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Original article

Synthesis of pyrazolo[1,5-*a*][1,3,5]triazine derivatives as inhibitors of thymidine phosphorylase

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ABSTRACT

Thymidine phosphorylase (TP) is an enzyme that promotes tumor growth and metastasis and therefore is an attractive druggable target. Using a reported TP inhibitor, 7-deazaxanthine (7DX), as the lead compound; this study was set up to evaluate whether pyrazolo[1,5-*a*][1,3,5]triazin-2,4-diones and pyrazolo[1,5-*a*][1,3,5]triazin-2-thioxo-4-ones would exhibit TP inhibitory activity. The pyrazolo[1,5-*a*][1,3,5]triazine nucleus was constructed using a reaction that annulated the 1,3,5-triazine ring onto a pyrazole scaffold. Among the 52 compounds synthesized and tested, it was found that 1,3-dihydro-pyrazolo[1,5-*a*][1,3,5]triazin-2-thioxo-4-ones exhibited various extent of inhibitory activity against TP. The best compound **17p**, which bears a para-substituted pentafluorosulfur group, showed an IC_{50} value of 0.04 μM, which was around 800 times more potent than the 7DX (IC_{50} = 32 μM) under the same bioassay conditions. The results of the study suggested that a substituent with +σ and +π properties inserted at position 4 of a phenyl ring that is attached to position 8 of the pyrazolo[1,5-*a*][1,3,5]triazin-2-thioxo-4-one scaffold would give excellent TP inhibitory action. In addition, **17p** was found to be a non-competitive inhibitor thus suggested that it might interact with TP at a position different from the substrate binding site.

1. Introduction

Thymidine phosphorylase (TP) is an enzyme that catalyzes the reverse phosphorolysis of pyrimidine nucleosides. It plays a key role in maintaining the balance of the nucleotide pool and controlling nucleic acid homeostasis by ensuring an ample supply of deoxyribonucleoside triphosphates (dNTPs) for DNA replication and repair [1].

However, besides pyrimidine nucleosides, TP can also induce the phosphorolysis of several nucleoside analog therapeutic agents thus reduce their bioavailability [2]. Therefore, it has been proposed that a co-administration of a nucleoside analog therapeutic agent together with a TP inhibitor might improve the biological efficacy of the therapeutic agent. TP has been also found to be associated with tumor angiogenesis [3] and metastasis [4,5], and it can promote tumor growth by preventing apoptosis as well [6]. Therefore, in addition to improving the biological efficacy of nucleoside analog therapeutic agents, the clinical implication of inhibiting TP has broadened to include controlling the development of tumor.

Structurally, most of the active TP inhibitors are derivatives of pyrimidine and its analogs which were designed to interact with the thymidine binding site. The best inhibitor found to date is 5-chloro-6-[1-(2-iminopyrrolidinyl)methyl] uracil hydrochloride (TPI) [7] which exhibits strong inhibitory activity as denoted by a reported IC_{50} value of 0.035 μM [8]. Besides pyrimidines, some purine analogs such as 7-deazaxanthine (7DX) [9] (Fig. 1) has been found to demonstrate inhibitory activity against TP too. However, 7DX (IC_{50} value = 40 μM [9]) is not as potent as TPI, and further structural modifications may lead to more potent purine-like TP inhibitors. In addition, some multisubstrate inhibitors designed for interacting with both the thymidine and phosphate binding sites have been reported in the literature [10–12]. However, these multisubstrate inhibitors showed weaker inhibition against TP, and they inhibited the enzyme in its opened conformation.

The pyrazolo[1,5-*a*][1,3,5]triazine heterocyclic system is recognized as an analog of purine in which the 9-N atom is translocated to position 5 of the bicyclic ring system. This heterocycle has been substituted for purine in the area of nucleoside chemistry and many biologically active agents have been developed [13].

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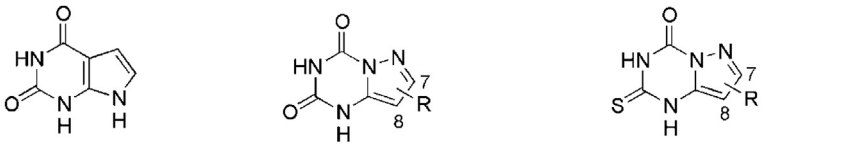


Fig. 1. 7DX and proposed substituted pyrazolo[1,5-*a*][1,3,5]triazine derivatives.

