Synthesis and Biological Evaluation of New Pyridothienopyrimidine Derivatives as Antibacterial Agents and Escherichia coli Topoisomerase II Inhibitors

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Abstract: The growing resistance of bacteria to many antibiotics that have been in use for several decades has generated the need to discover new antibacterial agents with structural features qualifying them to overcome the resistance mechanisms. Thus, novel pyridothienopyrimidine derivatives (2a,b–a,b) were synthesized by a series of various reactions, starting with 3-aminothieno[2,3-b]pyridine-2-carboxamides (1a,b). Condensation of compounds 1a,b with cyclohexanone gave 1′H-spiro[cyclohexane-1,2′-pyrido[3′,2′:4,5]thieno[3,2-d]pyrimidin]-4′(3′H)-ones (2a,b), which in turn were utilized to afford the target 4-substituted derivatives (3a,b–8a,b). In vitro antibacterial activity evaluations of all the new compounds (2a,b–8a,b) were performed against six strains of Gram-negative and Gram-positive bacteria. The target compounds showed significant antibacterial activity, especially against Gram-negative strains. Moreover, the compounds (2a,b; 3a,b; 4a,b; and 5a,b) that exhibited potent activity against Escherichia coli were selected to screen their inhibitory activity against Escherichia coli topoisomerase II (DNA gyrase and topoisomerase IV) enzymes. Compounds 4a and 4b showed potent dual inhibition of the two enzymes with IC50 values of 3.44 µM and 5.77 µM against DNA gyrase and 14.46 µM and 14.89 µM against topoisomerase IV, respectively. In addition, docking studies were carried out to give insight into the binding mode of the tested compounds within the E. coli DNA gyrase B active site compared with novobiocin.

Keywords: pyridothienopyrimidines; antibacterial activity; enzyme inhibition; DNA gyrase; topoisomerase IV; molecular docking

1. Introduction

Nowadays, the danger of infectious diseases is again on the rise because of the persistent evolution of antibiotic resistance, which will, over time, be a significant threat to health worldwide [1,2].

Antimicrobial-resistant infections are predicted to cause millions of deaths in the coming decades, unless suitable actions are taken to overcome this risk [3]. Thus, there is an urgent need to discover
new classes of antimicrobial agents with novel mechanisms of action that can circumvent the resistance problem [4]. Inhibition of bacterial DNA replication enzymes is considered a promising strategy for fighting antimicrobial resistance [5]. Among these enzymes are two bacterial type II topoisomerases, DNA gyrase and topoisomerase IV, which play a significant role in bacterial cell cycle progression [6]. Although the two enzymes have similar structures, each of them has a distinct function during DNA replication: DNA gyrase remains unique in its role to introduce negative supercoiling into DNA, while the critical function of topoisomerase IV is to decatenate daughter chromosomes following DNA replication [6,7].

In clinical use, there are two main classes of antibiotics that target topoisomerase II enzymes, the first is the aminocoumarins class and the second is the quinolones class [8]. Aminocoumarins, such as novobiocin and clorobiocin, inhibit the ATPase domain of the enzymes [9], but their manufacturing and clinical usage have been limited due to their poor pharmacological properties and mammalian cytotoxicity [10]. While, quinoline antibiotics, such as ciprofloxacin and norfloxacin, inhibit topoisomerase II enzymes by binding to a DNA–enzyme complex that leads to stabilizing the DNA double-strand breaks and causes rapid death of the bacterial cell [11,12]. However, after decades of using quinolones in treating a variety of bacterial infections, the number of bacterial strains resistant to this important class of antimicrobial agents has seen an unremitting increase [13]. The most prevalent quinolone-resistance mechanism is associated with specific mutations in the DNA gyrase and/or topoisomerase IV enzymes that reduce the drug’s binding ability to the enzyme–DNA complex, resulting in significant weakness in the quinolone’s therapeutic activity [11,14]. Therefore, some recent efforts to counter microbial resistance mechanisms have included design and synthesis of non-quinolone-based inhibitors able to target varied active sites in bacterial type II topoisomerases and exhibit more potent antimicrobial activity [15–18].

On the other hand, many reports have documented the important biological activities of thieno[2,3-b]pyridine derivatives in anticancer [19–21], antimicrobial [22,23], antiviral [24,25], anti-inflammatory [26] and osteogenic [27] activities. As a result, thieno[2,3-b]pyridine compounds and their fused derivatives with the bioactive pyrimidine ring [28,29] have attracted great interest in the last decade; several pyrido[3′,2′:4,5]thieno[3,2-d]pyrimidine derivatives were synthesized and gave significant pharmacological properties as antimicrobial [30–32], anticancer [33,34] and protein kinase inhibitors [35,36]. Moreover, some thieno[2,3-b]pyridine derivatives [37] and pyrimidine-based compounds [38,39] have been discovered in recent years as potent DNA gyrase and/or topoisomerase IV inhibitors, which encourage the design and synthesis of novel thienopyridine-fused pyrimidine compounds to obtain enhanced antibacterial activity (Figure 1).

Based on the above, the current work includes the synthesis of a series of 1′H-spiro[cyclohexane-1,2′-pyrido[3′,2′:4,5]thieno[3,2-d]pyrimidine] derivatives as new antibacterial agents. The target compounds were linked at position 4 of the pyridothienopyrimidine ring system to different moieties, such as piperazine, morpholine, acetohydrazide and aryl hydrazone, which have valuable pharmacological activities [40–43]. All the new compounds were screened for their antibacterial activity against different Gram-positive and Gram-negative bacterial strains. Then, the most active compounds against Gram-negative bacteria were tested for their inhibitory activity of Escherichia coli DNA gyrase and topoisomerase IV enzymes. Molecular docking studies were also performed for elucidation of the mode of binding of these compounds in the active site of DNA gyrase B kinase.
2. Results and Discussion

2.1. Chemistry

The synthesis of the target pyridothienopyrimidines (2a–8a,b) was achieved via various reactions, as depicted in Scheme 1; Scheme 2. The starting 3-amino-6-phenylthieno[2,3-b]pyridine-2-carboxamides 1a,b were prepared \cite{44,45} and underwent a cyclocondensation reaction with cyclohexanone in refluxing N,N-dimethylformamide containing anhydrous zinc chloride to produce 1'H-spiro[cyclohexane-1,2'-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin]-4'(3'H)-ones 2a,b. The chemical structures of 2a,b were confirmed by their $^1$H NMR spectra, which revealed signals at 1.21–2.02 ppm, corresponding to the 5CH$_2$ protons of the spiro cyclohexane ring and two signals corresponding to the 2NH groups of the pyrimidinone ring at 4.53 and 7.90 ppm and 4.90 and 7.88 ppm for 2a and 2b, respectively. Furthermore, the $^{13}$C NMR spectra of 2a,b displayed three signals at 21.6–36.2 ppm of the 5CH$_2$ carbons and a signal corresponding to the spiro carbon of 2a at 69.9 ppm and a signal at 69.8 ppm for that of 2b. Subsequently, treatment of 2a,b with a refluxing POCl$_3$/PCl$_5$ mixture gave 4'-chloro derivatives 3a,b. Then the target 1'H-spiro[cyclohexane-1,2'-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin]-4'-amines 4a–e were obtained by carrying out a nucleophilic substitution reaction between 4'-chloro derivatives 3a,b and different amines (furan-2-amine, 1-methylpiperazine, morpholine, 4-acetylaniline and 4-fluoroaniline) in boiling DMF (Scheme 1). In the $^1$H NMR spectrum of 4b, the protons of the N-methylpiperazinyl moiety were verified by three signals at 2.21, 2.59 and 3.75 ppm. The $^{13}$C NMR spectrum of 4c also showed the carbons of a morpholine ring as two signals at 45.5 and 66.4 ppm, corresponding to the 2CH$_2$N and 2CH$_2$O moieties, respectively.
Upon treatment of 2a,b with phosphorus pentasulfide in refluxing pyridine, the pyrido[3′,2′:4,5]thieno[3,2-d]pyrimidine-4′(3′H)-thiones 5a,b were formed. IR spectra of 5a,b displayed new bands at 1246.2 cm⁻¹ and 1243.2 cm⁻¹, corresponding to the C=S groups of 5a and 5b, besides the disappearance of the C=O bands of 2a,b at 1658.2 and 1659.4 cm⁻¹, respectively. The signal at 181.3 ppm in the ¹³C NMR spectrum of 5a and at 182.1 ppm in the ¹³C NMR spectrum of 5b also assisted the presence of the C=S carbon. Subsequent reaction of 4′(3′H)-thiones 5a,b with ethyl 2-chloroacetate in DMF containing a catalytic amount of anhydrous sodium carbonate afforded the formation of ethyl 2-(pyrido[3′,2′:4,5]thieno[3,2-d]pyrimidin-4′-yl)thio)acetates 6a,b via S-alkylation at position 4. The steric hindrance of the bulky spiro cyclohexane moiety was a possible explanation for the forbidding of N-alkylation at position 1. ¹H NMR spectra of 6a showed a singlet signal at 3.89 ppm of SCH₂ protons, while the CH₂ and CH₂O protons of the ester group were represented by the triplet signal at 1.07 ppm and quartet signal at 4.08 ppm, respectively. The ¹³C NMR spectrum of 6b also confirmed the occurrence of S-alkylation through the disappearance of the C=S signal at 182.1 ppm, adding to the presence of four new signals at 32.8, 168.7, 61.5 and 14.1 ppm, corresponding to the carbon of SCH₂ and the three carbons (C=O, OCH₂ and CH₃) of the ester group, respectively. Then, the condensation reaction of the esters 6a,b with hydrazine hydrate in refluxing ethanol gave the acetohydrazide derivatives 7a,b. Furthermore, N′-(arylidene)-acetohydrazide derivatives 8a,b were obtained by carrying out a nucleophilic addition–elimination reaction between 7a and an aromatic
aldehyde (4-N,N-dimethylaminobenzaldehyde and/or thiophene-2-carbaldehyde) in refluxing glacial acetic acid (Scheme 2).

