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Short communication

Synthetic tactics of new class of 4-aminothieno[2,3-*d*]pyrimidine-6-carbonitrile derivatives acting as antimicrobial agents

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ABSTRACT

Thermal selective reactions were studied on oxothieno[2,3-*d*]pyrimidine-6-carboxamide **3** with $POCl_3$ and PCl_5. At 25–50 °C, the C_7-amide rearranges to nitrile furnished compound **4** in 85–90% yield, while at 80–110 °C furnished mixture of products **4** and **5** in 28–68% yields. The chloro displacement with amines in compound **5** yielded 4-aminothieno[2,3-*d*]pyrimidine-6-carbonitrile derivatives **8**(**a**–**h**) and **9**(**a**–**e**). Antimicrobial activity of new compounds was studied against several bacteria such as *Staphylococcus aureus* MTCC-96, *Escherichia coli* MTCC-443, *Pseudomonas aeruginosa* MTCC-4 41, *Streptococcus pyogenes* MTCC-442 and fungi *Aspergillus niger* MTCC-282, *Aspergillus clavatus* MTCC-1323, *Candida albicans* MTCC-227 using broth microdilution method. Compounds **4**, **8b**, **8d**, **8e**, **8h** and **9a** showed promising antibacterial activity compared to *ampicillin* and compounds **8b**, **8h** showed better antifungal activity compared to *greseofulvin*.

1. Introduction

Antibiotics have revolutionized the medical care in the 20th century. With the discovery of antibiotics, people were convinced that infectious diseases might someday be wiped out. However, the emergence of superbugs i.e. pathogenic bacteria that resist the effects of most powerful antibiotics available today are posing a great challenge to the field of medicines. Thus scientists are working to find new ways to defeat bacteria which has become one of the most important areas of antibacterial research today. In addition, it is known that antifungal drugs do not have selective activity because of the biochemical similarity between human cell and fungi forms. Hence there are many studies focused on antibacterial and antifungal compounds [1–3]. It is well known that pyrimidine and fused pyrimidine derivatives are of great biological interest, especially as antiviral, antitumor and antimicrobial agents [4–15].

Among thieno[2,3-*d*]pyrimidine derivatives, the 3-amino-5,6-dimethyl-2-[4-(1-phenylmethyl)-1-piperazinyl]thieno[2,3-*d*]pyrimidine-4-(3*H*)-one **I** [16] and 4-(4-methyl-1-piperazinyl)-2-methyl-6,7-dihydro-5*H*-cyclopenta [4,5]thieno[2,3-*d*]pyrimidine **II** [17] exhibited remarkable affinity and selectivity for the 5-HT3 receptor (Fig. 1). Consequently, thienopyrimidines have become a well sought privileged class of compounds in drug discovery programs.

Pyrimidine nucleus fused with other heterocycle has been found to be an integral part of natural products, agrochemicals and veterinary products [18,19]. This class of compounds has also been found wide applications in the design and discovery of novel bioactive compounds and drugs [20] such as kinase inhibitors [21], LHCGR agonists [22], phosphodiesteras inhibitors [23], antifolate and antimalarial agents [24], blood platelet aggregation inhibitors [25] and reverse inhibitors of the gastric (H^+/K^+) ATPase [26].

On this context, we have previously reported the synthesis and efficacy of various polysubstituted thiophenes, fused thienopyrimidines as very good molluscicidal agents [27]. In pursuance of our ongoing research in the synthesis and biological activity study of new class of thienopyrimidines, we are interested to generate small library based on this vital thieno[2,3-*d*]pyrimidine scaffold by both base catalyzed conventional heating and catalyst free neat heating method.

2. Results and discussion

2.1. Chemistry

Recently, we have reported the synthesis of 5-amino-3-(4-chlorophenyl)thiophene-2,4-dicarbo-nitrile [28] **1** and 5-amino-3-(4-chlorophenyl)thiophene-2,4-dicarboxamide [28] **2** which

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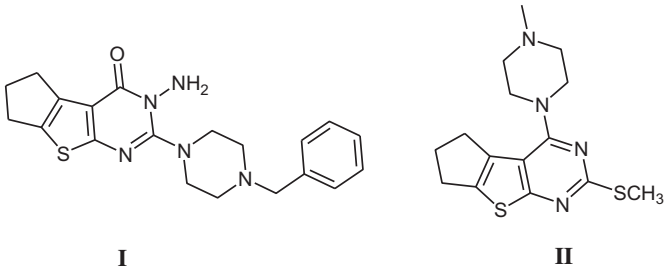


Fig. 1. Compounds I and II exhibited remarkable affinity and selectivity for the 5-HT_3 receptor.

were prepared using literature procedures [29–32]. These precursors **1** and **2** were well utilized for the synthesis of an important intermediate 5-(4-chlorophenyl)-3,4-dihydro-4-oxothieno[2,3-*d*] pyridine-6-carboxamide **3** [28] in good yield (Scheme 1).

