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Synthesis and Biological Evaluation of New 3-Substituted-pyrazolo[3,4-b]pyridine Derivatives as Antimicrobial Agents and DHFR Inhibitors

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Abstract

A new series of pyrazolo[3,4-*b*]pyridine compounds (**3a,b**–**9a-c**) was synthesized starting with 2-oxo-6-phenyl-1,2-dihydropyridine-3-carbonitriles **1a,b** which converted to their 2- chloro analogues **2a,b**. By further treatment of of **2a,b** with hydrazine hydrate, the key intermediates 3-amino-pyrazolopyridine derivatives **3a,b** were afforded. Whereas, the target 3-substituted-pyrazolopyridine derivatives (**4a-d**–**9a-c**) were obtained through treatment of **3a,b** with different reagents. All the new compounds were evaluated as antimicrobial agents against six bacterial and six fungal strains. The most potent antimicrobial activity was showed by compounds (**3a**, **3b**, **4a**, **4d**, **6a**, **6c**, **9a** and **9c**) with MIC values range (2-32) µg/mL. Moreover, the most active compounds were selected to be evaluated for their inhibition activity against the resistant bacteria methicillin-resistant *Staphylococcus aureus* (MRSA). In addition, the inhibitory activity of the potent compounds against dihydrofolate reductase (DHFR) was evaluated compared with Trimethoprim (TMP) as a reference DHFR inhibitor. The most potent inhibition of the target enzyme was also showed by compounds **4d**, **6c** and **9c of** IC$_{50}$ values 0.72, 0.95 and 1.09 µM, compared with the IC$_{50}$ value 5.54 µM of TMP. Also, molecular docking study showed that compounds **4d**, **6c** and **9c** having the most binding affinity in DHFR active site.

Keywords: Pyrazolo[3,4-*b*]pyridines; Antimicrobial activity; Anti-MRSA activity, DHFR inhibitors; Docking study.

1. Introduction

Over the last decades, the growing risk of microbial resistance has emerged as a serious threaten of the global health that resulted in an ever-increasing of bacterial and fungal infections that no longer susceptible to many frequently used antibiotics [1]. Antimicrobial Resistance can arise spontaneously through gene mutation and by horizontal gene transfer [2, 3]. However, the widespread of antibiotics in clinical and agriculture uses has implicated rapid evolution of antimicrobial resistance [4]. Also, the lack of the qualified knowledge of therapeutic doses

and the continuous exposure of the microbes to suboptimal plasma concentration of antibiotics appears to encourage antimicrobial resistance [5]. Another risk of antimicrobial resistance comes from antibiotic misuse as a result of the precocious hype about treatment of covid-19 by some known antibiotics [6]. The risk of antimicrobial resistance is associated mainly with the infections caused by multidrug-resistant (MDR) organisms, such as methicillin-resistant *Staphylococcus aureus* (MRSA), which causes a substantial health burden on hospitalized and health care patients [7]. Recent

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studies exhibited that MRSA strains have shown a diverse resistance pattern to many commonly used antibiotics of groups, fluoroquinolones, aminoglycosides, macrolides, tetracycline, and chloramphenicol [8, 9]. Also, antifungal resistance is becoming a significant risk, mainly for patients who have multiple comorbidities or severe immunosuppression. Recently, reports of a multidrug-resistant *Candida* and *Aspergillus* species are increasing with alarming frequency [10, 11]. Therefore, there is an urgent demand to find new antimicrobial agents having the ability to target and inhibit resistant microbes and can overcome the problem of antibiotic resistance.

Most clinically relevant antibiotics such as quinolones, β-lactams, carbapenems, and aminoglycosides act as inhibitors of microbial enzymes, they target specifically the enzymes that are responsible of vital functions like biosynthesis of cell wall, proteins, nucleic acids, and metabolites [12, 13]. However, these antibiotics are susceptible to high levels of resistance which preventing their binding to the target enzymes. Therefore, attention has been focused on new leading structures which may have the ability to defeat the resistance mechanisms and acts as potent enzymes inhibitors [14]. In last few decades, inhibition of dihydrofolate reductase (DHFR) has been specified as an attractive approach for anti-infectious drug development, due to its essential function in nucleic and amino acid synthesis. Bacterial DHFR catalyzes the reduction of dihydrofolate to tetrahydrofolate in the folic acid pathway, thereby it promotes thymidylate biosynthesis and controls cell proliferation [15, 16]. As a result, inhibitors of DHFR cause thymineless death of the bacterial cell and provide the clinical utility of antimicrobial agents [17, 18].

On the other hand, pyrazoles are an important class of nitrogen heterocyclic compounds, regarding to their vital role in the field of medicinal chemistry. Pyrazole derivatives have a variety of biological activities such as antimicrobial [19-21], anticancer [22-24], anti-inflammatory [25, 26], antidiabetic [27, 28] and antimalarial [29, 30] activities. Also, pyridine-based compounds have a broad spectrum of pharmacological properties include, antimicrobial [31-33], anticancer [34, 35], antimalarial [36] and antidiabetic [37] activities. Moreover, some recent studies showed that some pyrazole-based compounds (I-III) and pyridine-based compounds (IV-VI), exhibited potential antimicrobial activity as DHFR inhibitors [38-41] (Fig.

1). Therefore, combining both the pyrazole and pyridine cores in the same framework to synthesize pyrazolopyridine derivatives attracted great interest for designing a novel scaffold having enhanced pharmacological activities [42, 43]. Whereas, recent studies have been directed to the synthesis of various pyrazolo[3,4-*b*]pyridine derivatives as significant antimicrobial candidates [44, 45].

In view of the above bases, the present work includes designing of new 1*H*-pyrazolo[3,4-*b*]pyridine derivatives as antimicrobial agents and DHFR inhibitors (Fig. 1).

The target compounds were synthesized by cyclization of a pyrazole ring onto a pre-existing pyridine ring, starting with 2-chloro-nicotinonitrile derivatives **2a,b** to afford 1*H*-pyrazolo[3,4-*b*]pyridin-3-amine derivatives **3a,b**. By further treatment of **3a,b** with different reagents a new series of 3- substituted-1*H*-pyrazolo[3,4-*b*]pyridines (**4a-d-9a-c**) was obtained, which having at position-3 different structural motifs renewed with their significant pharmacological activities such as, Schiff bases, azetidin-2-one, urea, thiourea, thiazolidin-4-one and 1-thia-4-azaspiro[4.5]decan-3-one moieties [45-50]. All the target compounds (**3a,b-9a-c**) were evaluated as antimicrobial agents against a panel of six bacterial strains and six fungal strains. Moreover, the most active compounds against *Staphylococcus aureus* (**3a, 3b, 4a, 4d, 6a, 6c, 9a** and **9c**) were also tested for their *in vitro* inhibitory action on the resistant bacterial strain MRSA. Additionally, the compounds which showed the most potent antimicrobial activity (**3a, 3b, 4a, 4d, 6a, 6c, 9a** and **9c**) were evaluated for their inhibitory activity against DHFR enzyme. Also, molecular docking study was performed to predict the binding modes of the most promising derivatives in the active pocket of target enzyme DHFR.

2. Result and discussion

2.1. Chemistry

The new series of the 3-substituted-1*H*-pyrazolo[3,4-*b*]pyridine derivatives (**3a,b-9a-c**) was synthesized utilizing the routes outlined in **Schemes 1, 2**. The molecular structures of all the new compounds were confirmed via (^{1}H-NMR, ^{13}C-NMR, Mass and IR) spectral data, in addition to elemental microanalyses. The starting compounds 2-oxo-6-phenyl-1,2-dihydropyridine-3-carbonitriles **1a,b** were treated

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with refluxing $POCl_3/PCl_5$ mixture to afford the 2-chloro-6-phenyl-nicotinonitrile derivatives **2a,b**. By further reaction of **2a,b** with hydrazine hydrate in ethanol under reflux, the corresponding 1*H*-pyrazolo[3,4-*b*]pyridin-3-amine derivatives **3a,b** were formed. The structure of the 2-chloro-6-phenyl-nicotinonitrile derivatives **2a,b** were confirmed with their ¹H and ¹³C NMR spectra, which showed signals corresponding to all their protons and carbons in the expected regions. Moreover, the IR spectra of **2a,b** exhibited an absorption band at 2227 and 2223 cm⁻¹ corresponding to the CN group, respectively. While, the vanishing of the CN absorption band in the IR spectra of **3a,b** alongside the presence of new absorption bands at the region 3448-3176 cm⁻¹ related to the stretching vibrations of the pyrazole-NH and the

NH₂ at position-3 confirming the formation of the 1*H*-pyrazolo[3,4-*b*]pyridin-3-amine derivatives **3a,b**. Also, ¹H-NMR spectra of **3a** and **3b** revealed, beside the signals of the parent protons, two D₂O exchangeable signals at δ 4.59, 12.30 ppm and at δ 4.87, 12.46 ppm assignable to the NH₂ and the pyrazole-NH protons, respectively. The 3-amino-pyrazolopyridine derivatives **3a,b** act as key compounds for the synthesis of new 3-substituted-pyrazolopyridine derivatives, they reacted with different aldehydes namely; 5-methylfuran-2-carbaldehyde, thiophene-2-carbaldehyde, 4-methylbenzaldehyde and 4-methoxybenzaldehyde in refluxed glacial acetic acid to give the Schiff bases **4a-d**.
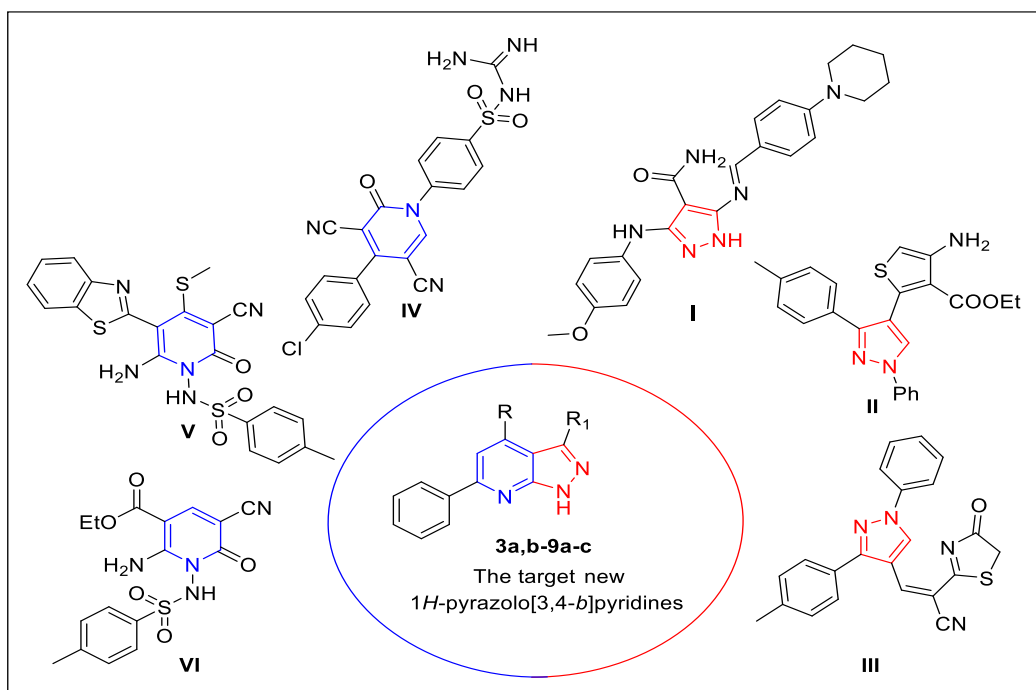

Fig. 1. Reported pyrazole-based (**I–III**) and pyridine-based (**IV–VI**) compounds as DHFR inhibitors and the design of the new 1*H*-pyrazolo[3,4-*b*]pyridines.