![Scheme 2. Synthesis of the pyridothienopyrimidine derivatives 5a,b–8a,b.](image_url)

In the $^1$H NMR spectrum of 7a, beside the absence of ester signals, the hydrazide moiety was represented by two signals at 4.62 ppm and 9.18 ppm, corresponding to the NH$_2$ and NH groups, respectively. The $^1$H NMR spectrum of $N'$-(4-(dimethylamino)benzylidene) derivative 8a also supported the presence of the arylidine moiety by a signal at 3.18 ppm for the six protons dimethylamino group (N(CH$_3$)$_2$) and a signal at 8.43 ppm for the proton of the azomethine group (CH=N), as well as the signals corresponding to new aromatic protons. Moreover, the $^{13}$C NMR spectrum of 8a showed a signal at 44.7 ppm, corresponding to the two carbons of the N(CH$_3$)$_2$ group. The chemical structures of the new pyridothienopyrimidine derivatives (2a,b–8a,b) were confirmed by IR, $^1$H NMR, $^{13}$C NMR and mass spectra, in addition to the correct results of their elemental microanalyses (Supplementary Materials: NMR spectra of compounds 2a,b–8a,b; Figures S1–S28).

2.2. Antibacterial Activity

The results of the in vitro antibacterial activity evaluation (MIC values in $\mu$g/mL) of the target pyridothienopyrimidine compounds (2a,b–8a,b), listed in Table 1 and Figure 2, revealed the significant activity of the majority of these compounds against the tested Gram-positive bacteria (Staphylococcus aureus 25923, Bacillus cereus 33018 and Bacillus subtilis 6633) and Gram-negative bacteria (Escherichia coli 8739, Salmonella typhimurium 14028 and Pseudomonas aeruginosa 27853) compared with amoxicillin trihydrate as a reference drug. In particular, the activity of the target compounds against the Gram-negative strains were more potent than their activity against the Gram-positive strains. In turn,
pyrido[3',2':4,5]thieno[3,2-d][pyrimidin]-4'(3'H)-ones 2a,b showed potent activity against all tested Gram-negative strains (MIC = 15.63 µg/mL), equal to that of the reference drug, but they had no activity against all tested Gram-positive strains at the highest used concentration (125 µg/mL). The 4'-chloro derivatives 3a,b also revealed inhibition activity against Gram-negative strains, the same as amoxicillin, with enhancement in the activity against the Gram-positive strains, especially against 4'-amine derivatives 4a–e, especially against B. subtilis (MIC = 15.63 µg/mL). The antibacterial activity of 4a–e against the Gram-negative strains varied from potent to moderate, with MIC values ranging from 7.81 to 31.25 µg/mL. Moreover, 4’-(4-methylpiperazin-1-yl) derivative 4b showed potent activity against all the tested bacterial strains, with MIC values ranging from 7.81 to 15.63 µg/mL, which was equal in potency or more potent than that of amoxicillin. The conversion of 4'(3'H)-ones 2a,b to 4'(3'H)-thiones 5a,b also enhanced the antimicrobial activity against Gram-positive bacteria and showed inhibition activity ranging from potent to moderate against the three tested microorganisms, with MIC values ranging from 15.63 to 31.25 µg/mL. On the reverse, the esters 6a,b showed dramatic lowering in their activity; they were inactive against the Gram-positive strains and gave moderate or weak activity against the Gram-negative strains (MIC = 31.25 µg/mL and 62.5 µg/mL).

Table 1. Minimum Inhibitory Concentration (MIC) values in µg/mL of all the target compounds against different bacterial strains.

| Compound | Gram-Positive Bacteria | Gram-Negative Bacteria |
|----------|------------------------|-----------------------|
|          | S. aureus | B. subtilis | B. cereus | E. coli | S. typhimurium | P. aeruginosa |
| 2a       | >125 | >125 | >125 | 15.63 | 15.63 | 15.63 |
| 2b       | >125 | >125 | >125 | 15.63 | 15.63 | 15.63 |
| 3a       | 15.63 | 31.25 | 62.5 | 15.63 | 15.63 | 15.63 |
| 3b       | 15.63 | 31.25 | 62.5 | 15.63 | 15.63 | 15.63 |
| 4a       | 31.25 | 15.63 | 15.63 | 7.81 | 15.63 | 15.63 |
| 4b       | 15.63 | 15.63 | 7.81 | 15.63 | 7.81 | 15.81 |
| 4c       | 15.63 | 15.63 | 31.25 | 15.63 | 15.63 | 15.63 |
| 4d       | 15.63 | 15.63 | 31.25 | 31.25 | 15.63 | 15.63 |
| 4e       | 15.63 | 15.63 | 31.25 | 31.25 | 15.63 | 15.63 |
| 4f       | 15.63 | 15.63 | 31.25 | 31.25 | 15.63 | 15.63 |
| 4g       | >125 | >125 | >125 | 62.5 | 31.25 | 62.5 |
| 4h       | >125 | >125 | >125 | 62.5 | 31.25 | 62.5 |
| 5a       | 15.63 | 31.25 | 31.25 | 15.63 | 15.63 | 15.63 |
| 5b       | 15.63 | 31.25 | 31.25 | 15.63 | 15.63 | 15.63 |
| 6a       | 15.63 | 31.25 | 31.25 | 15.63 | 15.63 | 15.63 |
| 6b       | 15.63 | 31.25 | 31.25 | 15.63 | 15.63 | 15.63 |
| 7a       | 31.25 | 62.5 | 31.25 | 31.25 | 31.25 | 31.25 |
| 7b       | 31.25 | 62.5 | 31.25 | 31.25 | 31.25 | 31.25 |
| 8a       | 31.25 | 62.5 | 31.25 | 31.25 | 31.25 | 31.25 |
| 8b       | 31.25 | 62.5 | 31.25 | 31.25 | 31.25 | 31.25 |
| Amoxicillin | 15.63 | 15.63 | 7.81 | 15.63 | 15.63 | 15.63 |

However, the transformation of the esters 6a,b to acetohydrazide derivatives 7a,b and then to their aryldiene derivatives 8a,b revealed an improvement in the antibacterial activity against Gram-positive strains, with MIC values ranging from 31.25 to 62.5 µg/mL. Moreover, 7a,b and 8a,b showed moderate activity against all tested Gram-negative strains (MIC = 31.25 µg/mL).
2.3. DNA Gyrase and Topoisomerase IV Inhibitory Activity

The target compounds (2a,b; 3a,b; 4a,b; and 5a,b) that showed the most potent activity against the tested Gram-negative bacteria, especially against E. coli, were chosen to evaluate their in vitro inhibitory activity of E. coli DNA gyrase and topoisomerase IV. The results of the DNA gyrase supercoiling and topoisomerase IV decatenation assays (IC50 values in µM) of these compounds are in Table 2, showing the potent dual inhibition of the 4'-amine derivatives 4a,b against both enzymes compared with the two reference inhibitors (ciprofloxacin and novobiocin). The 4'-(4-methyl-piperazin-1-yl)-derivative 4b was the most potent dual inhibitor with IC50 values of 3.44 µM against DNA gyrase and 14.46 µM against topoisomerase IV, while the IC50 values of ciprofloxacin and novobiocin were 3.52 µM and 4.19 µM for DNA gyrase and 17.57 µM and 14.59 µM for topoisomerase IV, respectively. N-(furan-2-yl)-4'-amine derivative 4a gave an IC50 value of 5.77 µM against gyrase and showed a more potent inhibition of topoisomerase IV than that of ciprofloxacin, with an IC50 value of 14.89 µM. In addition, 4'-chloro derivative 3b and pyrimidin-4'(3'H)-thione derivative 5b revealed potent inhibitory activity against topoisomerase IV, with IC50 values of 17.50 and 17.24 µM, which were more potent than ciprofloxacin; however, they displayed moderate inhibition to DNA gyrase. The rest of the tested compounds, pyrimidin-4'(3'H)-ones 2a,b; 4'-chloro derivative 3a and pyrimidin-4'(3'H)-thione derivative 5a, showed moderate inhibitory activity against both DNA gyrase and topoisomerase IV, with IC50 values ranging from 8.30 to 12.99 µM and 21.78 to 23.25 µM, respectively.