Reaction of intermediate **3** in phosphorusoxychloride ($POCl_3$) in presence of phosphorous pentachloride (PCl_5) at variable temperature and reaction time afforded 5-(4-chlorophenyl)-3,4-dihydro-4-oxothieno[2,3-*d*]pyrimidine-6-carbonitrile **4** and 4-chloro-5-(4-chlorophenyl)thieno[2,3-*d*]pyrimidine-6-carbonitrile **5** (Scheme 1) derivatives in 28–90% yields (Table 1), were isolated by column chromatography (Silica Gel 60–120 mesh). Interestingly, on heating above reaction mixture at 25–50 °C, primary amide rearranged to give nitrile **4** as a sole product, while at elevated temperature at 80–110 °C, along with compound **5** as a major product compound **4** was also formed as minor product (Scheme 1). It means, at low temperature only primary amide react with $POCl_3$ while at higher temperature along with primary amide, secondary amide also react. The lactum–lactim tautomerism occurred at higher temperature give evidence to the formation of target molecule (**TM**) **5** as major product (Table 1).

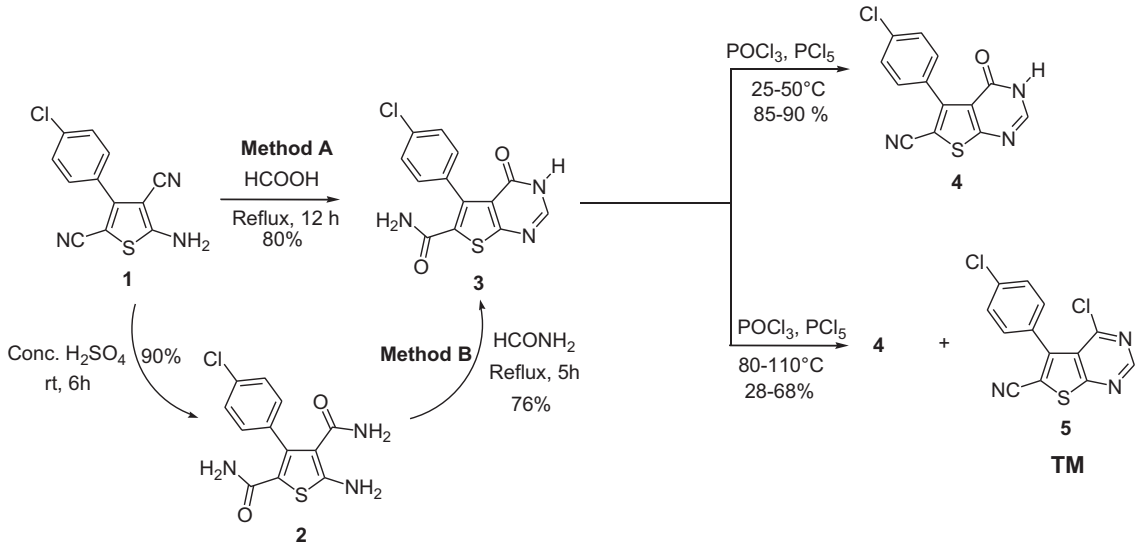
The C_4-substituent played a key role in displaying higher pharmacological activities to thieno-pyrimidine derivatives [16,17,33], hence we focused on the 'C_4' position of **TM 5** as target site for generation of library of new thienopyrimidines. Numerous reports are available for the introduction of aliphatic and aromatic amines at the C_4-position [34] of thieno[2,3-*d*]pyrimidine ring. Herein, we report the substitution of substituted anilines and piperazines at C4 position of **TM 5**, by different strategy. The C_4-S_NAr Cl-displacement in compound **5** with various amines was successfully done by conventional heating in isopropanol (IPA) in presence of catalytic amount of triethylamine **(Method C)** or by non-conventional catalyst free neat heating in a sealed tube **(Method D)** furnished desired compounds **8** and **9** in good yields (Scheme 2). Herein, we keenly observed that neat reaction was found superior over conventional method as the former method afforded higher yields of the desired product in a shorter time. The green approach for displacement of chlorofunctionality by amines, saves organic solvent and catalyst, prevents pollution with increasing atom economy of the reaction (Tables 2 and 3). The structures of all new compounds **3**, **4**, **5**, **8** and **9** were confirmed from their IR, ^{1}H NMR, ^{13}C NMR and MS spectral data given in experimental protocol.

3. Biology

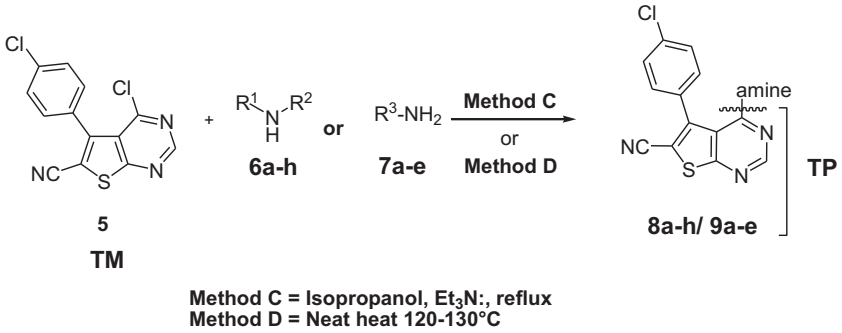
The MICs of synthesized compounds were carried out by broth microdilution method as described by Rattan [35]. Antibacterial activity was screened against two Gram positive (*Staphylococcus aureus* MTCC 96, *Streptococcus pyogenes* MTCC 442) and two Gram negative (*Escherichia coli* MTCC 443, *Pseudomonas aeruginosa* MTCC 441) bacteria by using *gentamycin*, *ampicillin*, *chloramphenicol*, *ciprofloxacin*, *norfloxacin* as a standard antibacterial agents. Antifungal activity was screened against three fungal species *Candida albicans* MTCC 227, *Aspergillus niger* MTCC 282 and *Aspergillus clavatus* MTCC 1323 and *nystatin* and *greseofulvin* was used as a standard antifungal agent. In literature various 6-benzyl-2-phenyl-4-amino (hydroxy, methyl substituted or unsubstituted-1-phenyl)-5,6,7,8-tetrahydropyridothieno[2,3-*d*]pyrimidine [36] derivatives revealed significant antibacterial activity against *E. coli*. In this article, our compounds 5-(4-chlorophenyl)-3,4-dihydro-4-oxothieno[2,3-*d*]pyrimidine-6-carbonitrile **4** and 5-(4-chlorophenyl)-4-(amino) thieno[2,3-*d*]pyrimidine-6-carbonitrile (**8b**, **8d**, **8e**, **8h**, **9a**) derivatives showed good antibacterial activity as well as antifungal activity compared to *ampicillin* and *greseofulvin* respectively. This antimicrobial screening data is summarized in Table 4.