The ¹H-NMR spectra of **4a-d** showed disappearance of the NH₂ signals and revealed a new singlet signal ascribed the azomethine N=CH proton at the range δ 8.94-9.04 ppm. In addition to the signals of the aromatic protons, the ¹H-NMR spectrum of the (*p*-tolyl)methanimine derivative **4c** showed singlet signal at δ 2.36 ppm corresponding to the CH₃ of the new *p*-tolyl moiety. Also, the signal at δ 3.78 ppm in the ¹H-NMR spectrum of the (4-methoxyphenyl)methanimine derivative **4d** confirmed the presence of the -OCH₃ of the new 4-methoxyphenyl moiety. Moreover, ¹³C-

NMR spectrum of **4c** showed signal at δ 21.75 ppm corresponding to CH₃ of the *p*-tolyl moiety and the signal at δ 55.95 ppm in the ¹³C-NMR spectrum of **4d** confirmed the presence of the -OCH₃ of the 4-methoxyphenyl moiety. Subsequent treatment of the Schiff bases **4b,c** with chloroacetyl chloride in dry dioxane containing triethyl amine afforded the 3-chloro-azetidin-2-one derivatives **5a,b**, respectively (**Scheme 1**). The IR spectra of **5a** and **5b** showed strong absorption band at 1668, 1662 cm⁻¹ assignable for the C=O group of the azetidin-2-one ring, respectively. The ¹H-NMR spectra of **5a,b** assisted the formation of the 3-chloro-azetidin-2-one ring by two

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doublet signals at range δ 3.52-4.05 ppm related to the CH-N and CH-Cl protons. Also, ^{13}C-NMR spectrum of **5a** showed signals at δ 60.93, 66.13 and 163.04

ppm corresponding to CH-N, CH-Cl, and C=O carbons of the 3-chloro-azetidin-2-one ring, respectively.
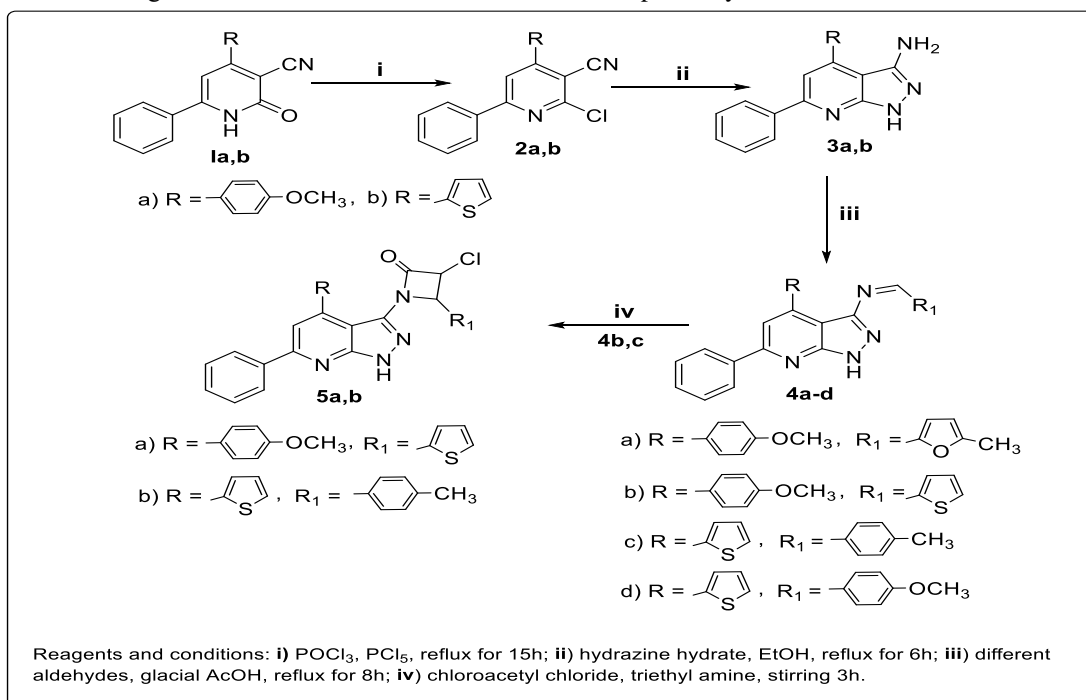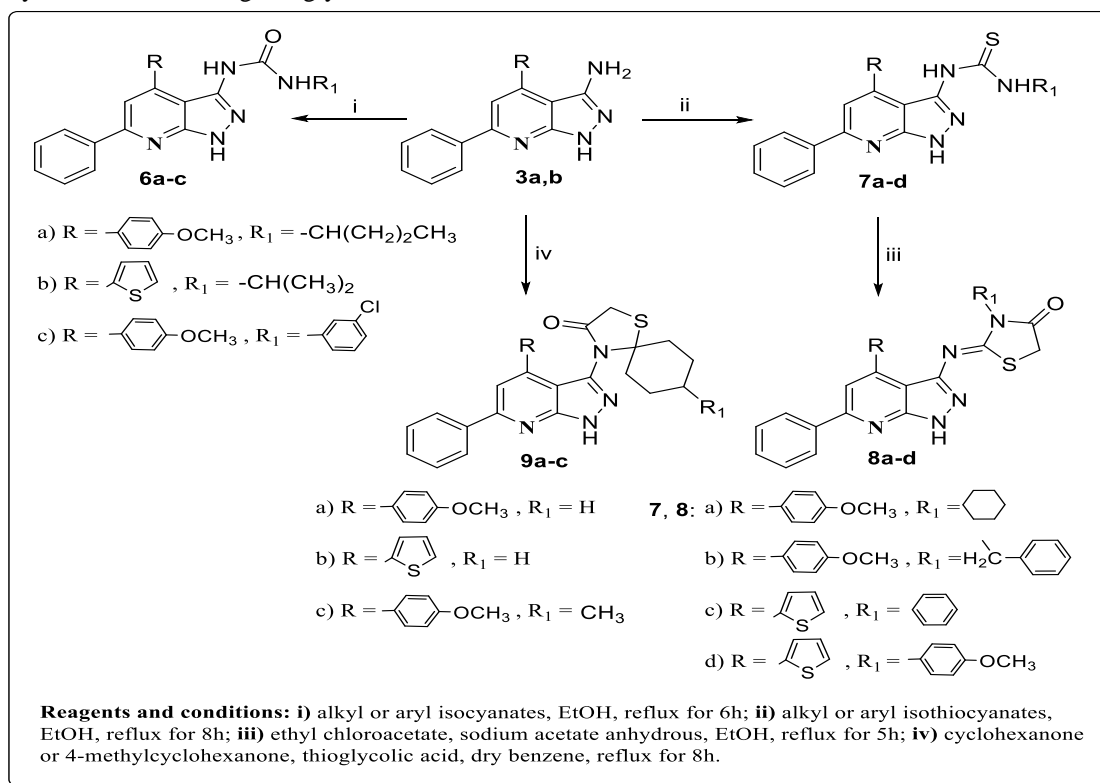

Scheme 1. Synthesis of new pyrazolo[3,4-*b*]pyridine compounds **3a,b–5a,b.**

Furthermore, the 3-amino-pyrazolopyridine derivatives **3a,b** treated with alkyl or aryl isocyanate namely; n-butyl, isopropyl and 3-chlorophenyl isocyanate in ethanol to afford the urea derivatives **6a-c**. The IR spectra of **6a-c** showed new strong absorption band at the region 1658-1649 cm^{-1} related to the urea C=O stretching vibration. The ^{1}H-NMR spectra of **6a-c** revealed two D$_2$O exchangeable signals at the range δ 6.27-10.98 ppm corresponding to the two NH groups of the urea side chain. Also, ^{1}H-NMR spectrum of the 1-butyl-urea derivative **6a** showed multiplet signals at the range δ 0.85-3.15 ppm assignable to the protons of the n-butyl moiety. Moreover, ^{13}C-NMR spectra of **6a** revealed four signals at the range δ 14.08-41.38 ppm corresponding to the four carbons of the n-butyl moiety along with the signal related to the urea C=O at δ 162.77 ppm. On further treatment of **3a,b** with alkyl or aryl isothiocyanates namely; cyclohexyl, benzyl, phenyl and 4-methoxyphenyl isothiocyanate in refluxed ethanol afforded the corresponding thiourea derivatives **7a-d**. The ^{1}H-NMR spectra of **7a-d** confirmed the presence of the thiourea side chain, they revealed all signals corresponding to the parent protons along with the new alkyl or aryl protons.

Whereas, ^{1}H-NMR spectrum of 1-cyclohexyl-thiourea derivative **7a** showed multiplet signals at the range δ 1.19-1.80 ppm corresponding to the cyclohexyl-protons and two D$_2$O exchangeable signals at δ 8.50 and 12.31 ppm related to the two NH groups of the thiourea side chain. Also, the ^{13}C-NMR spectra of **7a,b,d** showed the signal of the thiourea C=S carbon at the range δ 180.48-181.47 ppm alongside the alkyl or aryl carbons of the new thiourea side chain. Upon treatment of thiourea derivatives **7a-d** with ethyl 2-chloroacetate in refluxed ethanol containing anhydrous sodium acetate the corresponding 3-alkyl/aryl-2-((1*H*-pyrazolo[3,4-*b*]pyridin-3-yl)imino)thiazolidin-4-one derivatives **8a-d** were formed. The IR spectra of **8a-d** showed strong absorption band at the region 1718-1709 cm^{-1} related to the C=O stretching vibration. The singlet signal at the range δ 4.11-4.59 ppm in ^{1}H-NMR spectra of **8a-d** confirmed the presence of the SCH$_2$ of the thiazolidin-4-one ring. Additional support to the structures of **8a-d** was gained by the ^{13}C-NMR spectra of **8a** and **8d**, which revealed vanishing of the C=S signal and the presence of new signals related to the SCH$_2$ and C=O carbons of the thiazolidin-4-one ring at δ 38.21, 37.94 ppm and at δ 173.03, 171.89 ppm, respectively. In addition, the 3-amino-pyrazolopyridine

derivatives **3a,b** were condensed with cyclic ketones namely; cyclohexanone or 4-methylcyclohexanone in dry benzene containing thioglycolic acid to afford the 4-(1*H*-pyrazolo[3,4-*b*]pyridin-3-yl)-1-thia-4-azaspiro[4.5]decan-3-one derivatives **9a-c** (**Scheme 2**).

Reagents and conditions: i) alkyl or aryl isocyanates, EtOH, reflux for 6h; **ii)** alkyl or aryl isothiocyanates, EtOH, reflux for 8h; **iii)** ethyl chloroacetate, sodium acetate anhydrous, EtOH, reflux for 5h; **iv)** cyclohexanone or 4-methylcyclohexanone, thioglycolic acid, dry benzene, reflux for 8h.

Scheme 2. Synthesis of new pyrazolo[3,4-*b*]pyridine compounds **6a-c–9a-c**.

