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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

In vitro and in silico biological studies of novel thiazolo [3,2-*a*]pyrimidine-6-carboxylate derivatives

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Abstract In the present study, we report the synthesis, characterization of new series of thiazolo[3,2-*a*]pyrimidine-6-carboxylate derivatives **3a–f** and **4a–f**. The newly synthesized compounds were screened for in vitro antimicrobial and antiviral activities. The probable mode of action of these active compounds was determined through in silico docking study by docking the receptor methionyl-tRNA synthetase and human inosine-5′-monophosphate dehydrogenase (IMPDH) for antibacterial and antiviral activities, respectively. Among the compounds, **4c** exhibited excellent in vitro antimicrobial activity against all tested strains with binding and docking energies −35.6 and −12.4 kcal/mol, respectively. The antiviral studies were carried out for the selected compounds in which **4a** exhibited 73.69 and 54.42 % of inhibition of buffalopox and camelpox viruses, respectively. Furthermore, compound **4a** showed minimum docking and binding energy along with the maximum hydrogen/hydrophobic interaction with IMPDH. The study contributes towards identification and screening of potential antimicrobial and antiviral agent's against the pathogens.

Keywords Thiazolo[3,2-*a*]pyrimidine-6-carboxylate · In vitro antimicrobial activity · In vitro antiviral activity · In silico docking studies

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Introduction

Pyrimidine and its derivatives are widespread in nature and found to be used in a wide range of application in therapeutically active compounds. Pyrimidines and fused pyrimidines, being an integral part of DNA and RNA in it, play an important role in several biological activities. Nucleosides of pyrimidine bases have been extensively used as antiviral and anticancer agents (Donald *et al.*, 1987). Recently, fluoropyrimidines and fluorouracil-based combination therapy is used in the treatment of gastrointestinal cancer and solid tumors. Fluoropyrimidines are found to be metabolites of dihydropyrimidinones that are subtype-selective antagonists of the α_{1a}-adreginic receptor antagonists (Lagu *et al.*, 2000). Pyrimidine derivatives found to possess wide range of pharmacological properties such as analgesic, antiarrhythmic, anti-inflammatory, antiparkinsonian, and androgenic–anabolic activities (Nehad *et al.*, 2007; Amr *et al.*, 2006). Various analogs of thiopyrimidones exhibited antibacterial, antifungal, antiviral, and antileishmanial activities (Balalaie *et al.*, 2006; Ram *et al.*, 1984, 1987, 1994). The partially hydrogenated pyrimidine derivatives such as dihydropyrimidones and thiodihydropyrimidones have been used as key substrates to develop molecules as drug or potent leads in medicinal

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chemistry. Among them thiazolo[3,2-*a*]pyrimidine derivatives gained attention in drug discovery because of their wide range of biological activity such as calcium channel blocking (Kappe, 2003), antimalarial, and antitubercular (Geist *et al.*, 2010), acetlylcholine esterase inhibitory (Liu *et al.*, 2009), glutamate receptor antagonistic (Wichmann *et al.*, 1999), 5-HT2a receptor antagonistic (Awadallah, 2008) and anticancer activities (Rashad *et al.*, 2010).

Fungal and viral infections are growing problems in contemporary medicine, yet only a few effective chemotherapeutic agents are used in clinical practice. There is clearly a critical need for the development and evaluation of novel-specific antifungal and antiviral agents. The increasing need for novel antibiotics to overcome rapidly developing resistance mechanisms observed in clinical isolates of bacteria as well as virus has placed critical emphasis on the search for unique antibacterial/viral enzyme targets.

The most recent phase in the new drug discovery process has utilized the knowledge of the three dimensional structures of target macromolecules or of related proteins. The same strategy was used in the present study for the design of novel agents. The synthesis of lead compound by systematic chemistry in conjunction with binding studies was undertaken. From the experimental observation at the atomic level we theoretically predicted that how inhibitors bind the molecular targets, specific interactions that are important in the molecular recognition (Krovat *et al.*, 2005). The structural and mechanistic investigation of targets like to bacterial methionyl-tRNA synthetase (metRS) enzyme (Kim and Lee, 2003) and human Inosine-5′-monophosphate dehydrogenase (IMPDH) enzyme were used commonly to know the potential microbial inhibitors.

The present study describes the one pot synthesis and biological evaluation of some novel thiazolo[3,2-*a*]pyrimidine-6-carboxylate derivatives. The molecules are designed based on the preliminary docking studies by choosing the appropriate pharmacophoric groups. We herein report the synthesis of two different series of thiazolo[3,2-*a*]pyrimidine-6-carboxylate derivatives (**3a–f**, **4a–f**) and evaluation of their in vitro antibacterial, antifungal, and antiviral activity against selected pathogens of medical importance. Furthermore, in silico docking of the newly synthesized compounds to targets such as metRS and IMPDH enzymes are also presented.

Results and discussion

Chemistry

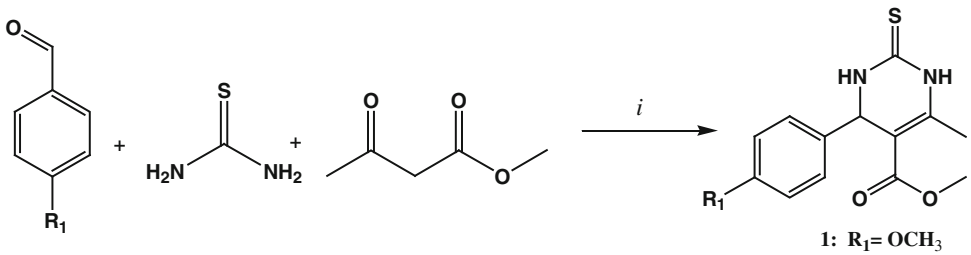
The key intermediates methyl-4-(4-methoxy/4-bromophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (**1** and **2**) were synthesized through optimized Biginelli reaction as shown in the Scheme 1. The homogeneous solution of 4-methoxy/4-bromo benzaldehyde, methylacetoacetate and thiourea in mixture of acetonitrile and dimethyl formamide was stirred in the presence of catalytic amount of silicon tetrachloride (Ramalingan and Kwak, 2008).

The structure of compounds **1** and **2** were confirmed by IR, ^{1}H, ^{13}C NMR, LC–MS, and elemental analysis. The absorption bands appeared at 3619, 3321, 3277, 2935, 1648, 1556, 1420, 1170 cm^{-1} were due to stretching of NH, CH, C=O of ester moety, C=N, C–O, and C–Br groups. The ^{1}H NMR of compound **2** showed signals at δ 2.28, a singlet for methyl protons and another singlet at δ 3.54 for methyl protons of ester moiety, respectively. The signal for hydrogen atom at the chiral carbon C-4 of dihydropyrimidone ring appeared as a doublet at δ 5.15–5.14 (d, 1H, $J = 3.72$ Hz). Aromatic protons of phenyl ring resonated at δ 7.17–7.14 (d, 2H, $J = 8.36$ Hz Ar–H), and 7.55–7.53 (d, 2H, $J = 8.28$ Hz Ar–H). The signals for the two NH protons of the pyrimidone ring appeared at 9.67 (d, 1H, $J = 1.64$ Hz –NH), and 10.39 (s, 1H, –NH) ppm respectively which disappeared on D_2O exchange. The down field shift of the signal was due to thiol thione tautomerism. Hence, the structure of the molecule **2** was confirmed. It was further supported by recording ^{13}C NMR spectrum. The signals appeared in the spectrum account for all the C-atoms present in molecule **2.** LC–MS of the compound **2** showed molecular ion peak at *m*/*z*, 341 (M+2), was in agreement with its molecular formula $C_{13}H_{13}BrN_2O_2S$. Similarly structure of **1** was also confirmed by analytical and spectral data.

