Synthesis of novel azole functionalized trifluoromethyl pyrido[2,3-*d*] pyrimidinone derivatives and their antimicrobial activity

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Abstract
Novel azole functionalized pyrido[2,3-d] pyrimidinone derivatives 6a-l were prepared starting from 2-Amino-6-(thiophen-2-yl)-4-(trifluoromethyl)nicotinonitrile 1 reacting with trifluoroacetic acid in the presence of conc. H_2SO_4 and further reaction with bromoethyl acetate to obtain 3a and 3b. Compounds 3a and 3b further reacted with hydrazine hydrate to afford hydrazide compounds 4a and 4b. Compounds 4a and 4b on treating with phenylisothiocyanate give compound 5a and b. Compound 5a and 5b on reaction with different reagents (NaOH, H_2SO_4 and $N_2H_4.H_2O$) produced different azole-substituted (triazolothione, thiadiazole, triazole) functionalized trifluoromethyl pyrido[2,3-d] pyrimidinone derivatives 6a-l respectively.

All the products 6a–l were screened against gram +ve, gram –ve bacteria and fungal strains. Compounds 6a-d showed promising activity against Bacillus subtilis microbial-type culture collection (MTCC) 121 at <25.6 micromolar concentration. Promising compounds were further screened for minimum bactericidal concentration against B. subtilis MTCC 121 using ciprofloxacin as standard reference and found to show very good activity. These compounds also screened for biofilm inhibition activity against B. subtilis MTCC 121 using erythromycin as standard confirming the high activity.

Keywords: Trifluoromethyl pyrido[2,3-*d*]pyrimidinone, triazolothione, thiadiazole, triazole, antimicrobial activity.

Introduction

Heterocyclic compounds play an important role in anti-cancer drug research. Nitrogen containing heterocycles are broadly distributed in nature and possess many pharmacological properties.[2,3,8,13,16,19,22] N-heterocycles are constituents in many biological important molecules such as in nucleic acids, vitamins, pharmaceuticals, antibiotics, dyes and agrochemicals. Adenine, thymine, guanine and cytosine (DNA and RNA base pair) are made up with N-heterocyclic compounds.[4,6,9-11,23]

Among heterocyclic compounds, heterocyclic bicyclic compounds were paid more attention. We focused on pyridopyrimidinone compounds designed with multi functional groups such as triazole, triazolothione, thiadiazole heterocyclic rings. Pyridopyrimidinone exhibits many biological activities as kinase inhibitors[5,18,21], anti-leukemic[14], against breast cancer[20] and antihypertensives[12]. Palbociclib drug was approved for the treatment of breast cancer (figure 1).

In our research we designed and synthesized novel triazole, thiadiazole, triazolothione functionalized trifluoromethyl pyrido[2,3-*d*] pyrimidinone derivatives. Trifluoro methyl group at particular position of molecule can increase lipophilicity, oral bioavailability and thermal stability. Fluorine/trifluoromethy group in medicinal chemistry has exploded from the past 20 years especially in the last decade.[17]

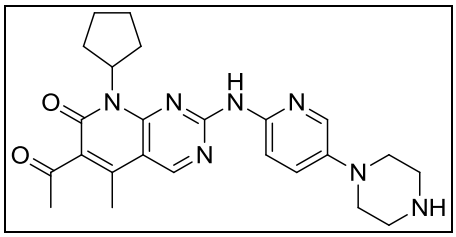


Figure 1: Palbociclib drug used for breast cancer

Considering all these and with the help of literature survey, here we reported azole functionalized trifluoromethyl pyrido[2,3-*d*] pyrimidinone derivatives submitted for antimicrobial activity. Compounds 6a, 6b, 6c and 6d showed promising activity.