The IR spectra of **9a-c** showed an absorption band at the region 1708-1998 cm^{-1} assignable for the C=O group. The ^{1}H-NMR spectra of **9a-c** revealed the signals corresponding to the cycloalkyl protons alongside the signal of the SCH$_2$ protons at the range δ 3.51-3.62 ppm. Also, the ^{13}C-NMR spectra of **9b** and **9c** revealed the signals corresponding to the cycloalkyl carbons and confirmed the formation of the 1-thia-4-azaspiro[4.5]decan-3-one moiety by the signal of the SCH$_2$ carbon at δ 41.62 and 41.23 ppm, the signal of the C=O at δ 171.23 and 170.95 ppm and the signal of the spiro carbon at δ 68.02.62 and 67,87 ppm, respectively.

Furthermore, the mass spectra of new 3-substituted-1*H*-pyrazolo[3,4-*b*]pyridine derivatives (**3a,b-9a-c**) gave additional support for their suggested structures by representing their correct molecular ion peaks.

2.2. *Antimicrobial Activity Evaluation*
2.2.1. In vitro antibacterial activity

The antibacterial activity of the new series of the 3-substituted-1*H*-pyrazolo[3,4-*b*]pyridine derivatives (**3a,b-9a-c**) was screened against three gram-positive bacterial strains (*Staphylococcus aureus* 25923, *Bacillus subtilis* 6633, *Bacillus cereus* 33018) and three gram-negative bacteria (*Escherichia coli* 8739, *Salmonella typhimurium* 14028, *Pseudomonas aeruginosa* 27853) compared with the reference antibiotic Gentamicin. The zones of inhibition in (mm) and the Minimum Inhibitory Concentration (MIC) values in (μg/mL) were determined for the target compounds and the reference drug (Table 1).

According to the MIC values, the target compounds showed significant antibacterial activity against both gram-positive and gram-negative bacterial strains. Whereas, the 3-amino-pyrazolopyridine derivatives **3a,b** showed antibacterial activity ranging from potent to moderate with MIC values range (4-32) μg/mL compared with the MIC values range (4-16) μg/mL of Gentamicin. However, the Schiff bases **4a-d** exhibited wide variability in their antibacterial potency of MIC values range (2-128) μg/mL. The most potent activity was revealed by the (4-methoxyphenyl)methanimine derivative **4d** with MIC values range (2-8) μg/mL, which more potent than Gentamicin against the six tested bacterial strains. Also, the (5-methylfuran-2-

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yl)methanimine **4a** showed potent activity against *B. subtilis* and *E. coli* equal to that of Gentamicin with MIC values 8 and 4 μg/mL, respectively. While, the other Schiff bases **4b** and **4c** showed lowering in potency with MIC values range (16-128) μg/mL. The cyclization of the azetidin-2-one ring to give **5a,b** led to an obvious increasing in the activity against the three gram-negative strains. Moreover, the urea derivatives **6a-c** showed significant activity, in particular the (3-Chlorophenyl) urea derivative **6c**, which showed more potent activity than Gentamicin against *S. aureus, B. cereus* and *E. coli* with MIC values 2, 8 and 2 μg/mL, respectively. Also, n-butyl-urea derivative **6a** showed potent activity equal to that of Gentamicin against most of the tested strains. However, the thiourea derivatives **7a-d** revealed dramatic lowering in the antibacterial activity, the

cyclohexyl thiourea derivative **7a** gave moderate activity with MIC values range (16-32) μg/mL and the other derivatives **7b-c** showed weak or no activity (NA). The cyclization of the thiazolidin-4-one ring enhanced the inhibition potency against both gram-positive and gram-negative strains. Moreover, the 3-cyclohexyl-thiazolidin-4-one derivative **8a** showed potent activity equal to that of Gentamicin against *B. subtilis* and *B. cereus* with MIC values 8 and 16 μg/mL, respectively. While, the 1-thia-4-azaspiro[4.5]decan-3-one derivatives **9a-c** showed antibacterial activity ranged from potent to moderate against the tested bacteria and the derivative **9c** was the most potent with MIC values 4 or 8 μg/mL the same as the activity of Gentamicin. Also, **9a** showed potent activity against *S. aureus, E. coli* and *P. aeruginosa* equal to that of Gentamicin.

Table 1: Antibacterial activities of the new pyrazolopyridine compounds represented as inhibition zone in mm and (MIC values in μg/mL).

Compd.	Gram-positive Bacteria			Gram-negative Bacteria		
	S. aureus	*B. subtilis*	*B. cereus*	*E. coli*	*S. typhimurium*	*P. aeruginosa*
3a	31 (4)	30 (8)	26 (32)	30 (4)	27 (16)	28 (16)
3b	32 (4)	31 (8)	30 (8)	29 (4)	29 (8)	26 (32)
4a	31 (8)	30 (8)	26 (32)	30 (4)	27 (16)	28 (16)
4b	28 (16)	29 (16)	22 (64)	25 (32)	19 (128)	18 (128)
4c	24 (32)	21 (64)	20 (64)	20 (64)	17 (128)	18 (128)
4d	33 (2)	38 (2)	30 (8)	34 (2)	37 (2)	32 (4)
5a	29 (16)	27 (16)	20 (64)	29 (8)	32 (4)	31 (8)
5b	25 (32)	26 (32)	21 (64)	27 (16)	28 (16)	26 (32)
6a	32 (4)	29 (16)	28 (16)	31(4)	31(8)	29 (8)
6b	28 (16)	27 (16)	28 (32)	29 (8)	27 (32)	27 (32)
6c	33 (2)	30 (8)	31 (8)	33 (2)	33 (4)	32 (8)
7a	28 (16)	27 (16)	27 (32)	25 (32)	29 (16)	26 (32)
7b	17 (128)	NA	16 (128)	17 (128)	NA	NA
7c	20 (64)	21 (64)	17(128)	21 (64)	24 (32)	18 (128)
7d	17 (128)	20 (128)	NA	18 (128)	17 (128)	NA
8a	28(32)	31 (8)	30(16)	29 (8)	27 (16)	28 (16)
8b	19 (64)	21 (64)	20 (64)	23 (32)	17 (128)	20 (64)
8c	31 (8)	28 (16)	24 (32)	24 (32)	25 (32)	26 (32)
8d	27 (32)	26 (32)	NA	20 (64)	22 (64)	23 (64)
9a	31(4)	27 (16)	26 (32)	29 (4)	29 (8)	29 (8)
9b	25 (32)	28 (16)	27 (32)	29 (8)	29 (16)	25 (32)
9c	33 (4)	30 (8)	30 (8)	30 (4)	30 (4)	29 (8)
Gentamicin	31 (4)	29 (8)	29 (16)	30 (4)	31 (4)	30 (8)

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2.2.2. *In vitro* antifungal activity

The antifungal activity of the new 3-substituted-1*H*-pyrazolo[3,4-*b*]pyridine compounds (**3a,b-9a-c**) was evaluated against three yeasts strains (*Candida tropicals* 750, *Candida albicans* 10231 and *Saccharomycese cerevisiae*) and three fungi (*Macrophomina Phaseolina, Aspergillus flavus* and *Aspergillus niger* EM77) compared with the reference drug Clotrimazole. The zones of inhibition in (mm) and the Minimum Inhibitory Concentration (MIC) values in (μg/mL) were determined for the target compounds and the reference drug (Table 2).

On the base of the MIC values, the (4-methoxyphenyl)methanimine derivative **4d**, the (3-Chlorophenyl) urea derivative **6c** and 1-thia-4-azaspiro[4.5]decan-3-one derivative **9c** showed potent antifungal activity with MIC values range (4-8) μg/mL, which equalized in potency with Clotrimazole against some strains and showed more potent activity than Clotrimazole against the others. Moreover, the 3-amino derivative **3b** revealed potent activity against *C. albicans* and *C. tropicals* with MIC values = 4 μg/mL compared with the MIC values 16 and 8 μg/mL of Clotrimazole, respectively. Also, the 3-cyclohexyl-thiazolidin-4-one derivative **8a** showed potent activity equal to that of Clotrimazole against *A. flavus* and *A. niger* with MIC value = 8 μg/mL. Moreover, the target compounds (**3a**, **4a**, **6a**, and **9a**) exhibited significant activity ranged from potent to moderate against the tested strains with MIC values range (8-32) μg/mL. While, the other target compounds showed lowering in the antifungal effect, which varied from moderate to weak or inactive with MIC values ranging from 32 μg/mL to > 128 μg/mL.

Table 1: Antifungal activities of the new pyrazolopyridine compounds represented as inhibition zone in mm and (MIC values in μg/mL).

	Yeasts			Fungi		
	C. albicans	*C. tropicals*	*S. cerevisiae*	*M. phaseolina*	*A. flavus*	*A. niger*
3a	26 (32)	30 (8)	30 (8)	28 (32)	29 (8)	30 (8)
3b	33 (4)	32 (4)	29 (16)	27 (32)	28 (16)	29 (16)
4a	28 (16)	26 (32)	28 (16)	30 (8)	25 (32)	24 (32)
4b	26 (32)	22 (64)	16 (128)	NA	NA)	NA
4c	20 (128)	21 (64)	19 (128)	NA	NA	NA
4d	40 (2)	34 (4)	37 (2)	33 (2)	32 (4)	30 (8)
5a	23 (32)	21 (64)	21 (64)	16 (128)	19 (128)	18 (128)
5b	34 (32)	25 (32)	24 (64)	23 (64)	22 (64)	25 (32)
6a	28 (8)	27 (16)	29 (8)	24(32)	23 (32)	26 (16)
6b	26 (32)	21 (64)	17(128)	24 (32)	23 (64)	18(128)
6c	31 (4)	32 (4)	29 (8)	31 (8)	29 (8)	29 (8)
7a	26 (32)	25 (32)	24 (64)	22 (64)	25 (32)	25 (32)
7b	16 (128)	17 (128)	NA	NA	NA	NA
7c	22 (64)	24 (64)	22 (64)	24(32)	25(32)	18(128)
7d	20 (128)	19 (128)	21 (128)	NA	NA	NA
8a	27 (32)	26 (32)	26 (32)	25 (32)	28 (8)	30 (8)
8b	21 (64)	20 (128)	NA	NA	NA	NA
8c	20 (64)	19(64)	26 (32)	NA	NA	NA
8d	23 (64)	20 (64)	20 (128)	25 (32)	21 (64)	18(128)
9a	28 (16)	27 (16)	28 (16)	28 (8)	29 (16)	27 (16)
9b	27 (32)	20 (64)	25 (32)	25 (32)	20 (64)	27 (32)
9c	29 (8)	31 (4)	29 (8)	29 (8)	31 (8)	34 (4)
Clotrimazole	28 (16)	30 (8)	29 (8)	29 (16)	29(8)	30 (8)

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2.2.3. Anti-MRSA activity

The new pyrazolopyridine compounds which showed the highest inhibitory activity against *S. aureus* (**3a**, **3b**, **4a**, **4d**, **6a**, **6c, 9a** and **9c**) were selected to be evaluated for their inhibition activity against the resistant bacteria methicillin-resistant *Staphylococcus aureus* (MRSA), compared with Gentamicin as a reference drug. The results of the MIC values in µg/mL of the tested compounds and the reference drug were listed in (Table 3). The (4-methoxyphenyl)methanimine derivative **4d** revealed the most potent activity with MIC value = 4 µg/mL, which exceeds the activity of Gentamicin of MIC value = 8 µg/mL. The 1-thia-4-azaspiro[4.5]decan-3-one derivative **9c** and the (3-Chlorophenyl) urea derivative **6c** showed activity against MRSA equal to that of Gentamicin with the same MIC value = 8 µg/mL. While, 4-(4-methoxyphenyl)-3-amino derivative **3a** showed less potent activity than Gentamicin with MIC value = 16 µg/mL. Also, more lowering in anti-MRSA activity was revealed by the 4-(thiophen-2-yl)-3-amino derivative **3b**, the Schiff base **4a** and **9a** with equal MIC value = 32 µg/mL. However, the less potent activity was showed by the n-butyl urea derivative **6a** with MIC value 64 µg/mL.

