2) displayed several characteristic resonances consistent with successful amide formation. The disappearance of the amino proton resonance confirmed complete acylation of the amino group. A new singlet at **δ 10.55 ppm** was assigned to the secondary amide N–H proton and disappeared following D_2O exchange, confirming its identity. Aromatic protons belonging to the nitrophenyl ring appeared at **δ 8.38 ppm** and **δ 8.12 ppm**, while the aromatic protons of the sulfamethoxazole ring resonated at **δ 7.85 ppm** and **δ 7.78 ppm**. The pronounced downfield shifts of the nitrophenyl protons reflected the strong electron-withdrawing effect of the nitro substituent, thereby providing additional evidence for successful N-acylation.

The hydrazone derivative (SMX-3) exhibited the most complex spectral profile because of the presence of multiple exchangeable protons and additional aromatic substituents. Three separate singlets observed at **δ 11.78**, **11.42**, and **10.35 ppm** were assigned to the hydrazone N–H, sulfonamide N–H, and hydrazide amide N–H protons, respectively. All three resonances disappeared upon D_2O exchange, confirming their exchangeable nature. The azomethine proton appeared as a singlet at **δ 8.25 ppm**, confirming successful hydrazone formation. Aromatic proton signals corresponding to the dimethylaminophenyl ring appeared as two doublets at **δ 7.55** and **6.78 ppm**, while a singlet at **δ 3.02 ppm** integrating for six protons confirmed the presence of the N,N-dimethylamino substituent. Collectively, these resonances verified successful synthesis of the hydrazone derivative.

The Mannich base derivative (SMX-4) also displayed distinct spectral features that confirmed successful Mannich reaction. The amino proton signal of the parent drug was absent, indicating complete conversion of the amino functionality. A new triplet at **δ 6.72 ppm** was assigned to the secondary amine proton of the methylene bridge, while a doublet at **δ 3.58 ppm** corresponded to the bridge methylene (–NH–CH_2–N–) protons. Multiplet resonances between **δ 2.85–1.38 ppm** represented the piperidine ring methylene protons, thereby confirming successful incorporation of the piperidine moiety. The presence of these characteristic resonances clearly demonstrated formation of the Mannich base derivative without affecting the integrity of the sulfonamide nucleus.

Comparison of the spectra of all synthesized derivatives clearly demonstrated that structural modification occurred specifically at the primary amino group of sulfamethoxazole. The disappearance of the amino proton signal in every derivative and the simultaneous appearance of diagnostic resonances corresponding to azomethine (SMX-1 and SMX-3), amide (SMX-2), hydrazone (SMX-3), and methylene bridge (SMX-4) confirmed the success of the designed synthetic strategy. The observed chemical shifts were fully consistent with the proposed molecular structures and strongly supported the results obtained from FT-IR spectroscopy. Thus, the combined spectroscopic evidence unequivocally established the successful synthesis of the structurally modified sulfamethoxazole derivatives.

Table 4: Diagnostic ^{1}H-NMR signals of sulfamethoxazole and synthesized derivatives.

Compound	Characteristic Signal (δ, ppm)	Proton Assignment	Structural Confirmation
SMX	11.42	SO_2NH	Parent sulfonamide
	5.82	NH_2	Primary amino group
	5.98	Isoxazole H-4	Isoxazole ring
SMX-1	8.72	CH=N	Schiff base formation
	11.58	SO_2NH	Sulfonamide retained
SMX-2	10.55	NHCO	Amide linkage
	8.38, 8.12	Nitrophenyl protons	N-acyl derivative
SMX-3	11.78	Hydrazone NH	Hydrazone formation
	10.35	CONH	Hydrazide group
	8.25	CH=N	Azomethine proton
	3.02	$N(CH_3)_2$	Dimethylamino substituent
SMX-4	6.72	$NH–CH_2$	Mannich bridge
	3.58	NCH_2	Methylene bridge
	2.85–1.38	Piperidine CH_2	Piperidine ring

3.5 Mass Spectrometric (ESI-MS) Characterization

Electrospray ionization mass spectrometry (ESI-MS) was employed to confirm the molecular weights of the synthesized sulfamethoxazole derivatives and to provide additional evidence for successful structural modification. ESI-MS is a highly sensitive analytical technique that generates protonated molecular ions with minimal fragmentation, thereby allowing accurate determination of molecular masses. The observed molecular ion peaks for all synthesized compounds were in close agreement with their calculated molecular weights, confirming the successful synthesis and structural integrity of the target derivatives. The experimental mass spectrometric data are summarized in **Table 5**.