Previous investigations on this scaffold have discovered numerous compounds that exhibit various therapeutic properties. In particular, many enzyme inhibitors were found to carry the pyrazolo[1,5-*a*][1,3,5]triazine scaffold, and a few of them have been approved as drugs. Some of the examples are included herein to illustrate the range of targeted enzymes. In 1985, Robins et al. [14] developed 8-phenyl-5-aza-9-deazahypoxanthine as a potent inhibitor of xanthine oxidase for the treatment of gout. Cyclin-dependent kinase, which is involved in the regulation of the cell cycle, was found to be inhibited by suitably substituted 2,4-diaminopyrazolo[1,5-*a*][1,3,5]triazines [15–17]. The class of cyclic nucleotide phosphodiesterases, which hydrolyze second messengers such as cAMP and cGMP, is another group of enzymes that can be inhibited by pyrazolo[1,5-*a*][1,3,5]triazines [18–21]. Finally, DNA gyrase, which is present in bacteria but not in human, and therefore a good target for antibacterial therapy, can also be selectively inhibited by some pyrazolo[1,5-*a*][1,3,5]triazines [22].

In summary, the pyrazolo[1,5-*a*][1,3,5]triazine scaffold has served as a good template for developing enzyme inhibitors as therapeutic agents. With appropriate structural modifications, this scaffold can be readily used to fulfill pharmacophoric requirements in the design stage of drug discovery. Together with the established approaches to chemical synthesis, libraries of compounds can be readily generated and evaluated for different biological activities.

In this study, using 7DX as the lead compound, it is hypothesized that 1,3-dihydro-pyrazolo[1,5-*a*][1,3,5]triazin-2,4-dione as well as 1,3-dihydro-pyrazolo[1,5-*a*][1,3,5]triazin-2-thioxo-4-one (Fig. 1) may possess TP inhibitory activity comparable to 7DX. In addition, the secondary aim of the study is to investigate whether separate substitution on positions 7 and 8 may lead to the enhancement of TP inhibitory activity of the above mentioned pyrazolo[1,5-*a*][1,3,5] triazines.

2. Results and discussion

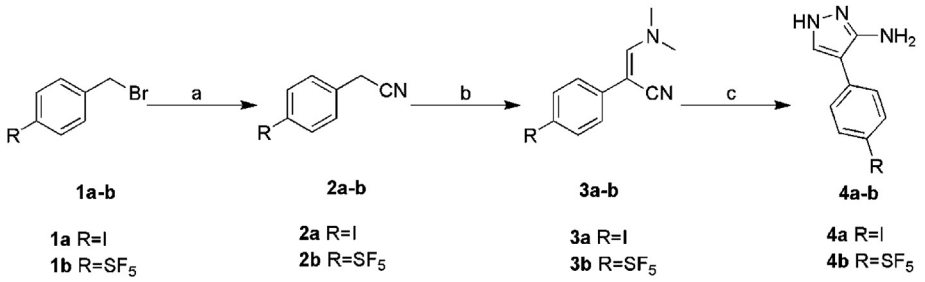
2.1. Chemistry

For the past 50 years, several methods have been reported for the construction of the pyrazolo[1,5-*a*][1,3,5]triazine ring system. The common approaches to the synthesis of pyrazolo[1,5-*a*][1,3,5] triazines can be categorized according to how the bicyclic fused ring system is formed. The following approaches have been reported in the literature, namely, (A) annulation of the 1,3,5-triazine ring onto a pyrazole scaffold; (B) annulation of the pyrazole ring onto a 1,3,5-triazine scaffold; (C) concurrent formation of both the 1,3,5-triazine and pyrazole ring; and (D) ring transformation reactions [13]. In the selection of the synthetic approach to be adopted for this study, commercial availability of the starting materials and synthetic practicality were important consideration factors. Since many 4- and 5-substituted 3-amino pyrazoles are either commercially available or can be easily synthesized; therefore it is believed that 7 or 8 substituted pyrazolo[1,5-*a*][1,3,5]triazines can be conveniently generated through the use of approach A. Hence this approach was adopted for synthesizing the target compounds in this study. By reacting respectively the substituted 3-amino pyrazoles with either ethoxycarbonyl isocyanate or ethoxycarbonyl isothiocyanate; both the 2,4-dione series and the 2-thioxo-4-one series can be readily synthesized in two steps. For the required aryl substituted 3-amino pyrazoles which were unavailable commercially, reported methods from the literature were used to synthesize them. The method of synthesis depended on positions where the final aryl substituents were located.

