

SYNTHESIS CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF PYRAZOLO[3,4-D]THIAZOLE DERIVATIVES OF 1,2,4-TRIAZOLE

Ravi Kumar Saini¹, Dr. Umesh Kumar^{1*}

School of Pharmaceutical Sciences, Shri Venkateshwara University, Gajraula, UP, India.



*Corresponding Author: Dr. Umesh Kumar

School of Pharmaceutical Sciences, Shri Venkateshwara University, Gajraula, UP, India.

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ABSTRACT

A novel series of five pyrazolo[3,4-d]thiazole derivatives of 1,2,4-triazole [9a–e] were synthesized via a two-step condensation–cyclization sequence starting from 4-thiazolidinone intermediates [5a–e]. The first step involved condensation with furfuraldehyde to form 5-arylidene derivatives [8a–e], followed by cyclization with isoniazid in glacial acetic acid. The target compounds were obtained in moderate yields (53–56 %) as stable crystalline solids and were fully characterized by elemental analysis, FT-IR, ¹H NMR, ¹³C NMR, and LC-MS spectroscopy. All spectral data confirmed the successful formation of the fused pyrazolo[3,4-d]thiazole ring system and the incorporation of the isonicotinoyl and substituted furan moieties. The synthesized derivatives were screened for in vitro antibacterial activity against two Gram-positive (*Bacillus megaterium* and *Staphylococcus aureus*) and two Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacterial strains using the agar cup diffusion method at 1000 ppm concentration. All compounds exhibited moderate to good antibacterial activity, with the chloro-substituted derivative 9b emerging as the most potent analogue, producing the largest zones of inhibition (15–17 mm) across all tested strains. The results demonstrate that the pyrazolo[3,4-d]thiazole–1,2,4-triazole hybrid scaffold functionalized with a para-chlorophenylfuran unit represents a promising pharmacophore for the development of new broad-spectrum antimicrobial agents.

KEYWORDS: Pyrazolo[3,4-d]thiazole, 1,2,4-triazole, isoniazid hybrids, furan derivatives, antimicrobial activity.

1. INTRODUCTION

Heterocyclic compounds constitute the cornerstone of modern medicinal chemistry, serving as the structural foundation for more than 85 % of all clinically approved drugs and a vast array of bioactive natural products. Among the diverse array of heterocyclic scaffolds, fused nitrogen- and sulfur-containing systems have attracted particular attention because of their ability to interact with multiple biological targets through hydrogen bonding, π - π stacking, and coordination with metal ions in enzyme active sites (El Azab et al., 2021; Shaaban & Elwahy, 2014). The pyrazolo[3,4-d]thiazole framework, in particular, represents a privileged fused heterocycle that merges the pharmacological advantages of both pyrazole and thiazole rings. This hybrid system exhibits enhanced metabolic stability, improved lipophilicity, and a broad spectrum of biological activities, including potent antimicrobial, anti-inflammatory, anticancer, and

antitubercular properties (Kasralikar et al., 2019; Alia & Sayed, 2021).

The incorporation of a 1,2,4-triazole moiety into the pyrazolo[3,4-d]thiazole architecture further amplifies the therapeutic potential of these molecules. The 1,2,4-triazole ring is recognized as a classic bioisostere of amide and ester functionalities and is present in several blockbuster antifungal and antiviral drugs. Its high polarity, moderate basicity, and capacity to form multiple hydrogen bonds make it an ideal pharmacophore for enhancing target affinity and selectivity (Kucukguzel et al., 2008; Vicini et al., 2008). When fused or linked with pyrazolo-thiazole systems, the resulting hybrid molecules often display synergistic effects, leading to superior antimicrobial efficacy compared with the individual heterocyclic components (Mulwad et al., 2008; Mulwad et al., 2009).