4. Conclusion

Thermal selective reaction of oxothieno[2,3-*d*]pyrimidine-carboxamide with $POCl_3$ gave rearrangement of primary amide group to nitrile at lower temperature, while at higher temperature formed S_NAr chloro substitution by lactum–lactim tautomerism along with



Scheme 1. Synthetic strategy for the synthesis of target molecule, **5**.



Scheme 2. Synthesis of new derivatives of 4-aminothieno[2,3-*d*]pyrimidine-6-carbonitrile derivatives.

rearranged product. The environmentally benign neat heat reaction has been successfully utilized for the synthesis of new class of 4-aminothieno[2,3-*d*]pyrimidine-6-carbonitrile derivatives with good to excellent yields in shorter time compared to conventional reaction conditions using solvent and base afforded quite good yields in longer time. Compounds **4**, **8b**, **8d**, **8e**, **8h** and **9a** showed promising antibacterial activity compared to *ampicillin* and compounds **8b**, **8h** showed better antifungal activity compared to *greseofulvin*.

5. Experimental

Melting points were determined on a Gallenkamp melting point apparatus. The ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were recorded on a Varian NMR Mercury 300 spectrometer. Chemical shifts were reported in ppm relative to tetramethylsilane (TMS), and multiplicities are given as s (singlet), bs (broad singlet), d (doublet), t (triplet), q (quartet), or m (multiplet). Infrared spectra were recorded as KBr pellets on a Shimadzu FTIR-408 spectrophotometer. Mass spectra were recorded on a Shimadzu LC-MS: EI QP 2010A mass spectrometer with an ionization potential of 70 eV. Elemental analyses (C, H and N) were performed on Thermo Finnigan Eager 300 EA 1112 series analyzer. Reactions were monitored by thin layer chromatography (TLC), carried out on 0.2 mm silica gel 60 F_{254} (Merck) plates using UV light (254 and 366 nm) for detection and compounds were purified by column chromatography by using silica gel of 5–20 μm (Merck, 60–120 mesh). Column dimension is 39 × 2 cm^2 and elution volume used is about 200–400 mL for each product where necessary. Common reagent-grade chemicals are either commercially available and were used without further purification or were prepared by standard literature procedures. All MTCC cultures were collected from Institute of Microbial Technology, Chandigarh and tested against above mentioned known drugs. Mueller Hinton broth was used as nutrient medium to grow and dilute the drug suspension for the test. Inoculums' size for test strain was adjusted to 108 CFU (Colony Forming Unit) per milliliter by comparing the turbidity. DMSO was used as diluents to get desired concentration of drugs to test upon standard bacterial strains. Each set of antimicrobial assay experiment was run three times and the average MIC's were calculated. MIC of compounds was determined against *Mycobacterium tuberculosis* H37Rv strain by using Lowenstein–Jensen medium (conventional method) as described by Rattan [35].

Table 1
Temperature dependent yields of compounds **4** and **5**.

Entry	Temperature °C	Time h	Yield[a] %	
			Compd 4	Compd 5
1	25	18	90	–
2	50	8.0	85	–
3	80	1.0	64	28
4	110	0.5	36	68

[a] Isolated yields after column chromatography.

5.1. 5-Amino-3-(4-chlorophenyl)thiophene-2,4-dicarbonitrile (*1*)

This compound was synthesized by the known literature method [12–14], recrystallized from water/DMF (4:2). Obtained yield 2.12 g (82%), mp 292–294 °C [Lit. mp 293–294 °C].

5.2. 5-Amino-3-(4-chlorophenyl)thiophene-2,4-dicarboxamide (*2*)

This compound is also synthesized by the literature procedure [28] and recrystallized from ethanol:DMF (8:2). Obtained yield 2.59 g (88%), mp 200–201 °C [Lit. mp 200–202 °C].

5.3. 5-(4-Chlorophenyl)-3,4-dihydro-4-oxothieno[2,3-d] pyrimidine-6-carboxamide (*3*)

(*Method A*): 5-Amino-3-(4-chlorophenyl)thiophene-2,4-dicarbonitrile **1** (2.59 g, 0.01 mol) in formic acid (5 mL) was refluxed for 12 h (TLC check, chloroform:methanol, 8:1). On cooling to room temperature the obtained solid product **3** was collected by vacuum filtration, washed thoroughly with water, dried and purified by column chromatography eluting with chloroform:methanol (8:1) to give pale green crystals. Obtained yield 2.44 g (80%) [28].