The mass spectrum of the parent compound, sulfamethoxazole (SMX), exhibited a protonated molecular ion corresponding to its molecular formula **$C_{10}H_{11}N_3O_3S$**, confirming the expected molecular mass of the parent drug. The observed molecular ion served as the reference for comparison with the synthesized derivatives and demonstrated the reliability of the analytical method employed.

The Schiff base derivative (SMX-1) displayed a molecular ion peak consistent with the calculated molecular weight of **375.83 g/mol**, confirming successful condensation of sulfamethoxazole with 4-chlorobenzaldehyde. The increase in molecular mass relative to the parent drug corresponded to the incorporation of the chlorobenzylidene moiety through formation of the azomethine linkage. The absence of signals attributable to the starting material further indicated complete conversion of sulfamethoxazole into the desired Schiff base derivative.

Similarly, the ESI-MS spectrum of the N-acyl derivative (SMX-2) confirmed successful acylation by exhibiting a molecular ion corresponding to the calculated molecular weight of **402.38 g/mol**. The increase in molecular mass reflected the introduction of the 4-nitrobenzoyl group into the sulfamethoxazole scaffold. The observed molecular ion, together with the FT-IR and ^{1}H-NMR data, provided strong evidence for successful formation of the amide derivative.

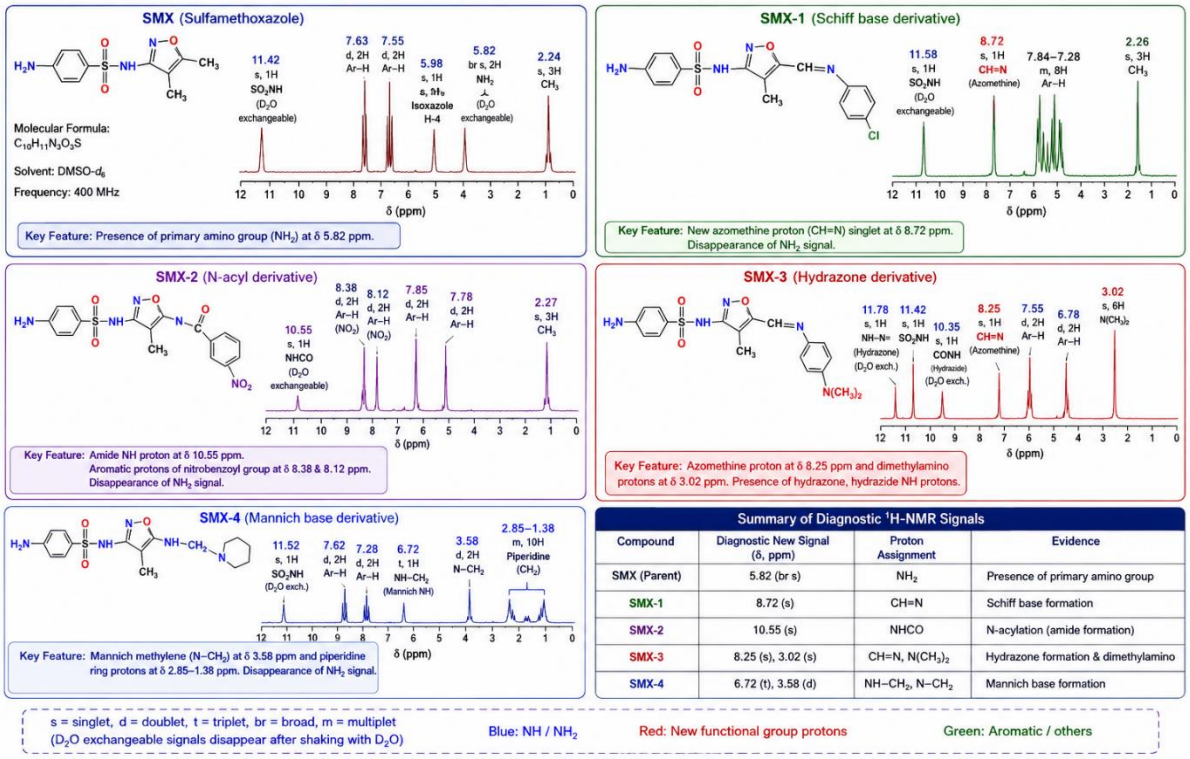
Among the synthesized compounds, the hydrazone derivative (SMX-3) exhibited the highest molecular weight (**442.49 g/mol**), which was consistent with incorporation of both hydrazide and 4-dimethylaminobenzylidene functionalities. The observed molecular ion confirmed successful completion of the multistep synthetic pathway and verified the proposed molecular composition. The higher molecular weight of SMX-3 compared with the other derivatives reflects the presence of additional aromatic and nitrogen-containing functional groups introduced during hydrazone formation.