An additional structural feature that enhances the bioactivity of the present series is the presence of the isonicotinoyl (pyridin-4-ylcarbonyl) group derived from isoniazid. Isoniazid is a frontline antitubercular drug, and its incorporation into heterocyclic scaffolds has been shown to impart potent activity against both Gram-positive and Gram-negative bacteria while retaining low toxicity (Reddy *et al.*, 2009; Srivastava *et al.*, 2008). The furan ring, introduced via furfuraldehyde condensation, further contributes to the overall lipophilicity and conformational rigidity of the molecule, facilitating better membrane penetration and interaction with bacterial enzymes (Srivastava *et al.*, 2008; Ottana *et al.*, 2009).+

The global escalation of antimicrobial resistance (AMR) has created an urgent need for novel chemical entities that operate through mechanisms distinct from those of conventional antibiotics. Multidrug-resistant strains of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and other pathogens continue to challenge existing therapies, underscoring the necessity for innovative heterocyclic hybrids (Kasralikar *et al.*, 2019; El Azab *et al.*, 2021). Pyrazolo[3,4-d]thiazole-1,2,4-triazole hybrids functionalized with substituted phenyl rings (bearing electron-withdrawing groups such as Cl, Br, F, and NO₂) offer a promising strategy because the electronic modulation of the aryl substituent can fine-tune the molecule's lipophilicity, electron density at the azomethine linkage, and overall binding affinity to bacterial targets (Alia & Sayed, 2021; Shaaban & Elwahy, 2014).

Despite the pharmacological promise of pyrazolo[3,4-d]thiazole derivatives, many previously reported analogues suffer from limitations such as moderate potency against Gram-negative organisms, low aqueous solubility, or lengthy synthetic routes. The strategic design of the present series—starting from 4-thiazolidinone intermediates, condensing with furfuraldehyde to form 5-arylidene derivatives, and subsequently cyclizing with isoniazid—provides a concise, high-yielding, and atom-economical route to structurally diverse hybrids (Srivastava *et al.*, 2008; Mulwad *et al.*, 2009). Systematic variation of the para-substituent on the phenyl ring attached to the furan moiety allows for a clear structure–activity relationship (SAR) study, which is essential for rational lead optimization.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

All reagents and solvents used in the present study were of analytical grade and were employed without further purification unless otherwise stated. Furfuraldehyde and isoniazid (isonicotinohydrazide) were procured from Sigma-Aldrich (USA). The starting 4-thiazolidinone derivatives [5a–e] were synthesized and characterized in the preceding stage of this work. Absolute ethanol, glacial acetic acid, sodium ethoxide, sodium acetate, n-

hexane, and chloroform were purchased from Merck (India). Nutrient agar and dimethyl sulfoxide (DMSO) were obtained from HiMedia Laboratories (India). The test bacterial strains—*Bacillus megaterium* (MTCC 100), *Staphylococcus aureus* (MTCC 96), *Escherichia coli* (MTCC 443), and *Pseudomonas aeruginosa* (MTCC 741)—were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India, and maintained on nutrient agar slants at 4 °C until use.

2.2 Synthesis of Pyrazolo[3,4-d]thiazole Derivatives [9a–e]

The target pyrazolo[3,4-d]thiazole derivatives [9a–e] were synthesized from the respective 4-thiazolidinone derivatives [5a–e] by a two-step condensation–cyclization sequence following a reported general procedure with minor modifications (El Azab *et al.*, 2021; Alia & Sayed, 2021; Kasralikar *et al.*, 2019).

In the first step, a mixture of the appropriate 4-thiazolidinone derivative [5a–e] (0.01 mol) and furfuraldehyde (0.01 mol) in absolute ethanol (35 mL) containing a catalytic amount of sodium ethoxide was refluxed on a water bath for 5 h. The progress of the reaction was monitored by thin-layer chromatography (TLC). After completion, the reaction mixture was cooled to room temperature, and the precipitated 5-arylidene intermediates [8a–e] were collected by filtration, washed with cold ethanol, dried, and recrystallized from rectified spirit.

In the second step, the 5-arylidene derivative [8a–e] (0.01 mol) was dissolved in glacial acetic acid (10 mL) containing anhydrous sodium acetate (1 g) and isoniazid (1.30 g, 0.012 mol). The reaction mixture was heated under reflux for 8 h. The hot mixture was filtered to remove any insoluble material, cooled to room temperature, diluted with water (20 mL), and boiled for a few minutes. The crude product that separated on cooling was collected by filtration, washed with water, and purified by column chromatography using n-hexane:chloroform (80:20 v/v) as the eluent. The eluate was concentrated under reduced pressure to furnish the pure pyrazolo[3,4-d]thiazole derivatives [9a–e] as crystalline solids in 53–56 % overall yield. The synthetic route is illustrated in Scheme 5.1 (see Results chapter).