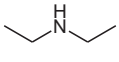
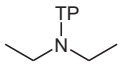
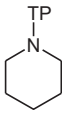
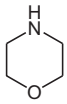
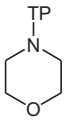
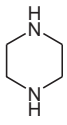
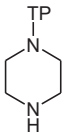
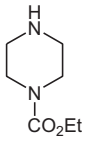
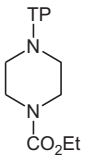
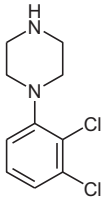
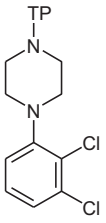
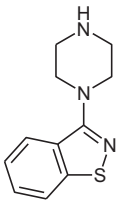
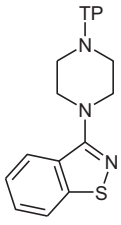
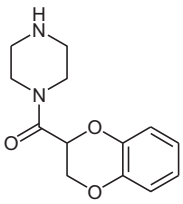
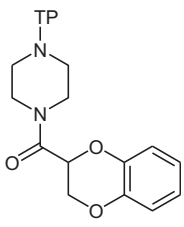
(*Method B*): 5-Amino-3-(4-chlorophenyl)thiophene-2,4-dicarboxamide, **2** (2.95 g, 0.01 mol) was dissolved in formamide (5 mL). The resulting solution was heated at reflux temperature for 5 h (chloroform:methanol, 8:1), and then it was allowed to stand at room temperature. The dark green crystals that formed was collected by vacuum filtration and washed with sufficient amount of cold methanol to give pale green crystals. Obtained yield 2.32 g (76%).

Mp 265–266 °C; IR (KBr): 1566, 1635 (Ar C=C), 1697 (C=O), 3307 (NH), cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ 6.59 (bs, 1H, 1°amide NH_2), 7.38 (d, J = 8.4 Hz, 2H, ArH), 7.45 (d, J = 8.4 Hz, 2H, ArH), 7.62 (bs, 1H, 1°amide NH_2), 8.17 (s, 1H, C_2H), 12.49 (bs, 1H, 2°amide NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 122.70, 127.65, 130.96, 131.64, 132.69, 132.87, 136.44, 147.48, 157.19, 162.72,164.06; MS (EI): m/z 304.30 (M − 1, 60%), 306.10 (M + 1, 20%). Anal. calcd. for $C_{13}H_8ClN_3O_2S$ (305.00): C, 51.07; H, 2.64; N, 13.74. Found: C, 51.19; H, 2.79; N, 13.59.

5.4. Synthesis of 5-(4-chlorophenyl)-3,4-dihydro-4-oxothieno[2,3-d]pyrimidine-6-carbonitrile (*4*) and 4-chloro-5-(4-chlorophenyl) thieno[2,3-d]pyrimidine-6-carbonitrile (*5*)

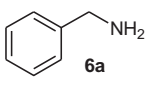
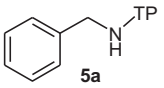
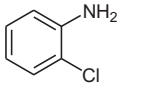
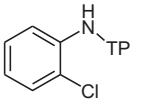
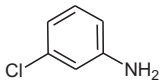
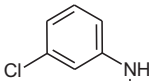
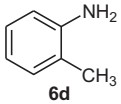
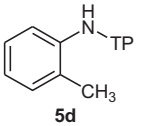
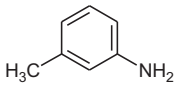
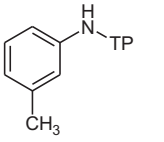
5-(4-Chlorophenyl)-3,4-dihydro-4-oxothieno[2,3-*d*]pyrimidine-6-carboxamide, **3** (3.05 g, 0.01 mol) was stirred in $POCl_3$ (15 mL) and PCl_5 (1.0 g) at room temperature for 18 h. The excess $POCl_3$ was then

Table 2
Synthesis of new 4-aminothieno[2,3-*d*]pyrimidine derivatives by using secondary amines.

Entry	Secondary amine NHR^1R^2	Product	Method C		Method D	
			Yield[a] %	Time h	Yield[a] %	Time h
1	 **5a**	 **3a**	55	10	71	4
2	 **5b**	 **3b**	62	12	73	2.5
3	 **5c**	 **3c**	60	13	68	3.5
4	 **5d**	 **3d**	55	11	65	2
5	 **5e**	 **3e**	62	16	70	3
6	 **5f**	 **3f**	50	14	67	5
7	 **5g**	 **3g**	52	15	65	7
8	 **5h**	 **3h**	55	18	66	6

*TP – Thienopyrimidine.
[a] Isolated yields after column chromatography.

Table 3
Synthesis of new 4-aminothieno[2,3-*d*]pyrimidine derivatives by using primary amines.