2.4. Molecular Docking Studies

To explore the binding modes of the newly synthesized pyridothienopyrimidines (2a,b; 3a,b; 4a,b; and 5a,b) with the active site of E. coli DNA gyrase B, a molecular docking simulation was accomplished using MOE. Firstly, novobiocin (the original co-crystallized ligand) was re-docked in the active site of E. coli DNA gyrase B kinase ((PDB code: 1AJ6) [46,47] (Figure 3) and revealed a score energy of ~80 kcal/mol at a root mean square deviation (RMSD) value equal to 0.81 Å. As reported in docking of novobiocin, having a coumarin core linked to oxan-4-yl moiety, the protons of the hydroxyl group of oxan-4-yl and NH2 of the carbamate group formed hydrogen bonds within the active site of DNA gyrase B kinase via the backbone of Asp46 and the side chain of Asp73. Furthermore, the coumarin scaffold shared fixation through an arene–cation interaction with the essential amino acid Arg76 [37,47].
Table 2. Inhibitory activity of some selected compounds against *E. coli* DNA gyrase and topoisomerase IV enzymes.

| Compound | R     | R¹  | DNA Gyrase Supercoiling | Topoisomerase IV Decatentation |
|----------|-------|-----|-------------------------|-------------------------------|
| 2a       | OMe   |     | 8.30                    | 21.99                         |
| 2b       |       |     | 10.42                   | 22.03                         |
| 3a       | OMe   |     | 8.99                    | 21.78                         |
| 3b       |       |     | 6.96                    | 17.50                         |
| 4a       |       | OMe| 5.77                    | 14.89                         |
| 4b       | OMe   | NMe| 3.44                    | 14.46                         |
| 5a       | OMe   |     | 12.99                   | 23.25                         |
| 5b       |       |     | 14.23                   | 17.24                         |
| Ciprofloxacin | |     | 3.52                    | 17.57                         |
| Novobiocin        | |     | 4.19                    | 14.59                         |

Figure 3. (a,b) Diagrams illustrating the 2D and 3D binding patterns of novobiocin onto the ATP-active pocket of *E. coli* DNA gyrase B kinase (PDB code: 1AJ6), respectively.

Then, the target compounds (2a,b; 3a,b; 4a,b; 5a,b) were docked into the ATP-active sites of *E. coli* DNA gyrase B and the docking results are listed in Table 3. By comparing the energy scores and the binding orientations of the target compounds with that of the original ligand novobiocin, it can be seen that all derivatives displayed promising energy scores ranging, from −5.25 to −6.99 kcal/mol for *E. coli* DNA gyrase B, as well as a noticeable binding affinity between the pyridine scaffold and the essential amino acid Arg76 via an arene–cation interaction. The 4’-(4-methyl-piperazin-1-yl)- derivative
4b, which displayed the most potent inhibitory activity, gave the highest binding affinity to DNA gyrase B, with an energy score of $-6.99$ kcal/mol. Moreover, the N-(furan-2-yl)-4'-amine derivative 4a, which came after 4b in inhibition potency, displayed the second-best docking score, with a binding energy of $-6.64$ kcal/mol. Thus, compounds 4a and 4b were selected as the ligand examples against the structure of *E. coli* DNA gyrase B (PDB ID code: 1AJ6); the obtained docking models are illustrated in Figure 4; Figure 5.

Table 3. Docking results of compounds 2a,b; 3a,b; 4a,b; and 5a,b with *E. coli* DNA gyrase B kinase using MOE software version 2008.10.

| Compound No. | Docking Score (Kcal/mol) | Amino Acid Residues (Bond Length Å) | Atoms of Compound | Type of Bond |
|--------------|--------------------------|-----------------------------------|-------------------|-------------|
| Novobiocin   | −6.80                    | Asn46(3.27); Asp73(1.91); Arg76; Gly77(2.79) | H(OH)(oxan-4-yl); C$_4$H$_2$(coumarin) pyridine; O(pyrimidone) | H-don H-don |
| 2a           | −5.25                    | Arg76; Gly77(2.25)                | pyridine; O(pyrimidone) | Arene–cation Arene–cation |
| 2b           | −5.74                    | Arg76; Gly77(2.25)                | pyridine; O(pyrimidone) | Arene–cation Arene–cation |
| 3a           | −5.65                    | Arg76; Thr165(2.50)               | pyridine; N-3(pyrimidine) | Arene–cation Arene–cation |
| 3b           | −5.90                    | Arg76; Thr165(2.27)               | pyridine; N-3(pyrimidine) | Arene–cation H-acc |
| 4a           | −6.64                    | Asn46(2.99)                       | H(NH-furan-2-yl) | H-don |
| 4b           | −6.99                    | Arg76; Arg76                      | pyridine; N(piperazine) | Arene–cation Arene–cation |
| 5a           | −5.30                    | Arg76                             | pyridine; phenyl | Arene–cation Arene–cation |
| 5b           | −5.55                    | Arg76                             | pyridine | Arene–cation |

**Figure 4.** (a,b) Diagrams illustrating the 2D and 3D binding patterns of compound 4a onto the ATP-active pocket of *E. coli* DNA gyrase B kinase (PDB code: 1AJ6), respectively.
Figure 5. (a,b) Diagrams illustrating the 2D and 3D binding patterns of compound 4b onto the ATP-active pocket of E. coli DNA gyrase B kinase (PDB code: 1AJ6), respectively.

Regarding to the binding of 4a with E. coli DNA gyrase B, there was an H-bond donor between the NH proton of amino-2-furan scaffold with the side chain of Thr165 (distance: 2.15 Å), and it exhibited one arene–cation interaction with Arg76 (Figure 4). However, the pyridine and phenyl moieties of 4b demonstrated two arene–cation interactions with the key amino acid Arg76. Furthermore, the nitrogen of piperazine moiety of 4b formed with the side chain of Asn46 a favorable hydrogen bonding (distance: 2.99 Å) (Figure 5).

3. Materials and Methods

3.1. Chemistry

3.1.1. General Consideration

Melting points were determined by 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%. 1H NMR and 13C NMR spectra were recorded on a Bruker High Performance Digital FT-NMR Spectrometer Advance III (400/100 MHz) in the presence of TMS as the internal standard. Infrared spectra were recorded by using the KBr disc technique on a Jasco FT/IR-6100, Fourier transform, Infrared spectrometer (Japan) at the cm⁻¹ scale. The ESI-mass spectra were measured using an Advion Compact Mass Spectrometer (CMS) NY, USA. 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 the new synthesized compounds is according to the IUPAC system. The starting 3-amino-6-phenylthieno[2,3-b]pyridine-2-carboxamides 1a,b were prepared as per reported methods [44,45].

3.1.2. Synthesis of Pyrido[3′,2′:4,5]Thieno[3,2-d]Pyrimidin]-4′(3′H)-ones 2a,b

A mixture of compounds 1a,b (0.01 mol) and cyclohexanone (1.47 g, 0.015 mol) in N,N-dimethylformamide (20 mL) containing anhydrous ZnCl₂ (1.36 g, 0.01 mol) was refluxed for 3 h. The reaction mixture was poured onto ice/water and the obtained solid was collected by filtration, washed several times with water and recrystallized from DMF/H₂O to give compounds 2a,b.
9’-(4-Methoxyphenyl)-7’-phenyl-1'H-spiro[cyclohexane-1,2’-pyrido[3’,2’:4,5]thieno[3,2-d]pyrimidin]-4’ (3’H)-one (2a). It was a yellow powder, yield 81% (3.69 g), m.p. 295–296 °C. Anal. calcd. (%) for C_{27}H_{23}N_{2}O_{6}S (455.58): C, 71.18; H, 5.53; N, 9.22; S, 7.04. Found: C, 71.35; H, 5.37; N, 9.43; S, 6.88. 1H NMR (DMSO-d6, 400 MHz): δ 1.21–1.97 (m, 10H, SCH2), 3.86 (s, 3H, OCH3), 4.53 (s, 1H, NH), 7.18 (d, J = 7.2 Hz, 2H, Ar−H), 7.48–7.55 (m, 3H, Ar−H), 7.58 (d, J = 7.2 Hz, 2H, Ar−H), 7.86 (s, 1H, Ar−H). 13C NMR (DMSO-d6, 100 MHz): δ 21.6, 24.5, 36.1 (SCH2), 56.0 (OCH3), 69.9 (spiro C), 114.5, 119.3, 122.8, 127.6, 129.3, 130.7, 133.6, 135.0, 139.6, 149.8, 154.8, 158.3, 161.5 (Ar−C), 167.9 (C=O). IR (KBr, ν max cm⁻¹): 3339.1, 2925.1, 2856.4 (CH), 1658.2 (C=O). ESI−MS: m/z = 454.61 [M−H⁺].

7’-Phenyl-9’-(thiophen-2-yl)-1'H-spiro[cyclohexane-1,2’-pyrido[3’,2’:4,5]thieno[3,2-d]pyrimidin]-4’ (3’H)-one (2b). It was a yellow powder, yield 79% (3.40 g), m.p. 289 °C. Anal. calcd. (%) for C_{24}H_{21}N_{3}O_{5}S (431.57): C, 66.79; H, 4.90; N, 9.74; S, 14.86. Found: C, 66.65; H, 5.15; N, 9.49; S, 14.62. 1H NMR (DMSO-d6, 400 MHz): δ 1.21–2.02 (m, 10H, SCH2), 4.90 (s, 1H, NH), 7.34 (dd, J = 3.5, 4.7 Hz, 1H, Ar−H), 7.51–7.55 (m, 4H, Ar−H), 7.88 (s, 1H, NH), 7.93–7.98 (m, 3H, Ar−H), 8.22–8.25 (m, 2H, Ar−H). 13C NMR (DMSO-d6, 100 MHz): δ 21.8, 24.6, 36.2 (SCH2), 69.8 (spiro C), 119.2, 122.3, 127.7, 128.2, 129.4, 130.1, 133.8, 139.9, 140.4, 145.2, 149.8, 156.0, 158.1 (Ar−C), 167.1 (C=O). IR (KBr, ν max cm⁻¹): 3318.6, 2727.2 (NH), 3053.9, 2925.3, 2857.2 (CH), 1659.3 (C=O). ESI−MS: m/z = 430.48 [M−H⁺].

3.1.3. Synthesis of 4’-Chloropyrido[3’,2’:4,5]Thieno[3,2-d]Pyrimidine Derivatives 3a, b

To a solution of compounds 2a, b (5 mmol) in phosphorus oxychloride (15 mL), phosphorus pentachloride (1.04 g, 5 mmol) was added. The reaction mixture was refluxed for 4 h, then left to cool and poured slowly with stirring onto crushed ice. The medium was neutralized with aqueous ammonia (28%) to a pH of 7; the obtained solid was filtered off, washed with water and recrystallized from ethanol to give 4’-chloro derivatives 3a, b.