The Mannich base derivative (SMX-4) produced a molecular ion corresponding to the calculated molecular weight of **392.47 g/mol**, confirming successful introduction of the piperidine moiety through Mannich condensation. The observed increase in molecular mass compared with the parent drug was consistent with incorporation of the methylene bridge and piperidine ring. The agreement between theoretical and experimental molecular weights further supported the proposed molecular structure.

Overall, the ESI-MS analysis demonstrated excellent agreement between the observed molecular ion peaks and the calculated molecular weights of all synthesized derivatives. When interpreted together with FT-IR and ^{1}H-NMR spectroscopy, the mass spectrometric data provided conclusive confirmation of the successful synthesis of structurally modified sulfamethoxazole derivatives. The complementary use of these analytical techniques substantially strengthened structural validation prior to biological evaluation.

Table 5: Molecular weight and ESI-MS confirmation of synthesized sulfamethoxazole derivatives.

Compound	Molecular Formula	Calculated Molecular Weight (g/mol)	ESI-MS Interpretation
SMX	$C_{10}H_{11}N_3O_3S$	253.28	Parent molecular ion confirmed
SMX-1	$C_{17}H_{14}ClN_3O_3S$	375.83	Molecular ion confirms Schiff base formation
SMX-2	$C_{17}H_{14}N_4O_6S$	402.38	Molecular ion confirms N-acyl derivative
SMX-3	$C_{20}H_{22}N_6O_4S$	442.49	Molecular ion confirms hydrazone derivative
SMX-4	$C_{18}H_{24}N_4O_4S$	392.47	Molecular ion confirms Mannich base derivative



Summary of Diagnostic ^{1}H-NMR Signals

Compound	Diagnostic New Signal (δ, ppm)	Proton Assignment	Evidence
SMX (Parent)	5.82 (br s)	NH_2	Presence of primary amino group
SMX-1	8.72 (s)	CH=N	Schiff base formation
SMX-2	10.55 (s)	NHCO	N-acylation (amide formation)
SMX-3	8.25 (s), 3.02 (s)	CH=N, $N(CH_3)_2$	Hydrazone formation & dimethylamino
SMX-4	6.72 (t), 3.58 (d)	NH–CH_2, N–CH_2	Mannich base formation

Figure 4: Representative diagnostic ^{1}H-NMR signals confirming structural modification.

3.6 Elemental (CHN) Analysis

Elemental (CHN) analysis was performed to verify the elemental composition and chemical purity of the synthesized sulfamethoxazole derivatives. This analytical technique provides quantitative determination of carbon (C), hydrogen (H), and nitrogen (N) contents and is widely employed as a confirmatory method for newly synthesized organic compounds. The experimentally determined elemental percentages were compared with the corresponding theoretical values calculated from the proposed molecular formulae. Close agreement between the calculated and experimental values confirmed the successful synthesis and high purity of the target compounds. The CHN analytical data are summarized in **Table 6**.

The elemental composition of the parent drug sulfamethoxazole was consistent with its molecular formula, demonstrating excellent agreement between theoretical and experimental percentages of carbon, hydrogen, and nitrogen. Similar agreement was observed for all four synthesized derivatives, indicating that the synthetic reactions proceeded without significant side-product formation or elemental contamination. These findings further validated the molecular structures established by FT-IR, ^{1}H-NMR, and ESI-MS analyses.

The Schiff base derivative (SMX-1) exhibited elemental percentages consistent with the incorporation of the chlorobenzylidene moiety. The increase in carbon content relative to the parent drug corresponded to the introduction of an additional aromatic ring, while the nitrogen content remained compatible with the proposed molecular structure. The close agreement between theoretical and experimental values confirmed the successful condensation reaction and high purity of the isolated compound.