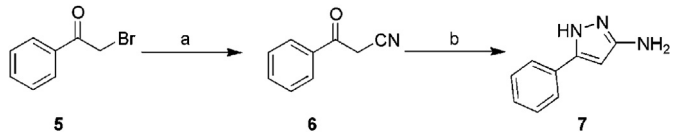
3-amino pyrazoles with aryl substituents located at position 4 was prepared via a three step synthetic scheme [23–25] (Scheme 1). 4-Substituted-aryl acetonitriles (**2**) were synthesized by treating correspondingly substituted bromomethylbenzenes **1** with potassium cyanide (Yield 56% for **2a**; 69% for **2b**) and then subsequently reacted with dimethoxymethyldimethylamine to give 3-dimethylamino-2-aryl-acrylonitriles **3** (Yield 80% for **3a**, 85% for **3b**). The target 4-aryl-3-amino pyrazoles **4** were obtained via a reaction between hydrazine and the corresponding acrylonitriles (Yield 29% for **4a**, 19% for **4b**).

5-Phenyl-1*H*-pyrazol-3-ylamine **7** was prepared by a two-step synthetic reaction [26] (Scheme 2). 3-Oxo-3-phenylpropionitrile **6** was first synthesized by treating 2-bromo-1-phenylethanone **5** with potassium cyanide (Yield 76%) and followed by a reaction with hydrazine to generate the target amine **7** (Yield 16%).

Target compounds 1,3-dihydro-pyrazolo[1,5-*a*][1,3,5]triazin-2,4-diones **10** and **13** were synthesized via a two-step synthetic scheme [27] as presented in Scheme 3. The corresponding amines **7**,



Scheme 1. Synthesis of 4-aryl-1*H*-pyrazol-3-ylamines. Reagents and conditions: (a) KCN, ethanol, reflux, yields 56% (**2a**) and 69% (**2b**); (b) dimethoxymethyldimethylamine, DMF, reflux, yields 80% (**3a**) and 85% (**3b**); (c) NH_2NH_2, ethanol, reflux, yields 29% (**4a**) and 19% (**4b**).



Scheme 2. Synthesis of 5-phenyl-1*H*-pyrazol-3-ylamine. Reagents and conditions: (a) KCN, ethanol, 50 °C, yield 76%; (b) NH_2NH_2, ethanol, reflux, yield 16%.

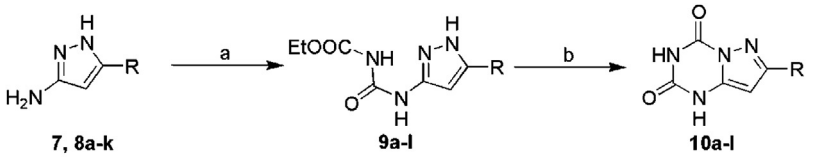
8, and **11** were reacted separately with ethoxycarbonyl isocyanate at room temperature in anhydrous DMF to give *N*-ethoxycarbonyl-*N*′-(pyrazol-3-yl) ureas **9** and **12** in good yields (Yields 41%–91% for **9a**–**l**, Yields 40%–76% for **12a**–**l**). This was followed subsequently by an intramolecular ring annulation reaction under the catalysis of sodium ethoxide to generate the target compounds (Yields 41%–91% for **10a**–**l**, Yields 51%–87% for **13a**–**l**).

On the other hand, target compounds1,3-dihydropyrazolo[1,5-*a*][1,3,5]triazin-2-thioxo-4-ones **15** and **17** were synthesized similarly by a two-step synthesis as illustrated in Scheme 4 [27]. The corresponding amines **4**, **7**, **8**, and **11** were reacted separately with ethoxycarbonyl isothiocyanate instead at room temperature in anhydrous DMF to give *N*-ethoxycarbonyl-*N*′-(pyrazol-3-yl)thioureas **14** and **16** in good yields (Yields 82%–98% for **14a**–**l**, Yields 64%–95% for **16a**–**p**). Compared to the same reaction between amines and ethoxycarbonyl isocyanate, it was found that the reactions between amines and ethoxycarbonyl isothiocyanate took shorter time to complete and gave higher yields. This could be due to the presence of the higher electrophilic center in the reactant ethoxycarbonyl isothiocyanate. An intramolecular ring annulation reaction of compounds **14** and **16** was carried out under the catalysis of sodium ethoxide to produce the eventual target compounds of **15** and **17** (Yields 54%–89% for **15a**–**l**, Yields 43%–94% for **17a**–**p**).