Table 3: *In vitro* inhibitory activity of the most potent compounds against MRSA.

Compd.	MRSA MIC values (µg/mL)
3a	16
3b	32
4a	32
4d	4
6a	64
6c	8
9a	32
9c	8
Gentamicin	8

2.3. Inhibitory Activity of DHFR

The most potent pyrazolopyridine compounds (**3a**, **3b**, **4a**, **4d**, **6a**, **6c**, **9a** and **9c**) against the tested microbial strains were selected to further investigation for their inhibition profiles against the target DHFR enzyme, compared with Trimethoprim (TMP) as bacterial DHFR inhibitor. The results of the new compounds and the reference inhibitor were expressed as IC_{50} values (µM) (Table 4). Interestingly, most of the tested derivatives showed more potent inhibitory activity than that of Trimethoprim with IC_{50} values range (0.72-7.03) µM. The Schiff base **4d** showed the most potent inhibitory activity with IC_{50} = 0.72 µM, compared with the IC_{50} = 5.54 µM of Trimethoprim. Moreover, the (3-Chlorophenyl) urea derivative **6c** and the 1-thia-4-azaspiro[4.5]decan-3-one derivative **9c** showed nearly equipotent inhibition of DHRF, higher than that of TMP, with IC_{50} values 0.95 and 1.09 µM, respectively. The target compounds **3a**, **6a** and **9a** revealed lowering in the inhibition potency of IC_{50} values range (2.29-3.36) µM, but they are still more potent than TMP. While, detectable drop in the activity was recognized by the 4-(thiophen-2-yl)-3-amino derivative **3b** and the Schiff base **4a**, which showed less potent inhibitory activity than TMP with IC_{50} values 5.99 and 7.03 µM, respectively.

Table 4: *In vitro* inhibitory activity of some selected compounds against DHFR enzyme.

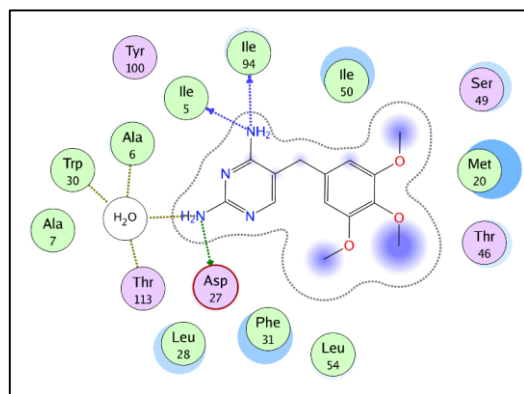| Compd.	DHFR IC_{50} values (µM)
3a	3.36 ± 0.16
3b	5.99 ± 0.29
4a	7.03 ± 0.34
4d	0.72 ± 0.04
6a	2.29 ± 0.11
6c	0.95 ± 0.05
9a	2.78 ± 0.14
9c	1.09 ± 0.05
TMP	5.54 ± 0.31

2.4. Molecular Docking Studies

Molecular docking simulations were performed to study the binding of the most active compounds (**3a**, **3b**, **4a**, **4d**, **6a**, **6c**, **9a** and **9c**) in *E. coli* DHFR active site to predict their binding pattern and to rationalize their promising binding affinity.
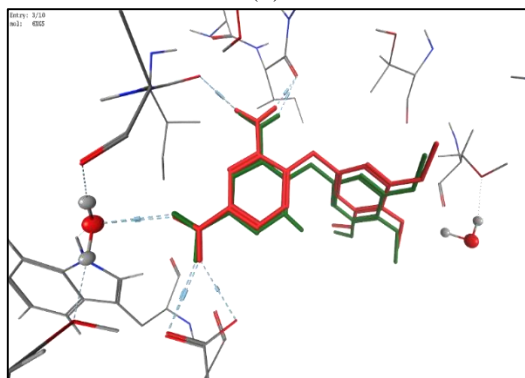
The molecular docking protocol was initially validated through self-docking of the co-crystallized Trimethoprim in *E. coli* DHFR active site. The self-docking validation accurately reproduced the binding mode of the co-crystallized ligand (Trimethoprim) in the target protein active site indicating the suitability of the used docking setup for the intended molecular docking study. This was confirmed by the small RMSD between the co-crystallized and the docked ligand poses in DHFR active site (0.568Å), giving a

docking pose with an energy score (S) = −12.09 kcal/mol and by the capability of the obtained docking poses to reproduce the key interactions accomplished by the co-crystallized ligand with the amino acids in DHFR active site either directly or through water molecule bridge (Ile5, Ala6, Asp27, Trp30, Ile94, and Thr113). (Fig. 2).
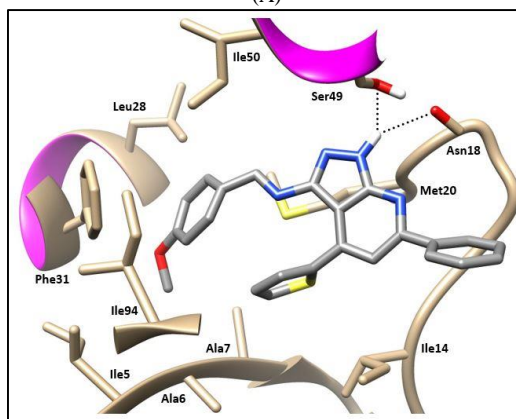
chains of the amino acids Ile5, Ala6, Ala7, Leu28, Phe31, and Ile94. Whereas the phenyl substitution on position 6 interacts through hydrophobic interaction with Ile14 (Fig 3, 4).

Table 5: Docking energy scores (S) in kcal/mol for the target compounds and the reference TMS in DHFR active site.

Compd.	Energy score (S) kcal/mol
3a	−11.10
3b	−10.39
4a	−10.55
4d	−15.21
6a	−12.14
6c	−14.44
9a	−13.47
9c	−16.52
TMP	−12.09

(A)

(B)

Fig. 2. 2D diagram (A) and 3D diagram (B) of Trimethoprim showing its interaction with DHFR active site.

The investigated pyrazolopyridine compounds adapted comparable binding patterns in DHFR active site and their docking energy scores (S) in kcal/mol were recorded in Table 5. The compound binding pattern involves the accommodation of their central pyrazolopyridine scaffold in Trimethoprim's trimethoxyphenyl ring binding region accomplishing hydrogen bond interactions with Asn18 and/or Ser49 directly by its NH in position 1. It was obvious from the docking data that compounds **4d**, **6c** and **9c**, with the highest DHFR inhibitory activities, showed the best binding style.

In compounds **4d** and **6c**, substitutions on positions 3 and 4 of the pyrazolopyridine scaffold are directed towards Trimethoprim's diaminopyrimidine ring binding region achieving multiple hydrophobic interactions with the surrounding hydrophobic side


(A)

(B)

Fig. 3. 2D diagram (A) and 3D diagram (B) of **4d** showing its interaction with DHFR active site.

In the target compound **9c**, the thiazolidinone substitution on position 3 interacts through multiple hydrogen bond interactions with the surrounding amino acids Met20, Gly96, Gly97, and Thr123.

Moreover, the substation on position 4 interacts through hydrophobic interactions with the surrounding amino acids hydrophobic side chains Ile5, Ala6, and Ala7. The phenyl substitution on position 6 interacts through hydrophobic interaction with Leu28, Phe31, and Ile50 (Fig. 5).
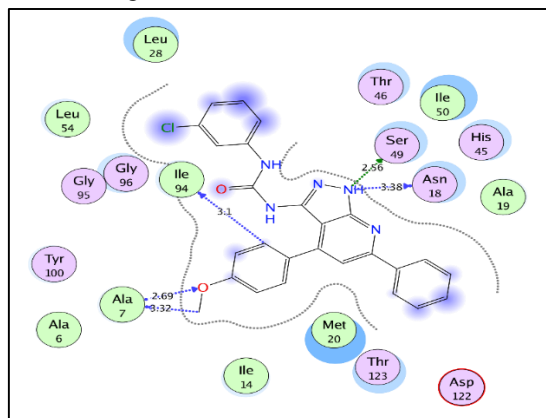
(A)
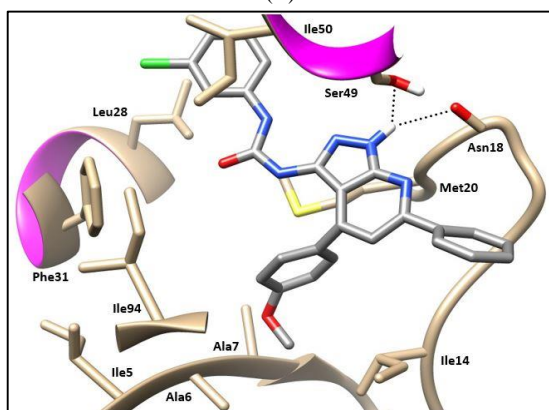
(B)

Fig. 4. 2D diagram (A) and 3D diagram (B) of **6c** showing its interaction with DHFR active site.

While, the other derivatives **3a**, **3b**, **4a**, **6a** and **9a** showed lower binding affinity with energy scores ranging from −10.39 to −13.47 kcal/mol, compared with the energy score −12.09 kcal/mol of Trimethoprim.

3. Experimental

3.1. Chemistry

The melting points were determined by an open glass capillary tubes using an Electro thermal IA9100 digital melting point apparatus and were uncorrected. Elemental microanalyses were carried out at the Micro Analytical Unit at Cairo University and were found within ± 0.5%. ^{1}H NMR and ^{13}C NMR spectra were carried out at the National research Centre and were recorded on JEOL (Japan) High Performance Digital

FT-NMR S (500/125 MHz) in the presence of TMS as internal standard. Infrared spectra were carried out at Cairo University and were recorded by using KBr disc technique on a Jasco FT/IR-6100, Fourier transform, Infrared spectrometer (Japan) at cm^{-1} scale. Mass spectra (MS) were performed at 70 e.v by GCMS-QP1000 EX spectrometer using the Electron Ionization Technique (EI) at Al-Azhar University. Follow up of the reactions and checking the purity of the compounds were made by TLC on silica gel aluminum sheets (Type 60, F 254, Merck, Darmstadt, Germany) and the spots were illustrated by exposure to UV analysis lamp at λ 254/366 nm or by iodine vapor. The nomenclature of new synthesized compounds is according to the IUPAC system. The starting 2-oxo-6-phenyl-1,2-dihydropyridine-3-carbonitriles **1a,b** were prepared as the reported method [51].
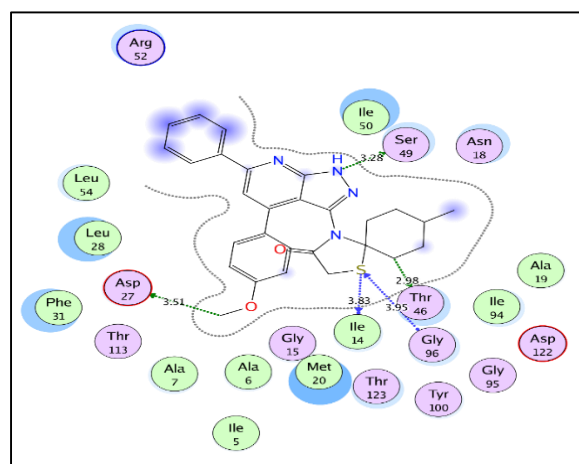
(A)
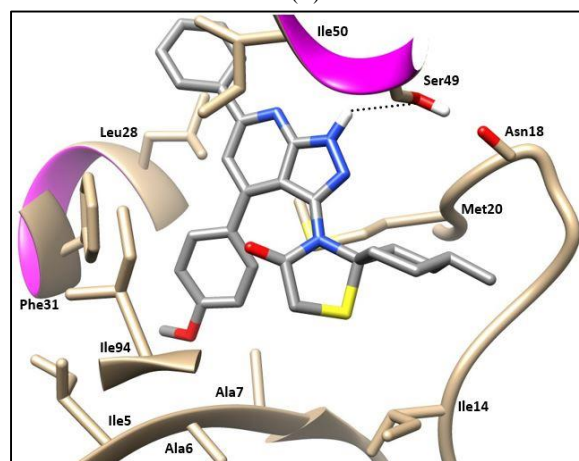
(B)

Fig. 5. 2D diagram (A) and 3D diagram (B) of **9c** showing its interaction with DHFR active site.