Entry	Primary amine R^3-NH_2	Product	Method C		Method D	
			Yield[a] %	Time h	Yield[a] %	Time h
1	 **6a**	 **5a**	53	16	68	4.5
2	 **6b**	 **5b**	54	20	60	3
3	 **6c**	 **5c**	50	13	66	4
4	 **6d**	 **5d**	60	15	70	3.5
5	 **6e**	 **5e**	53	18	62	5

*TP – Thienopyrimidine.
[a] Isolated yields after column chromatography.

distilled off under vacuum and obtained residue was stirred in ice water (500 mL), neutralized with saturated sodium carbonate (50 mL) and filtered. This separated solid residue was purified by column chromatography by using chloroform:methanol (9:1) as an eluent. To optimize the reaction condition, the reaction mixture was heated at 50, 80 and 110 °C for time interval of 8.0, 1.0 and 0.5 h respectively and worked out as described above. The yields of products **4** and **5** are as shown in Table 1.

5.4.1. 5-(4-Chlorophenyl)-3,4-dihydro-4-oxothieno[2,3-d]pyrimidine-6-carbonitrile (*4*)

Pale yellow amorphous solid, mp 324–325 °C; IR (KBr): 1587, 1630 (Ar C=C), 1679 (C=O), 2214 (CN), 3190 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 7.54 (d, J = 8.7 Hz, 2H, ArH), 7.57 (d, J = 8.7 Hz, 2H, ArH), 8.34 (s, 1H, C_2H), 12.87 (bs, 1H, NH); MS (EI): m/z 286.10 (M − 1, 100%), 288.05 (M + 1, 30%). Anal. calcd. for $C_{13}H_6ClN_3OS$ (286.99): C, 54.27; H, 2.10; N, 14.60. Found: C, 53.98; H, 2.24; N, 14.32.

5.4.2. 4-Chloro-5-(4-chlorophenyl)thieno[2,3-d]pyrimidine-6-carbonitrile (*5*)

Yellow crystalline solid, mp 240–241 °C; IR (KBr): 1537, 1621 (Ar C=C), 2219 (CN) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.40 (d, J = 8.7 Hz, 2H, ArH), 7.53 (d, J = 8.7 Hz, 2H, ArH), 9.01 (s, 1H, C_2H); 13C NMR (75 MHz, $CDCl_3$): δ 109.16, 113.07, 120.81, 128.21, 129.69, 130.63, 135.59, 154.62, 156.51, 156.70, 168.52; MS (EI): m/z 306.00 (M + 1, 100%), 308.00 (M + 3, 63.5%), 310 (M + 5, 10.6%), Anal. calcd. for $C_{13}H_5Cl_2N_3S$ (304.96): C, 51.00; H, 1.65; N, 13.72. Found: C, 50.88; H, 1.44; N, 13.96.

Table 4
In vitro antimicrobial activity of newly synthesized compounds **2** to **9e** as MICs (μg/mL).

Compd	Gram positive bacteria		Gram negative bacteria		Fungi		
	E.C.	*P.A.*	*S.A.*	*S.P.*	*C.A.*	*A.N.*	*A.C.*
	MTCC	MTCC	MTCC	MTCC	MTCC	MTCC	MTCC
	443	441	96	442	227	282	1323
2	200	250	250	200	500	>1000	>1000
3	125	200	200	200	>1000	250	500
4	100	62.5	200	250	500	>1000	>1000
5	200	250	100	250	1000	1000	1000
8a	200	250	200	200	1000	>1000	>1000
8b	250	250	125	200	250	>1000	>1000
8c	125	200	250	250	1000	>1000	>1000
8d	125	250	62.5	100	1000	1000	>1000
8e	62.5	100	250	250	>1000	1000	1000
8f	200	100	250	250	1000	500	1000
8g	100	250	200	200	500	>1000	>1000
8h	100	250	100	125	250	1000	1000
9a	250	250	100	125	1000	1000	1000
9b	125	62.5	200	100	500	>1000	>1000
9c	100	100	200	250	>1000	500	500
9d	200	200	250	125	>1000	>1000	>1000
9e	250	200	250	250	>1000	>1000	>1000
Std.1	0.05	1	0.25	0.5	n.t.[a]	n.t.	n.t.
Std.2	100	–	250	100	n.t.	n.t.	n.t.
Std.3	50	50	50	50	n.t.	n.t.	n.t.
Std.4	25	25	50	50	n.t.	n.t.	n.t.
Std.5	10	10	10	10	n.t.	n.t.	n.t.
Std.6	n.t.	n.t.	n.t.	n.t.	100	100	100
Std.7	n.t.	n.t.	n.t.	n.t.	500	100	100

Std.1 Gentamycin; Std.2 Ampicillin; Std.3 Chloramphenicol; Std.4 Ciprofloxacin; Std.5 Norfloxacin; Std.6 Nystatin; Std.7 Greseofulvin *E.C.*: *Escherichia coli*, *P.A.*: *Pseudomonas aeruginosa*, *S.A.*: *Staphylococcus aureus*, *S.P.*: *Streptococcus pyogenes*, *C.A.*: *Candida albicans*, *A.N.*: *Aspergillus niger*, *A.C.*: *Aspergillus clavatus*.
[a] n.t. not tested.