Among the 52 target compounds synthesized; compounds **10a** [27], **10f** [28], **10i** [28], **10k** [28], **10l** [29], **13e** [30], **15a** [27], **15l** [29], **17a** [15] and **17e** [14] have been previously reported; however, full characterization of these compounds were not disclosed and their TP inhibitory activity was never investigated. The rest of the 42 target compounds, which have not hitherto been reported in literature, were synthesized and fully characterized by ^1H, ^{13}C NMR and mass spectrometry. Their purity was also determined by reverse phase HPLC (to be >95% pure) and found to be suitable for biological evaluation.

2.2. Structure activity relationship (SAR) based on results of in vitro TP enzyme assay

All the 1,3-dihydropyrazolo[1,5-*a*][1,3,5]triazin-2,4-diones (**10** and **13**) and 1,3-dihydro-pyrazolo[1,5-*a*][1,3,5]triazin-2-thioxo-4-

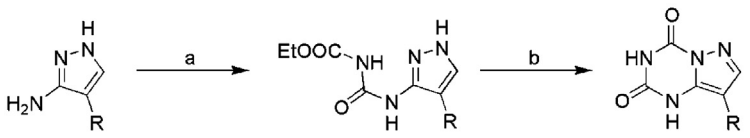


7, 8a-k **9a-l** **10a-l**

8a R=H
8b R=CH_3
8c R=$C(CH_3)_3$
8d R=CF_3
8e R=4-fluoro-phenyl
8f R=4-chloro-phenyl
8g R=4-bromo-phenyl
8h R=4-trifluoromethyl-phenyl
8i R=4-methyl-phenyl
8j R=4-*tert*-butyl-phenyl
8k R=4-methoxy-phenyl
7 R=phenyl

9a R=H
9b R=CH_3
9c R=$C(CH_3)_3$
9d R=CF_3
9e R=4-fluoro-phenyl
9f R=4-chloro-phenyl
9g R=4-bromo-phenyl
9h R=4-trifluoromethyl-phenyl
9i R=4-methyl-phenyl
9j R=4-*tert*-butyl-phenyl
9k R=4-methoxy-phenyl
9l R=phenyl

10a R=H
10b R=CH_3
10c R=$C(CH_3)_3$
10d R=CF_3
10e R=4-fluoro-phenyl
10f R=4-chloro-phenyl
10g R=4-bromo-phenyl
10h R=4-trifluoromethyl-phenyl
10i R=4-methyl-phenyl
10j R=4-*tert*-butyl-phenyl
10k R=4-methoxy-phenyl
10l R=phenyl



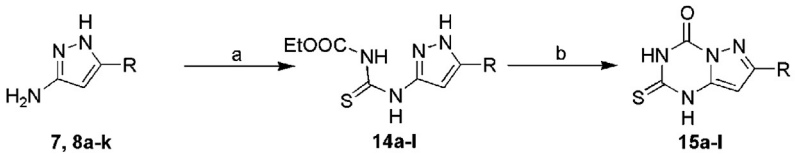
11a-l **12a-l** **13a-l**

11a R=CH_3
11b R=Cl
11c R=Br
11d R=I
11e R=phenyl
11f R=4-fluoro-phenyl
11g R=4-chloro-phenyl
11h R=4-bromo-phenyl
11i R=4-trifluoromethyl-phenyl
11j R=4-methyl-phenyl
11k R=4-*tert*-butyl-phenyl
11l R=4-methoxy-phenyl

12a R=CH_3
12b R=Cl
12c R=Br
12d R=I
12e R=phenyl
12f R=4-fluoro-phenyl
12g R=4-chloro-phenyl
12h R=4-bromo-phenyl
12i R=4-trifluoromethyl-phenyl
12j R=4-methyl-phenyl
12k R=4-*tert*-butyl-phenyl
12l R=4-methoxy-phenyl

13a R=CH_3
13b R=Cl
13c R=Br
13d R=I
13e R=phenyl
13f R=4-fluoro-phenyl
13g R=4-chloro-phenyl
13h R=4-bromo-phenyl
13i R=4-trifluoromethyl-phenyl
13j R=4-methyl-phenyl
13k R=4-*tert*-butyl-phenyl
13l R=4-methoxy-phenyl