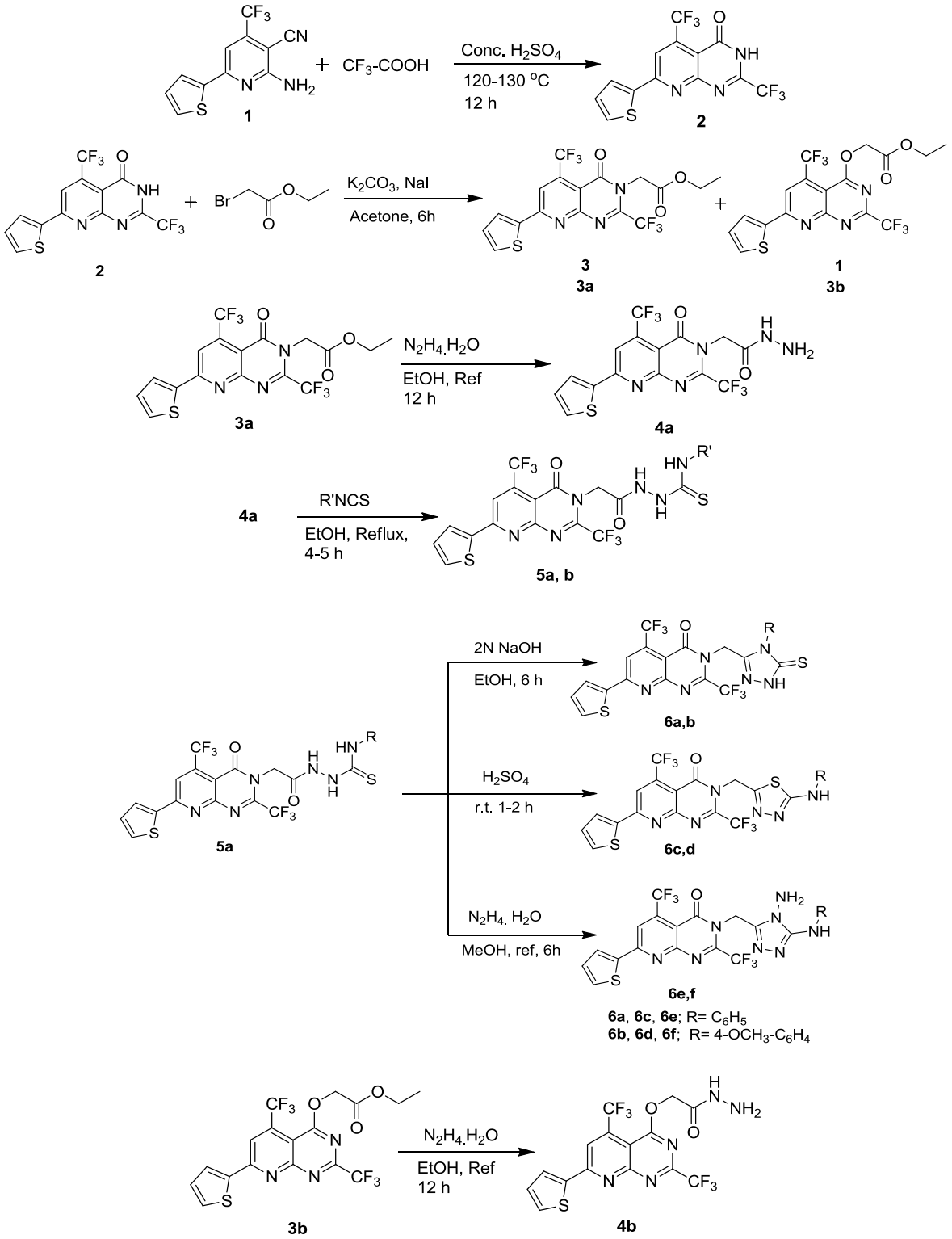
Material and Methods

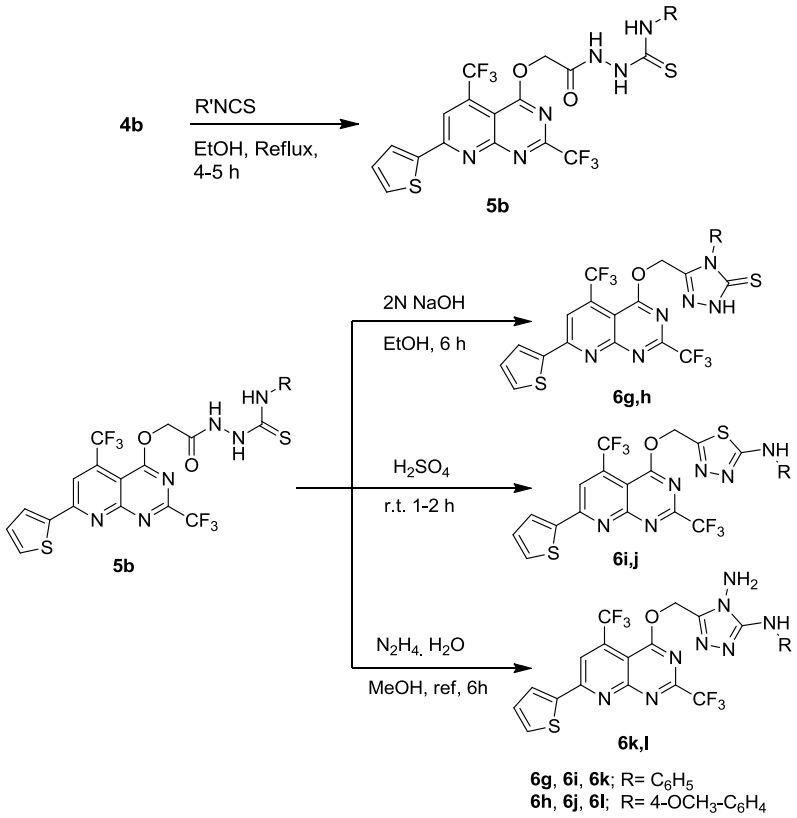
Experimental: Melting points were recorded on Casia-Siamia (VMP-AM) melting point apparatus and are uncorrected. Infrared spectra were recorded on a Perkin Elmer FTIR 240-C spectrophotometer using KBr optics. 1H NMR spectra were recorded on Bruker AV 300 MHz in $CDCl_3$ and dimethyl sulfoxide (DMSO)-d^6 using tetramethylsilane as internal standard. Electron impact and chemical ionization mass spectra were recorded on a VG 7070 H instrument at 70 eV. All high-resolution spectra were recorded on QSTARXL hybrid MS/MS system (Applied Biosystems, USA) under electrospray ionization.