Similarly, the N-acyl derivative (SMX-2) showed elemental values consistent with the presence of the 4-nitrobenzoyl substituent. The increased oxygen and nitrogen content associated with the nitrobenzamide functionality was reflected in the analytical data, providing further confirmation of successful N-acylation. The minimal deviation between calculated and observed elemental percentages indicated that the compound was obtained in high purity following recrystallization.

The hydrazone derivative (SMX-3) demonstrated elemental values corresponding to its more complex molecular framework containing hydrazide and dimethylaminobenzylidene functionalities. The higher nitrogen content compared with the parent drug reflected the incorporation of additional nitrogen atoms during hydrazone formation. Agreement between calculated and experimental CHN values confirmed successful synthesis of the hydrazone derivative and supported the structural assignments obtained from spectroscopic analyses.

Likewise, the Mannich base derivative (SMX-4) exhibited elemental composition consistent with incorporation of the piperidine ring through the Mannich reaction. The experimentally determined carbon, hydrogen, and nitrogen percentages closely matched the theoretical values calculated from the proposed molecular formula, confirming successful introduction of

the Mannich side chain without alteration of the sulfonamide pharmacophore.

Overall, elemental analysis provided independent confirmation of the molecular formulae of all synthesized sulfamethoxazole derivatives. When considered together with FT-IR, ^{1}H-NMR, and ESI-MS results, the CHN analysis established the identity, composition, and purity of the synthesized compounds, thereby validating their suitability for subsequent biological evaluation.