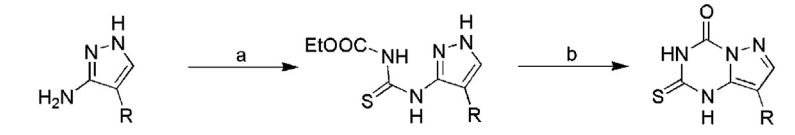
Scheme 3. Synthesis of 1,3-dihydro-pyrazolo[1,5-*a*][1,3,5]triazin-2,4-diones. Reagents and conditions: (a) ethoxycarbonyl isocyanate, DMF, rt, yields 41%–91% (**9a**–**l**) and 40%–76% (**12a**–**l**); (b) EtONa, ethanol, reflux, yields 41%–91% (**10a**–**l**) and 49%–87% (**13a**–**l**).



8a R=H
8b R=CH_3
8c R=$C(CH_3)_3$
8d R=CF_3
8e R=4-fluoro-phenyl
8f R=4-chloro-phenyl
8g R=4-bromo-phenyl
8h R=4-trifluoromethyl-phenyl
8i R=4-methyl-phenyl
8j R=4-*tert*-butyl-phenyl
8k R=4-methoxy-phenyl
7 R=phenyl

14a R=H
14b R=CH_3
14c R=$C(CH_3)_3$
14d R=CF_3
14e R=4-fluoro-phenyl
14f R=4-chloro-phenyl
14g R=4-bromo-phenyl
14h R=4-trifluoromethyl-phenyl
14i R=4-methyl-phenyl
14j R=4-*tert*-butyl-phenyl
14k R=4-methoxy-phenyl
14l R=phenyl

15a R=H
15b R=CH_3
15c R=$C(CH_3)_3$
15d R=CF_3
15e R=4-fluoro-phenyl
15f R=4-chloro-phenyl
15g R=4-bromo-phenyl
15h R=4-trifluoromethyl-phenyl
15i R=4-methyl-phenyl
15j R=4-*tert*-butyl-phenyl
15k R=4-methoxy-phenyl
15l R=phenyl



11a R=CH_3
11b R=Cl
11c R=Br
11d R=I
11e R=phenyl
11f R=4-fluoro-phenyl
11g R=4-chloro-phenyl
11h R=4-bromo-phenyl
11i R=4-trifluoromethyl-phenyl
11j R=4-methyl-phenyl
11k R=4-*tert*-butyl-phenyl
11l R=4-methoxy-phenyl
11m R=4-ethoxy-phenyl
11n R=4-nitro-phenyl
4a R=4-iodo-phenyl
4b R=4-pentafluorosulfur-phenyl

16a R=CH_3
16b R=Cl
16c R=Br
16d R=I
16e R=phenyl
16f R=4-fluoro-phenyl
16g R=4-chloro-phenyl
16h R=4-bromo-phenyl
16i R=4-trifluoromethyl-phenyl
16j R=4-methyl-phenyl
16k R=4-*tert*-butyl-phenyl
16l R=4-methoxy-phenyl
16m R=4-ethoxy-phenyl
16n R=4-nitro-phenyl
16o R=4-iodo-phenyl
16p R=4-pentafluorosulfur-phenyl

17a R=CH_3
17b R=Cl
17c R=Br
17d R=I
17e R=phenyl
17f R=4-fluoro-phenyl
17g R=4-chloro-phenyl
17h R=4-bromo-phenyl
17i R=4-trifluoromethyl-phenyl
17j R=4-methyl-phenyl
17k R=4-*tert*-butyl-phenyl
17l R=4-methoxy-phenyl
17m R=4-ethoxy-phenyl
17n R=4-nitro-phenyl
17o R=4-iodo-phenyl
17p R=4-pentafluorosulfur-phenyl

Scheme 4. Synthesis of 1,3-dihydro-pyrazolo[1,5-*a*][1,3,5]triazin-2-thioxo-4-ones. Reagents and conditions: (a) ethoxycarbonyl isothiocyanate, DMF, rt, yields 82%–98% (**14a**–**l**) and 64%–95% (**16a**–**p**); (b) EtONa, ethanol, reflux, yields 32%–89% (**15a**–**l**) and 43%–94% (**17a**–**p**).

ones (**15** and **17**) were evaluated for inhibitory activity using a modified TP bioassay. A continuous UV spectrophotometric enzyme assay [14] was employed in the evaluation. The bioassay used recombinant *Escherichia coli* TP (EC Number 2.4.2.4), expressed in *E. coli* as the enzyme and thymidine as the substrate. In the bioassay, the rate of decrease in absorbance monitored at 290 nm was taken to be the rate of the enzymatic activity. A comparison of the rate of enzymatic reaction in the absence and presence of an inhibitor or test compound would give the relative extent of enzyme inhibition.