After confirming the structure of these two compounds they were allowed to react with different aromatic aldehydes and chloroacetic acid in the presence of sodium acetate, acetic acid and acetic anhydride to afford the title thiazolo[3,2-*a*]pyrimidine-6-carboxylate derivatives **3a–f** and **4a–f** (Scheme 2).

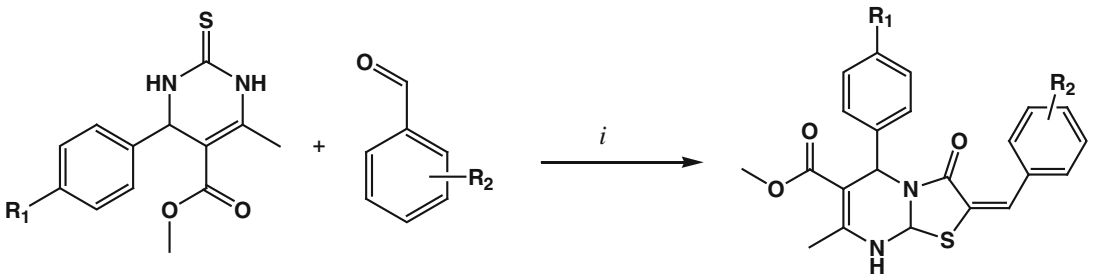
The structure of **3a–f** and **4a–f** was confirmed by analytical and spectral data. The IR absorption bands appeared at 3274, 2968, 1657, 1446, 1178, 1110, 690 cm^{-1}. The absence of bands due NH in the compound confirmed the cyclization. ^{1}H NMR was recorded in the DMSO-d_6 (400 MHz) for compound **3a.** The ^{1}H NMR of compound **3a** showed three singlet signals at δ 2.38, 3.59, and 3.70 for methyl, methyl protons of ester moiety and methoxy protons, respectively. The signal for hydrogen atom at C-4 appeared as a singlet at δ 5.98. The aromatic proton of the methoxy phenyl ring appeared as two doublets in the region δ 6.89 (d, 2H, $J = 8.72$ Hz, Ar–H), 7.21 (d, 2H, $J = 8.68$ Hz, Ar–H). The signals for three protons of chloro fluoro substituted phenyl appeared as multiplets at δ 7.70–7.65 (m, 1H, Ar–H), 7.95–7.91 (m, 1H, Ar–H), 8.16–8.14 (m, 1H, Ar–H). This

Scheme 1 (*i*) Silicon tetrachloride, DMF/ACN, 16 h



1: R_1= OCH_3

2: R_1= Br



(1a-1f) and **(2a-2f)**

2 a: R_1=Br, R_2=2-Cl, 6-F
2 b: R_1=Br, R_2=3-CN, 4-F
2 c: R_1=Br, R_2=3,4-difluoro
2 d: R_1=Br, R_2=4-Cl, 3-CF_3
2 e: R_1=Br, R_2=4-OMe
2 f: R_1=Br, R_2=2-F, 4-OMe

1 a: R_1=OMe, R_2=2-Cl, 6-F
1 b: R_1=OMe, R_2=3-CN, 4-F
1 c: R_1=OMe, R_2=3,4-difluoro
1 d: R_1=OMe, R_2=4-Cl, 3-CF_3
1 e: R_1=OMe, R_2=4-OMe
1 f: R_1=OMe, R_2=2-F, 4-OMe

Scheme 2 (*i*) Chloro acetic acid, NaOAc, AcOH, Ac_2O, 130 °C, 3–5 h

complex pattern could be attributed to the H–F coupling. The signal appeared at δ 7.78 was due to exocyclic methylene proton. The absence of the signals due to NH protons in this product indicated the formation of thiazolopyrimidine ring. The LC–MS spectrum showed a molecular ion peak at *m/z* 473 (M+1), which corresponded to its molecular formula $C_{23}H_{18}ClFN_2O_4S$. In addition, the molecular structure of the compound **3f** was established by single-crystal X-ray diffraction studies (Fun *et al.*, 2011). The ORTEP view of the molecular structure showing the spatial atomic positions of the compound **3f** is given in Fig. 1. The crude product (**3a–f** and **4a–f)** was purified on a Biotage parallel column purifier using EtOAc/Pet ether (3:2) as eluant. The purity of the final compounds was determined by HPLC and all the compounds possessed a purity of >95 %.

Biological activities

In vitro antibacterial and antifungal activities

The newly synthesized compounds **3a–f**; **4a–f** were screened for their antibacterial activity against *Escherichia coli, Staphyllococcus aureus* (*Smith*), *Bacillus subtilis*, and *Salmonella typhi* bacterial strains in vitro. Antifungal activity for the newly synthesized compounds was also carried out against fungal strains *Aspergillus niger* and *Candida albicans* by disk diffusion method (Cruickshank *et al.*, 1975; Warnock *et al.*, 1998). The zone of inhibition and MIC were noted and data are given in Table 1 and 2.

All the tested compounds (**3a–f**, **4a–f)** showed comparable activity with the standard compounds Ciprofloxacin and Fluconazole for antibacterial and antifungal activities, respectively. Among them **4c** showed significant activity with zone of inhibition 24 mm (MIC 26 µg/mL) for *S. aureus*, 26 mm (MIC 26 µg/mL) for *B. subtilis*, 23 mm (30 µg/mL) *S. typhi* and 24 mm (30 µg/mL) for *E. coli* (24mm, 30µg/mL), respectively. The zone of inhibition and MIC of **4c** against *A. niger* was found to be 21 mm and 25 µg/mL and against *C. albicans* is 22 mm and 30 µg/mL. The compounds **3c**, **3f**, and **4e** were inactive against *C. albicans* even though they exhibited good activity against other tested microbial strains.

Cytotoxicity and antiviral activity

Usually cell lines such as primary chick embryo, chick kidney, calf kidney, Madin Darby Canine Kidney