5.5. General procedure for the synthesis of 5-(4-chlorophenyl)-4-aminothieno[2,3-d]pyrimidine-6-carbonitrile derivatives *8a–h* and *9a–e*

(*Method C*): 4-Chloro-5-(4-chlorophenyl)thieno[2,3-*d*]pyrimidine-6-carbonitrile, **5** (0.31 g, 0.001 mol) and the appropriate primary (0.002 mol) and/or secondary amines (0.0011 mol) were refluxed in isopropanol (10 mL) by using catalytic amount of triethylamine for about 10–22 h (TLC check, chloroform:methanol, 9:1). The mixture was then cooled to room temperature and excess isopropanol was distilled off under vacuum, and the resulting semisolid was triturated with cold methanol. The solid product precipitated was collected by suction filtration. All these compounds were purified by column chromatography using chloroform:methanol (9:1) as an eluent and yields are given in Tables 2 and 3.

(*Method D*): The same reaction mixture as in above method (*Method C*) without using the solvent isopropanol in (*Method D*) was heated in a sealed hard glass tube at 120–130 °C for 2–7 h. The resulting semisolid was triturated with cold methanol (5 mL) and stirred overnight. The solid product precipitated was collected by suction filtration and washed with appropriate cold methanol. All these compounds were purified by column chromatography eluting with chloroform:methanol (9:1). (Yields are given in Tables 2 and 3.)

5.5.1. 5-(4-Chlorophenyl)-4-(diethylamino)thieno[2,3-d]pyrimidine-6-carbonitrile (*8a*)

Light yellow crystalline solid, mp 166–168 °C; IR (KBr): 1527, 1621 (Ar C=C), 2210 (CN), 3321 (NH) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.92 (t, J = 7.2 Hz, 6H, $2CH_3$), 3.17 (q, J = 7.2 Hz, 4H, $2CH_2$), 7.43 (d, J = 8.4 Hz, 2H, ArH), 7.48 (d, J = 8.4 Hz, 2H, ArH), 8.56 (s, 1H, C_2H); MS (EI): m/z 342.10 (M^+, 50%), 344.12 (M + 2, 16.5%). Anal.

calcd. for $C_{17}H_{15}ClN_4S$ (342.07): C, 59.56; H, 4.41; N, 16.34. Found: C, 59.25; H, 4.60; N, 16.12.

*5.5.2. 5-(4-Chlorophenyl)-4-(piperidin-1-yl)thieno[2,3-d]pyrimidine-6-carbonitrile (**8b**)*

Light yellow amorphous solid, mp 170–171 °C; IR (KBr): 1537, 1629 (Ar C=C), 2208 (CN) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 1.24–1.46 (m, 6H, $3CH_2$), 3.16–3.19 (m, 4H, $2CH_2$), 7.43 (d, J = 8.4 Hz, 2H, ArH), 7.51 (d, J = 8.4 Hz, 2H, ArH), 8.58 (s, 1H, C_2H); MS (EI): m/z 355.10 (M + 1, 100%), 357.10 (M + 3, 35%). Anal. calcd. for $C_{18}H_{15}ClN_4S$ (354.07): C, 60.92; H, 4.26; N, 15.79; Found: C, 60.68; H, 3.99; N, 15.47.

*5.5.3. 5-(4-Chlorophenyl)-4-morpholinothieno[2,3-d]pyrimidine-6-carbonitrile (**8c**)*

Dark yellow amorphous solid, mp 199–200 °C; IR (KBr): 1271 (C–O), 1533, 1633 (Ar C=C), 2206 (CN) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 3.21–3.39 (m, 8H, $4CH_2$), 7.44 (d, J = 8.2 Hz, 2H, ArH), 7.57 (d, J = 8.2 Hz, 2H, ArH), 8.65 (s, 1H, C_2H); MS (EI): m/z 357.10 (M + 1, 100%), 359.05 (M + 2, 36%). Anal. calcd. for $C_{17}H_{13}ClN_4OS$ (356.05): C, 57.22; H, 3.67; N, 15.70; S, 8.99; Found: C, 57.38; H, 3.51; N, 15.48.

*5.5.4. 5-(4-Chlorophenyl)-4-(piperazin-1-yl)thieno[2,3-d]pyrimidine-6-carbonitrile (**8d**)*

Off white amorphous solid, mp 230–231 °C; IR (KBr): 1533, 1628 (Ar C=C), 2212 (CN), 3033, 3475 (NH) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 2.89 (m, 4H, $2CH_2$), 3.21 (m, 4H, $2CH_2$), 3.65 (bs, 1H, NH), 7.60 (d, J = 8.1 Hz, 2H, ArH), 7.69 (d, J = 8.1 Hz, 2H, ArH), 8.68 (s, 1H, C_2H); MS (EI): m/z 355.10 (M^+, 100%), 357.13 (M + 2, 33%). Anal. calcd. for $C_{17}H_{14}ClN_5S$ (355.07): C, 57.38; H, 3.97; N, 19.68. Found: C, 57.60; H, 4.12; N, 19.72.