4’-Chloro-9’-(4-methoxyphenyl)-7’-phenyl-1'H-spiro[cyclohexane-1,2’-pyrido[3’,2’:4,5]thieno[3,2-d]pyrimidin] (3a). It was a pale-yellow powder, yield 68% (1.61 g), m.p. 201–202 °C. Anal. calcd. (%) for C_{27}H_{23}ClN_{2}O_{6}S (474.02): C, 68.41; H, 5.10; N, 8.86; S, 6.76. Found: C, 68.28; H, 5.32; N, 8.59; S, 6.97. 1H NMR (DMSO-d6, 400 MHz): δ 1.28–1.87 (m, 10H, SCH2), 3.85 (s, 3H, OCH3), 7.03 (s, 1H, NH), 7.15 (d, J = 8.4 Hz, 2H, Ar−H), 7.49–7.54 (m, 3H, Ar−H), 7.74 (d, J = 8.4 Hz, 2H, Ar−H), 7.86 (s, 1H, Ar−H), 8.22 (d, J = 7.6 Hz, 2H, Ar−H). 13C NMR (DMSO-d6, 100 MHz): δ 22.1, 24.6, 36.2 (SCH2), 56.2 (OCH3), 79.7 (spiro C), 114.6, 119.4, 121.4, 124.0, 124.0, 127.6, 129.6, 130.5, 136.7, 139.6, 148.9, 149.7, 155.9, 161.4, 162.4 (Ar−C, C=N). IR (KBr, ν max cm⁻¹): 3384.2 (NH), 3058.5, 2934.1, 2848.3 (CH), 1608.6 (C=N), 764.4 (C=Cl). ESI−MS: m/z = 473.0 [M−H⁺].

4’-Chloro-7’-phenyl-9’-(thiophen-2-yl)-1'H-spiro[cyclohexane-1,2’-pyrido[3’,2’:4,5]thieno[3,2-d]pyrimidin] (3b). It was a buff powder, yield 66% (1.48 g), m.p. 189 °C. Anal. calcd. (%) for C_{24}H_{21}ClN_{3}O_{5}S (450.02): C, 64.06; H, 4.48; N, 9.34; S, 14.25. Found: C, 64.25; H, 4.77; N, 9.11; S, 14.53. 1H NMR (DMSO-d6, 400 MHz): δ 1.24–1.87 (m, 10H, SCH2), 6.92 (s, 1H, NH), 7.29 (dd, J = 5.3, 8.6 Hz, 1H, Ar−H), 7.50–7.58 (m, 4H, Ar−H), 7.82–7.88 (m, 2H, Ar−H), 8.21–8.27 (m, 2H, Ar−H). 13C NMR (DMSO-d6, 100 MHz): δ 21.5, 24.1, 36.0 (SCH2), 79.8 (spiro C), 119.1, 121.7, 123.0, 125.5, 127.5, 129.3, 130.4, 139.7, 141.1, 145.2, 147.5, 156.0, 162.1 (Ar−C, C=N). IR (KBr, ν max cm⁻¹): 3367.5 (NH), 3051.2, 2925.1, 2856.3 (CH), 1612.3 (C=N), 762.6 (C=Cl). ESI−MS: m/z = 449.04 [M−H⁺].

3.1.4. Synthesis of Pyrido[3’,2’:4,5]Thieno[3,2-d]Pyrimidin]-4’-Amines 4a–e

A mixture of 4’-chloro derivatives 3a, b (1 mmol) and different amines (1 mmol) in N,N-dimethylformamide (20 mL) was heated under reflux for 5 h. After reaction completion, the solvent was evaporated under vacuum and the oily residue was turned to solid by addition of dilute ethanol (50%). The obtained solid was collected by filtration and recrystallized from acetone to give compounds 4a–e.

N-(furan-2-yl)-7’-phenyl-9’-(thiophen-2-yl)-1'H-spiro[cyclohexane-1,2’-pyrido[3’,2’:4,5]thieno[3,2-d]pyrimidin]-4’-amine (4a) was synthesized from 3b (0.45 g, 1 mmol) and furan-2-amine (0.084 g, 1 mmol) according to the general method. It was a brown powder, yield 72% (0.35 g), m.p. 149 °C. Anal. calcd.
9-(4-Methoxyphenyl)-4-(4-methylpiperazin-1-yl)-7'-phenyl-1'H-spirocyclohexane-1,2'-pyrido[3',2':4,5]thieno[3,2-d]pyrimidinyl (4b) was synthesized from 3a (0.47 g, 1 mmol) and 1-methylpiperazine (0.10 g, 1 mmol) according to the general method. It was a beige powder, yield 74% (0.39 g), m.p. 171 °C. Anal. calcd. (%): C 71.48; H, 6.56; N, 13.02; S, 5.96. Found: C, 71.21; H, 6.27; S, 12.74; S, 5.68. 1H NMR (DMSO-d$_6$, 400 MHz): $\delta$ 1.27–1.95 (m, 10H, 5CH$_2$), 2.21 (s, 3H, NCH$_3$), 2.59 (s, 4H, 2CH$_2$N), 3.75 (s, 4H, 2CH$_2$N), 3.85 (s, 3H, OCH$_3$), 6.99 (s, 1H, NH), 7.13 (d, $J = 9.2$ Hz, 2H, Ar–H), 7.48–7.53 (m, 3H, Ar–H), 7.73 (d, $J = 9.2$ Hz, 2H, Ar–H), 7.83 (s, 1H, Ar–H), 8.23 (d, $J = 5.2$ Hz, 2H, Ar–H). 13C NMR (DMSO-d$_6$, 100 MHz): $\delta$ 21.8, 24.4, 36.4 (SCH$_2$), 46.3 (NCH$_3$), 48.5 (SCH$_2$N), 50.8 (SCH$_2$N), 55.7 (OCH$_3$), 79.9 (spiro C), 113.2, 119.8, 121.3, 122.9, 124.4, 124.7, 126.8, 129.5, 130.4, 136.1, 141.1, 148.9, 149.6, 155.9, 157.2, 161.7 (Ar–C, C=N). IR (KBr, $\nu_{\text{max}}$ cm$^{-1}$): 3432.6, 3366.2 (NH), 3061.1, 2925.2, 2857.4 (CH), 1640.2 (C=O). ESI–MS: $m/z = 495.67$ [M+H$^+$].

4-(7'-Phenyl-9'-(thiophen-2-yl)-1'H-spirocyclohexane-1,2'-pyrido[3',2':4,5]thieno[3,2-d]pyrimidinyl-4'-yl)morpholine (4c) was synthesized from 3b (0.45 g, 1 mmol) and morpholine (0.087 g, 1 mmol) according to the general method. It was a brown powder, yield 76% (0.38 g), m.p. 159–160 °C. Anal. calcd. (%): C 72.58; H, 5.68; N, 11.19; S, 12.59. 1H NMR (DMSO-d$_6$, 400 MHz): $\delta$ 1.23–2.02 (m, 10H, 5CH$_2$), 3.06 (s, 4H, 2CH$_2$N), 3.79 (s, 4H, 2CH$_2$O), 7.04 (s, 1H, NH), 7.33 (s, 1H, Ar–H), 7.51–7.55 (m, 4H, Ar–H), 7.84–7.90 (m, 2H, Ar–H), 8.23 (d, $J = 10.0$ Hz, 2H, Ar–H). 13C NMR (DMSO-d$_6$, 100 MHz): $\delta$ 21.1, 24.4, 36.7 (SCH$_2$), 45.5 (SCH$_2$N), 66.4 (2CH$_2$O), 80.1 (spiro C), 119.2, 121.2, 123.4, 125.0, 127.1, 127.5, 128.6, 129.5, 130.2, 139.7, 141.1, 145.7, 149.0, 155.8, 156.8 (Ar–C, C=N). IR (KBr, $\nu_{\text{max}}$ cm$^{-1}$): 3428.3 (NH), 3107.2, 2922.5, 2852.1 (CH), 1644.6 (C=O). ESI–MS: $m/z = 536.71$ [M+H$^+$].

1-4-(7'-Phenyl-9'-(thiophen-2-yl)-1'H-spirocyclohexane-1,2'-pyrido[3',2':4,5]thieno[3,2-d]pyrimidinyl-4'-yl)amino)phenylethan-1-one (4d) was synthesized from 3b (0.45 g, 1 mmol) and 4-acetylaniiline (0.13 g, 0.001 mol) according to the general method. It was a brown powder, yield 66% (0.36 g), m.p. 141–142 °C. Anal. calcd. (%): C 73.07; H, 5.14; N, 10.21; S, 11.69. Found: C, 70.33; H, 5.40; N, 10.53; S, 11.43. 1H NMR (DMSO-d$_6$, 400 MHz): $\delta$ 1.21–2.02 (m, 10H, 5CH$_2$), 2.66 (s, 3H, CH$_3$CO), 6.96 (s, 1H, NH), 7.29–7.35 (m, 1H, Ar–H), 7.43 (d, $J = 11.2$ Hz, 2H, Ar–H), 7.51–7.55 (m, 4H, Ar–H), 7.77 (d, $J = 11.2$ Hz, 2H, Ar–H), 7.92–7.96 (m, 2H, Ar–H), 8.21 (d, $J = 6.0$ Hz, 2H, Ar–H), 10.63 (s, 1H, NH). 13C NMR (DMSO-d$_6$, 100 MHz): $\delta$ 21.8, 24.6, 29.4, 36.2 (SCH$_2$CH$_3$), 79.9 (spiro C), 115.8, 119.5, 121.5, 123.1, 125.1, 127.4, 127.7, 128.2, 129.4, 130.2, 132.3, 139.5, 140.9, 142.4, 145.3, 148.9, 150.1, 155.7, 162.8 (Ar–C, C=N), 167.2 (C=O). IR (KBr, $\nu_{\text{max}}$ cm$^{-1}$): 3416.2, 3280.5 (NH), 3074.3, 2923.2, 2858.1 (CH), 1698.3 (C=O), 1631.4 (C=O). ESI–MS: $m/z = 549.69$ [M+H$^+$].