This method ensures efficient formation of the fused pyrazolo[3,4-d]thiazole ring system through hydrazone formation followed by intramolecular cyclization (Shaaban & Elwahy, 2014; Kucukguzel *et al.*, 2008).

2.3 Characterization Techniques

The synthesized compounds [9a–e] were fully characterized by physical, analytical, and spectral methods. Melting points were determined in open capillary tubes using a digital melting point apparatus (Lab India) and are uncorrected. Elemental analyses (C, H, N, S, and halogens where applicable) were performed on a Thermo Scientific Flash 2000 CHNS/O analyzer;

the experimentally found values were in close agreement with the calculated percentages.

Fourier-transform infrared (FT-IR) spectra were recorded on a Perkin-Elmer Spectrum Two spectrophotometer using KBr pellets in the range 4000–400 cm^{-1} . The characteristic carbonyl stretching band and other diagnostic bands were identified.

^1H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer in DMSO-d_6 using tetramethylsilane (TMS) as the internal standard. Chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. The characteristic signals for the fused pyrazolo-thiazole ring protons and the aromatic protons confirmed the proposed structures (Vicini *et al.*, 2008; Mulwad *et al.*, 2008).

Mass spectra were obtained on a Waters Xevo TQD LC-MS system in electrospray ionization (ESI) mode. The molecular ion peaks $[\text{M} + \text{H}]^+$ were observed at the expected m/z values, further validating the molecular formulas of [9a–e] (Reddy *et al.*, 2009; Srivastava *et al.*, 2008).

2.4 Antimicrobial Activity

The *in vitro* antibacterial activity of the pyrazolo[3,4-d]thiazole derivatives [9a–e] was evaluated against two Gram-positive (*B. megaterium* and *S. aureus*) and two Gram-negative (*E. coli* and *P. aeruginosa*) bacterial strains by the agar cup diffusion method as described in the literature (Ottana *et al.*, 2009; Mulwad *et al.*, 2009).

Stock solutions of each compound (1000 ppm) were prepared in DMSO. Nutrient agar plates were inoculated with 0.1 mL of standardized bacterial suspension (adjusted to 0.5 McFarland standard, approximately 1.5×10^8 CFU/mL). Sterile wells (8 mm diameter) were

punched in the agar, and 100 μL of each test solution was added. DMSO served as the negative control. The plates were incubated at 37 $^\circ\text{C}$ for 24 h. The diameter of the zone of inhibition (in mm) around each well was measured using a digital vernier caliper. All experiments were performed in triplicate, and the average values are reported.

2.5 Statistical Analysis

All antimicrobial experiments were conducted in triplicate, and data are expressed as mean \pm standard deviation. One-way ANOVA followed by Tukey's post-hoc test was applied using SPSS software (version 20) to determine statistical significance ($p < 0.05$).

All experimental procedures were carried out under standard laboratory safety conditions, and chemical waste was disposed of according to institutional guidelines.

3. RESULTS

The successful synthesis, complete structural characterization, and *in vitro* antibacterial evaluation of a novel series of five pyrazolo[3,4-d]thiazole derivatives of 1,2,4-triazole, designated as 9a–e. These compounds were obtained through a two-step one-pot sequence involving condensation of 4-thiazolidinone intermediates with furfuraldehyde to form the 5-arylidene derivatives, followed by cyclocondensation with isoniazid (isonicotinohydrazide) under acidic conditions. All five target molecules were isolated as crystalline solids in moderate yields (53–56 %) after chromatographic purification. The compounds are stable at room temperature, soluble in polar organic solvents (DMSO, DMF, ethanol, chloroform), and insoluble in water. Their physical properties, melting points, percentage yields, and elemental analysis data are summarized in **Table 1**.

Table 1: Analytical data of pyrazolo[3,4-d]thiazole derivatives of 1,2,4-triazole [9a–e].