Synthesis of 2-chloro-6-phenylnicotinonitriles 2a,b

To a solution of compounds **1a,b** (0.03 mol) in phosphorus oxychloride (50 mL), phosphorus pentachloride (6.25 g, 0.03 mol) was added. The reaction mixture was refluxed for 15h, then left to cool and poured slowly with stirring onto crushed ice. The obtained solid was collected by filtration, washed several times with water, and recrystallized from ethanol to give the chloro compounds **2a,b**.

2-Chloro-4-(4-methoxyphenyl)-6-phenyl-nicotinonitrile (2a)

Yield 91%, pale yellow powder, m.p. 189 °C (Lit. m.p. = 189-190 °C [52]). IR (KBr, ν_{max} cm^{-1}): 3036, 2924 (CH), 2227 (C≡N), 751 (C–Cl). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.82 (s, 3H, -OCH$_3$), 7.11 (d, J = 8.5 Hz, 2H, Ar-H), 7.52 (m, 3H, Ar-H), 7.74 (d, J = 8.5 Hz, 2H, Ar-H), 8.14 (s, 1H, Ar-H), 8.17 (d, J = 7.5 Hz, 2H, Ar-H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 56.48 (-OCH$_3$), 106.38, 114.87, 116.14, 119.69, 127.65, 128.14, 129.57, 131.08, 131.73, 136.25, 153.12, 156.42, 159.29, 161.67 (Ar-C., CN). MS, m/z (%): 320 (M$^{+\cdot}$, 67). Analysis for C$_{19}$H$_{13}$ClN$_2$O (320.78) Calcd.: C, 71.14; H, 4.09; N, 8.73%. Found: C, 71.42; H, 4.36; N, 9.03%.

2-Chloro-6-phenyl-4-(thiophen-2-yl)nicotinonitrile (2b)

Yield 93%, beige powder, m.p. 122 °C. IR (KBr, ν_{max} cm^{-1}): 3100 (CH), 2223 (C≡N), 730 (C–Cl). ^{1}H NMR (DMSO-d_6, 500 MHz): 7.29-7.32 (m, 1H, Ar-H), 7.52-7.58 (m, 3H, Ar-H), 7.68 (d, J =13.3 Hz, 1H, Ar-H), 7.98 (d, J =14.8 Hz, 1H, Ar-H), 8.04 (s, 1H, Ar-H), 8.15 (d, J = 7.6 Hz, 2H, Ar-H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 104.32, 116.25, 118.31, 128.14, 128.93, 129.28, 129.90, 138.87, 139.27, 148.65, 149.91, 153.90, 159.50 (Ar-C, CN). MS, m/z (%): 296 (M$^{+\cdot}$, 48). Analysis for C$_{16}$H$_9$ClN$_2$S (296.77) Calcd.: C, 64.76; H, 3.06; N, 9.44; S, 10.80%. Found: C, 64.45; H, 2.89; N, 9.69; S, 10.54%.

Synthesis of 6-phenyl-1*H*-pyrazolo[3,4-b]pyridin-3-amine derivatives 3a,b

A mixture of the chloro derivatives **2a,b** (0.02 mol) and hydrazine hydrate 100% (3 mL, excess) in absolute ethanol (60 mL) was refluxed for 8h. After reaction completion, the solvent was evaporated till dryness under reduced pressure. The obtained solid was treated with water, collected by filtration and recrystallized from EtOH to give the amines **3a,b**.

4-(4-Methoxyphenyl)-6-phenyl-1H-pyrazolo [3,4-b]pyridin-3-amine (3a)

Yield 79%, yellow powder, m.p. 201-202 °C. IR (KBr, ν_{max} cm^{-1}): 3448, 3289, 3176 (NH, NH$_2$), 3058, 2965 (CH). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.82 (s, 3H, -OCH$_3$), 4.59 (s, 2H, NH$_2$, D$_2$O exchangeable), 7.10 (d, J = 8.6 Hz, 2H, Ar-H), 7.41-7.47 (m, 4H, Ar-H), 7.62 (d, J = 8.6 Hz, 2H, Ar-H), 8.11 (d, J =10.5 Hz, 2H, Ar-H), 12.30 (s, 1H, NH, D$_2$O exchangeable). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 55.78 (-OCH$_3$), 102.62, 114.81, 119.54, 127.08, 127.69, 129.23, 129.92, 131.32, 139.54, 145.76, 147.76, 153.94, 156.05, 160.44 (Ar-C). MS, m/z (%): 316 (M$^{+\cdot}$, 78). Analysis for C$_{19}$H$_{16}$N$_4$O (316.36) Calcd.: C, 72.13; H, 5.10; N, 17.71%. Found: C, 72.39; H, 5.34; N, 18.01%.

6-Phenyl-4-(thiophen-2-yl)-1H-pyrazolo[3,4-b]pyridin-3-amine (3b)

Yield 81%, yellow powder, m.p. 196 °C. IR (KBr, ν_{max} cm^{-1}): 3431, 3292, 3180 (NH, NH$_2$), 3069 (CH). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 4.87 (s, 2H, NH$_2$, D$_2$O exchangeable), 7.26-7.29 (m, 1H, Ar-H), 7.44-7.80 (m, 6H, Ar-H), 8.09 (d, J = 8.8 Hz, 2H, Ar-H), 12.46 (s, 1H, NH, D$_2$O exchangeable). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 102.35, 121.95, 127.49, 127.74, 128.41, 128.69, 129.53, 138.54, 139.08, 142.35, 150.72, 151.01, 154.69 (Ar-C). MS, m/z (%): 292 (M$^{+\cdot}$, 81). Analysis for C$_{16}$H$_{12}$N$_4$S (292.36) Calcd.: C, 65.73; H, 4.14; N, 19.16; S, 10.97%. Found: C, 65.99; H, 4.40; N, 19.38; S, 10.69%.

Synthesis of 1*H*-pyrazolo[3,4-*b*]pyridine Schiff bases 4a-d

A mixture of the 3-amino derivatives **3a,b** (2 mmol) and the appropriate aldehyde (2 mmol) in glacial acetic acid (20 mL) was refluxed for 8h. Then the excess solvent was evaporated under vacuum and the residue was treated with dil. EtOH. The formed solid was collected by filtration and recrystallized from EtOH to give the Schiff bases **4a-d**.

N-(4-(4-Methoxyphenyl)-6-phenyl-1H-pyrazolo[3,4-b]pyridin-3-yl)-1-(5-methylfuran-2-yl)methanimine (4a)

Yield 71%, brown powder, m.p. 225-226 °C. IR (KBr, ν_{max} cm^{-1}): 3428 (NH), 3040, 2926, 2838 (CH), 1603 (C=N). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 2.22 (s, 3H, CH$_3$), 3.80 (s, 3H, -OCH$_3$), 6.50 (d, J = 7.9 Hz, 1H, Ar-H), 7.01 (d, J = 8.6 Hz, 2H, Ar-H), 7.08 (d, J = 7.9 Hz, 1H, Ar-H), 7.45-7.50 (m, 4H, Ar-H), 7.58 (d, J = 8.5 Hz, 2H, Ar-H), 8.17 (d, J = 7.6 Hz, 2H, Ar-H), 8.95 (s, 1H, CH=N), 13.55 (s, 1H, NH, D$_2$O exchangeable). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 13.87 (CH$_3$), 56.60 (-OCH$_3$), 100.13, 107.61, 113.66, 114.81, 119.58, 127.46, 127.87, 129.33, 129.89, 130.67, 138.49, 145.70, 147.76, 148.02, 149.96, 153.74, 156.43, 160.37 (Ar-C, CH=N). MS, m/z (%): 408 (M$^{+\cdot}$, 52). Analysis for

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$C_{25}H_{20}N_4O_2$ (408.46) Calcd.: C, 73.51; H, 4.94; N, 13.72%. Found: C, 73.83; H, 5.22; N, 13.49%.

N-(4-(4-Methoxyphenyl)-6-phenyl-1H-pyrazolo[3,4-b]pyridin-3-yl)-1-(thiophen-2-yl)methanimine (4b)

Yield 74%, pale yellow powder, m.p. 233 °C. IR (KBr, v_{max} cm^{-1}): 3427 (NH), 3050, 2923, 2840 (CH), 1595 (C=N). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.80 (s, 3H, -OCH$_3$), 7.03 (d, J = 7.3 Hz, 2H, Ar-H), 7.51-7.86 (m, 7H, Ar-H), 8.18 (d, J =7.6 Hz, 2H, Ar-H), 9.04 (s, 1H, CH=N), 13.56 (s, 1H, NH, D$_2$O exchangeable). ^{13}C NMR (DMSO-d_6, 125 MHz): 56.77 (-OCH$_3$), 102.87, 114.56, 119.51, 127.09, 127.65, 128.13, 128.85, 129.44, 130.01, 130.79, 131.40, 139.43, 141.66, 145.54, 147.02, 151.82, 156.10, 160.50 (Ar-C, CH=N). MS, m/z (%): 410 (M$^{+\cdot}$, 64). Anal. Calcd. (%) for $C_{24}H_{18}N_4OS$ (410.50): C, 70.22; H, 4.42; N, 13.65; S, 7.81. Found: C, 70.53; H, 4.75; N, 13.31; S, 7.44.