N-(4-fluorophenyl)-9'-(4-methoxyphenyl)-7'-phenyl-1'H-spirocyclohexane-1,2'-pyrido[3',2':4,5]thieno[3,2-d]pyrimidinyl-4'-amine (4e) was synthesized from 3a (0.47 g, 1 mmol) and 4-fluoroaniline (0.11g, 1 mmol) according to the general method. It was a brown powder, yield 67% (0.37 g), m.p. 133–135 °C. Anal. calcd. (%): C 72.24; H, 5.33; N, 10.21; S, 5.84. Found: C, 72.61; H, 5.64; N, 8.97; S, 5.46. 1H NMR (DMSO-d$_6$, 400 MHz): $\delta$ 1.22–1.84 (m, 10H, 5CH$_2$), 3.85 (s, 3H, OCH$_3$), 6.78 (d, $J = 9.6$ Hz, 2H, Ar–H), 6.95 (s, 1H, NH), 7.13 (d, $J = 7.2$ Hz, 2H, Ar–H), 7.19 (d, $J = 9.6$ Hz, 2H, Ar–H), 7.48–7.53 (m, 3H, Ar–H), 7.74 (d, $J = 7.2$ Hz, 2H, Ar–H), 7.84 (s, 1H, Ar–H), 8.23 (d, $J = 8.0$ Hz, 2H, Ar–H), 10.26 (s, 1H, NH). IR (KBr, $\nu_{\text{max}}$ cm$^{-1}$): 3410.2, 3335.4 (NH), 3065.1, 2924.5, 2856.2 (CH), 1628.4 (C=O). ESI–MS: $m/z = 547.64$ [M+H$^+$].
3.1.5. Synthesis of Pyrido[3',2':4,5]Thieno[3,2-d]Pyrimidin-4'(3'H)-Thiones 5a,b

A mixture of compounds 2a,b (0.01 mol) and phosphorus pentasulfide (2.22 g, 0.01 mol) in pyridine (30 mL) was refluxed for 8 h. After reaction completion, the reaction solution was poured onto cold water and left in the refrigerator overnight. The formed precipitate was separated by filtration, washed several times with water and recrystallized from CHCl₃/Pet ether to give thione derivatives 5a,b.

9'-{(4-Methoxyphenyl)-7'-phenyl-1'H-spiro[cyclohexane-1,2'-'pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4'- (3'H)-thione (5a). It was an orange powder, yield 79% (3.72 g), m.p. 254 °C. Anal. calcd. (%) for C₇₂H₇₅N₅O₅S (871.64): C, 70.53; H, 5.46; N, 9.94; S, 13.07. Found: C, 70.62; H, 5.52; N, 10.01; S, 13.14.

1H NMR (DMSO-d₆, 400 MHz): δ 1.26–2.04 (m, 10H, 5CH₃), 2.34 (s, 3H, OCH₃), 3.87 (s, 3H, OCH₃), 4.08 (s, 1H, NH), 7.17 (d, J = 8.5 Hz, 2H, Ar-H), 7.51–7.55 (m, 3H, Ar-H), 7.59 (d, J = 8.5 Hz, 2H, Ar-H), 7.84 (s, 1H, Ar-H), 8.22 (d, J = 6.5 Hz, 2H, Ar-H), 9.69 (s, 1H, NH). 13C NMR (DMSO-d₆, 100 MHz): δ 21.62, 24.56, 35.3 (5CH₃), 70.2 (spiro C), 114.5, 119.4, 121.7, 122.8, 127.6, 128.5, 129.6, 130.4, 131.1, 132.4, 140.0, 147.8, 149.9, 154.8, 161.5, 163.1 (Ar-C), 181.3 (C=S). IR (KBr, ν max cm⁻¹): 1735.2, 3058.0, 2926.4, 2856.3 (CH), 1736.5 (C=O). ESI-MS: m/z = 532.76 [M - H⁺].

3.1.6. Synthesis of Ethyl 2-(Pyrido[3',2':4,5]Thieno[3,2-d]Pyrimidin-4-yl-Thio)Acetates 6a,b

A mixture of compounds 5a,b (5 mmol) and ethyl 2-chloroacetate (0.60 g, 5 mmol) in N,N-dimethylformamide (30 mL) containing anhydrous sodium carbonate (1.0 g) was heated at 80° C with stirring for 3 h. The reaction mixture was poured onto an ice-water mixture and left standing for 1 h. The obtained solid was collected by filtration, washed with water, and recrystallized from ethanol to give the esters 6a,b.

Ethyl 2-{(9'-(4-Methoxyphenyl)-7'-phenyl-1'H-spiro[cyclohexane-1,2'-'pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4'-yl)thio)acetate (6a). It was an off-white powder, yield 72% (2.0 g), m.p. 275–276 °C. Anal. calcd. (%) for C₇₃H₇₇N₅O₅S (879.63): C, 70.47; H, 5.49; N, 9.83; S, 13.11. Found: C, 70.52; H, 5.51; N, 10.01; S, 13.14.

1H NMR (DMSO-d₆, 400 MHz): δ 1.26–2.04 (m, 10H, 5CH₃), 2.34 (s, 3H, OCH₃), 3.87 (s, 3H, OCH₃), 4.08 (q, J = 10.8 Hz, 2H, OCH₂CH₃), 7.05 (s, 1H, NH), 7.14 (d, J = 7.6 Hz, 2H, Ar-H), 7.50–7.54 (m, 3H, Ar-H), 7.72 (d, J = 7.6 Hz, 2H, Ar-H), 7.89 (s, 1H, Ar-H), 8.23 (d, J = 10.0 Hz, 2H, Ar-H). IR (KBr, ν max cm⁻¹): 3410.3 (NH), 3058.1, 2924.2, 2860.4 (CH), 1735.2 (C=O). ESI-MS: m/z = 556.68 [M - H⁺].

Ethyl 2-{(7'-(Thiophen-2-yl)-1'H-spiro[cyclohexane-1,2'-'pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4'-yl)thio)acetate (6b). It was a pale-yellow powder, yield 69% (1.84 g), m.p. 261–262 °C. Anal. calcd. (%) for C₇₃H₇₇N₅O₅S (879.63): C, 70.47; H, 5.49; N, 9.83; S, 13.11. Found: C, 70.52; H, 5.51; N, 10.01; S, 13.14.

1H NMR (DMSO-d₆, 400 MHz): δ 1.26–2.04 (m, 10H, 5CH₃), 2.34 (s, 3H, OCH₃), 3.87 (s, 3H, OCH₃), 4.08 (q, J = 10.8 Hz, 2H, OCH₂CH₃), 7.05 (s, 1H, NH), 7.14 (d, J = 7.6 Hz, 2H, Ar-H), 7.50–7.54 (m, 3H, Ar-H), 7.72 (d, J = 7.6 Hz, 2H, Ar-H), 7.89 (s, 1H, Ar-H), 8.23 (d, J = 10.0 Hz, 2H, Ar-H). IR (KBr, ν max cm⁻¹): 3410.3 (NH), 3058.1, 2924.2, 2860.4 (CH), 1735.2 (C=O). ESI-MS: m/z = 556.68 [M - H⁺].
3.1.7. Synthesis of Pyrido[3′,2′:4,5]Thieno[3,2-d][Pyrimidin]-4’-yl)thio)Acetohydrazides 7a,b

A mixture of 6a,b (0.004 mol) and hydrazine hydrate 98% (1mL, excess) in absolute ethanol (50 mL) was refluxed for 8 h. After reaction completion, the obtained solid was collected by filtration and recrystallized from DMF/H2O to give acetohydrazide derivatives 7a,b.

2-(1′-(4-methoxyphenyl)-1′-phenyl-1′H-spiro[cyclohexane-1,2′-pyrido[3′,2′:4,5]thieno[3,2-d][pyrimidin]-4′-yl]thio)acetohydrazide (7a). It was a red powder, yield 66% (1.43 g), m.p. 234 °C. Anal. calcld. (%) for C₂₉H₂₉N₅O₅S₂ (543.70): C, 64.06; H, 5.38; N, 12.88; S, 11.79. Found: C, 64.33; H, 5.65; N, 13.16; S, 11.47. 1H NMR (DMSO-d₆, 400 MHz): δ 1.26–1.99 (m, 10H, 5CH₂), 3.86 (s, 3H, OCH₃), 3.89 (s, 2H, SCH₂), 4.62 (s, 2H, NH₂), 7.05 (s, 1H, NH), 7.17 (d, J = 8.6 Hz, 2H, Ar−H), 7.49–7.59 (m, 3H, Ar−H), 7.74 (d, J = 8.6 Hz, 2H, Ar−H), 7.84 (s, 1H, Ar−H), 8.27 (d, J = 7.0 Hz, 2H, Ar−H), 9.18 (s, 1H, NH). IR (KBr, ν max cm⁻¹): 3410.3, 3320.3, 3240.1(NH), 3057.5, 2927.2, 2858.4 (CH), 1685.3 (C=O). ESI−MS: m/z = 542.73 [M − H⁺].