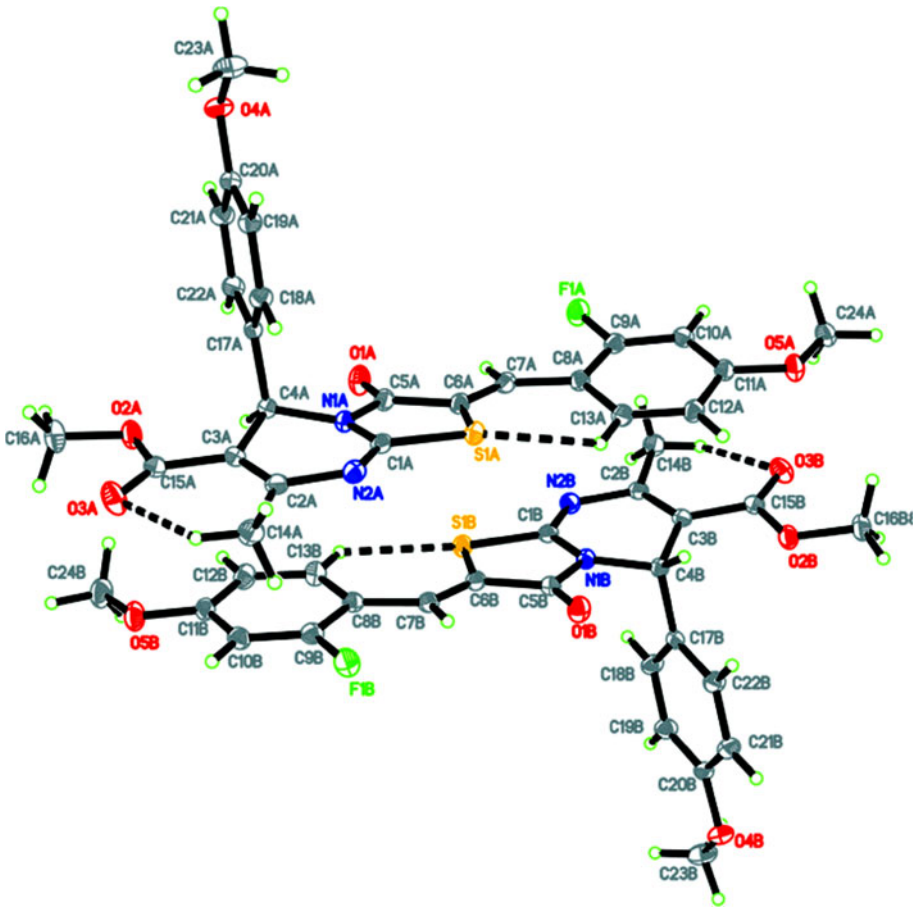


Fig. 1 ORTEP diagram showing the X-ray crystal structure of **3f**

Table 1 Antimicrobial activity of thiazolopyrimidine carboxylate derivatives **3a–f**; **4a–f** (zone of inhibition in mm)

Compd. conc. (40 μg)	Bacterial strains				Fungal strains	
	Staphylococcus aureus	*Bacillus subtilis*	*Salmonella typhi*	*Escherichia coli*	*Aspergillus niger*	*Candida albicans*
3a	23	21	22	23	23	20
3b	21	22	22	22	24	22
3c	21	22	24	22	24	R
3d	23	22	23	23	23	22
3e	22	23	24	22	23	22
3f	24	24	23	25	25	R
4a	22	24	22	24	24	22
4b	23	21	23	22	23	22
4c	24	26	23	24	21	22
4d	22	22	23	22	22	22
4e	23	22	23	22	24	R
4f	22	24	22	20	23	21
Standard[a]	23	23	24	25	23	24

Compounds used: (40 μg/ml)

[a] Standard drug used: bacteria (ciprofloxacin); fungal (fluconazole) (40 μg/ml)

(MDCK), mink lung, and human respiratory epithelial cells could be used for in vitro antiviral assays (Sidwell and Smee, 2000). But Vero cells are preferred because; pox viruses grow well in this cell line. The effect of various concentrations of DMSO on Vero cell monolayers was tested by dye exclusion method assay, resulting in near to

Table 2 Minimum inhibitory concentration (MIC in μg/ml)

Compd. conc. 10–50 (μg/ml)	Bacterial strains				Fungal strains	
	Staphylococcus aureus	*Bacillus subtilis*	*Salmonella typhi*	*Escherichia coli*	*Aspergillus niger*	*Candida albicans*
3a	30	30	40	30	30	30
3b	30	40	30	30	40	40
3c	23	23	30	30	35	R
3d	30	40	40	30	40	30
3a	30	30	30	40	40	40
3f	40	30	30	40	30	R
4a	30	30	40	20	30	40
4b	40	20	30	30	30	30
4c	26	26	30	30	25	30
4d	40	30	30	30	30	30
4a	30	30	30	20	30	R
4f	30	30	40	40	30	40
Standard[a]	22	23	24	22	23	24

[a] Standard drug used: bacteria (ciprofloxacin); fungal (fluconazole) (40 lg/ml)

100 % cell survival at more than 1 % DMSO after 48 h incubation (data not shown). Moreover, potential interference of DMSO in the assay throughout the screening process was assessed for every plate with the addition of 0.2 μL of DMSO to three wells, volume that was equivalent to those of compounds.

Selection of camelpox (CMLV) and buffalopox (BPXV) inhibitors may be of value in obtaining antiviral agents against pox viruses (De Clercq and Neyts, 2004) in general and smallpox virus in particular, which has re-emerged as a threat to use in bioterrorism (Breman and Henderson, 1998).

Prior to evaluating antiviral activities of the compounds, the cytotoxicity of the synthetic compounds on host cell (Vero cell) was carried out. The maximum non-toxic concentration/dose (MNTC/MNTD) for **3a**, **3c**, **4a,** and **4c** was carried out and is shown in Table 3. There was no visible toxicity to the cells at these concentrations. These compounds at MNTCs were utilized for antiviral efficacy testing against CMLV and BPXV. But there was no significant inhibition in the replication of these viruses in vitro by the tested compounds. The titers of both the viruses viz; BPXV and CMLV both in control and treated (virus+compound) wells are presented in Table 3. In this investigation, among the tested compounds, **4a** showed in vitro inhibition of 73.29 and 54.42 % against two pox viruses (DNA viruses) viz., BPXV and CMLV, respectively at their MNTC.

Docking studies

Docking calculations with ICM^{TM} (Internal Coordinate Mechanics) dock All the docking calculations of compounds in this article were performed using the ICM^{TM} docking module with the default setup as earlier mentioned (Abagyan *et al.*, 1994; Khan *et al.*, 2009).

Preparations of the inhibitors and target molecules The 2D structures of the compounds (in mol file formats) have been converted to 3D and energy minimized at the 3D space of ICM environment. The formal charges and 3D topology were assigned by the atom types using local

Table 3 Cytotoxicity and inhibitory effects of different synthetic compounds on Vero cells, BPXV, and CMLV viruses

Compd.	Amount (mg)	DMSO (μl)	MNTC (μg/ml)	CMLV control	Inhibitory titer (virus+extract)	Percent of inhibition (PI)	Buffalo pox control (TCID50/ml)	Inhibitory titer (virus + extract) (TCID50/ml)	Percent of inhibition (PI)
CR 22	3	200	250	$10^{6.5}$	$10^{6.5}$	0	$10^{6.74}$	$10^{6.74}$	0
3a	2	200	31.2	$10^{6.5}$	$10^{6.16}$	54.29	$10^{6.74}$	$10^{6.74}$	0
3c	2	200	31.2	$10^{6.5}$	$10^{6.5}$	0	$10^{6.74}$	$10^{6.74}$	0
4a	4	300	20.8	$10^{6.5}$	$10^{6.16}$	54.42	$10^{6.74}$	$10^{6.16}$	73.69
4c	2	300	05.2	$10^{6.5}$	$10^{6.5}$	0	$10^{6.74}$	$10^{6.74}$	0

chemical environment, Merck Molecular Force Field (MMFF) (Halgren, 1996a, b, c, d, e; Halgren and Nachbar, 1996f).

Docking process All the docking calculations were performed using the "interactive docking" menu at the ICM environment. After docking the stack of docking poses were checked visually. Multiple stack conformations were selected based on their docking energies, rmsd values (compared between the docked model and X-ray conformation with similarities to closely related X-ray crystal structures from PDB). Then the best conformations for each of the compounds were finally chosen, and their binding energies were calculated using ICM script.

Putative molecular interactions with metRS The compounds **1**, **3a–f**, **2** and **4a–f** have been docked into the active site of the metRS (PDB ID: 1A8H).