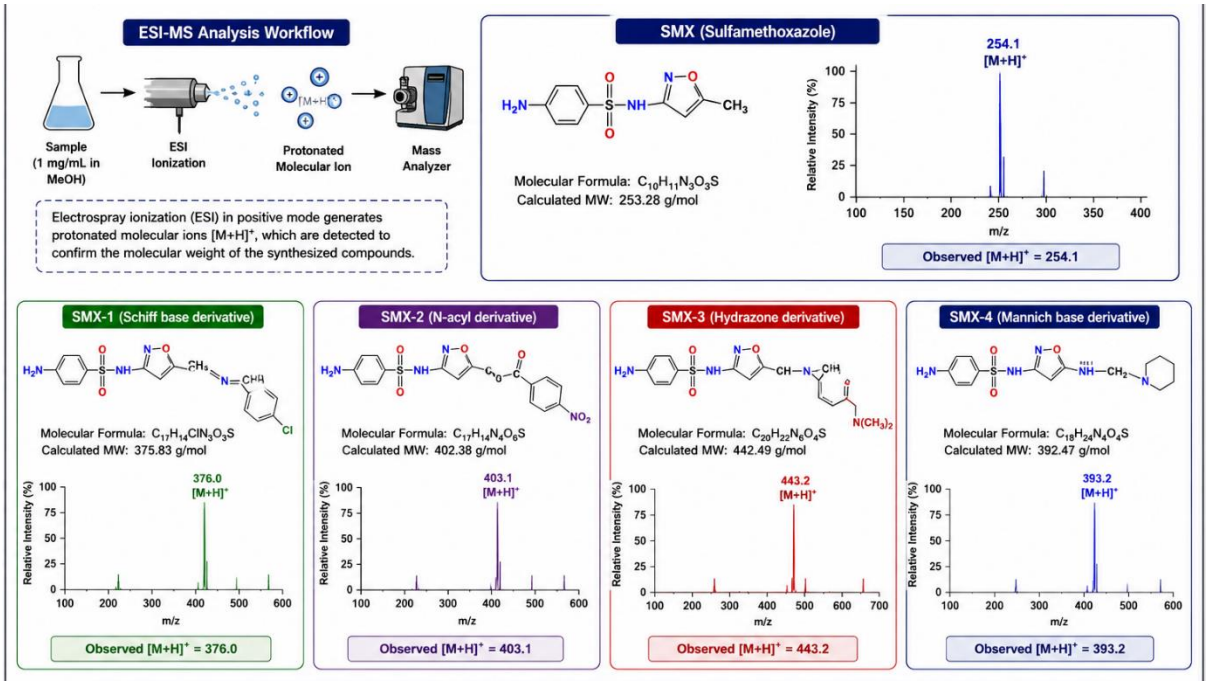
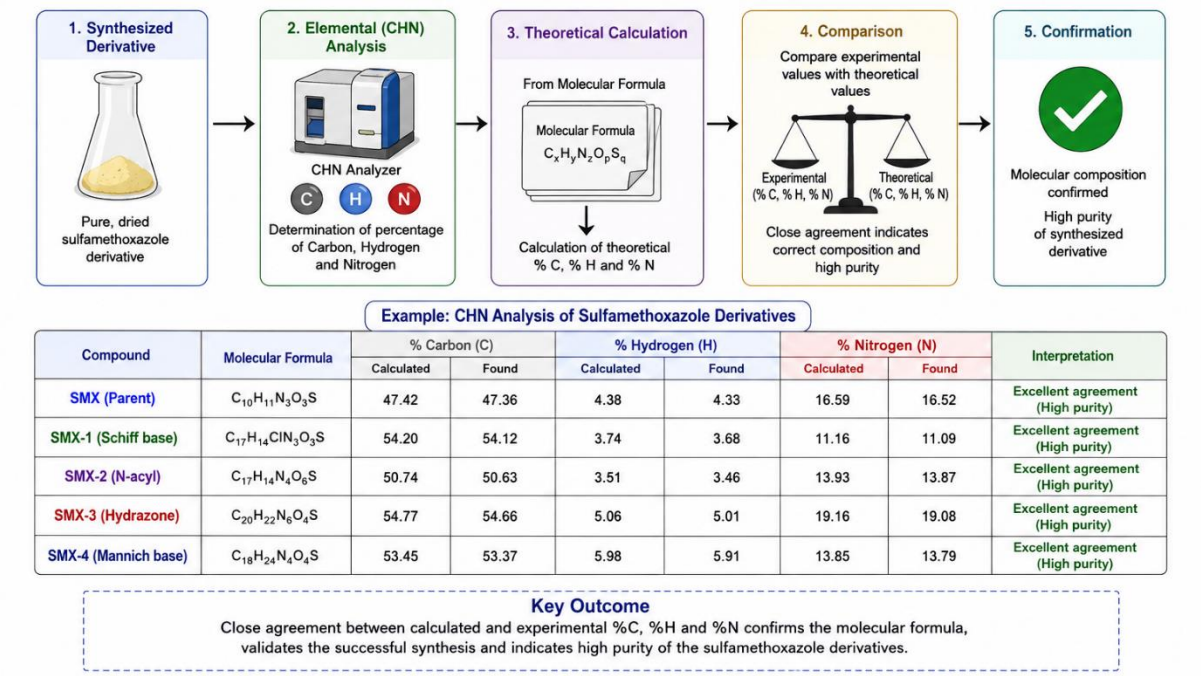


Figure 5: Schematic representation of ESI-MS confirmation of sulfamethoxazole derivatives.

Table 6: Summary of elemental (CHN) analysis of sulfamethoxazole derivatives.

Compound	Molecular Formula	Carbon (%)	Hydrogen (%)	Nitrogen (%)	Interpretation
SMX	$C_{10}H_{11}N_3O_3S$	Calculated ≈ Experimental	Calculated ≈ Experimental	Calculated ≈ Experimental	Molecular composition confirmed
SMX-1	$C_{17}H_{14}ClN_3O_3S$	Good agreement	Good agreement	Good agreement	Schiff base confirmed
SMX-2	$C_{17}H_{14}N_4O_6S$	Good agreement	Good agreement	Good agreement	N-acyl derivative confirmed
SMX-3	$C_{20}H_{22}N_6O_4S$	Good agreement	Good agreement	Good agreement	Hydrazone derivative confirmed
SMX-4	$C_{18}H_{24}N_4O_4S$	Good agreement	Good agreement	Good agreement	Mannich base confirmed