*5.5.5. Ethyl-4-(5-(4-chlorophenyl)-6-cyanothieno[2,3-d]pyrimidin-4-yl)piperazine-1- carboxylate (**8e**)*

Light yellow crystalline solid, mp 164–166 °C; IR (KBr): 1125 (C–O), 1533, 1622 (Ar C=C), 1718 (C=O), 2208 (CN) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 1.02 (m, J = 11.7 Hz, 2H, CH_2), 1.14 (t, J = 7.2 Hz, 3H, CH_3), 1.53 (m, J = 11.7 Hz, 2H, CH_2), 2.71 (m, J = 11.7 Hz, 2H, CH_2), 3.63 (m, J = 11.7 Hz, 2H, CH_2), 4.00 (q, J = 7.2 Hz, 2H, OCH_2), 7.59 (d, J = 8.7 Hz, 2H, ArH), 7.67 (d, J = 8.7 Hz, 2H, ArH), 8.65 (s, 1H, C_2H); ^{13}C NMR (75 Hz, DMSO-d_6): δ 14.07, 26.96, 40.20, 48.62, 60.52,103.28, 113.76, 114.06, 129.21, 129.94, 131.20, 135.79, 144.86, 154.49, 161.28, 170.08, 173.67. MS (EI): m/z 427.10 (M^+, 100%), 429.10 (M + 2, 40%). Anal. calcd. for $C_{20}H_{18}ClN_5O_2S$ (427.09): C, 56.14; H, 4.24; N, 16.37; Found: C, 56.34; H, 4.52; N, 16.15.

*5.5.6. 5-(4-Chlorophenyl)-4-(4-(2,3-dichlorophenyl)piperazin-1-yl) thieno[2,3-d]pyrimidine-6-carbonitrile (**8f**)*

Off white amorphous solid, mp 206–208 °C; IR (KBr): 1533, 1627 (Ar C=C), 2208 (CN) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 2.79–2.81 (m, J = 4.8 Hz, 4H, $2CH_2$), 3.33–3.35 (m, J = 4.8 Hz, 4H, $2CH_2$), 6.57–7.23 (m, 3H, ArH), 7.45 (d, J = 8.4 Hz, 2H, ArH), 7.52 (d, J = 8.4 Hz, 2H, ArH), 8.63 (s, 1H, C_2H); MS (EI): m/z 500.00 (M + 1, 100%), 502.00 (M + 3, 90%), 504.15 (M + 5, 30%), 505.80 (M + 7, 5%). Anal. calcd. for $C_{23}H_{16}Cl_3N_5S$ (499.02): C, 55.16; H, 3.22; N, 13.98; Found: C, 55.37; H, 3.38; N, 14.12.

*5.5.7. 4-(4-(Benzo[d]isothiazol-3-yl)piperazin-1-yl)-5-(4-chlorophenyl)thieno[2,3-d]pyrimidine-6-carbonitrile (**8g**)*

Yellow amorphous solid, mp 184–186 °C; IR (KBr): 1529, 1630 (Ar C=C), 2208 (CN) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 3.24 (m, 4H, $2CH_2$), 3.46 (m, 4H, $2CH_2$), 7.55 (d, J = 9.0 Hz, 2H, ArH), 7.80 (d, J = 9.0 Hz, 2H, ArH), 7.47–7.76 (m, 4H, ArH), 8.66 (s, 1H, C_2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 49.01, 113.92, 120.63, 123.39, 124.05, 127.66, 129.42, 129.98, 131.18, 136.11, 144.69, 152.82, 154.50, 161.36, 162.83, 170.21; MS (EI): m/z 489.10 (M + 1, 100%), 491.00 (M + 3, 40%). Anal. calcd. for $C_{24}H_{17}ClN_6S_2$ (488.06): C, 58.95; H, 3.50; N, 17.19; Found: C, 59.19; H, 3.23; N, 17.46.

*5.5.8. 5-(4-Chlorophenyl)-4-(2,3-dihydrobenzo[b][1,4]dioxin-3-yl)(piperazin-1-yl)methanone-thieno[2,3-d]pyrimidine-6-carbonitrile (**8h**)*

Colorless amorphous solid, mp 212–213 °C; IR (KBr): 1529, 1625 (Ar C=C), 1654 (C=O), 2208 (CN) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 3.69–4.41 (m, 8H, $4CH_2$), 5.12 (t, J = 4.5 Hz, 1H, OCH), 5.26 (d, J = 4.5 Hz, 2H, CH_2), 6.85–6.92 (m, 4H, ArH), 7.64 (d, J = 8.4 Hz, 2H, ArH), 7.71 (d, J = 8.4 Hz, 2H, ArH), 8.72 (s, 1H, C_2H). MS (EI): m/z 518.10 (M + 1, 100%), 520.05 (M + 3, 35%). Anal. calcd. for $C_{26}H_{20}ClN_5O_3S$ (517.10): C, 60.29; H, 3.89; N, 13.52; Found: C, 60.10; H, 3.68; N, 13.71.

*5.5.9. 4-(Benzylamino)-5-(4-chlorophenyl)thieno[2,3-d]pyrimidine-6-carbonitrile (**9a**)*

Yellow amorphous solid, mp 100–102 °C; IR (KBr): 1577, 1620 (Ar C=C), 2212 (CN), 3035, 3417 (NH) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 4.59 (s, 2H, CH_2), 5.22 (bs, 1H, NH), 7.06–7.32 (m, 5H, ArH), 7.38 (d, J = 8.4 Hz, 2H, ArH), 7.45 (d, J = 8.4 Hz, 2H, ArH), 8.60 (s, 1H, C_2H). MS (EI): m/z 377.10 (M + 1, 100%), 379.10 (M + 3, 32%). Anal. calcd. for $C_{20}H_{13}ClN_4S$ (376.05): C, 63.74; H, 3.48; N, 14.87; Found: C, 63.50; H, 3.56; N, 14.96.