The calculated docking and binding (ΔG) energies (in Kcal/mol) of the compounds are shown in Table 4. Docking energy gives an idea about the energy required to cover the entire protein by a ligand molecule, whereas the binding energy gives the information about the putative interaction of the molecules at the active site of the enzyme. The molecular interactions between the compounds and the active site residues of metRS at 3D space are shown in Fig. 2, different compounds in different panels, accordingly. The S1 atom of the compound **1** showed strong hydrogen bonding interactions with O, C, and CA atoms of Glu138 residue at the active site of metRS and most of the other parts of the compounds showed short and long distance hydrophobic interactions with different residues of the active sites like Val140, Lys137, Pro145, Leu139, Tyr134, Ile146, Arg132 and Thr55. Same S1 atom of compound **3a** showed strong hydrogen bonding interactions with the CD1 atom of Ile146 and other atoms showed hydrophobic interactions with Tyr134, Arg132, Glu138, Pro145, and Val140. The S1 atom of compound **3b** showed long distance (3.19Å) hydrogen bond with the OH atom of Tyr134 and others showed hydrophobic interactions with Val140, Pro145, Arg132, and Thr55. O4 and C14 atoms of compound **3c** showed strong hydrogen bonding interactions with the CG1 atom of Val140 and others showed hydrophobic interactions with Glu138, Pro145, Ile146, and Thr55. Compound **3d** did not show any hydrogen bonding with any residues of the active site of metRS, but showed several hydrophobic interactions with different residues of the active sites like Ile146, Thr55, Val140, Pro145, Leu139, Glu138, Tyr134, and Arg132. Again S1 atom of the compound **3e** showed strong hydrogen bonding interactions with the NE2 atom of His147 and hydrophobic interactions with Ile146, Arg58, Thr55, and Ala59. Compound **3f** did not show any hydrogen bonding, but showed some hydrophobic interactions with Ile146, His147, Thr55, Arg132, and Glu138.

Compound **2** did not show any hydrogen bonding, but showed some hydrophobic interactions with Ile146, Pro145, Glu138, Tyr134, and Arg132. Compound **4a** showed hydrophobic interactions with only Pro145 and Ile146. Compound **4b** showed several hydrophobic interactions with Thr55, Arg132, Tyr134, Ile146, Glu138, Pro145, and Val140. Compound **4c** showed hydrophobic interactions with only Ile146, Arg132, and Thr55. Compound **4d** showed several hydrophobic interactions with Thr55, Ile146, Pro145, Arg132, Val140, Tyr134, and Glu138. Compound **4e** also showed several hydrophobic interactions with Thr55, Ile146, Pro145, and Val140. Compound **4f** showed hydrophobic interactions with only Ile146, Arg132, Glu138, and Thr55. Docking energy gives an idea about the energy required to cover the entire protein by a ligand molecule, whereas the binding energy gives the information about the putative interaction of the molecules at the active site of the enzyme. Lower the value of these energies, efficient will be the molecule. All the compounds showed minimum docking and binding energy, among them compound **1**, **2**, **4d**, and **4e** could be effective binders to metRS.

Table 4 Calculated docking and binding (ΔG) energies of the compounds against metRS

Compd.	Docking	Binding (ΔG)
1	−44.3	−10.9
3a	−13.8	−11.5
3b	−14.2	−12.4
3c	−22.1	−12.0
3d	−6.6	−11.0
3e	−9.6	−13.1
3f	−36.3	−7.2
2	−37.3	−7.2
4a	−15.9	−10.9
4b	−20.5	−11.9
4c	−35.6	−12.4
4d	−35.9	−13.2
4e	−23.4	−10.8
4f	−10.2	−11.6

Calculated energies (in Kcal/mol)

Putative molecular interactions with the human IMPDH The compounds **3a** and **4a** have been docked into the active site of the human IMPDH (PDB ID: 1NFB), where Cys331 is the catalytic residue, based on their in vitro antiviral results mentioned in Table 3 against BPXV and CMLV. The calculated docking (DE) and binding (ΔG) energies of **3a** were −54.2 and −8.7 kcal/mol,

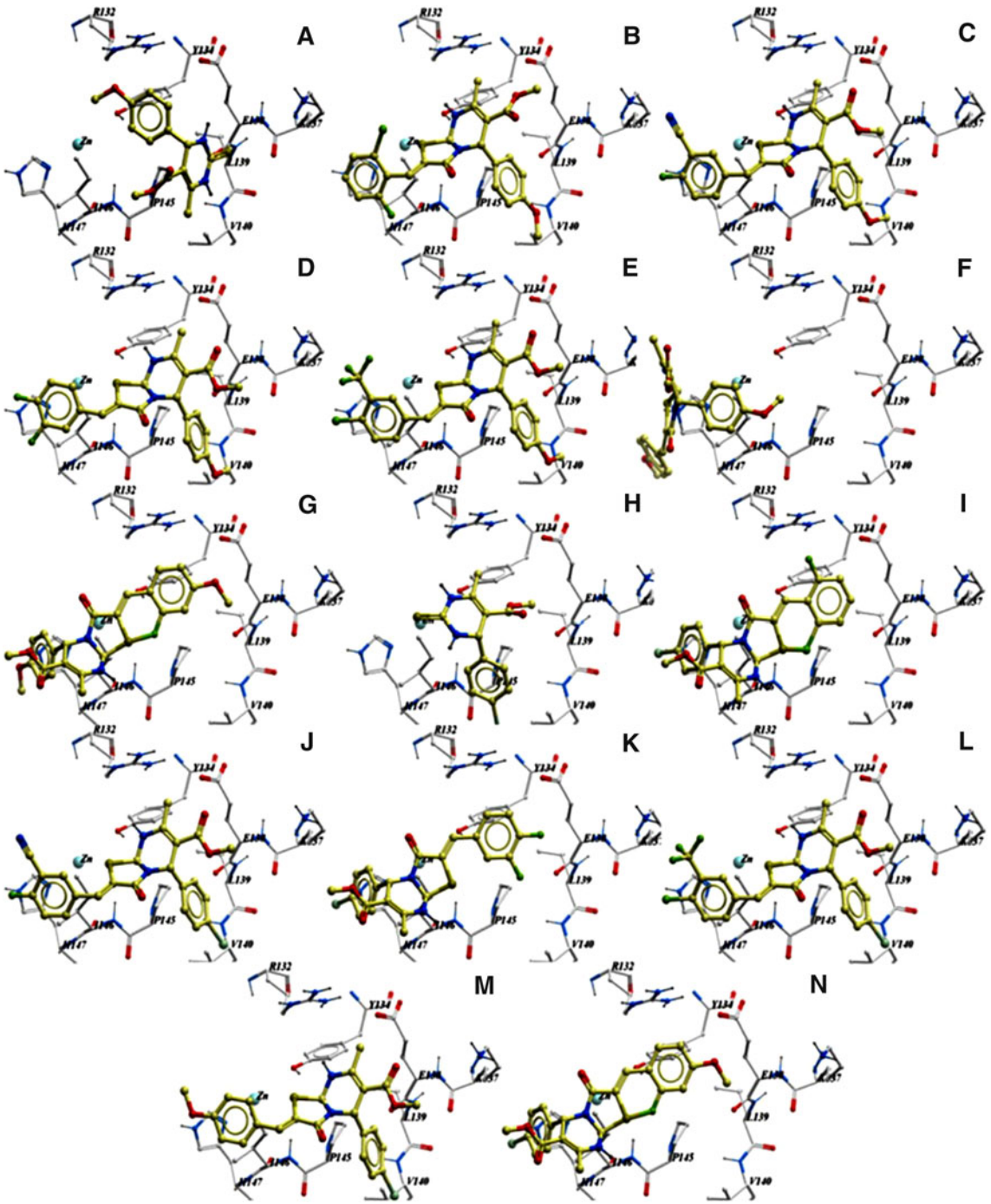


Fig. 2 Molecular interactions with of the compounds **1** (**A**), **3a** (**B**), **3b** (**C**), **3c** (**D**), **3d** (**E**), **3e** (**F**), **3f** (**G**), **2** (**H**), **4a** (**I**), **4b** (**J**), **4c** (**K**), **4d** (**L**), **4e** (**M**), and **4f** (**N**) against methionyl-tRNA synthetase (metRS) (PDB ID: 1A8H)

respectively, whereas for compound **4a** the calculated energy values were −58.6 and −10.4 kcal/mol, respectively. O4 oxygen atom of compound **3a** exhibited long distance (3.24 Å) hydrogen-bond interactions with the N atom of the residue Met414 of human IMPDH and most of the atoms of the molecule showed hydrophobic interactions with Cys331, Met70, His93, Asn94, and Cys95. These intermolecular interactions are shown in the panel A of Fig. 3. Whereas compound **4a** exhibited at least 3 different long distance hydrogen-bond interactions. Atom O1 showed with NH_2 atom of Arg259 with a distance of 3.06 Å, O3 showed with the NE2 atom of His93 with a distance of 2.87 Å, and Cl atom showed with the O atom of Pro69 with a distance of 2.87 Å. Other atoms of the molecule