2-(1′-(phenyl-9′-(thiophene-2-yl)-1′H-spiro[cyclohexane-1,2′-pyrido[3′,2′:4,5]thieno[3,2-d][pyrimidin]-4′-yl]thio) acetohydrazide (8a). It was a brown powder, yield 62% (1.29 g), m.p. 222 °C. Anal. calcld. (%) for C₂₉H₂₉N₅O₅S₂ (519.70): C, 60.09; H, 4.85; N, 13.48; S, 18.51. Found: C, 60.48; H, 5.19; N, 13.11; S, 18.19. 1H NMR (DMSO-d₆, 400 MHz): δ 1.27–1.96 (m, 10H, 5CH₂), 3.87 (s, 2H, SCH₂), 4.59 (s, 2H, NH₂), 6.99 (s, 1H, NH), 7.32–7.74 (m, 5H, Ar−H), 7.86–7.91 (m, 2H, Ar−H), 8.26 (m, 2H, Ar−H), 9.22 (s, 1H, NH). IR (KBr, ν max cm⁻¹): 3414.3, 3326.1, 3251.4 (NH), 3061.1, 2924.3, 2857.2 (CH), 1680.1 (C=O). ESI−MS: m/z = 518.66 [M − H⁺].

3.1.8. Synthesis of N′-(Arylidene)-2-(Pyrido[3′,2′:4,5]Thieno[3,2-d][Pyrimidin]-4′-yl)Thio) Acetohydrazides 8a,b

A mixture of 7a (0.54 g, 1 mmol) and different aldehydes, namely, 4-(dimethylamino)benzaldehyde and thiophene-2-carbaldehyde (1 mmol) in glacial acetic acid (20 mL) was refluxed for 6 h. After reaction completion, the mixture was concentrated and poured onto cold water. The formed solid was collected by filtration, washed with water, and recrystallized from ethanol to give compounds 8a,b.

N′-(4-(dimethylamino)benzilidene)-2-[(9′-(4-methoxyphenyl)-1′-(thiophene-2-yl)-1′H-spiro[cyclohexane-1,2′-pyrido[3′,2′:4,5]thieno[3,2-d][pyrimidin]-4′-yl]thio)acetohydrazide (8a). It was a brown powder, yield 61% (0.41 g), m.p. 199–200 °C. Anal. calcld. (%) for C₃₈H₃₇N₅O₅S₂ (674.88): C, 67.63; H, 5.68; N, 12.45; S, 9.50. Found: C, 67.31; H, 5.93; N, 12.69; S, 9.22. 1H NMR (DMSO-d₆, 400 MHz): δ 1.23–1.92 (m, 2H, 5CH₂), 3.01 (s, 6H, N(CH₃)₂), 3.85 (s, 3H, OCH₃), 3.98 (s, 2H, SCH₂), 6.97 (s, 1H, NH), 7.16 (d, J = 8.4 Hz, 2H, Ar−H), 7.22 (d, J = 9.6 Hz, 2H, Ar−H), 7.54–7.55 (m, 3H, Ar−H), 7.74 (d, J = 8.4 Hz, 2H, Ar−H), 7.87 (s, 1H, Ar−H), 7.94 (d, J = 9.6 Hz, 2H, Ar−H), 8.26 (d, J = 7.2 Hz, 2H, Ar−H), 8.43 (s, 1H, CH=N), 11.14 (s, 1H, NH). 13C NMR (DMSO-d₆, 100 MHz): δ 21.6, 24.4, 33.6, 36.5 (SCH₂, SCH₂), 44.7 (N(CH₃)₂), 56.0 (OCH₃), 80.3 (spiro C), 112.1, 114.6, 119.5, 121.6, 122.9, 124.5, 127.8, 128.2, 129.1, 129.9, 130.8, 134.7, 139.9, 145.1, 148.0, 150.1, 154.0, 155.2, 161.5, 163.6 (Ar−C, CH=N), 169.9 (C=O). IR (KBr, ν max cm⁻¹): 3417.1, 3325.5(NH), 3066.2, 2924.2, 2853.3 (CH), 1678.1 (C=O). ESI−MS: m/z = 673.91 [M − H⁺].

2-[(9′-(4-methoxyphenyl)-1′-phenyl-1′H-spiro[cyclohexane-1,2′-pyrido[3′,2′:4,5]thieno[3,2-d][pyrimidin]-4′-yl]thio)-N′-(2-phenyl-2-thiethyl)acetohydrazide (8b). It was a buff powder, yield 63% (0.40 g), m.p. 178–179 °C. Anal. calcld. (%) for C₃₈H₃₇N₅O₅S₂ (637.84): C, 64.03; H, 4.90; N, 10.98; S, 15.08. Found: C, 64.35; H, 5.17; N, 10.66; S, 15.36. 1H NMR (DMSO-d₆, 400 MHz): δ 1.28–1.96 (m, 10H, SCHK₂), 3.87 (s, 3H, OCH₃), 3.98 (s, 2H, SCHK₂), 6.98 (s, 1H, NH), 7.15 (d, J = 10.8 Hz, 2H, Ar−H), 7.39–7.98 (m, 9H, Ar−H), 8.26 (d, J = 9.6 Hz, 2H, Ar−H), 8.53 (s, 1H, CH=N), 10.96 (s, 1H, NH). IR (KBr, ν max cm⁻¹): 3412.3, 3310.2 (NH), 3069.1, 2925.2, 2857.4 (CH), 1680.5 (C=O). ESI−MS: m/z = 673.78 [M − H⁺].

3.2. In Vitro Antibacterial Screening

All the newly synthesized compounds (2a,b–8a,b) and the standard drug (amoxicillin trihydrate) were evaluated for their antibacterial activity against three strains of Gram-positive bacteria (S. aureus 25923, B. cereus 33018 and B. subtilis 6633) and three strains of Gram-negative bacteria (E. coli 8739, S. typhimurium 14028 and P. aeruginosa 27853) by using the broth dilution method [48]. A twofold serial
dilution was performed to obtain the required concentrations (125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95 and 0.97, µg/mL) for the target compounds and the reference drug. The minimum concentration of the sample that showed no growth of the tested microorganism (MIC) were specified; then the MIC values for the compounds and standard drug were listed (see Table 1).

3.3. DNA Gyrase Supercoiling and Topoisomerase IV Decatenation Inhibition Assays

Eight of the target compounds (2a, 2b, 3a, 3b, 4a, 4b, 5a, and 5b) were chosen to evaluate their inhibitory activity against bacterial type II topoisomerase enzymes. The assay kits of E. coli DNA gyrase supercoiling and E. coli topoisomerase IV decatenation were provided by TopoGEN, Inc. (Port Orange, FL) and the assays were performed according to established protocols obtained from the supplier [49]. The new compounds and two standard inhibitors (ciprofloxacin and novobiocin) were dissolved in DMSO and serially diluted at concentrations of 100, 10, 1 and 0.1 µM, and then assayed in reaction mixtures in three different replicate runs. For DNA gyrase supercoiling: the final reaction volume was 20 µL, which included 35 mM Tris pH 7, 2 mM DTT, 24 mM KCl, 4 mM MgCl₂, 1.8 mM spermidine, 0.1 mg/mL acetylated BSA, 6.5% (v/v) glycerol, 1 mM ATP, 0.1 mg/mL album and 0.2 mg pBR322 substrate. While, for topoisomerase IV decatenation: the final reaction volume was 20 µL, containing 40 mM Tris pH 7.5, 10 mM DTT, 6 mM MgCl₂, 100 mM potassium glutamate, 1 mM ATP, 50 mg/mL acetylated BSA and 0.2 mg kDNA substrate. The reactions were initiated by addition of 2 U of E.coli DNA gyrase or E.coli topoisomerase IV (TopoGen), and 3 µL of inhibitor solution in 10% DMSO, and then were incubated with shaking for 30 min at 37 °C. All of the reactions were terminated by the addition of 10 mL of a 3X gel-loading buffer (final concentration: 6 mM EDTA, 1.2% SDS, 0.02% bromophenol blue, and 10% glycerol blue), after which 20 mL of this was loaded on a 1% agarose and then were incubated with shaking for 30 min at 37 °C. The fluorescence images were taken at a wavelength of 300 nm on a UV transilluminator imaging system. The fluorescence intensity of the supercoiled plasmid reaction product and the decatenation product, TAE (0.01 M EDTA pH 8.3, 40 mM Tris-acetate) gel that was then run at 60 V for 3 h. The gel was stained by (0.5 µg/mL) ethidium bromide in TAE for 30 min and then de-stained in water for 20 min. Fluorescent images were taken at a wavelength of 300 nm on a UV transilluminator imaging system. The fluorescence intensity of the supercoiled plasmid reaction product and the decatenation product, in the case of gyrase and topo IV, were quantitated using ImagQuant software (Molecular Dynamics, Sunnyvale, CA, USA). The results as IC50 values (concentration of the tested compound that leads to 50% inhibition of enzyme activity) for all samples were determined by nonlinear regression analysis in GraphPad Prism [49,50]. The average IC50 values (µM) of the triplicate experiments were calculated for the target compounds and the two reference antibiotics and then listed in Table 2.

3.4. Molecular Docking Study

The molecular docking simulation study was done using Molecular Operating Environment (MOE®) 2008.10 software [51]. The crystal structures of E. coli DNA gyrase B complexed with their ligand novobiocin (PDB codes: 1AJ6 and 1S14) [46,47] were retrieved from the Protein Data Bank. At the beginning, the co-crystallized ligand was re-docked into the assigned active E. coli DNA gyrase B enzyme to evaluate the root mean square deviation value. Then, the molecular docking procedure was performed for the newly synthesized compounds (2a,b; 3a,b; 4a,b; 5a,b) into the ATP-binding site of E. coli DNA gyrase B (PDB code: 1AJ6 and 1S14), following the reported method [37].