All the reactions were monitored by thin-layer chromatography on precoated silica gel 60 F254 (mesh); spots were visualized with UV light. Merck silica gel (60–120 mesh) was used for column chromatography. CHN analysis was recorded on a Vario EL analyzer.

2-Amino-6-(thiophen-2-yl)-4-(trifluoromethyl) nicotinenitrile 1 on reaction with trifluoroacetic acid in the presence of conc. H_2SO_4 at 120-130 °C produced 2-methyl-7-(thiophen-2-yl)-5-(trifluoromethyl) pyrido[2,3-*d*]pyrimidin-4(3H)-one 2. Compound 2 on reaction with bromoethyl acetate in the presence of basic condition produced 3a and 3b at the ratio of 3:1. Compound 3a and 3b on further reaction with hydrazine hydrate produced hydrazide compounds 4a and 4b.

Compound 4a on reaction with different substituted phenyl iso thiocyanate in the presence of EtOH refluxing condition gave thiourea derivatives 5a and b. Compound 5a and 5b on reaction under different conditions with different reagents (NaOH, H_2SO_4 and $N_2H_4.H_2O$) produced different azole-substituted triazolothione, thiadiazole, triazole functionalized trifluoromethyl pyrido[2,3-*d*] pyrimidinone derivatives 6a-l. Synthetic sequence is drawn in scheme 1.





Scheme 1: Synthesis of triazolothione, thiadiazole, triazole functionalized pyrido[2,3-*d*] pyrimidinone derivatives 6a-l

Table 1
Antimicrobial activity (6a-l)

S. N.	Test Compounds	Minimum Inhibitory Concentration (µg/ml)							
		Micrococcus luteus **MTCC 2470**	***Staphylococcus aureus*** **MTCC 96**	***Staphylococcus aureus*** **MLS-16 MTCC 2940**	***Bacillus subtilis*** **MTCC 121**	***Escherichia coli*** **MTCC 739**	***Pseudomonas aeruginosa*** **MTCC 2453**	***Klebsiella planticola*** **MTCC 530**	***Candida albicans*** **MTCC 3017**
1	**6a**	>125.0	>125.0	>125.0	25.6	>125.0	>125.0	>125.0	>125.0
2	**6b**	>125.0	>125.0	>125.0	25.6	>125.0	>125.0	>125.0	>125.0
3	**6c**	>125.0	>125.0	>125.0	8.5	>125.0	>125.0	>125.0	>125.0
4	**6d**	>125.0	>125.0	>125.0	8.5	>125.0	>125.0	>125.0	>125.0
5	**6e**	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0
6	**6f**	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0
7	**6g**	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0
8	**6h**	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0
9	**6i**	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0
10	**6j**	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0
11	**6k**	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0
12	**6l**	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0	>125.0
Ciprofloxacin (Standard)		0.9	0.9	0.9	0.9	0.9	0.9	0.9	-
Miconazole (Standard)		-	-	-	-	-	-	-	7.8

Results and Discussion

Antimicrobial activity and structure activity relationship: Compounds 6a–l were screened for antimicrobial activity against seven bacterial organisms such as *Micrococcus luteus* microbial-type culture collection (MTCC) 2470, *Staphylococcus aureus* MTCC 96, *S. aureus* MLS-16 MTCC 2940, *Bacillus subtilis* MTCC 121, *Escherichia coli* MTCC 739, *Pseudomonas aeruginosa* MTCC 2453, *Klebsiella planticola* MTCC 530 and one fungal strain *Candida albicans* MTCC 3017. Out of 12 compounds screened, 4 compounds 6a, 6b, 6c and 6d showed promising activity against gram +ve *B. subtilis* MTCC 121.

Table 2
Bactericidal activity results

S.N.	Test compound	Minimum Bactericidal Concentration (µg/ml)
		***Bacillus subtilis* MTCC 121**
1	**6a**	25.6
2	**6b**	25.6
3	**6c**	8.5
4	**6d**	8.5
Ciprofloxacin (Standard)		1.17

Table 3
Bio-film inhibition assay results

S.N.	Test compound	IC_{50} values in (µg/ml)
		***Bacillus subtilis* MTCC 121**
1	**6a**	17.9 ± 0.26
2	**6b**	14.5 ± 0.46
3	**6c**	6.3± 0.38
4	**6d**	5.5 ± 0.32
Erythromycin (Standard)		0.2 ± 0.08

Structure verses activity relationship revealed that specific variation in structure showed enhanced activity. If we consider compounds 6a, b series of 1,2,4- triazolothione derivatives, compounds 6a and 6b showed moderate activity, minimum inhibitory concentration (MIC) 25.6 µg/mL and compounds 6c and 6d showed promising activity, MIC 8.5 µg/mL. Compounds 6a–d (1,3,4-triazolothione, thiadiazole derivatives) played a crucial role to promote antibacterial activity against gram +ve *B. subtilis* MTCC 121. Among all the derivatives, compounds 6c and 6d showed high activity i.e. MIC is 8.5 µg/mL. However, compounds 6e-6l could not show activity up to the concentration of 125 µg/mL against all the organisms. The details of activity data are outlined in tables 1-3.