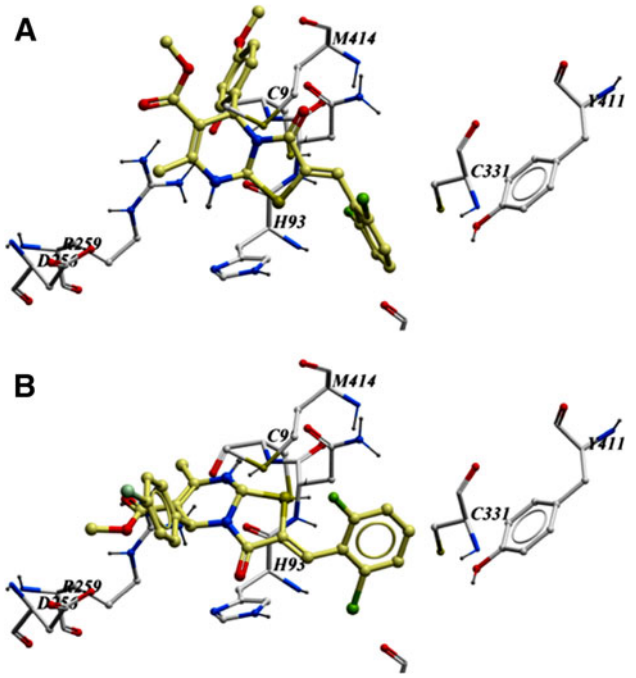


Fig. 3 Intermolecular interactions with of the compounds **3a** (**A**) and **4a** (**B**) at the active site of human inosine monophosphate dehydrogenase (IMPDH) type II (PDB ID: 1NFB), where Cys331 is the catalytic residue. Both the compounds exhibited imperative hydrogen bonding with a number of explicit residues of the active site of IMPDH

showed several important hydrophobic interactions against Asp256, Asn94, Met414, Asp71, and Tyr411 with different distances. These intermolecular interactions are shown in the panel B of Fig. 3. These results showed that the relatively higher antiviral activity of compound **4a** in comparison **3a** may due to lower docking and binding energies as well as higher hydrogen and hydrophobic interactions. Earlier, in a review by Bhanuprakash *et al.* (2010) an effective anti-poxvirus compounds which inhibited IMP dehydrogenase, such as ribavarin, and tyrosine kinase inhibitor, STI-571/imatinib mesylate, or Gleevec were reported.

Conclusions

Two new series of thiazolopyrimidine carboxylate derivatives **3a**–**f** and **4a**–**f** were synthesized with good yield by a three component MCR reaction, characterized by spectral and elemental analysis. All the tested compounds exhibited promising antimicrobial (fungal and bacterial) activities. However, compound methyl(2Z)-5-(4-bromophenyl)-2-[(3,4-difluorophenyl)methylidene]-7-methyl-3-oxo-2,3-dihydro-5H[1,3]thiazolo[3,2-*a*]pyrimidine-6-carboxylate **4c** exhibited excellent in vitro antimicrobial activity. Among the tested compounds for antiviral activity, **4a** exhibited maximum anti-pox virus (BPXV and CMLV) activity. The molecular docking studies of the compound **4c** and **4a** with target for metRS and human IMPDH showed minimum binding and docking energies, respectively coupled with more hydrogen bonding and hydrophobic interaction, which gives impetus for further work on their antimicrobial/viral activities. This preliminary study has opened up an interesting area to evaluate thiazolpyrimidine carboxylate derivatives as possible antimicrobial and antiviral drug candidates.

Experimental

All the chemicals were analytical grade and purchased from Sigma-Aldrich. Final purifications were carried out using Quad biotage Flash purifier. TLC experiments were performed on alumina-blocked silica gel 40 F254 plates. Melting points were determined using Buchi-540 by open capillary and are uncorrected. Elemental analysis was carried out on an automatic Flash EA 1112 Series, CHNSO Analyzer (Thermo). X-ray diffraction studies were carried out on Oxaford Cryosystems Cobra open flow nitrogen cryostat (Cosier and Glazer, 1986) operating at 100.0 (1) K (Universiti Sains Malaysia). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX 400 or Bruker DPX 300 spectrometer, with 5 mm PABBO BB-1H TUBES LCMS were obtained using Agilent 6200 series. The chemical shifts are reported in ppm (δ), the signals are designated as follows: s, singlets; d, doublets; dd, doublet of doublets; t, triplets; brs, broad singlet, m, and multiplet. LC and Micromass zQ spectrometer.

Synthesis of methyl-4-(4-methoxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (**1**)

To a homogeneous solution 4-methoxy/4-bromo benzaldehyde (5.0 g, 0.0367 mol) in mixture of dry acetonitrile and *N*,*N*′-dimethylformamide (2:1, 100 mL), were added methylacetoacetate (4.26 g, 0.0367 mol) and thiourea (3.35 g, 0.0440 mol) at room temperature. The reaction mixture was cooled to 0 °C and silicon tetrachloride (0.62 g, 0.00367 mol) was added in drops. The reaction mass was stirred at 25–28 °C for 16 h. After the completion of the reaction, the reaction mass was quenched with chilled water (100 mL) and stirred for 30 min. The solids precipitated out were collected by filtration, washed with water, and dried under suction to get the title compound (8 g, 75 %) as a white solid.

1 : Appearance: off white solid; yield 75 %; m.p: 180–182 °C; IR (KBr, cm^{-1}) ν_{max}: 3318, 3270,1657; ^{1}H NMR (DMSO-d$_6$, 400 MHz), δ: 2.06 (s, 3H, –CH_3), 3.53 (s, 3H, –CO_2CH_3), 3.71 (s, 3H, –OCH_3), 5.10 (d, 1H,

$J = 3.72$ Hz, pyrimidine-CH–), 6.88 (dd, 2H, $J = 2.00$, 6.88 Hz, Ar–H), 7.11 (dd, $J = 1.92$, 8.68 Hz, 2H, Ar–H), 9.61 (d, 1H, $J = 1.80$ Hz, –NH), 10.31 (s, 1H, –NH) ppm; ^{13}C (DMSO-d_6, 100 MHz), 17.17, 51.05, 53.29, 55.07, 100.63, 113.9, 127.53, 135.47, 145.03, 158.73, 165.64, 174.01 ppm; Anal. for $C_{14}H_{16}N_2O_3S$; cal: C 57.52, H 5.52, N 9.58; found; C 57.47, H 5.55, N 9.50; LC/MS (ESI–MS) *m/z* 293.0 (M+1).