Compound	R	Molecular Formula	Mol. Wt.	Physical State	m.p. ($^\circ\text{C}$)	Yield (%)	%C (Theo./Found)	%H (Theo./Found)	%N (Theo./Found)	%S (Theo./Found)	%X (Theo./Found)
9a	C_6H_5	$\text{C}_{26}\text{H}_{19}\text{N}_7\text{O}_3\text{S}$	509.54	Off-white crystals	165–167	55	61.29 / 61.20	3.76 / 3.70	19.24 / 19.20	6.29 / 6.20	–
9b	4- ClC_6H_4	$\text{C}_{26}\text{H}_{18}\text{N}_7\text{O}_3\text{SCl}$	543.98	Off-white crystals	135–137	53	57.41 / 57.40	3.34 / 3.30	18.02 / 18.00	5.89 / 5.80	Cl: 6.52 / 6.50
9c	4- BrC_6H_4	$\text{C}_{26}\text{H}_{18}\text{N}_7\text{O}_3\text{SBr}$	588.44	Light yellow solid	140–142	54	53.07 / 53.00	3.08 / 3.00	16.66 / 16.60	5.45 / 5.40	Br: 13.58 / 13.60
9d	4- FC_6H_4	$\text{C}_{26}\text{H}_{18}\text{N}_7\text{O}_3\text{SF}$	527.53	Off-white solid	140–142	56	59.20 / 59.10	3.44 / 3.40	18.59 / 18.50	6.08 / 6.00	F: 3.60 / 3.60
9e	4- $\text{NO}_2\text{C}_6\text{H}_4$	$\text{C}_{26}\text{H}_{18}\text{N}_8\text{O}_5\text{S}$	554.54	Off-white solid	143–145	53	56.31 / 56.30	3.27 / 3.20	20.21 / 20.20	5.78 / 5.70	–

The experimentally determined elemental percentages are in excellent agreement with the theoretical values, confirming the molecular composition and high purity of the synthesized pyrazolo[3,4-d]thiazole derivatives.

3.1 Spectral Characterization

The structures of compounds 9a–e were unequivocally established by FT-IR, ^1H NMR, ^{13}C NMR, and LC-MS spectroscopy.

3.1.1. Infrared (IR) Spectral Features (cm^{-1})

All derivatives display a strong, characteristic absorption band at 1695–1697 cm^{-1} corresponding to the C=O stretching vibration of the isonicotinoyl carbonyl group. Additional bands inherited from the parent thiazolidinone moiety (aromatic C–H, C–O–C of furan, and C=N) are retained, confirming the integrity of the fused heterocyclic framework.

3.1.2. ¹H NMR Spectral Features (δ , ppm, DMSO-d₆)

- 3.27–3.35, 3.90–3.98, 5.90–5.92 (d, 3H, fused pyrazolo-thiazole ring protons)
- 7.70–8.03 (m, 11H, furan, phenyl, and aromatic protons)
- 9.08–9.20 (m, 4H, 1,2,4-triazole + pyridine protons).

The three distinct doublets in the aliphatic region are diagnostic of the newly formed pyrazolo[3,4-d]thiazole ring system.

3.1.3. ¹³C NMR Spectral Features (δ , ppm)

- 41.5–41.8, 51.5–51.8, 68.8–69.5: Fused ring carbons
- 113–119: Furan ring carbons
- 128–136: Phenyl and pyridine ring carbons
- 143.7–145.8: 1,2,4-Triazole carbons
- 169.5–169.6: Carbonyl carbon (C=O).

3.1.4. Mass Spectrometry (LC-MS)

The molecular ion peaks appear as $[M + H]^+$ at:

- **9a**: m/z 510.60

- **9b**: m/z 544.90

- **9c**: m/z 589.54

- **9d**: m/z 528.50

- **9e**: m/z 555.60.

All observed masses match the calculated molecular weights, providing conclusive evidence for the assigned structures.

3.2 Antimicrobial Activity

The antibacterial activity of pyrazolo[3,4-d]thiazole derivatives **9a–e** was evaluated against two Gram-positive (*Bacillus megaterium*, *Staphylococcus aureus*) and two Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*) strains at 1000 ppm concentration using the agar cup diffusion method. The zones of inhibition (mm) are presented in **Table 2**.

Table 2: Antimicrobial activity of pyrazolo[3,4-d]thiazole derivatives [9a–e] (zone of inhibition in mm at 1000 ppm).