N-(6-Phenyl-4-(thiophen-2-yl)-1H-pyrazolo[3,4-b]pyridin-3-yl)-1-(p-tolyl)methanimine (4c)

Yield 81%, buff powder, m.p. 241-242 °C. IR (KBr, v_{max} cm^{-1}): 3431 (NH), 3094, 2921, 2852 (CH), 1590. (C=N). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 2.36 (s, 3H, CH$_3$), 7.24-7.25 (m, 1H, Ar-H), 7.32 (d, J = 7.6 Hz, 2H, Ar-H), 7.47-7.54 (m, 3H, Ar-H), 7.78 (s, 1H, Ar-H), 7.79 (d, J = 3.8 Hz, 1H, Ar-H), 7.84 (d, J = 7.6 Hz, 2H, Ar-H), 8.16 (d, J = 6.7 Hz, 2H, Ar-H), 8.27 (d, J = 2.9 Hz, 1H, Ar-H), 8.94 (s, 1H, CH=N), 13.66 (s, 1H, NH, D$_2$O exchangeable). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 21.75 (CH$_3$), 105.39, 113.78, 127.75, 127.98, 128.53, 129.41, 129.55, 129.78, 130.14, 132.08, 133.90, 138.55, 138.93, 142.59, 150.69, 154.70, 156.72, 161.16 (Ar-C, CH=N). MS, m/z (%): 394 (M$^{+\cdot}$, 43). Anal. Calcd. (%) for $C_{24}H_{18}N_4S$ (394.50): C, 73.07; H, 4.60; N, 14.20; S, 8.13. Found: C, 73.44; H, 4.87; N, 14.49; S, 7.79.

1-(4-Methoxyphenyl)-N-(6-phenyl-4-(thiophen-2-yl)-1H-pyrazolo[3,4-b]pyridin-3-yl)methanimine (4d)

Yield 71%, pale yellow powder, m.p. 209 °C. IR (KBr, v_{max} cm^{-1}): 3432 (NH), 3069, 2934, 2848 (CH), 1601 (C=N). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.78 (s, 3H, -OCH$_3$), 7.11 (d, J = 6.5 Hz, 2H, Ar-H), 7.21 (dd, J = 5.2, 4.8 Hz, 1H, Ar-H), 7.47-7.69 (m, 5H, Ar-H), 7.76 (s, 1H, Ar-H), 7.81 (d, J = 6.5 Hz, 2H, Ar-H), 8.19 (d, J = 5.5 Hz, 2H, Ar-H), 8.96 (s, 1H, CH=N), 13.54 (s, 1H, NH, D$_2$O exchangeable). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 55.95 (-OCH$_3$), 104.16, 114.17, 119.29, 127.15, 127.55, 127.74, 128.11, 128.85, 129.23, 129.57, 129.72, 130.44, 139.09, 141.72, 145.58, 147.02, 139.43, 141.66, 145.54,

147.12, 150.79, 151.70, 155.02, 159.15, 163.16 (Ar-C, CH=N). MS, m/z (%): 410 (M$^{+\cdot}$, 61). Anal. Calcd. (%) for $C_{24}H_{18}N_4OS$ (410.50): C, 70.22; H, 4.42; N, 13.65; S, 7.81. Found: C, 69.97; H, 4.09; N, 13.94; S, 8.06.

Synthesis of 3-chloro-1-(1*H*-pyrazolo[3,4-b]pyridin-3-yl)-azetidin-2-one derivatives 5a,b

To a stirred solution of the Schiff bases **4b,c** (1 mmol) in dry dioxane (30 mL) at 0-5 °C, chloroacetyl chloride (0.11 g, 1 mmol) in (10 mL) dry dioxane was added dropwise. Then triethyl amine (0.1 mL) was added to the reaction mixture and the stirring was continued for 3h. The reaction mixture was left over night at room temperature. The solvent evaporated under vacuum and the remained solid was treated with cold water, separated by filtration and recrystallized from acetone to give **5a,b**.

3-Chloro-1-(4-(4-methoxyphenyl)-6-phenyl-1H-pyrazolo[3,4-b]pyridin-3-yl)-4-(thiophen-2-yl)azetidin-2-one (5a)

Yield 69%, brown powder, m.p. 256-257 °C. IR (KBr, v_{max} cm^{-1}): 3429 (NH), 3056, 2924 (CH), 1668 (C=O), 771 (C–Cl). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.52 (d, J = 7.6 Hz, 1H, CH-N), 3.80 (s, 3H, -OCH$_3$), 3.95 (d, J = 7.6 Hz, 1H, CH-Cl), 7.01 (d, J = 8.6 Hz, 2H, Ar-H), 7.45-7.5 6 (m, 8H, Ar-H), 7.62 (d, J = 8.5 Hz, 1H, Ar-H), 8.18 (d, J = 9.2 Hz, 2H, Ar-H), 13.55 (s, 1H, NH, D$_2$O exchangeable). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 55.96 (-OCH$_3$), 60.93 (CH-N), 66.13 (CH-Cl), 101.36, 114.49, 119.45, 127.11, 127.62, 127.98, 128.79, 129.38, 129.97, 130.66, 131.39, 139.51, 145.61, 149.67, 150.33, 156.11, 160.48 (Ar-C) 163.04 (C=O). MS, m/z (%): 486 (M$^{+\cdot}$, 67). Analysis for $C_{26}H_{19}ClN_4O_2S$ (486.97) Calcd.: C, 64.13; H, 3.93; N, 11.51; S, 6.58%. Found: C, 63.87; H, 3.67; N, 11.27; S, 6.83%.

3-Chloro-1-(6-phenyl-4-(thiophen-2-yl)-1H-pyrazolo[3,4-b]pyridin-3-yl)-4-(p-tolyl)azetidin-2-one (5b)

Yield 81%, brown powder, m.p. 280 °C. IR (KBr, v_{max} cm^{-1}): 3421 (NH), 3087, 2925 (CH), 1662 (C=O), 769 (C-Cl). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 2.47 (s, 3H, CH$_3$), 3.59 (d, J =10.0 Hz, 1H, CH-N), 4.05 (d, J =10.0 Hz, 1H, CH-Cl), 6.90 (m, 1H, Ar-H), 7.30 (d, J = 7.9 Hz, 1H, Ar-H), 7.53-7.61 (m, 6H, Ar-H), 7.84-7.88 (m, 3H, Ar-H), 8.16 (d, J = 10.5 Hz, 2H, Ar-H), 12.72 (s, 1H, NH, D$_2$O exchangeable). MS, m/z (%): 470 (M$^{+\cdot}$, 46). Analysis for $C_{26}H_{19}ClN_4OS$ (470.98) Calcd.: C, 66.31; H, 4.07; N, 11.90; S, 6.81%. Found: C, 66.64; H, 4.45; N, 11.57; S, 7.11%.

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Synthesis of 1-alkyl/aryl-3-(1H-pyrazolo[3,4-b]pyridin-3-yl)urea derivatives 6a-c

A mixture of the 3-amino derivatives **3a,b** (1 mmol) and the appropriate alkyl or aryl isocyanate (1 mmol) in absolute ethanol (20 mL) was refluxed for 6h. Then the solvent was evaporated under vacuum and the residue was treated with water. The obtained solid was collected by filtration and recrystallized from EtOH to give the urea derivatives **6a-c**.

1-Butyl-3-(4-(4-methoxyphenyl)-6-phenyl-1H-pyrazolo[3,4-b]pyridin-3-yl)urea (6a)

Yield 61%, yellow powder, m.p. 224 °C. IR (KBr, ν max cm^{-1}): 3443, 3294, 3181 (NH), 3059, 2930, 2867 (CH), 1658 (C=O). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 0.85-1.18 (m, 7H, C$\underline{H}_3$(CH$_2$)$_2$), 2.97-3.15 (m, 2H, NHC$\underline{H}_2$), 3.80 (s, 3H, -OCH$_3$), 6.27 (s, 1H, NH$_2$, D$_2$O exchangeable), 7.09 (d, J = 7.6 Hz, 2H, Ar-H), 7.40-7.46 (m, 4H, Ar-H), 7.61 (d, J = 7.6 Hz, 2H, Ar-H), 8.12 (d, J = 7.6 Hz, 2H, Ar-H), 8.94 (s, 1H, NH, D$_2$O exchangeable), 12.32 (s, 1H, NH, D$_2$O exchangeable). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 14.08 (CH$_3$), 19.21, 29.22 (2CH$_2$), 41.38 (NHCH$_2$), 55.78 (-OCH$_3$), 102.66, 114.83, 119.73, 127.68, 129.21, 129.60, 129.90, 130.68, 139.58, 145.75, 147.82, 153.99, 156.08, 160.46 (Ar-C), 162.77 (C=O). MS, m/z (%): 415 (M$^{+\cdot}$, 74). Analysis for C$_{24}$H$_{25}$N$_5$O$_2$ (415.50) Calcd.: C, 69.38; H, 6.07; N, 16.86%. Found: C, 69.64; H, 6.35; N, 16.53%.

1-Isopropyl-3-(6-phenyl-4-(thiophen-2-yl)-1H-pyrazolo[3,4-b]pyridin-3-yl)urea (6b)

Yield 64%, brown powder, m.p. 230-231 °C. IR (KBr, ν$_{max}$ cm^{-1}): 3442, 3291, 3189 (NH), 3067, 2927, 2861 (CH), 1649 (C=O). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 1.21 (d, J = 7.4 Hz, 6H, CH(C$\underline{H}_3$)$_2$), 3.87 (m, 1H, C$\underline{H}$(CH$_3$)$_2$), 6.94 (s, 1H, NH, D$_2$O exchangeable), 7.32-7.35 (m, 1H, Ar-H), 7.53-7.60 (m, 4H, Ar-H), 7.87 (d, J = 8.0 Hz, 1H, Ar-H), 8.06 (d, J = 8.5 Hz, 2H, Ar-H), 8.87, 12.69 (2s, 2H, 2NH, D$_2$O exchangeable). MS, m/z (%): 377 (M$^{+\cdot}$, 35). Analysis for C$_{20}$H$_{19}$N$_5$OS (377.47) Calcd.: C, 63.64; H, 5.07; N, 18.55; S, 8.49%. Found: C, 63.41; H, 4.76; N, 18.89; S, 8.19%.

1-(3-Chlorophenyl)-3-(4-(4-methoxyphenyl)-6-phenyl-1H-pyrazolo[3,4-b]pyridin-3-yl)urea (6c)

Yield 65%, yellow powder, m.p. 255 °C. IR (KBr, ν$_{max}$ cm^{-1}): 3447, 3289, 3177 (NH), 3069, 2928 (CH), 1652 (C=O). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.83 (s, 3H, -OCH$_3$), 7.11 (d, J = 7.6 Hz, 2H, Ar-H), 7.32-7.87 (m, 10H, Ar-H), 8.12 (d, J = 8.5 Hz, 2H, Ar-H), 9.15, 10.98, 12.32 (3s, 3H, 3NH, D$_2$O exchangeable). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 55.74 (-OCH$_3$),

102.58, 114.77, 119.54, 120.03, 121.98, 127.22, 127.69, 127.98, 128.01, 129.23, 129.92, 131.32, 136.05, 139.03, 139.67, 145.72, 147.71, 153.88, 155.97, 160.23 (Ar-C), 162.18 (C=O). MS, m/z (%): 469 (M$^{+\cdot}$, 38). Analysis for C$_{26}$H$_{20}$ClN$_5$O$_2$ (469.93) Calcd.: C, 66.45; H, 4.29; N, 14.90%. Found: C, 66.76; H, 4.56; N, 14.66 %.

Synthesis of 1-alkyl/aryl-3-(1H-pyrazolo[3,4-b]pyridin-3-yl)thiourea derivatives 7a-d

A mixture of the 3-amino derivatives **3a,b** (1 mmol) and the appropriate alkyl or aryl isothiocyanate (1 mmol) in absolute ethanol (20 mL) was refluxed for 8h. The formed solid was collected by filtration and recrystallized from dioxane to give the thiourea derivatives **7a-d**.