2: Appearance: off white solid; yield 73 %; m.p: 168.3–170.7 °C; IR (ν cm^{-1}): 3619, 3321, 3277, 2935, 1648, 1556, 1420, 1170; ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 2.28 (s, 3H, –CH_3), 3.54 (s, 3H, –CO_2CH_3), 5.13 (d, 1H, $J = 3.72$ Hz, pyrimidine CH–), 7.15 (d, 2H, $J = 8.36$ Hz, Ar–H), 7.55 (d, 2H, $J = 8.36$ Hz, Ar–H), 9.69 (s, 1H, –NH), 10.41 (s, 1H, –NH) ppm; ^{13}C (DMSO-d_6, 100 MHz), 17.21, 51.12, 53.36, 99.95, 120.84, 128.57, 131.54, 142.56, 145.62, 165.47, 174.26; Anal. for $C_{13}H_{13}BrN_2O_2S$; cal: C 45.76, H 3.84, N 8.21; found; C 45.65, H 3.81, N 8.24; LC/MS (ESI–MS) *m/z* 343 (M+2).

General procedure for synthesis of methyl(2Z)-2-(substituted benzylidene)-5-(4-methoxy/bromophenyl)-7-methyl-3-oxo-2,3-dihydro-5H-[1,3]-thiazolo[3,2-*a*]pyrimidine-6-carboxylates. **3a**–**3f** and **4a**–**4f**

A mixture of methyl 4-(4-methoxyphenyl)/(4-bromophenyl)-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate **1/2** (2.59 mmol), chloro acetic acid (0.29 g, 3.11 mmol), an aldehyde (3.11 mmol) and sodium acetate (10 mmol) in 1:1 mixture glacial acetic acid/acetic anhydride (10 mL) were heated to 130 °C for 3–5 h. After the completion of the reaction, the reaction mass was cooled to room temperature and quenched to ice cooled water. The solids precipitated out were collected by filtration and purified on a Biotage parallel column purifier using EtOAc/Pet ether (3:2) as eluant to afford title compounds (60–75 %).

*Methyl(2Z)-2-[(2-chloro-6-fluorophenyl)methylidene]-5-(4-methoxyphenyl)-7-methyl-3-oxo-2,3-dihydro-5H-[1,3]thiazolo[3,2-a]pyrimidine-6-carboxylate (**3a**)*

Appearance: yellow solid; yield 70 %; m.p: 152.5–154.5 °C; IR (ν cm^{-1}): 3274, 2968, 1657, 1446, 1178, 1110, 690; ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 2.38 (s, 3H, –CH_3), 3.59 (s, 3H, –CO_2CH_3), 3.70 (s, 3H, –OCH_3), 5.98 (s, 1H pyrimidine-CH–), 6.89 (d, 2H, $J = 8.72$ Hz, Ar–H), 7.21 (d, 2H, 8.68 Hz, Ar–H), 7.70–7.65 (m, 1H, Ar–H), 7.78 (s, 1H Ar–CH=), 7.95–7.91 (m, 1H, Ar–H), 8.16–8.14 (m, 1H, Ar–H) ppm; Anal. for $C_{23}H_{18}ClFN_2O_4S$; cal: C 58.41, H 3.84, N 5.92; found; C 58.36, H 3.82, N 5.96; LC/MS (ESI–MS) *m/z* 473.0 (M+1).

*Methyl(2Z)-2-[(3-cyano-4-fluorophenyl)methylidene]-5-(4-methoxyphenyl)-7-methyl-3-oxo-2,3-dihydro-5H-[1,3]thiazolo[3,2-a]pyrimidine-6-carboxylate (**3b**)*

Appearance: yellow solid; yield 67 %; m.p: 172.3–173 °C; IR (ν cm^{-1}): 3278, 2976, 2364, 2189, 1656, 1448, 1175, 1107; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 2.36 (s, 3H, –CH_3), 3.58 (s, 3H, –CO_2CH_3), 3.71 (s, 3H, –OCH_3), 5.98 (s, 1H pyrimidine-CH–), 6.91(d, 2H, $J = 8.72$ Hz, Ar–H), 7.22 (dd, 2H, $J = 1.88$, 8.72 Hz, Ar–H), 7.41–7.37 (m, 1H, Ar–H), 7.48 (d, 1H, $J = 7.44$ Hz, Ar–H), 7.58–7.52 (m, 1H, Ar–H), 7.68 (s, 1H, Ar–CH=) ppm. Anal. for $C_{24}H_{18}FN_3O_4S$; cacld; C 62.19, H 3.91, N 9.07; found; C 62.26, H 3.87, N 9.02; LC/MS (ESI–MS) *m/z*, 464.0 (M+1).

*Methyl(2Z)-2-[(3,4-difluorophenyl)methylidene]-5-(4-methoxyphenyl)-7-methyl-3-oxo-2,3-dihydro-5H-[1,3]thiazolo[3,2-a]pyrimidine-6-carboxylate (**3c**)*

Appearance: pale yellow solid; yield 68 %; m.p: 197.5–198 °C; IR (ν cm^{-1}): 3274, 2968, 1657, 1446, 1176, 1110, 690; ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 2.37 (s, 3H, –CH_3), 3.60 (s, 3H, –CO_2CH_3), 3.71 (s, 3H, –OCH_3), 5.99 (s, 1H pyrimidine-CH–), 6.87 (dd, 2H, $J = 1.96$, 8.72 Hz, Ar–H), 7.20 (dd, 2H, $J = 1.92$, 8.76 Hz, Ar–H), 7.46–7.43 (m, 1H, Ar–H), 7.64–7.57 (m, 1H, Ar–H), 7.73–7.68 (m, 1H, Ar–H), 7.77 (s, 1H, Ar–CH=) ppm; ^{13}C (DMSO-d_6, 100 MHz), 22.33, 51.47, 54.29, 55.05, 108.90, 113.94, 114.03, 118.55, 118.72, 119.17, 119.35, 121.05, 121.07, 126.73, 126.76, 128.68, 130.52, 130.58, 130.62, 130.69, 132.08, 148.27, 148.40, 149.00, 149.12, 150.73, 150.87, 150.94, 151.63,154.88, 159.29, 164.01, 165.33 ppm; Anal. for $C_{23}H_{18}F_2N_2O_4S$; cacld; C 60.52, H 3.97, N 6.14; found; C 60.46, H 3.94, N 6.18; LC/MS (ESI–MS) *m/z*, 457.0 (M+1).

*Methyl(2Z)-2-[(4-chloro-3-trifluoromethane phenyl)methylidene]-5-(4-methoxyphenyl)-7-methyl-3-oxo-2,3-dihydro-5H-[1,3]thiazolo[3,2-a]pyrimidine-6-carboxylate (**3d**)*

Appearance: yellow solid; yield 65 %; m.p: 176.8–178.1 °C; IR (ν cm^{-1}): 3287, 2986, 1657, 1450, 1175, 1104; ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 2.38 (s, 3H, –CH_3), 3.59 (s, 3H, –CO_2CH_3), 3.70 (s, 3H, –OCH_3), 5.99 (s, 1H pyrimidine-CH–), 6.89 (d, 2H, $J = 8.64$ Hz, Ar–H), 7.22 (d, 2H, $J = 8.64$ Hz, Ar–H), 7.86–7.81 (m, 2H, Ar–H), 7.88 (s, 1H, Ar–CH=), 8.09. (s, 1H, Ar–H) ppm. Anal. for $C_{24}H_{18}ClF_3N_2O_4S$; cacld; C 55.12, H 3.47, N 5.36; found; C 55.23, H 3.40, N 5.33; LC/MS (ESI–MS) *m/z*, 523.0 (M+1).