General Procedure: 2-(2-(4-oxo-7-(thiophen-2-yl)-2,5-bis (trifluoromethyl) pyrido[2,3-d]pyrimidin-3(4H)-yl)acetyl)-N-phenyl hydrazine carbothioamide (5a / b) (0.01 mol) in 2N NaOH was refluxed for about 4–6 h. The resulting solution was cooled to room temperature and acidified to pH 3–4 with 37% hydrochloricacid. The precipitate formed was filtered, washed with distilled water and dried to afford compound 6a, 6b, 6g and 6h.

3-((4-phenyl-5-thioxo-4,5-dihydro-1H-1,2,4-triazol-3-yl)methyl)-7-(thiophen-2-yl)-2,5 bis(trifluoromethyl) pyrido[2,3-d]pyrimidin-4(3H)-one (6a): IR (KBr, cm^{-1}): 3521, 1632 (-NHCS-); ^{1}H NMR (DMSO-d_6, 300 MHz): δ 4.26 (s, 2H, -CH_2-), 7.19 (dd, *J* = 4.91, 1H, Ar-H), 7.31-7.35 (m, 3H, Ar-H), 7.46 (dd, *J* = 4.91, 1H, Ar-H), 7.71-7.75 (m, 2H, Ar-H), 7.82 (dd, *J* = 3.73, 1H, Ar-H), 8.24 (s, 1H, Ar-H), 12.96 (br, s, 1H, -NHCO-); ^{13}C NMR (DMSO-d_6, 300 MHz): 48.23, 118.17, 120.54, 121.61, 122.41, 123.11, 123.72, 124.74, 125.23, 126.54, 128.62, 130.37, 132.56, 136.42, 138.62, 140.81, 142.55, 146.21, 158.25, 160.34; MS (ESI): m/z [$(M+H)^+$]: 555. Anal. calc. for $C_{22}H_{12}F_6N_6OS_2$: C 47.65, H 2.18, N 15.16 %. Found: C 47.66, H 2.19, N 15.18 %

3-((4-(4-methoxyphenyl)-5-thioxo-4,5-dihydro-1H-1,2,4-triazol-3-yl)methyl)-7-(thiophen-2-yl)-2,5-bis(trifluoro-methyl)pyrido[2,3-d]pyrimidin-4(3H)-one (6b): IR (KBr, cm^{-1}): 3518, 1631 (-NHCS-); ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.82(s, 3H, -OCH_3) 4.23 (s, 2H, -CH_2-), 7.21 (dd, *J* = 4.89, 1H, Ar-H), 7.29 (d, 2H, Ar-H), 7.36 (dd, *J* = 4.89, 1H, Ar-H), 7.48 (d, 2H, Ar-H), 7.61 (dd, *J* = 3.72, 1H, Ar-H), 8.11 (s, 1H, Ar-H), 12.92 (br, s, 1H, -NHCO-); ^{13}C NMR (DMSO-d_6, 300 MHz): 48.24, 54.61, 119.32, 120.11, 121.74, 122.25, 123.52, 124.46, 125.37, 126.47, 128.35, 131.22, 132.45, 135.24, 138.75, 140.65, 142.37, 146.48, 159.21, 160.34; MS (ESI): m/z [$(M+H)^+$]: 585. Anal. calc. for $C_{23}H_{14}F_6N_6O_2S_2$: C 47.26, H 2.41, N 14.38 %. Found: C 47.27, H 2.42, N 14.39 %

General procedure for the synthesis of 3-[(5-(Phenylamino)-1,3,4-thiadiazol-2-yl)methyl)-7-(thiophen-2-yl)-2,5-bis (trifluoromethyl) pyrido [2,3-d]pyrimidin-4(3H)-one derivatives (6c, 6d, 6i and **6j)]:** A mixture of 0.001 mol of 2-(2-(4-oxo-7-(thiophen-2-yl)-2,5-bis (trifluoromethyl) pyrido[2,3-*d*] pyrimidin-3(4H)-yl)acetyl)-N-phenyl hydrazine carbothioamide (5a/5b) and concentrated H_2SO_4 (1 mL) was stirred at room temperature for about 1 h. Then after, the reaction mixture was poured over crushed ice. The precipitated solid was washed with sodium carbonate solution followed by water to obtain compound 6c, 6d, 6i and 6j.