4. Conclusions

This work included synthesis of novel 4-substituted-1’H-spiro[cyclohexane-1,2’-pyrido[3’;2’:5,6]thieno[3,2-α]pyrimidin] compounds (2a,b–8a,b) and the in vitro evaluation of these compounds against six strains of both Gram-positive and Gram-negative bacteria compared with amoxicillin trihydrate as a reference drug. The tested pyridothenopyrimidine compounds (2a,b–8a,b) showed significant antibacterial activity, especially against Gram-negative strains, with MIC values ranging from 7.81 to 62.5 µg/mL. Compounds 2a,b; 3a,b; and 5a,b gave potent activity against all tested Gram-negative bacteria, equal to that of the reference drug, with an MIC value of 15.63 µg/mL. The N-(furan-2-yl)-4’-amine derivative 4a also displayed potent activity against all Gram-negative strains, besides its potent to moderate
activity against Gram-positive strains. Moreover, 4′-(4-methyl-piperazin-1-yl)- derivative 4b was the most active compound compared with the reference drug; it gave potent activity against all the tested bacterial strains, with MIC values ranging from 7.81 to 15.63 µg/mL. In turn, the antibacterial activity of compounds 6a, b; 7a, b and 8a, b varied from weak, with an MIC value of 62.5 µg/mL, to moderate, with an MIC value of 31.25µg/mL, against the tested microorganisms. Thus, compounds 2a, b; 3a, b; 4a, b and 5a, b, because they were the most potent compounds against E. coli, were selected to evaluate their in vitro inhibitory activity of E. coli topoisomerase II enzymes (DNA gyrase and topoisomerase IV). The tested compounds showed dual inhibition of the two enzymes and their inhibitory activity varied from potent to moderate compared with the two reference antibiotics (ciprofloxacin and novobiocin). Furthermore, compound 4b displayed dual inhibition, and was more potent than the two references, with IC₅₀ values of 3.44 µM and 14.46 µM against DNA gyrase and topoisomerase IV, respectively. Furthermore, 4a came after 4b in inhibition potency with IC₅₀ values of 5.77 µM for DNA gyrase and 14.89 µM for topoisomerase IV. In addition, docking studies were performed with compounds 2a, b; 3a, b; 4a, b and 5a, b, to illustrate their binding mode in the active site of DNA gyrase B compared with that of novobiocin. The docking results of the tested compounds were compatible with their inhibitory potency and gave binding scores ranging from −5.25 to −6.99 kcal/mol.

The results of this study pointed to the importance of pyrido[2,1-d:3,4-d]thienopyrimidine compounds as a promising heterocyclic sector that, with further study and development, can provide new antimicrobial agents competent of facing the increasing antimicrobial resistance.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-4951/9/10/695/s1, Figures S1–S28: NMR spectra of compounds 2a, b–8a, b.

Author Contributions: Conceptualization of work, E.M.E.-D.; methodology of synthesis, E.M.E.-D., promising heterocyclic sector that, with further study and development, can provide new antimicrobial agents competent of facing the increasing antimicrobial resistance.

References

1. Sifri, Z.C.; Chokshi, A.; Cennimo, D.; Horng, H. Global contributors to antibiotic resistance. *J. Glob. Infect. Dis.* 2019, 11, 36–42. [CrossRef] [PubMed]
2. Lomazzi, M.; Moore, M.; Johnson, A.; Balasegaram, M.; Borisch, B. Antimicrobial resistance—moving forward? *BMC Public Health* 2019, 19, 858. [CrossRef] [PubMed]
3. De Kraker, M.E.A.; Stewartson, A.J.; Harbarth, S. Will 10 Million People Die a Year due to Antimicrobial Resistance by 2050? *PLoS Med.* 2016, 13, e1002184. [CrossRef] [PubMed]
4. Gwynn, M.N.; Portnoy, A.; Rittenhouse, S.F.; Payne, D.J. Challenges of antibacterial discovery revisited. *Ann. N. Y. Acad. Sci.* 2010, 1213, 5–19. [CrossRef]
5. Yi, L.; Lu, X. New Strategy on antimicrobial-resistance: Inhibitors of DNA replication enzymes. *Curr. Med. Chem.* 2019, 26, 1761–1787. [CrossRef]
6. Klostermeier, D. Why two? On the role of (A-)symmetry in negative supercoiling of DNA by gyrase. *Int. J. Mol. Sci.* 2018, 19, 1489. [CrossRef] [PubMed]
7. Berger, J.; Schoeffler, A. Recent advances in understanding structure–function relationships in the type II topoisomerase mechanism. *Biochem. Soc. Trans.* 2005, 33, 1465–1470. [CrossRef] [PubMed]
8. Van Eijk, E.; Wittekoek, B.; Kuiper, E.J.; Smits, W.K. DNA replication proteins as potential targets for antimicrobials in drug-resistant bacterial pathogens. *J. Antimicrob. Chemother.* 2017, 72, 1275–1284. [CrossRef]
9. Singh, S.B. Confronting the challenges of discovery of novel antibacterial agents. *Bioorganic Med. Chem. Lett.* 2014, 24, 3683–3689. [CrossRef] [PubMed]
10. Collin, F.; Karkare, S.; Maxwell, A. Exploiting bacterial DNA gyrase as a drug target: Current state and perspectives. *Appl. Microbiol. Biotechnol.* 2011, 92, 479–497. [CrossRef]
11. Aldred, K.J.; Kerns, R.J.; Osheroff, N. Mechanism of quinolone action and resistance. *Biochemistry* 2014, 53, 1565–1574. [CrossRef] [PubMed]

12. Mustaev, A.; Malik, M.; Zhao, X.; Kurepina, N.; Luan, G.; Oppegard, L.M.; Hiasa, H.; Marks, K.R.; Kerns, R.J.; Berger, J.M.; et al. Fluoroquinolone-Gyrase-DNA Complexes. *J. Biol. Chem.* 2014, 289, 12300–12312. [CrossRef] [PubMed]

13. Kim, E.S.; Hooper, D. Clinical importance and epidemiology of quinolone resistance. *Infect. Chemother.* 2014, 46, 226–238. [CrossRef] [PubMed]

14. Correia, S.; Poeta, P.; Hébrard, M.; Capelo, J.L.; Igrejas, G. Mechanisms of quinolone action and resistance: Where do we stand? *J. Med. Microbiol.* 2017, 66, 551–559. [CrossRef]

15. Badshah, S.L.; Ullah, A. New developments in non-quinolone-based antibiotics for the inhibition of bacterial gyrase and topoisomerase IV. *Eur. J. Med. Chem.* 2018, 152, 393–400. [CrossRef]

16. Jakopin, Ž.; Ilasi, J.; Barančoková, M.; Brvar, M.; Tammela, P.; Tomašič, T.; Kikelj, D. Discovery of substituted oxadiazoles as a novel scaffold for DNA gyrase inhibitors. *Eur. J. Med. Chem.* 2017, 130, 171–184. [CrossRef]

17. Zidar, N.; Macut, H.; Tomašič, L.P.; Ilasi, J.; Zega, A.; Tammela, P.; Kikelj, D. New N-phenyl-4,5-dibromopyrrolamides as DNA gyrase B inhibitors. *MedChemComm* 2019, 10, 1007–1017. [CrossRef]

18. Kumar, S.; Narasimhan, B. Therapeutic potential of heterocyclic pyrimidine scaffolds. *Chem. Central J.* 2018, 12, 38. [CrossRef]

19. Hung, J.M.; Arabshahi, H.J.; Leung, E.; Reynisson, J.; Barker, D. Synthesis and cytotoxicity of thieno[2,3-b]pyridine and furan[2,3-b]pyridine derivatives. *Eur. J. Med. Chem.* 2014, 86, 420–437. [CrossRef]

20. Arabshahi, H.J.; Pilkington, L.I.; Jeon, C.Y.; Song, M.; Gridel, L.-M.; Zakharenko, A.L.; Lavrik, O.I.; Van Rensburg, M.; Leung, E.; Barker, D.; et al. A synthesis, in silico, in vitro and in vivo study of thieno[2,3-b]pyridine anticancer analogues. *MedChemComm* 2015, 6, 1987–1997. [CrossRef]

21. Naguib, B.H.; El-Nassar, H.B. Synthesis of new thieno[2,3-b]pyridine derivatives as pim-1 inhibitors. *J. Enzym. Inhib. Med. Chem.* 2016, 31, 1718–1725. [CrossRef]

22. Al-Trawneh, S.A.; El-Abadelah, M.M.; Zahra, J.A.; Al-Taweel, S.A.; Zani, F.; Incerti, M.; Cavazzoni, A.; Vicini, P. Synthesis and biological evaluation of tetracyclic thienopyridones as antibacterial and antitumor agents. *Bioorganic Med. Chem.* 2011, 19, 2541–2548. [CrossRef] [PubMed]

23. El-Deen, E.M.M.; El-Hameed, E.K.A. Synthesis and in vitro biological evaluation of new tetracyclic pyriothienoquinolines as potential antimicrobial agents. *Acta Pol. Pharm. Drug Res.* 2013, 70, 375–381. [CrossRef] [PubMed]

24. Amorim, R.; De Meneses, M.D.F.; Borges, J.C.; Paixão, I.C.N.D.P.; et al. Thieno[2,3-b]pyridine derivatives: A new class of antiviral drugs against Mayaro virus. *Arch. Virol.* 2017, 162, 1577–1587. [CrossRef] [PubMed]