*Methyl(2Z)-2-[(4-methoxy phenyl)methylidene]-5-(4-methoxyphenyl)-7-methyl-3-oxo-2,3-dihydro-5H-[1,3]thiazolo[3,2-a]pyrimidine-6-carboxylate (**3e**)*

Appearance: yellow solid; yield 68 %; m.p: 188.1–189.3 °C; IR (ν cm^{-1}): 3275, 2975, 1658, 1449, 1172; ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 2.38 (s, 3H, –CH_3), 3.60 (s, 3H, –CO_2CH_3), 3.71 (s, 3H), 3.81 (s, 3H, –OCH_3), 5.99 (s, 1H pyrimidine-CH–), 6.89 (d, 2H, J = 8.64 Hz, Ar–H), 7.10 (d, 2H, J = 8.8 HZ), 7.21 (d, 2H, J = 8.64 Hz, Ar–H), 7.86–7.81 (m, 2H, Ar–H), 7.88 (s, 1H, Ar–CH=), 8.09. (s, 1H, Ar–H) ppm. Anal. for $C_{24}H_{22}N_2O_6S$; cacld; C 63.98, H 4.92, N 6.22; found; C 63.90, H 4.90, N 6.19; LC/MS (ESI–MS) *m/z*, 451 (M+1).

*Methyl(2Z)-2-[(2-fluoro-4-methoxy phenyl)methylidene]-5-(4-methoxyphenyl)-7-methyl-3-oxo-2,3-dihydro-5H-[1,3]thiazolo[3,2-a]pyrimidine-6-carboxylate (**3f**)*

Appearance: yellow solid; yield 70 %; m.p: 204.9–205.5 °C; IR (ν cm^{-1}): 3278, 2980, 1657, 1444, 1173, 1107; ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 2.39 (s, 3H, –CH_3), 3.60 (s, 3H, –CO_2CH_3), 3.71 (s, 3H, –OCH_3), 3.84 (s, 3H, –OCH_3), 6.00 (s, 1H pyrimidine-CH–), 6.90 (d, 2H, J = 8.6 Hz, Ar–H), 7.07–6.98 (m, 1H), 7.23 (d, 2H, 8.6 Hz, Ar–H), 7.51 (t, 1H, J = 8.76 Hz, Ar–H), 7.70 (s, 1H, Ar–CH=) ppm. Anal.for $C_{24}H_{21}FN_2O_5S$; cacld; C 61.53, H 4.52, N 5.98; found; C 61.45, H 4.49, N 5.94; LC/MS (ESI–MS) *m/z*, 469.0 (M+1).

*Methyl(2Z)-5-(4-bromophenyl)-2-[(2-chloro-6-fluorophenyl)methylidene]-7-methyl-3-oxo-2,3-dihydro-5H-[1,3]thiazolo[3,2-a]pyrimidine-6-carboxylate (**4a**)*

Appearance: brown solid; yield 75 %; m.p: 147.8–148.8 °C; IR (ν cm^{-1}): 3092, 2917, 2358, 1724, 1689, 1342, 1163,784; ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 2.36 (s, 3H, –CH_3), 3.58 (s, 3H, –CO_2CH_3), 6.00 (s, 1H, pyrimidine-CH–), 7.28 (d, 2H, J = 8.4 Hz, Ar–H), 7.42–7.38 (m, 1H, Ar–H), 7.48 (d, 1H, J = 7.88 Hz, Ar–H), 7.59–7.53 (m, 3H, Ar–H), 7.69 (s, 1H, Ar–CH=) ppm.**^{13}C** (DMSO-d_6, 100 MHz), 22.41, 51.54, 54.69, 108.52, 115.32, 115.53, 119.95, 121.91, 124.04, 126.26, 127.34, 129.80, 131.74, 132.90, 133.00, 134.16, 139.22, 151.09, 154.74, 157.92, 160.28, 163.28, 165.14.; Anal. for $C_{22}H_{15}BrClFN_2O_3S$; cacld; C 50.64, H 2.90, N 5.37; found; C 50.55, H 2.88, N 5.34; LC/MS (ESI–MS) *m/z*, 523.8 (M+2).

*Methyl(2Z)-5-(4-bromophenyl)-2-[(3-cyano-4-fluorophenyl)methylidene]-7-methyl-3-oxo-2,3-dihydro-5H-[1,3]thiazolo[3,2-a]pyrimidine-6-carboxylate (**4b**)*

Appearance: yellow solid; yield 70 %; m.p: 152.3–153.3 °C; IR (ν cm^{-1}): 3405, 2944, 2155, 1709, 1369, 1158, 821; ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 2.38 (s, 3H, –CH_3), 3.59 (s, 3H, –CO_2CH_3), 6.01 (s, 1H, pyrimidine-CH–), 7.26 (d, 2H, J = 8.4 Hz, Ar–H), 7.55 (d, 2H, J = 8.4 Hz), 7.76–7.66 (m, 1H, Ar–H), 7.79 (s, 1H, Ar–CH=), 7.97–7.93 (m, 1H, Ar–H), 8.18–8.16 (m, 3H, Ar–H) ppm; ^{13}C (DMSO-d_6, 100 MHz), 22.45, 51.55, 54.51, 101.45, 101.60, 108.36, 113.26, 117.69, 117.89, 121.89, 129.67, 129.87, 130.49, 130.52, 131.69, 135.88, 136.17, 136.26, 139.23, 151.39, 155.00, 161.33, 163.88, 163.93, 165.14; Anal. for $C_{23}H_{15}BrFN_3O_3S$; cacld; C 53.92, H 2.95, N 8.20; found; C 53.84, H 2.92, N 8.18; LC/MS (ESI–MS) *m/z*, 514.0 (M+2).

*Methyl(2Z)-5-(4-bromophenyl)-2-[(3,4-difluorophenyl)methylidene]-7-methyl-3-oxo-2,3-dihydro-5H-[1,3]thiazolo[3,2-a]pyrimidine-6-carboxylate (**4c**)*

Appearance: yellow solid; yield 72 %; m.p: 188.3–189.0 °C; IR (ν cm^{-1}): 2934, 2376, 1711, 1690, 1332, 1134, 788; ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 2.38 (s, 3H, –CH_3), 3.59 (s, 3H, –CO_2CH_3), 6.01 (s, 1H, pyrimidine-CH–), 7.26 (d, 2H, J = 8.4 Hz, Ar–H), 7.48–7.46 (m, 1H, Ar–H), 7.56 (d, 2H, J = 8.4 Hz), 7.65–7.58 (m, 1H, Ar–H), 7.74–7.69 (m, 1H, Ar–H), 7.78 (s, 1H, Ar–CH=) ppm. Anal.for $C_{22}H_{15}BrF_2N_2O_3S$; cacld; C 52.29, H 2.99, N 5.54; found; C 52.34, H 2.96, N 5.62; LC/MS (ESI–MS) *m/z*, 507.0 (M+2).

*Methyl(2Z)-5-(4-bromophenyl)-2-{[4-chloro-3-(trifluoromethyl)phenyl]methylidene}-7-methyl-3-oxo-2,3-dihydro-5H-[1,3]thiazolo[3,2-a]pyrimidine-6-carboxylate (**4d**)*

Appearance: yellow solid; yield 68 %; m.p: 186.3–187.5 °C; IR (ν cm^{-1}): 2942, 2353, 1714, 1687, 1316, 1132, 784; ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 2.38 (s, 3H, –CH_3), 3.59 (s, 3H, –CO_2CH_3), 6.03 (s, 1H, pyrimidine-CH–), 7.27 (d, 2H, J = 8.4 Hz, Ar–H), 7.56 (d, 2H, J = 8.4 Hz), 7.91–7.84 (m, 3H, Ar–H), 8.12 (s, 1H, Ar–H) ppm. Anal. for $C_{22}H_{15}BrClF_3N_2O_3S$; cacld; C 48.31, H 2.64, N 4.90; found; C 48.25, H 2.63, N 4.95; LC/MS (ESI–MS) *m/z*, 574.0 (M+2).