3-((5-(Phenylamino)-1,3,4-thiadiazol-2-yl)methyl)-7-(thiophen-2-yl)-2,5-bis (trifluoromethyl) pyrido [2,3-d]pyrimidin-4(3H)-one (6c): IR (KBr, cm^{-1}): 3412 (-NH-); ^{1}H NMR (DMSO-d_6, 300 MHz): δ 4.21 (s, 2H, -CH_2-), 7.11 (br. s., 1H, -NH-), 7.19 (dd, *J* = 4.86, 1H, Ar-H), 7.26-7.31 (m, 3H, Ar-H), 7.36 (dd, *J* = 4.86, 1H, Ar-H), 7.42-7.45 (m, 2H, Ar-H), 7.63 (dd, *J* = 3.71, 1H, Ar-H), 8.12 (s, 1H, Ar-H); ^{13}C NMR (DMSO-d_6, 300 MHz): 39.11, 118.27, 120.42, 121.33, 122.63, 123.75, 124.55, 125.47, 126.05, 129.41, 131.15, 132.38, 136.10, 138.45, 140.37, 142.28, 142.96,

146.35, 148.21, 159.64,; MS (ESI): m/z [(M+H)$^+$]: 555. Anal. calc. for $C_{22}H_{12}F_6N_6OS_2$: C 47.65, H 2.18, N 15.16 %. Found: C 47.66, H 2.19, N 15.17 %

3-((5-((4-Methoxyphenyl)amino)-1,3,4-thiadiazol-2-yl) methyl)-7-(thiophen-2-yl)-2,5-bis(trifluoromethyl)pyrid o[2,3-d]pyrimidin-4(3H)-one (6d): IR (KBr, cm^{-1}): 3415 (-NH-); ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.82 (s, 3H, -OCH_3), 4.24 (s, 2H, -CH_2-), 7.08 (br. s., 1H, -NH-), 7.18 (dd, *J* = 4.81, 1H, Ar-H), 7.28 (dd, 2H, Ar-H), 7.36 (dd, *J* = 4.81, 1H, Ar-H), 7.42-7.45 (m, 2H, Ar-H), 7.63 (dd, *J* = 3.71, 1H, Ar-H), 8.12 (s, 1H, Ar-H); ^{13}C NMR (DMSO-d_6, 300 MHz): 39.44, 56.28, 118.27, 120.42, 121.33, 122.63, 123.75, 124.55, 125.47, 126.05, 129.41, 131.15, 132.38, 136.10, 138.45, 140.37, 142.28, 142.96, 146.35, 148.21, 159.64,; MS (ESI): m/z [(M+H)$^+$]: 585. Anal. calc. for $C_{22}H_{12}F_6N_6OS_2$: C 47.65, H 2.18, N 15.16 %. Found: C 47.66, H 2.19, N 15.17 %

General procedure for the synthesis of 5-(3-amino-6-(thiophen-2-yl)-4- (trifluoromethyl) furo[2,3-b]pyridin-2-yl) -N3-phenyl-4H-1,2,4-triazole-3,4-diamine (6e, 6f, 6k and 6l): 2-(2-(4-oxo-7-(thiophen-2-yl)-2,5-bis (trifluoromethyl) pyrido[2,3-*d*]pyrimidin-3(4H)-yl)acetyl)-N-phenyl hydrazine carbothioamide (5a / 5b) was mixed with $N_2H_4.H_2O$ (0.01 mol) and MeOH (1 mL). The solution was refluxed for 5–6 h. After cooling to room temperature, ice cold water (10 mL) was added to the reaction mixture, then neutralized with 3N HCl to form a precipitate. The precipitate was separated by filtration to afford the triazole derivatives 6e, 6f, 6k and 6l.

3-((4-Amino-5-(phenylamino)-4H-1,2,4-triazol-3-yl)met hyl)-7-(thiophen-2-yl)-2,5-bis (trifluoromethyl) pyrido [2,3-d]pyrimidin-4(3H)-one (6e): IR (KBr, cm^{-1}): 3321, 3386(-NH_2), 3415 (-NH-); ^{1}H NMR (DMSO-d_6, 300 MHz): δ 4.20 (s, 2H, -CH_2-), 6.26 (br s, 2H, –NH_2), 7.01 (br. s., 1H, –NH–),7.25(dd, *J* = 4.83, 1H, Ar-H), 7.29-7.32 (m, 3H, Ar-H), 7.38 (dd, *J* = 4.83, 1H, Ar-H), 7.43-7.46 (m, 2H, Ar-H), 7.67 (dd, *J* = 3.72, 1H, Ar-H), 8.12 (s, 1H, Ar-H); ^{13}C NMR (DMSO-d_6, 300 MHz): 42.21, 119.12, 120.35, 121.45, 122.61, 123.86, 124.15, 125.37, 126.27, 130.53, 132.38, 135.45, 138.17, 140.44, 142.47, 143.85, 146.52, 148.15, 151.47, 152.47,; MS (ESI): m/z [(M+H)$^+$]: 553. Anal. calc. for $C_{22}H_{14}F_6N_8OS$: C 47.83, H 2.55, N 20.28 %. Found: C 47.84, H 2.56, N 20.29 %