1-Cyclohexyl-3-(4-(4-methoxyphenyl)-6-phenyl-1H-pyrazolo[3,4-b]pyridin-3-yl)thiourea (7a)

Yield 82%, pale yellow powder, m.p. 217-218 °C. IR (KBr, ν$_{max}$ cm^{-1}): 3454, 3384, 3191 (NH), 3059, 2925 (CH), 1596 (C=N), 1112 (C=S). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 1.19-1.61 (m, 10H, 5CH$_2$-cyclohexyl), 1.80 (m, 1H, C$\underline{H}$-cyclohexyl), 3.81 (s, 3H, -OCH$_3$), 7.10 (d, J = 8.6 Hz, 2H, Ar-H), 7.44-7.50 (m, 4H, Ar-H), 7.61 (d, J = 8.6 Hz, 2H, Ar-H), 8.17 (d, J = 9.8 Hz, 2H, Ar-H), 8.50 (s, 1H, NH, D$_2$O exchangeable), 12.31 (s, 1H, NH, D$_2$O exchangeable), 13.52 (s, 1H, NH, D$_2$O exchangeable). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 25.07, 25.69, 26.02, 33.10 (5$\underline{C}$H$_2$-cyclohexyl), 54.63 ($\underline{C}$H-cyclohexyl), 55.73 (-OCH$_3$), 106.25, 114.00, 119.20, 127.21, 127.86, 129.45, 129.81, 132.65, 139.92, 147.58, 153.74, 155.42, 160.37 (Ar-C), 180.48 (C=S). MS, m/z (%): 457 (M$^{+\cdot}$, 78). Analysis for C$_{26}$H$_{27}$N$_5$OS (457.60) Calcd.: C, 68.24; H, 5.95; N, 15.30; S, 7.01%. Found: C, 68.61; H, 5.68; N, 14.91; S, 7.35%.

1-Benzyl-3-(4-(4-methoxyphenyl)-6-phenyl-1H-pyrazolo[3,4-b]pyridin-3-yl)thiourea (7b)

Yield 79%, yellowish powder, m.p. 226 °C. IR (KBr, ν$_{max}$ cm^{-1}): 3445, 3394, 3214 (NH), 3078, 2921 (CH), 1597 (C=N), 1176 (C=S). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.81 (s, 3H, -OCH$_3$), 4.68 (s, 2H, C$\underline{H}_2$-Ph), 7.04 (d, J = 8.6 Hz, 2H, Ar-H), 7.17-7.27 (m, 5H, Ar-H), 7.46-7.50 (m, 3H, Ar-H), 7.64 (s, 1H, Ar-H), 7.67 (d, J = 8.6 Hz, 2H, Ar-H), 8.17 (d, J = 7.6 Hz, 2H, Ar-H), 8.68 (broad s, 1H, 1NH, D$_2$O exchangeable), 9.00 (broad s, 1H, 1NH, D$_2$O exchangeable), 13.59 (s, 1H, NH, D$_2$O exchangeable). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 48.43 ($\underline{C}$H$_2$-Ph), 55.88 (-OCH$_3$), 106.02, 114.78, 114.93, 119.49, 127.42, 127.88, 128.75, 129.36, 130.07, 130.80, 139.18, 139.43, 139.59, 145.46, 153.44, 156.97, 160.53 (Ar-C), 181.47 (C=S).

MS, m/z (%): 465 (M$^{+\cdot}$, 59). Analysis for $C_{27}H_{23}N_5OS$ (465.58) Calcd.: C, 69.66; H, 4.98; N, 15.04; S, 6.89%. Found: C, 69.31; H, 4.69; N, 15.32; S, 6.61%.

1-phenyl-3-(6-phenyl-4-(thiophen-2-yl)-1H-pyrazolo[3,4-b]pyridin-3-yl)thiourea (7c)

Yield 76%, yellowish powder, m.p. 232-233 °C. IR (KBr, v_{max} cm^{-1}): 3448, 3400, 3187 (NH), 3040, 2923, (CH), 1586 (C=N), 1169 (C=S). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 7.08-7.23 (m, 6H, Ar-H), 7.47-7.53 (m, 3H, Ar-H), 7.71 (s, 1H, Ar-H), 7.75 (d, J = 2.8 Hz, 1H, Ar-H), 7.80 (d, J = 4.7 Hz, 1H, Ar-H), 8.15 (d, J = 7.6 Hz, 2H, Ar-H), 9.76 (broad s, 2H, 2NH, D_2O exchangeable), 13.76 (s, 1H, NH, D_2O exchangeable). MS, m/z (%): 427 (M$^{+\cdot}$, 67). Analysis for $C_{23}H_{17}N_5S_2$ (427.54) Calcd.: C, 64.61; H, 4.01; N, 16.38; S, 15.00%. Found: C, 64.93; H, 4.39; N, 16.04; S, 14.68%.

1-(4-Methoxyphenyl)-3-(6-phenyl-4-(thiophen-2-yl)-1H-pyrazolo[3,4-b]pyridin-3-yl)thiourea (7d)

Yield 79%, pale yellow powder, m.p. 219 °C. IR (KBr, v_{max} cm^{-1}): 3440, 3399, 3211 (NH), 3090, 2936, (CH), 1543 (C=N), 1174 (C=S). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.73 (s, 3H, CH_3O), 6.81-6.98 (m, 3H, Ar-H), 7.24-7.51 (m, 6H, Ar-H), 7.70-7.81 (m, 2H, Ar-H), 8.14 (d, J = 6.6 Hz, 2H, Ar-H), 9.53 (broad s, 2H, 2NH, D_2O exchangeable), 13.72 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 56.08 (CH_3O), 106.78, 114.00, 114.20, 120.21, 126.62, 127.40, 127.74, 129.43, 130.08, 138.93, 140.03, 142.94, 150.21, 154.92, 156.58, 157.08, 160.02 (Ar-C), 180.83 (C=S). MS, m/z (%): 457 (M$^{+\cdot}$, 33). Analysis for $C_{24}H_{19}N_5OS_2$ (457.57) Calcd.: C, 63.00; H, 4.19; N, 15.31; S, 14.01%. Found: C, 63.32; H, 4.50; N, 15.64; S, 14.38 %.

Synthesis of 3-alkyl/aryl-2-((1H-pyrazolo[3,4-b]pyridin-3-yl)imino)thiazolidin-4-one derivatives 8a-d

A mixture of the thiourea derivatives **7a-d** (1 mmol) and ethyl 2-chloroacetate (0.12 g, 1 mmol) in absolute ethanol (30 mL) containing anhydrous sodium acetate (0.5 g) was refluxed for 5h. The formed solid was collected by filtration, washed with water and recrystallized from acetone to give the thiazolidin-4-one derivatives **8a-d**.

3-Cyclohexyl-2-((4-(4-methoxyphenyl)-6-phenyl-1H-pyrazolo[3,4-b]pyridin-3-yl)imino)thiazolidin-4-one (8a)

Yield 72%, yellowish white powder, m.p. 238 °C. IR (KBr, v_{max} cm^{-1}): 3432 (NH), 3087, 2924, 2852 (CH), 1709 (C=O), 1588 (C=N). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 1.02-2.06 (m, 10H, $5CH_2$-cyclohexyl), 3.79 (s, 3H, -OCH_3), 4.10-4.13 (m, 1H, C<u>H</u>-cyclohexyl), 4.59 (s, 2H, SCH_2), 7.04 (d, J = 8.6 Hz, 2H, Ar-H), 7.46-7.50 (m, 3H, Ar-H), 7.58 (s, 1H, Ar-H), 7.63 (d, J = 8.6 Hz, 2H, Ar-H), 8.17 (d, J = 6.7 Hz, 2H, Ar-H), 13.46 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 25.33, 25.80, 28.02, 33.04 (5<u>C</u>H_2-cyclohexyl), 38.21 (SCH_2), 55.66 (-OCH_3), 61.21 (<u>C</u>H-cyclohexyl), 55.73 (-OCH_3), 102.63, 114.12, 119.14, 127.59, 127.82, 129.31, 129.94, 131.42, 139.25, 139. 57, 146.22, 147.16, 153.88, 156.52, 157.62, 160.26 (Ar-C, C=N), 173.03 (C=O). MS, m/z (%): 497 (M$^{+\cdot}$, 69). Analysis for $C_{28}H_{27}N_5O_2S$ (497.62) Calcd.: C, 67.58; H, 5.47; N, 14.07; S, 6.44%. Found: C, 67.32; H, 5.15; N, 14.35; S, 6.77%.

3-Benzyl-2-((4-(4-methoxyphenyl)-6-phenyl-1H-pyrazolo[3,4-b]pyridin-3-yl)imino)thiazolidin-4-one (8b)

Yield 81%, white powder, m.p. 241°C. IR (KBr, v_{max} cm^{-1}): 3443 (NH), 3088, 2927 2846 (CH), 1712 (C=O), 1596 (C=N). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.82 (s, 3H, -OCH_3), 4.43 (s, 2H, SCH_2), 5.67 (s, 2H,C<u>H</u>$_2$-Ph), 7.08 (d, J = 7.6 Hz, 2H, Ar-H), 7.21-7.31 (m, 5H, Ar-H), 7.48-7.53 (m, 3H, Ar-H), 7.66 (s, 1H, Ar-H), 7.74 (d, J = 7.6 Hz, 2H, Ar-H), 8.11 (d, J = 6.4 Hz, 2H, Ar-H), 13.44 (s, 1H, NH, D_2O exchangeable). MS, m/z (%): 505 (M$^{+\cdot}$, 31). Analysis for $C_{29}H_{23}N_5O_2S$ (505.60) Calcd.: C, 68.89; H, 4.59; N, 13.85; S, 6.34%. Found: C, 69.19; H, 4.81; N, 14.11; S, 6.77 %.

3-Phenyl-2-((6-phenyl-4-(thiophen-2-yl)-1H-pyrazolo[3,4-b]pyridin-3-yl)imino)thiazolidin-4-one (8c)

Yield 81%, pale yellow powder, m.p. 258-259 °C. IR (KBr, v_{max} cm^{-1}): 3390 (NH), 3094, 2923(CH), 1710 (C=O), 1585 (C=N). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 4.13 (s, 2H, SCH_2), 7.39-7.78 (m, 12H, Ar-H), 8.10 (d, J = 8.6 Hz, 2H, Ar-H), 13.60 (s, 1H, NH, D_2O exchangeable). MS, m/z (%): 467 (M$^{+\cdot}$, 53). Analysis for $C_{25}H_{17}N_5OS_2$ (467.57) Calcd.: C, 64.22; H, 3.66; N, 14.98; S, 13.71%. Found: C, 63.94; H, 3.34; N, 15.25; S, 13.96 %.