*Methyl(2Z)-5-(4-bromophenyl)-2-[(4-methoxyphenyl)methylidene]-7-methyl-3-oxo-2,3-dihydro-5H-[1,3]thiazolo[3,2-a]pyrimidine-6-carboxylate (**4e**)*

Appearance: yellow solid; yield 67 %;m.p: 173.3–174.1 °C; IR (ν cm^{-1}): 2918, 2364, 1719, 1688, 1316, 1109, 815; ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 2.37 (s, 3H, –CH_3), 3.59 (s, 3H, –CO_2CH_3), 3.81 (s, 3H, –OCH_3), 6.01 (s, 1H, pyrimidine-CH–), 7.11 (d, 2H, J = 8.84 Hz, Ar–H), 7.26 (d, 2H, J = 1.84, 6.68 Hz, Ar–H), 7.58–7.52 (m, 4H), 7.75 (s, 1H, Ar–CH=) ppm. Anal.for $C_{23}H_{19}BrF_2N_2O_4S$; cacld; C 55.32, H 3.83, N 5.61; found; C 55.25, H 3.86, N 5.66; LC/MS (ESI–MS) *m/z*, 501.0 (M+2).

*Methyl(2Z)-5-(4-bromophenyl)-2-[(2-fluoro-4-methoxyphenyl)methylidene]-7-methyl-3-oxo-2,3-dihydro-5H-[1,3]thiazolo[3,2-a]pyrimidine-6-carboxylate (**4f**)*

Appearance: yellow solid; yield 72 %; m.p: 197.8–198.1 °C; IR (ν cm^{-1}): 2938, 2344, 1712, 1688, 1332, 1134, 790; ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 2.37 (s, 3H, –CH_3), 3.58 (s, 3H, –CO_2CH_3), 3.83 (s, 3H, –OCH_3), 6.01 (s, 1H, pyrimidine-CH–), 7.06–6.97 (m, 2H, Ar–H), 7.26 (dd, 2H, $J = 1.92$, 6.68 Hz, Ar–H), 7.55–7.48 (m, 3H, Ar–H), 7.69 (s, 1H, Ar–CH=) ppm; ^{13}C (DMSO-d_6, 100 MHz), 22.49, 51.51, 54.41, 56.16, 102.14, 102.39, 108.04, 111.94, 118.70, 121.82, 124.26, 129.64, 129.92, 131.69, 139.46, 151.62, 155.48, 163.11, 164.16, 165.21; Anal. for $C_{23}H_{18}BrFN_2O_4S$; cacld; C 53.39, H 3.51, N 5.41; found; C 53.30, H 3.55, N 5.38; LC/MS (ESI–MS) *m/z*, (*m/z*, %), 519.0 (M+2).

Antimicrobial activity

The antimicrobial activity of synthesized compounds was carried out using agar well-diffusion method. The bacterial strains were collected from different infectious status of patients who had not administered any antibacterial drugs for at least two weeks with the suggestions of an authorized physician, in Kiran diagnostic health centre of Chitradurga, Karnataka state, India. Fungal strains were procured from the culture maintained at National College of Pharmacy Shimoga. The in vitro antimicrobial activity was carried out against 24 h culture of four bacterial strains Gram-positive *S. aureus, B. subtilis,* Gram-negative, *S. typhi, E. coli.* Two fungal strains used were *A. niger and C. albicans*. The stock solution of compounds was made at 50 μg/100 μL and desired concentrations were prepared using stock solution. DMSO was used as a solvent to dissolve compounds. Ciprofloxacin (40 μg in 100 μL) and Fluconazole (50 μg in 100 μL) were used as standard drugs for antibacterial and antifungal activities, respectively. The zone of inhibition was compared with standard drug after 24 h of incubation at 37 °C for antibacterial activity and 72 h at 25 °C for antifungal activity. The zone of inhibition was recorded in mm and given in Table 1.

Minimum inhibitory concentrations (MIC)

The MIC of all synthesized compounds **3a–f**; **4a–f** was determined by a micro dilution method. The respective clinical strain was spread separately on the medium. The wells were created using a stainless steel sterilized cork borer under aseptic conditions. The compounds at different concentrations viz. 10, 20, 30, 40, and 50 μg in 100 μL of DMSO and later was loaded into corresponding wells. The standard drug Ciprofloxacin (50 μg in 100 μL) and Fluconazole (50 μg in 100 μL) were used. The zone of inhibition was compared with standard drug after 24 h of incubation at 37°C for antibacterial activity and 72 h at 25 °C for antifungal activity. The data are given in Table 2.

Cytotoxicity assay: determination of the maximum non-toxic concentration (MNTC)

The cytotoxicity of each compound to Vero cells was determined using the method developed by Bhanuprakash *et al.* (2008) with small modifications. In brief, initially the compounds were dissolved in DMSO and different concentrations of compound with two-fold dilutions were prepared in Eagle's minimum maintenance medium (EMEM). Later, added to the 24 h confluent Vero cell monolayer in 96-well tissue culture plates and incubated in an atmosphere of 5 % CO_2 at 37 °C for 4 days. Each concentration was tested in triplicate along with controls. Cells were examined daily for morphological changes (rounding, degeneration, cell lysis), if any. Cells were observed at every 24 h interval for visible morphological changes under inverted microscope (Leitz), cell morphology was compared between treated and untreated cultures (control). The highest concentration of the synthetic compound that showed no cellular morphological changes was considered as the maximum non-toxic concentration/dose (MNTC/MNTD). Furthermore, at the end of incubation period, the test medium was removed from the plate and washed gently with Phosphate buffered saline (0.1 M).Then, the wells were stained with crystal violet (1 %) in formalin (10 %) for 15 min. The cell viability was evaluated as the percentage of the mean value of optical density resulting from the 6 cell controls, which was set at 100 %. The 50 % cytotoxic concentration (CC_{50}) was calculated from the mean dose-response of 3 independent assays. The concentration of synthetic compounds at which cell viability was above 80 % was further used in the study for assessment of antiviral activity of the synthetic compound against camelpox/buffalopox viruses in a dose-dependent manner (Darshan Raj *et al.*, 2012a, b).

Antiviral screening assay (cytopathic effect inhibitory assay)

Antiviral activity was determined for the compounds **3a**, **3c**, **4a**, and **4c** by reduction of virus titers using $TCID_{50}$ determinations. Vero cells were grown in cell culture plates containing 96 wells for 48 h. After decanting the growth medium, the cells were treated with chemicals at their respective MNTCs (50 μL/well) and immediately logarithmic dilutions of virus (CMLV/BPXV) were added at 50 μL/well in treated and untreated cell cultures and incubated at 37 °C in CO_2 (5 %) for 24 h. After 24 h of

simultaneous incubation, both chemical and the virus were discarded and the wells were replenished with fresh maintenance medium. Subsequent change of medium was done regularly at 48 h interval and the virus titers ($TCID_{50}$/mL) were calculated after 6 days. The experiment was carried out in triplicates. The antiviral activity was expressed as percentage of inhibition (PI) using antilogarithm values of $TCID_{50}$, as—PI = [1 − (T antilogarithm/C antilogarithm)] × 100. A chemical was considered active when the PI is >80 % at its MNTC (Darshan Raj *et al.*, 2012a early online).

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