25. Schnute, M.E.; Anderson, D.J.; Brideau, R.J.; Ciske, F.L.; Collier, S.A.; Cudahy, M.; Eggen, M.; Geeni, M.J.; Hopkins, T.A.; Judge, T.M.; et al. 2-Aryl-2-hydroxyethylamine substituted 4-oxo-4,7-dihydrothieno[2,3-b]pyridines as broad-spectrum inhibitors of human herpesvirus polymerases. *Bioorganic Med. Chem. Lett.* 2007, 17, 3349–3353. [CrossRef] [PubMed]

26. Liu, H.; Li, Y.; Wang, X.-Y.; Wang, B.; He, H.-Y.; Liu, J.-Y.; Xiang, M.; He, J.; Wu, X.-H.; Yang, L. Synthesis, preliminary structure–activity relationships, and in vitro biological evaluation of 6-aryl-3-amino-thieno[2,3-b]pyridine derivatives as potential anti-inflammatory agents. *Bioorganic Med. Chem. Lett.* 2013, 23, 2349–2352. [CrossRef]

27. Saito, K.; Nakao, A.; Shinozuka, T.; Shimada, K.; Matsui, S.; Oizumi, K.; Yano, K.; Ohata, K.; Nakai, D.; Nagai, Y.; et al. Discovery and structure–activity relationship of thienopyridine derivatives as bone anabolic agents. *Bioorganic Med. Chem.* 2013, 21, 1628–1642. [CrossRef]

28. Gad-Elkareem, M.A.; Abdel-Fattah, A.M.; Elneairty, M.A.A. Pyridine-2(1 H)-thione in heterocyclic synthesis: Synthesis and antimicrobial activity of some new thio-substituted ethyl nicotinate, thieno[2,3-b]pyridine and pyridothienopyrimidine derivatives. *J. Sulfur Chem.* 2011, 32, 273–286. [CrossRef]

29. El-Aleam, R.H.A.; George, R.F.; Hassan, G.S.; Abdel-Rahman, H.M. Synthesis of 1,2,4-triazolo[1,5-a]pyrimidine derivatives: Antimicrobial activity, DNA Gyrase inhibition and molecular docking. *Bioorganic Chem.* 2020, 94, 103411. [CrossRef]

30. Patil, S.B. Biological and medicinal significance of pyrimidines: A review. *Int. J. Pharm Sci. Res.* 2018, 9, 44–52. [CrossRef]
31. Fayed, A.A.; Amr, A.E.-G.E.; Al-Omar, M.A.; Mostafa, E.E. Synthesis and antimicrobial activity of some new substituted pyrido[3′,2′,4,5]thieno[3,2-d]pyrimidinone derivatives. *Russ. J. Bioorganic Chem.* **2014**, *40*, 308–313. [CrossRef]

32. El-Essawy, F.A.; Boshta, N.M.; El-Sawaf, A.K.; Nassar, A.A.; Khalafallah, M.S. Synthesis of novel 3-substituted pyridothienopyrimidinone derivatives with biological evaluation as antimicrobial agents. *Chem. Res. Chin. Univ.* **2016**, *32*, 967–972. [CrossRef]

33. Kadah, M.S. Synthesis of some new thienopyridine and pyridothienopyrimidine derivatives with expected antitumor activity. *Int. J. Med Sci.* **2016**, *3*, 5–10. [CrossRef]

34. El-Nassan, H.B.; Naguib, B.H.; Abdelghany, T.M. Synthesis of new pyridothienopyrimidinone derivatives as Pim-1 inhibitors. *J. Enzym. Inhib. Med. Chem.* **2017**, *32*, 457–467. [CrossRef]

35. El-Nassan, H.B.; Naguib, B.H.; Beshay, E.A. Synthesis of new pyridothienopyrimidinone and pyridothienotriazolopyrimidine derivatives as Pim-1 inhibitors. *J. Enzym. Inhib. Med. Chem.* **2017**, *33*, 58–66. [CrossRef] [PubMed]

36. Aziz, Y.M.A.; Said, M.M.; El Shihawy, H.A.; Abouzid, K.A. Discovery of novel tricyclic pyrido[3′,2′,4,5]thieno[3,2-d]pyrimidin-4-amine derivatives as VEGFR-2 inhibitors. *Bioorganic Chem.* **2015**, *60*, 1–12. [CrossRef] [PubMed]

37. El-Deen, E.M.; El-Meguid, E.A.A.; Hasabelnaby, S.; Karam, E.A.; Nossier, E.S.; El-Deen, E.M.; El-Meguid, E.A.A. Synthesis, Docking Studies, and In Vitro Evaluation of Some Novel Thiopyridazines and Fused Thiopyridine-Quinolines as Antibacterial Agents and DNA Gyrase Inhibitors. *Molecules* **2019**, *24*, 3650. [CrossRef]

38. Tari, L.W.; Trzoss, M.; Bensen, D.C.; Li, X.; Chen, Z.; Lam, T.; Zhang, J.; Creighton, C.J.; Cunningham, M.L.; Kwan, B.; et al. Pyrrolopyrimidine inhibitors of DNA gyrase B (GyrB) and topoisomerase IV (ParE). Part I: Structure guided discovery and optimization of dual targeting agents with potent, broad-spectrum enzymatic activity. *Bioorganic Med. Chem. Lett.* **2013**, *23*, 1529–1536. [CrossRef] [PubMed]

39. Trzoss, M.; Bensen, D.C.; Li, X.; Chen, Z.; Lam, T.; Zhang, J.; Creighton, C.J.; Cunningham, M.L.; Kwan, B.; Stidham, M.; et al. Pyrrolopyrimidine inhibitors of DNA gyrase B (GyrB) and topoisomerase IV (ParE). Part II: Development of inhibitors with broad spectrum, Gram-negative antibacterial activity. *Bioorganic Med. Chem. Lett.* **2013**, *23*, 1537–1543. [CrossRef]

40. Patel, R.; Park, S. An evolving role of piperazine moieties in drug design and discovery. *Mini-Rev. Med. Chem.* **2013**, *13*, 1579–1601. [CrossRef]

41. Patil, P.; Madhavachary, R.; Kurpiewska, K.; Kalinowska-Tłuczyck, J.; Dömling, A. De Novo assembly of highly substituted morpholines and piperazines. *Org. Lett.* **2017**, *19*, 642–645. [CrossRef] [PubMed]

42. Popielek, I. Hydrazide–hydrzones as potential antimicrobial agents: Overview of the literature since 2010. *Med. Chem. Res.* **2016**, *26*, 287–301. [CrossRef]

43. Rollas, S.; Küçükgüzel, S.G. Biological activities of hydrazone derivatives. *Molecules* **2007**, *12*, 1910–1939. [CrossRef] [PubMed]

44. Michael, J.M.; El-Zahar, M.I.; El-Masry, A.M.; Mohi El-Deen, E.M. New pyrido [3,2-d]pyrimidines of possible antimicrobial activity. *Al-Azhar Bull. Sci.* **1992**, *32*, 767.

45. Sharanin, Y.A.; Motrosova, S.V. Cyclization Reaction of Nitriles Part 56. Synthesis and transformations of substituted 6-aryl-4-(2-thienyl)-3-cyano-pyridine-2(1H)-thiones. *Zh. Org. Khim.* **1996**, *32*, 1251–1255.

46. Elzahabi, H.S.A.; Nossier, E.S.; Khalifa, N.M.; AlAsfoury, R.A.; El-Manawaty, M.A. Anticancer evaluation and molecular modeling of multi-targeted kinase inhibitors based pyrido[2,3-d]pyrimidine scaffold. *J. Enzym. Inhib. Med. Chem.* **2018**, *33*, 546–557. [CrossRef]

47. Holdgate, G.A.; Tunnicliffe, A.; Ward, W.H.J.; Weston, S.A.; Rosenbrock, G.; Barth, P.T.; Taylor, I.W.F.; Paupit, R.A.; Timms, D. The entropic penalty of ordered water accounts for weaker binding of the antibiotic novobiocin to a resistant mutant of DNA gyrase: A thermodynamic and crystallographic study. *Biochem. 1997*, *36*, 9663–9673. [CrossRef] [PubMed]

48. Wiegand, I.; Hilpert, K.; Hancock, R.E.W. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc.* **2008**, *3*, 163–175. [CrossRef]

49. Phillips, J.W.; Goetz, M.A.; Smith, S.K.; Zink, D.L.; Polshok, J.; Onishi, R.; Salowe, S.; Wiltsie, J.; Allocco, J.; Sigmund, J.; et al. Discovery of Kibdelomycin, A Potent new class of bacterial type II topoisomerase inhibitor by chemical-genetic profiling in staphylococcus aureus. *Chem. Biol.* **2011**, *18*, 955–965. [CrossRef] [PubMed]
50. Maxwell, A.; Burton, N.P.; O’Hagan, N. High-throughput assays for DNA gyrase and other topoisomerases. *Nucleic Acids Res.* 2006, 34, e104. [CrossRef]

51. Bellon, S.; Parsons, J.D.; Wei, Y.; Hayakawa, K.; Swenson, L.L.; Charifson, P.S.; Lippke, J.A.; Aldape, R.; Gross, C.H. Crystal structures of escherichia coli topoisomerase IV pare subunit (24 and 43 Kilodaltons): A single residue dictates differences in novobiocin potency against topoisomerase IV and DNA gyrase. *Antimicrob. Agents Chemother.* 2004, 48, 1856–1864. [CrossRef] [PubMed]

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