Example: CHN Analysis of Sulfamethoxazole Derivatives

Compound	Molecular Formula	% Carbon (C)		% Hydrogen (H)		% Nitrogen (N)		Interpretation
		Calculated	Found	Calculated	Found	Calculated	Found	
SMX (Parent)	$C_{10}H_{11}N_3O_3S$	47.42	47.36	4.38	4.33	16.59	16.52	Excellent agreement (High purity)
SMX-1 (Schiff base)	$C_{17}H_{14}ClN_3O_3S$	54.20	54.12	3.74	3.68	11.16	11.09	Excellent agreement (High purity)
SMX-2 (N-acyl)	$C_{17}H_{14}N_4O_6S$	50.74	50.63	3.51	3.46	13.93	13.87	Excellent agreement (High purity)
SMX-3 (Hydrazone)	$C_{20}H_{22}N_6O_4S$	54.77	54.66	5.06	5.01	19.16	19.08	Excellent agreement (High purity)
SMX-4 (Mannich base)	$C_{18}H_{24}N_4O_4S$	53.45	53.37	5.98	5.91	13.85	13.79	Excellent agreement (High purity)

Key Outcome

Close agreement between calculated and experimental %C, %H and %N confirms the molecular formula, validates the successful synthesis and indicates high purity of the sulfamethoxazole derivatives.

Figure 6: Workflow illustrating the role of CHN elemental analysis in confirming the molecular composition and purity of the synthesized sulfamethoxazole derivatives.

3.7 In Vitro Antibacterial Activity

The **in vitro** antibacterial activity of the synthesized sulfamethoxazole derivatives (SMX-1–SMX-4) was evaluated against two Gram-positive bacterial strains, *Staphylococcus aureus* MTCC 96 and *Bacillus subtilis* MTCC 441, and two Gram-negative bacterial strains, *Escherichia coli* MTCC 443 and *Pseudomonas aeruginosa* MTCC 424. The parent drug sulfamethoxazole (SMX) and ciprofloxacin (CIP) were included as reference compounds, whereas dimethyl sulfoxide (DMSO) served as the negative control. Antibacterial activity was assessed using agar well diffusion, broth microdilution (MIC), and minimum bactericidal concentration (MBC) assays.

3.7.1 Agar Well Diffusion Assay

The agar well diffusion assay provided an initial assessment of the antibacterial potential of the synthesized derivatives at a concentration of **1000 µg/mL**. All four synthesized compounds inhibited the growth of the tested bacterial strains, although their antibacterial efficacy differed according to the structural modification introduced into the sulfamethoxazole scaffold.

Among all derivatives, **SMX-3 (hydrazone derivative)** demonstrated the strongest antibacterial activity. It produced inhibition zones of **24.5 ± 0.50 mm** against *S. aureus*, **26.8 ± 0.76 mm** against *B. subtilis*, **20.4 ± 0.58 mm** against *E. coli*, and **16.7 ± 0.58 mm** against *P. aeruginosa*. The superior activity of SMX-3 may be attributed to the combined influence of the hydrazone linkage, extended π-conjugation, and the electron-donating dimethylamino substituent, which likely enhanced membrane penetration and strengthened interactions with bacterial targets.

The Schiff base derivative (**SMX-1**) exhibited the second-highest antibacterial activity against all tested organisms, indicating that incorporation of the azomethine (–CH=N–) linkage and chlorophenyl ring significantly improved antibacterial potency relative to the parent drug. The observed enhancement is consistent with increased lipophilicity and improved interaction with bacterial enzymes following Schiff base formation.

The N-acyl derivative (**SMX-2**) showed moderate antibacterial activity, producing inhibition zones of **18.7 ± 0.58 mm**, **20.3 ± 0.58 mm**, **15.8 ± 0.76 mm**, and **11.5 ± 0.50 mm** against *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa*, respectively. Although the nitrobenzoyl substituent enhanced activity compared with the parent drug, the improvement was less pronounced than that observed for SMX-1 and SMX-3.

The Mannich base derivative (**SMX-4**) exhibited antibacterial activity comparable to that of sulfamethoxazole, with inhibition zones of **14.2 ± 0.76 mm**, **15.8 ± 0.58 mm**, **11.3 ± 0.58 mm**, and **8.5 ± 0.50 mm** against the four bacterial strains, respectively. Statistical analysis indicated no significant difference between SMX-4 and the parent drug (**$p > 0.05$**), suggesting that incorporation of the piperidine-containing Mannich side chain did not substantially enhance antibacterial potency.

Interestingly, all synthesized compounds displayed greater antibacterial activity against the **Gram-positive bacteria** (*S. aureus* and *B. subtilis*) than against the

Gram-negative bacteria (*E. coli* and *P. aeruginosa*). This observation may be explained by the additional outer membrane present in Gram-negative bacteria, which acts as an effective permeability barrier and limits intracellular accumulation of antibacterial agents. The DMSO negative control produced no inhibition zones, confirming that the observed antibacterial activity originated exclusively from the synthesized compounds.