3-((4-Amino-5-((4-methoxyphenyl)amino)-4H-1,2,4-tri azol-3-yl)methyl)-7-(thiophen-2-yl)-2,5-bis(trifluoromet hyl)pyrido[2,3-d]pyrimidin-4(3H)-one (6f): IR (KBr, cm^{-1}): 3318, 3365(-NH_2), 3402 (-NH-); ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.83(s, 3H, -OCH_3), 4.21 (s, 2H, -CH_2-), 6.25 (br s, 2H, –NH_2), 7.06 (br. s., 1H, –NH–),7.28 (dd, *J* = 4.81, 1H, Ar-H), 7.31 (dd, 2H, Ar-H), 7.38 (dd, *J* = 4.81, 1H, Ar-H), 7.45 (dd, 2H, Ar-H), 7.68 (dd, *J* = 3.72, 1H, Ar-H), 8.11 (s, 1H, Ar-H); ^{13}C NMR (DMSO-d_6, 300 MHz): 42.14, 56.32, 118.24, 120.75, 121.65, 122.28, 123.34, 124.11, 125.54, 128.12, 129.35, 131.68, 132.47, 136.28, 138.34, 140.41, 142.25, 146.56, 148.37, 150.27, 151.89; MS (ESI): m/z [(M+H)$^+$]: 583. Anal. calc. for $C_{23}H_{16}F_6N_8O_2S$: C 47.43, H 2.77, N 19.24 %. Found: C 47.44, H 2.78, N 19.26 %

4-Phenyl-3-(((7-(thiophen-2-yl)-2,5-bis(trifluoromethyl) pyrido[2,3-d]pyrimidin-4-yl) oxy)methyl)-1H-1,2,4-tri azole-5(4H)-thione (6g): IR (KBr, cm^{-1}): 3518, 1621 (-NHCS-); ^{1}H NMR (DMSO-d_6, 300 MHz): δ 5.61 (s, 2H, -CH_2-), 7.17 (dd, *J* = 4.91, 1H, Ar-H), 7.30-7.36 (m, 3H, Ar-H), 7.52 (dd, *J* = 4.91, 1H, Ar-H), 7.68-7.72 (m, 2H, Ar-H), 7.83 (dd, *J* = 3.71, 1H, Ar-H), 8.21 (s, 1H, Ar-H), 12.95 (br, s, 1H, -NHCO-); ^{13}C NMR (DMSO-d_6, 300 MHz): 71.21, 119.58, 120.57, 121.56, 122.35, 123.18, 124.11, 124.78, 125.23, 126.45, 128.36, 131.85, 133.31, 136.42, 139.65, 140.47, 142.35, 146.26, 158.47, 160.12; MS (ESI): m/z [(M+H)$^+$]: 555. Anal. calc. for $C_{22}H_{12}F_6N_6OS_2$: C 47.65, H 2.18, N 15.16 %. Found: C 47.64, H 2.17, N 15.18 %

4-(4-Methoxyphenyl)-3-(((7-(thiophen-2-yl)-2,5-bis(trifle oromethyl)pyrido[2,3-d] pyrimidin-4-yl)oxy)methyl)-1H-1,2,4-triazole-5(4H)-thione (6h): IR (KBr, cm^{-1}): 3515, 1633 (-NHCS-); ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.80 (s, 3H, -OCH_3) 5.65 (s, 2H, -CH_2-), 7.18 (dd, *J* = 4.88, 1H, Ar-H), 7.32 (d, 2H, Ar-H), 7.38 (dd, *J* = 4.88, 1H, Ar-H), 7.49 (d, 2H, Ar-H), 7.66 (dd, *J* = 3.72, 1H, Ar-H), 8.17 (s, 1H, Ar-H), 12.95 (br, s, 1H, -NHCO-); ^{13}C NMR (DMSO-d_6, 300 MHz): 48.52, 54.85, 119.25, 120.24, 121.34, 122.74, 123.35, 124.71, 125.65, 126.31, 128.61, 131.21, 132.40, 136.24, 138.31, 141.25, 142.52, 146.68, 149.32, 159.28 ; MS (ESI): m/z [(M+H)$^+$]: 585. Anal. calc. for $C_{23}H_{14}F_6N_6O_2S_2$: C 47.26, H 2.41, N 14.38 %. Found: C 47.27, H 2.42, N 14.39 %