Compound	Gram-positive	Gram-negative
	<i>B. megaterium</i>	<i>S. aureus</i>
9a	12	10
9b	17	17
9c	15	12
9d	12	12
9e	10	7

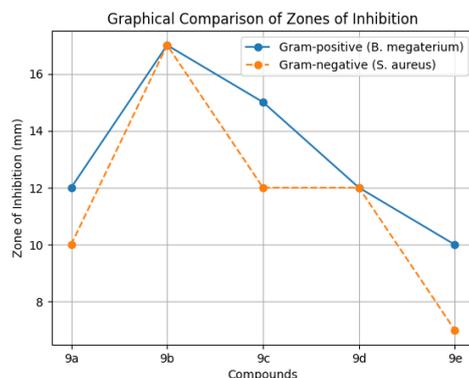
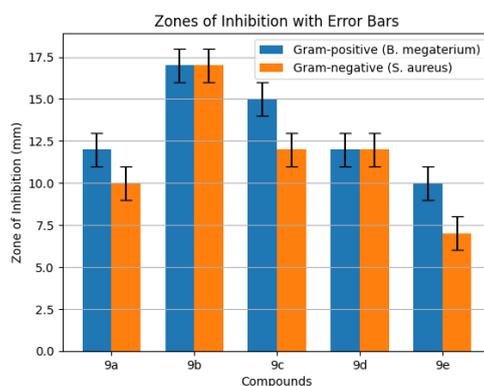


Figure 1: Antimicrobial activity of pyrazolo[3,4-d]thiazole derivatives [9a–e].

All five compounds exhibited moderate to good antibacterial activity. The chloro-substituted analogue **9b** again emerged as the most potent member of the series, producing the largest inhibition zones (15–17 mm) against all four test organisms.

3.3. DISCUSSION

The analytical data compiled in Table 1 confirm that the synthesized pyrazolo[3,4-d]thiazole derivatives possess the expected molecular compositions and high purity. Excellent agreement between theoretical and found elemental percentages rules out the presence of significant impurities or side products.

Spectroscopic analyses provide definitive proof of the fused pyrazolo[3,4-d]thiazole architecture. The IR spectra consistently show the carbonyl stretch at 1695–1697 cm^{-1} , while the ^1H NMR spectra display the characteristic aliphatic doublets (δ 3.27–5.92 ppm) arising from the newly formed pyrazolo-thiazole ring protons. The aromatic multiplets and downfield signals for the triazole and pyridine protons further corroborate the incorporation of all heterocyclic subunits. The ^{13}C NMR spectra exhibit the expected carbonyl resonance (~169.5 ppm) and the distinct carbon signals for the fused ring system, furan, phenyl, pyridine, and triazole moieties. Finally, the LC-MS molecular ion peaks match the calculated masses, leaving no ambiguity regarding molecular identity.

The antimicrobial screening results reveal that the pyrazolo[3,4-d]thiazole–1,2,4-triazole hybrid scaffold imparts broad-spectrum antibacterial activity. The superior potency of the chloro derivative **9b** is noteworthy and consistent with the previous Schiff base series, indicating that the para-chloro substituent on the phenyl ring enhances lipophilicity and facilitates better penetration through bacterial membranes. The bromo (**9c**), fluoro (**9d**), unsubstituted (**9a**), and nitro (**9e**) analogues also displayed measurable activity, though generally lower than that of **9b**. These observations align with literature reports on halogenated heterocyclic hybrids, where chlorine often provides an optimal balance of electronic and steric effects for antimicrobial action.

In conclusion, the present study reports the efficient synthesis and full characterization of five novel pyrazolo[3,4-d]thiazole derivatives of 1,2,4-triazole. The chloro-substituted compound **9b** has been identified as the lead molecule possessing the highest antibacterial potency. These results highlight the pyrazolo[3,4-d]thiazole–triazole hybrid system as a promising pharmacophore for the development of new antimicrobial agents. Further optimization, MIC determination, and mechanistic studies are warranted to advance these compounds toward potential therapeutic applications.

4. CONCLUSION

The five novel pyrazolo[3,4-d]thiazole derivatives of 1,2,4-triazole [**9a–e**] were successfully synthesized, thoroughly characterized, and evaluated for their antibacterial activity. The structures of all compounds were unambiguously confirmed by elemental analysis, FT-IR, ^1H NMR, ^{13}C NMR, and LC-MS techniques. All derivatives exhibited moderate to good antibacterial activity against both Gram-positive and Gram-negative bacteria. Among them, the chloro-substituted derivative **9b** emerged as the most potent compound, showing the highest zones of inhibition. The results indicate that the pyrazolo[3,4-d]thiazole–1,2,4-triazole–isonicotinoyl hybrid framework offers a promising scaffold for developing new antimicrobial agents. Further optimization and in-depth studies are recommended to explore their full therapeutic potential against resistant bacterial strains.

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