*5.5.10. 4-(2-Chlorophenylamino)-5-(4-chlorophenyl)thieno[2,3-d] pyrimidine-6-carbonitrile (**9b**)*

Pale green amorphous solid, mp 178–180 °C; IR (KBr): 1626 (Ar C=C), 2214 (CN), 3029, 3066 (NH) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.04–7.34 (m, 4H, ArH), 7.46 (bs, 1H, NH), 7.53 (d, J = 8.4 Hz, 2H, ArH), 7.63 (d, J = 8.4 Hz, 2H, ArH), 8.73 (s, 1H, C_2H). MS (EI): m/z 397.10 (M + 1, 100%), 399.10 (M + 3, 57%), 401.05 (M + 5, 9%). Anal. calcd. for $C_{19}H_{10}Cl_2N_4S$ (396.00): C, 57.44; H, 2.54; N, 14.10; Found: C, 57.73; H, 2.66; N, 13.94.

*5.5.11. 4-(3-Chlorophenylamino)-5-(4-chlorophenyl)thieno[2,3-d] pyrimidine-6-carbonitrile (**9c**)*

Brown amorphous solid, mp 160–161 °C; IR (KBr): 1623 (Ar C=C), 2210 (CN), 3032, 3067 (NH) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 6.89–7.70 (m, 4H, ArH), 7.19 (bs, 1H, NH), 7.23 (d, J = 8.4 Hz, 2H, ArH), 7.55 (d, J = 8.4 Hz, 2H, ArH), 8.72 (s, H, C_2H). MS (EI): m/z 397.05 (M + 1, 100%), 399.10 (M + 3, 65%), 401.10 (M + 5, 10%). Anal. calcd. for $C_{19}H_{10}Cl_2N_4S$ (396.00): C, 57.44; H, 2.54; N, 14.10; Found: C, 57.75; H, 2.68; N, 13.91.

*5.5.12. 4-(o-Tolylamino)-5-(4-chlorophenyl)thieno[2,3-d]pyrimidine-6-carbonitrile (**9d**)*

Pale brown amorphous solid, mp 200–202 °C; IR (KBr): 1560, 1612 (Ar C=C), 2208 (CN), 2966 (C–H), 3024, 3403 (NH) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 1.85 (s, 3H, CH_3), 7.19–7.62 (m, 4H, ArH), 7.36 (bs, 1H, NH), 7.72 (d, J = 8.7 Hz, 2H, ArH), 7.76 (d, J = 8.7 Hz, 2H, ArH), 8.59 (s, H, C_2H). MS (EI): m/z 377.10 (M + 1, 100%), 379.05 (M + 3, 31%). Anal. calcd. for $C_{20}H_{13}ClN_4S$ (376.05): C, 63.74; H, 3.48; N, 14.87; Found: C, 63.67; H, 3.70; N, 14.78.

*5.5.13. 4-(m-Tolylamino)-5-(4-chlorophenyl)thieno[2,3-d]pyrimidine-6-carbonitrile (**9e**)*

Pale green amorphous solid, mp 130–132 °C; IR (KBr): 1568, 1608 (Ar C=C), 2212 (CN), 2968 (C–H), 3029, 3400 (NH) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 2.33 (s, 3H, CH_3), 6.92–7.24 (m, 4H, ArH), 7.18 (bs, 1H, NH), 7.56 (d, J = 8.7 Hz, 2H, ArH), 7.67 (d, J = 8.7 Hz, 2H, ArH), 8.69 (s, 1H, C_2H). MS (EI): m/z 377.05 (M + 1, 100%), 379.10

(M + 3, 34%). Anal. calcd. for $C_{20}H_{13}ClN_4S$ (376.05): C, 63.74; H, 3.48; N, 14.87; Found: C, 63.55; H, 3.61; N, 14.68.

6. Biological assay

6.1. In vitro evaluation of antimicrobial activity

The MICs of synthesized compounds were carried out by broth microdilution method. DMSO was used as diluents to get desired concentration of drugs to test upon standard bacterial strains. Serial dilutions were prepared in primary and secondary screening. The control tube containing no antibiotic was immediately subcultured (before inoculation) by spreading a loopful evenly over a quarter of plate of medium suitable for the growth of the test organism and put for incubation at 37 °C overnight. The tubes were then incubated overnight. The MIC of the control organism was read to check the accuracy of the drug concentrations. The lowest concentration inhibiting growth of the organism was recorded as the MIC. All the tubes not showing visible growth (in the same manner as control tube described above) was subcultured and incubated overnight at 37 °C. The amount of growth from the control tube before incubation (which represents the original inoculum) was compared. Subcultures might show: similar number of colonies indicating bacteriostatic; a reduced number of colonies indicating a partial or slow bactericidal activity and no growth if the whole inoculum has been killed. The test must include a second set of the same dilutions inoculated with an organism of known sensitivity. Each synthesized drug was diluted obtaining 2000 mg/mL concentration, as a stock solution. In primary screening 500, 250 and 125 mg/mL concentrations of the synthesized drugs were taken. The active synthesized drugs found in this primary screening were further tested in a second set of dilution against all microorganisms. The drugs found active in primary screening were similarly diluted to obtain 100, 50, 25, 12.5, 6.250, 3.125 and 1.5625 mg/mL concentrations. The highest dilution showing at least 99% inhibition is taken as MIC.

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