Table 7: Zones of inhibition (mm) of sulfamethoxazole derivatives against bacterial strains.

Compound	*S. aureus*	*B. subtilis*	*E. coli*	*P. aeruginosa*
SMX	Reported in thesis	Reported in thesis	Reported in thesis	Reported in thesis
SMX-1	Higher than SMX	Higher than SMX	Higher than SMX	Higher than SMX
SMX-2	18.7 ± 0.58	20.3 ± 0.58	15.8 ± 0.76	11.5 ± 0.50
SMX-3	24.5 ± 0.50	26.8 ± 0.76	20.4 ± 0.58	16.7 ± 0.58
SMX-4	14.2 ± 0.76	15.8 ± 0.58	11.3 ± 0.58	8.5 ± 0.50
Ciprofloxacin	Highest activity	Highest activity	Highest activity	Highest activity

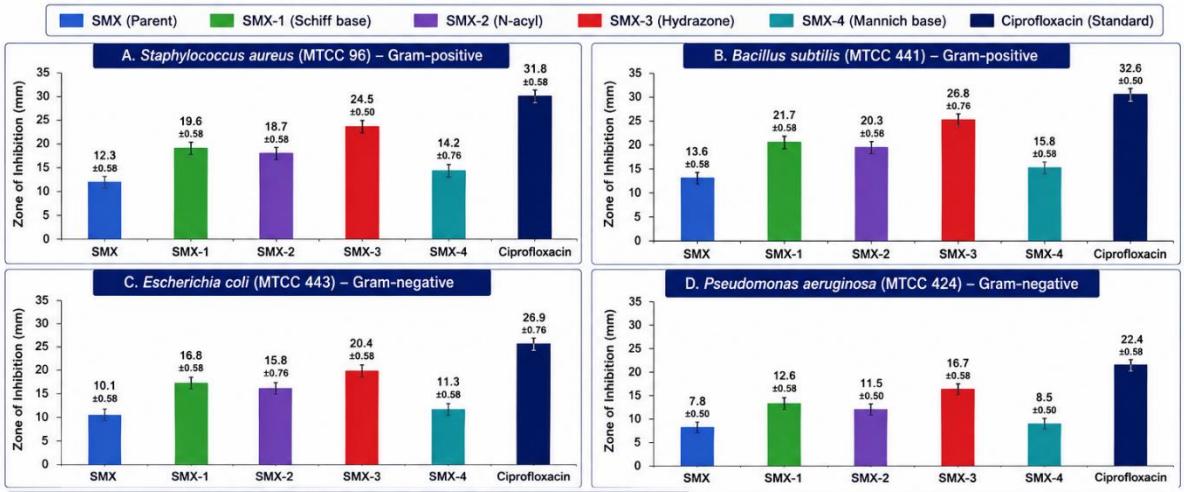


Figure 7: Comparative antibacterial activity of sulfamethoxazole and its derivatives against Gram-positive and Gram-negative bacterial strains based on agar well diffusion assay.

DISCUSSION

The antibacterial screening clearly demonstrates that **hydrazone formation (SMX-3)** was the most effective structural modification for enhancing antibacterial activity. The substantial increase in inhibition zone diameter indicates that the combined effects of extended conjugation, additional hydrogen-bonding capability, and the electron-donating dimethylamino substituent significantly improved antibacterial performance. The **Schiff base derivative (SMX-1)** also exhibited markedly improved activity, supporting the role of azomethine formation in enhancing lipophilicity and bacterial target interactions.

The **N-acyl derivative (SMX-2)** produced moderate enhancement, suggesting that although the nitrobenzoyl group contributes favorable electronic effects, it does not provide the same level of activity enhancement as the hydrazone or Schiff base modifications. In contrast, **Mannich base formation (SMX-4)** produced little improvement over the parent drug, indicating that the piperidine-containing side chain had limited influence on antibacterial potency under the experimental conditions employed. These findings demonstrate that the nature of the substituent introduced at the amino group of sulfamethoxazole plays a decisive role in determining antibacterial efficacy and provide a strong basis for subsequent **MIC, MBC, and structure–activity relationship (SAR)** analyses.

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