3-(4-Methoxyphenyl)-2-((6-phenyl-4-(thiophen-2-yl)-1H-pyrazolo[3,4-b]pyridin-3-yl)imino)thiazolidin-4-one (8d)

Yield 71%, white powder, m.p. 247 °C. IR (KBr, v_{max} cm^{-1}): 3398 (NH), 3047, 2921, 2856.3 (CH), 1718 (C=O), 1590 (C=N). ^{1}H NMR (DMSO-d6, 500 MHz): δ 3.68 (s, 3H, -OCH_3), 4.11 (s, 2H, SCH_2), 7.08

(d, J = 8.6 Hz, 2H, Ar-H), 7.22-7.24 (m, 1H, Ar-H), 7.28 (d, J = 8.6 Hz, 2H, Ar-H), 7.47-7.53 (m, 3H, Ar-H), 7.70 (s, 1H, Ar-H), 7.74 (d, J = 3.8 Hz, 1H, Ar-H), 7.80 (d, J = 4.8 Hz, 1H, Ar-H), 8.14 (d, J = 7.6 Hz, 2H, Ar-H), 13.72 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 37.94 (SCH$_2$), 55.77 (-OCH$_3$), 106.48, 114.63, 120.72, 126.54, 127.44, 127.66, 127.80, 128.75, 128.98, 129.24, 129.45, 129.88, 130.73, 138.94, 139.15, 141.35, 146.57, 153.92, 156.75 (Ar-C, C=N), 171.89 (C=O). MS, m/z (%): 497 (M$^+$, 41). Analysis for $C_{26}H_{19}N_5O_2S_2$ (497.59) Calcd.: C, 62.76; H, 3.85; N, 14.07; S, 12.89%. Found: C, 62.41; H, 3.56; N, 13.79; S, 13.18 %.

Synthesis of 4-(1*H*-pyrazolo[3,4-*b*]pyridin-3-yl)-1-thia-4-azaspiro[4.5]decan-3-one derivatives 9a-c

A mixture of the 3-amino derivatives **3a,b** (2 mmol), cyclohexanone or 4-methylcyclohexanone (2 mmol) and thioglycolic acid (0.184 g, 2 mmol) in dry benzene (50 mL) was refluxed with stirring for 8h. The excess solvent was evaporated till dryness under reduced pressure. The oily residue was dissolved in hot CHCl$_3$ (30 mL), then petroleum ether 40/60 (20 mL) was added with stirring. The obtained solid was filtered off and recrystallized from ethanol to give **9a-c**.

4-(4-(4-Methoxyphenyl)-6-phenyl-1H-pyrazolo[3,4-b]pyridin-3-yl)-1-thia-4-azaspiro[4.5]decan-3-one (9a)

Yield 61%, brown powder, m.p. 181-182 °C. IR (KBr, ν$_{max}$ cm^{-1}): 3421 (NH), 3072, 2929, 2857 (CH), 1701 (C=O). ^1H NMR (DMSO-d_6, 500 MHz): δ 1.32-1.97 (m, 10H, 5CH$_2$), 3.51 (s, 2H, SCH$_2$), 3.82 (s, 3H, -OCH$_3$), 7.09 (d, J = 7.2 Hz, 2H, Ar-H), 7.43-7.52 (m, 4H, Ar-H), 7.64 (d, J = 7.2 Hz, 2H, Ar-H), 8.12 (d, J = 8.5 Hz, 2H, Ar-H), 12.39 (s, 1H, NH, D_2O exchangeable). MS, m/z (%): 470 (M$^+$, 38). Analysis for $C_{27}H_{26}N_4O_2S$ (470.59) Calcd.: C, 68.91; H, 5.57; N, 11.91; S, 6.81%. Found: C, 68.77; H, 5.22; N, 11.64; S, 6.59 %.

4-(6-Phenyl-4-(thiophen-2-yl)-1H-pyrazolo[3,4-b]pyridin-3-yl)-1-thia-4-azaspiro[4.5]decan-3-one (9b)

Yield 59%, brick red powder, m.p. 172 °C. IR (KBr, ν$_{max}$ cm^{-1}): 3432 (NH), 3078, 2923, 2856 (CH), 1698 (C=O). ^1H NMR (DMSO-d_6, 500 MHz): δ 1.29-2.12 (m, 10H, 5CH$_2$), 3.58 (s, 2H, SCH$_2$), 7.24-7.29 (m, 1H, Ar-H), 7.47-7.82 (m, 6H, Ar-H), 8.11 (d, J = 8.6 Hz, 2H, Ar-H), 12.32 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 25.96, 26.74, 34.98 (5CH$_2$), 41.62 (SCH$_2$), 68.02 (spiro C), 101.02, 121.89, 127.38, 127.80, 128.39, 128.76, 129.49, 138.67, 139.04, 142.43, 150.69, 150.98, 154.55 (Ar-C), 171.23 (C=O). MS, m/z (%):

446 (M$^+$, 39). Analysis for $C_{24}H_{22}N_4OS_2$ (446.59) Calcd.: C, 64.55; H, 4.97; N, 12.55; S, 14.36 %. Found: C, 64.82; H, 4.68; N, 12.31; S, 14.65%.

4-(4-(4-Methoxyphenyl)-6-phenyl-1H-pyrazolo[3,4-b]pyridin-3-yl)-8-methyl-1-thia-4-azaspiro[4.5]decan-3-one (9c)

Yield 62%, scarlet red powder, m.p. 175-176 °C. IR (KBr, ν$_{max}$ cm^{-1}): 3424 (NH), 3063, 2923 (CH), 1708 (C=O). ^1H NMR (DMSO-d_6, 500 MHz): δ 0.87 (d, J = 7.7 Hz, 3H, CH$_3$), 1.23-2.05 (m, 9H, 4CH$_2$, -CH), 3.62 (s, 2H, SCH$_2$), 3.82 (s, 3H, -OCH$_3$), 7.10 (d, J = 8.6 Hz, 2H, Ar-H), 7.41-7.49 (m, 4H, Ar-H), 7.61 (d, J = 8.6 Hz, 2H, Ar-H), 8.12 (d, J = 7.0 Hz, 2H, Ar-H), 12.41 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 22.67 (CH$_3$), 26.35, 26.88, 35.39 (4CH$_2$, CH), 41.23 (SCH$_2$), 55.82 (-OCH$_3$), 67.87 (spiro C), 100.01, 114.84, 119.29, 127.69, 127.88, 129.21, 129.69, 130.70, 139.53, 145.77, 147.56, 153.96, 156.09, 160.49 (Ar-C), 170.95 (C=O). MS, m/z (%): 484 (M$^+$, 55). Analysis for $C_{28}H_{28}N_4O_2S$ (484.62) Calcd.: C, 69.40; H, 5.82; N, 11.56; S, 6.62%. Found: C, 69.77; H, 6.12; N, 11.31; S, 6.34%.

3.2. Antimicrobial and Anti-MRSA Assay

All the new pyrazolopyridine derivatives (**3a,b-9a-c**) were screened for there *in vitro* antibacterial activity against six bacterial strains (*S. aureus* 25923, *B. subtilis* 6633, *B. cereus* 33018, *E. coli* 8739, *S. typhimurium* 14028 *P. aeruginosa* 27853) compared with the reference antibiotic Gentamicin. Also, they were screened for their antifungal activity against six fungal strains (*C. albicans* 10231, *C. tropicals* 750, *S. cerevisiae, M. Phaseolina, A. flavus, A. niger EM77*). In addition, the most active compounds (**3a**, **3b**, **4a**, **4d**, **6a**, **6c**, **9a** and **9c**) were evaluated against MRSA. The diameter of inhibition zone (DIZ) assay was performed by agar disk diffusion method [53]. The Minimum Inhibitory Concentration (MIC) of the compounds was then evaluated against bacterial strains, fungal strains and MRSA, using broth dilution method [54]. Whereas, two-fold serial dilution at the concentrations (128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 µg/ml) was used to investigate the MIC values. The determined values of DIZ in (mm) and the MIC values in (µg/mL) for the target compounds and the reference drug against the tested bacterial and fungal strains were listed in Table 1 and Table 2, respectively. While, the MIC values of the selected compounds against MRSA were listed in Table 3.

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3.3. DHFR Inhibition Assay

The inhibitory activity of the selected compounds (**3a**, **3b**, **4a**, **4d**, **6a**, **6c**, **9a** and **9c**) against DHFR enzyme were screened by using DHFR kit (BioVision, Catalog #K247-100). The assay was applied as indicated in the DHFR assay kit. The solutions of the tested compounds and the reference inhibitor TMP were prepared by dissolving each tested compounds in DMSO and then diluted to different concentrations, (2 μL) of the tested compounds were added into wells of 96-well clear plate. Prepare a 15-fold dilution of DHFR substrate (i.e. Dilute 40 μl of DHFR stock substrate with 560 μl DHFR Assay Buffer), vortex briefly and keep on ice. Add 60 μl of diluted DHFR substrate to each well containing the test samples and enzyme control, the total volume should be 200 μl. The absorbance was measured immediately at 340 nm in kinetic mode for 10-20 min at room temperature. The results were expressed as the mean of the IC_{50} values (the concentration of the tested compound that inhibits 50% of DHFR) in (μM) and standard deviation of triplicate samples [55]. The determined IC_{50} values are listed in Table 4.

3.4. Molecular Docking

The molecular docking studies were performed using Molecular Operating Environment (MOE, 2020.0901) software. All structural geometry optimization and energy minimizations were performed with MOE until an RMSD gradient of 0.05 $kcal\cdot mol^{-1}Å^{-1}$ with MMFF94x force field and the partial charges were automatically calculated. The X-ray crystallographic structure of dihydrofolate reductase in complex with trimethoprim (PDB ID: 6XG5) was retrieved from the protein data bank. All water molecules and ligands that are not involved in the inhibitor (trimethoprim) binding were first removed, then the protein was prepared for docking using *LigX* protocol in MOE using the default options Triangle Matcher placement method and London dG scoring function were used for docking [56].

4. Conclusion

A new series of 3-substituted-pyrazolopyridine compounds (**3a,b–9a-c**) was synthesized and evaluated for their antimicrobial activity against several bacterial and fungal strains. The obtained results of the MIC values showed significant antimicrobial activity of the tested compounds. The most potent activity was showed by the

3-amino- derivatives **3a,b**; the Schiff bases **4a,d**; the urea derivatives **6a,c**; and the 1-thia-4-azaspiro[4.5]decan-3-one derivatives **9a,c**; which revealed activity equal or more potent than the reference Gentamicin against most of the tested microorganisms with MIC value ranging from 2 μg/mL to 32 μg/mL. Moreover, the most potent compounds (**3a,b**; **4a,d**; **6a,c** and **9a,c**) were tested against methicillin-resistant *Staphylococcus aureus* (MRSA). Compounds **4d**, **6c** and **9c** showed the most potent anti-MRSA activity with MIC values 4, 8, 8 μg/mL, respectively. Also, the inhibitory activity against dihydrofolate reductase (DHFR) of the most potent compounds was determined. The tested compounds showed significant inhibition to the target enzyme with IC_{50} values ranging from 0.72 ± 0.04 to 7.03 ± 0.34 μM, compared with the IC_{50} value 5.54 ± 0.31 μM of the reference inhibitor (Trimethoprim). Whereas, **4d**, **6c** and **9c** exhibited the most potent inhibition to DHFR with IC_{50} values 0.72 ± 0.04, 0.95 ± 0.05 and 1.09 ± 0.05 μM, respectively. In addition, molecular docking studies were performed to the most active new pyrazolopyridine compounds to determine the binding affinity and their binding pattern in the DHFR active sites. The results of the molecular study revealed the promising binding affinity of the tested compounds with energy scores range from −10.39 to −16.52 kcal/mol. Whereas, the highest binding affinity was revealed by the target compounds **4d**, **6c** and **9c** of the energy scores −15.21, −14.44 and −16.52, respectively.

The present study showed the significant antimicrobial activity of the target 3-substituted-pyrazolopyridine compounds, especially compounds **4d**, **6c** and **9c** which revealed broad spectrum antimicrobial activity and potent inhibitory activity to the target DHFR enzyme.

5. Conflicts of interest
"There are no conflicts to declare".

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