N-Phenyl-5-(((7-(thiophen-2-yl)-2,5-bis(trifluoromethyl) pyrido[2,3-d]pyrimidin-4-yl)oxy)methyl)-1,3,4-thiadiazo l-2-amine (6i): IR (KBr, cm^{-1}): 3421 (-NH-); ^{1}H NMR (DMSO-d_6, 300 MHz): δ 5.60 (s, 2H, -CH_2-), 7.11 (br. s., 1H, -NH-), 7.18 (dd, *J* = 4.81, 1H, Ar-H), 7.26-7.30 (m, 3H, Ar-H), 7.37 (dd, *J* = 4.81, 1H, Ar-H), 7.36-7.39 (m, 2H, Ar-H), 7.61 (dd, *J* = 3.71, 1H, Ar-H), 8.12 (s, 1H, Ar-H); ^{13}C NMR (DMSO-d_6, 300 MHz): 54.23, 119.25, 120.41, 121.24, 122.35, 123.41, 124.56, 125.37, 126.18, 128.64, 131.28, 132.75, 136.63, 138.45, 140.47, 142.36, 142.74, 146.55, 148.18, 159.47,; MS (ESI): m/z [(M+H)$^+$]: 555. Anal. calc. for $C_{22}H_{12}F_6N_6OS_2$: C 47.65, H 2.18, N 15.16 %. Found: C 47.66, H 2.19, N 15.18%

N-(4-methoxyphenyl)-5-(((7-(thiophen-2-yl)-2,5-bis(trif luoromethyl)pyrido[2,3-d] pyrimidin-4-yl)oxy) methyl)-1,3,4-thiadiazol-2-amine (6j): IR (KBr, cm^{-1}): 3413 (-NH-); ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.82 (s, 3H, -OCH_3), 5.62 (s, 2H, -CH_2-), 7.05 (br. s., 1H, -NH-), 7.19 (dd, *J* = 4.81, 1H, Ar-H), 7.26 (d, 2H, Ar-H), 7.32 (dd, *J* = 4.81, 1H, Ar-H), 7.41 (d, 2H, Ar-H), 7.63 (dd, *J* = 3.71, 1H, Ar-H), 8.10 (s, 1H, Ar-H); ^{13}C NMR (DMSO-d_6, 300 MHz): 49.15, 55.28, 118.47, 120.42, 121.33, 122.63, 123.75, 124.55, 125.47, 126.05, 129.41, 131.15, 132.38, 136.15, 138.45, 140.37, 142.28, 142.96, 146.36, 148.25, 159.61,;

MS (ESI): m/z [$(M+H)^+$]: 585. Anal. calc. for $C_{22}H_{12}F_6N_6OS_2$: C 47.65, H 2.18, N 15.16 %. Found: C 47.66, H 2.19, N 15.17 %

N3-phenyl-5-(((7-(thiophen-2-yl)-2,5-bis(trifluoromethyl)pyrido[2,3-d]pyrimidin-4-yl)oxy) methyl)-4H-1,2,4-triazole-3,4-diamine (6k):IR (KBr, cm^{-1}): 3322, 3385 (-NH_2), 3411 (-NH-); ^{1}H NMR (DMSO-d_6, 300 MHz): δ 5.60 (s, 2H, -CH_2-), 6.25 (br s, 2H, –NH_2), 7.05 (br. s., 1H, –NH–),7.26 (dd, *J* = 4.83, 1H, Ar-H), 7.30-7.35 (m, 3H, Ar-H), 7.39 (dd, *J* = 4.83, 1H, Ar-H), 7.44-7.46 (m, 2H, Ar-H), 7.68 (dd, *J* = 3.72, 1H, Ar-H), 8.12 (s, 1H, Ar-H); ^{13}C NMR (DMSO-d_6, 300 MHz): 55.24, 119.28, 120.36, 121.75, 122.26, 123.41, 124.28, 125.36, 126.44, 130.15, 132.36, 135.46, 138.27, 140.41, 142.43, 143.75, 146.15, 149.24, 151.24, 153.55; MS (ESI): m/z [$(M+H)^+$]: 553. Anal. calc. for $C_{22}H_{14}F_6N_8OS$: C 47.83, H 2.55, N 20.28 %. Found: C 47.84, H 2.56, N 20.29 %

N3-(4-Methoxyphenyl)-5-(((7-(thiophen-2-yl)-2,5-bis(trifluoromethyl)pyrido[2,3-d] pyrimidin-4-yl)oxy)methyl)-4H-1,2,4-triazole-3,4-diamine (6l): IR (KBr, cm^{-1}): 3314, 3362(-NH_2), 3405 (-NH-); ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.84 (s, 3H, -OCH_3), 5.61 (s, 2H, -CH_2-), 6.26 (br s, 2H, –NH_2), 7.05 (br. s., 1H, –NH–),7.26 (dd, *J* = 4.82, 1H, Ar-H), 7.32 (dd, 2H, Ar-H), 7.39 (dd, *J* = 4.82, 1H, Ar-H), 7.46 (dd, 2H, Ar-H), 7.69 (dd, *J* = 3.72, 1H, Ar-H), 8.12 (s, 1H, Ar-H); ^{13}C NMR (DMSO-d_6, 300 MHz): 48.89, 56.35, 118.35, 120.22, 121.48, 122.37, 123.62, 124.18, 125.27, 128.48, 129.61, 130.18, 132.75, 136.37, 138.48, 140.38, 142.41, 146.54, 148.24, 151.24, 152.27; MS (ESI): m/z [$(M+H)^+$]: 583. Anal. calc. for $C_{23}H_{16}F_6N_8O_2S$: C 47.43, H 2.77, N 19.24 %. Found: C 47.44, H 2.78, N 19.26 %

Antibacterial assay: The antimicrobial activity of the synthesized compounds was determined using a well diffusion method[1] against different pathogenic Bacterial and Candida strains procured from the MTCC and Gene Bank, CSIR-Institute of Microbial Technology, Chandigarh, India.

The pathogenic reference strains were seeded on surface of the media Petri plates containing Mueller-Hinton agar with 0.1 mL of previously prepared microbial suspensions individually containing $1.5X10^8$ cfu/mL (equal to 0.5 McFarland). Wells of 6.0 mm diameter were prepared in the media plates using a cork borer and the synthesized compounds dissolved in 10% DMSO at a dose range of 125–0.97 µg were added in each well under sterile conditions in a laminar air flow chamber.

Standard antibiotic solutions of neomycin (bacterial strains) and miconazole (Candida strains) at a dose range of 125–0.97 µg/well served as positive controls while the well containing DMSO served as negative control. The plates were incubated for 24 h at 30 °C and the well containing least concentration showing the inhibition zone is considered as the minimum inhibitory concentration. All experiments were performed in duplicate and mean values are represented.

Minimum bactericidal concentration assay: Bactericidal assay[15] (NCCLS, 2000) was performed in sterile 2.0 mL microfuge tubes against a panel of pathogenic bacterial strains including *M. luteus* MTCC 2470, *S. aureus* MTCC 96, *S. aureus* MLS-16 MTCC 2940, *B. subtilis* MTCC 121, *E. coli* MTCC 739, *P. aeruginosa* MTCC 2453 and *K. planticola* MTCC 530, cultured overnight in Mueller-Hinton broth. Serial dilutions of test compounds were prepared in Mueller-Hinton broth with different concentrations ranging from 0 to 150 µg/mL. To the test compounds, 100 µL of overnight cultured bacterial suspensions was added to reach a final concentration of 1.5 X 10^8 cfu/ mL (equal to 0.5 McFarland) and incubated at 37 °C for 24 h. After 24 h of incubation, the minimum bactericidal concentration (MBC) was determined by sampling 10 µL of suspension from the tubes onto Mueller-Hinton agar plates and incubated for 24 h at 37 °C to observe the growth of test organisms. MBC is the lowest concentration of test compound required to kill a particular bacterial strain. All the experiments were performed in duplicate.

Biofilm inhibition assay: The test compounds were screened in sterile 96-well polystyrene microtiter plates using the modified biofilm inhibition assay,[7] against a panel of pathogenic bacterial strains including *S. aureus* MTCC 96, *S. aureus* MLS16 MTCC 2940, *B. subtilis* MTCC121, *P. aeruginosa* MTCC 2453 and *K. planticola* MTCC 530 which were cultured overnight in tryptone soy broth (supplemented with 0.5% glucose). The test compounds of predetermined concentrations ranging from 0 to 250 µg/mL were mixed with the bacterial suspensions having an initial inoculum concentration of 5 X 10^5 cfu/mL. Aliquots of 100 µL were distributed in each well and then incubated at 37 °C for 24 h under static conditions.

The medium was then discarded and washed with phosphate buffered saline to remove the nonadherent bacteria. Each well of the microtiter plate was stained with 100 µL of 0.1% crystal violet solution followed by 30 min incubation at room temperature.

Later, the crystal violet solution from the plates was discarded, thoroughly washed with distilled water for 3 to 4 times and air-dried at room temperature. The crystal violet stained biofilm was solubilised in 95% ethanol (100 µL) and the absorbance was recorded at 540 nm using TRIAD multimode reader (Dynex Technologies, Inc, Chantilly, VA, USA). Blank wells were used as background check. The inhibition data were interpreted from the dose–response curves where IC_{50} value is defined as the concentration of inhibitor required to inhibit 50% of biofilm formation under the above assay conditions. All the experiments were performed in triplicate and the values are indicated as mean ± SD.

Conclusion

In conclusion, we designed and synthesized novel triazolothione, thiadiazole, triazole functionalized

trifluoromethyl pyrido[2,3-*d*] pyrimidine derivatives 6a-l. All compounds screened against gram +ve, gram –ve bacterial and fungal strains.

Compounds 6a–d showed high activity against *B. subtilis* MTCC 121 at < 25.6 micromolar concentration. Promising compounds were further screened for MBC against *B. subtilis* MTCC 121 using ciprofloxacin as standard and found to show moderate to very good activity.

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