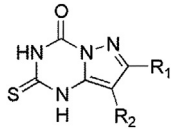
First, compounds **10a**–**l**, **13a**–**l**, **15a**–**l** and **17a**–**l** were screened at concentration of 50 μM for their inhibitory effects against the enzymatic activity. The screening results, expressed as percent inhibition, showed that none of the 1,3-dihydropyrazolo[1,5-*a*][1,3,5]triazin-2,4-diones (**10** and **13**) exhibited more than 50% inhibition at the concentration of 50 μM, thus these two series were considered not active against TP. However, 21 out of the 24 1,3-dihydropyrazolo[1,5-*a*][1,3,5]triazin-2-thioxo-4-ones (**15** and **17**) were found to exhibit a range of 50%–100% inhibition at the concentration of 50 μM. Therefore, this screening experiment suggested that the bioisosteric substitution of oxygen with sulfur at position 2 of the pyrazolo[1,5-*a*][1,3,5]triazine scaffold was important for the TP inhibitory activity.

To further investigate the structure and TP inhibitory activity relationship of the 1,3-dihydro-pyrazolo[1,5-*a*][1,3,5]triazin-2-thioxo-4-ones (**15** and **17**), the IC_{50} values for TP inhibition of all the compounds in this series were subsequently determined. The two libraries of 26 compounds (**15a**–**l**, **17a**–**n**) exhibited IC_{50} values ranging from 87.3 μM to 0.24 μM, with 8 out of the 26 compounds having IC_{50} values below 10 μM (Table 1).

Depending on the substituents at position 7 and position 8, the inhibitory activity of the pyrazolo[1,5-*a*][1,3,5]triazin-2-thioxo-2ones can vary significantly. In comparison, compound **15a**, which did not possess any substituent at either position 7 or position 8, was found to be about half as active as the reference compound **7DX**. However, with the introduction of certain substituents on **15a** at either 7 or 8 position, the inhibitory activity was found to be better than **7DX** with respect to IC_{50} values.

In particular, placing a phenyl ring at either position 7 or 8 appeared to enhance the TP inhibition. For instance, based on a comparison between compounds **15a** & **15l**, **15b** & **15i**, **15c** & **15j** and

Table 1
TP inhibitory activity of 1,3-dihydro-pyrazolo[1,5-*a*][1,3,5]triazin-2-thioxo-4-ones.



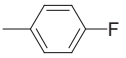
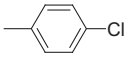
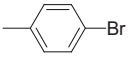
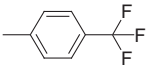
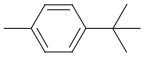
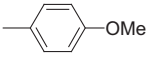
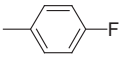
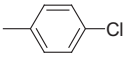
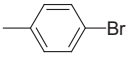
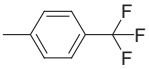
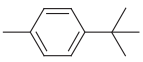
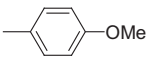
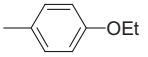
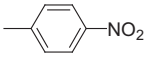
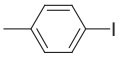
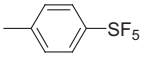
Compound	R_1	R_2	IC_{50}[a](μM)
15a	H	H	68.2 ± 2.7
15b	Me	H	79.4 ± 8.2
15c		H	29.1 ± 4.6
15d		H	32.6 ± 5.7
15e		H	41.7 ± 0.5
15f		H	33.5 ± 3.8
15g		H	34.9 ± 6.3
15h		H	9.9 ± 1.9
15i		H	19.3 ± 2.3
15j		H	6.6 ± 1.6
15k		H	28.4 ± 2.0
15l		H	46.2 ± 1.8
17a	H	Me	87.3 ± 6.0
17b	H	Cl	27.6 ± 1.3
17c	H	Br	44.4 ± 8.6
17d	H	I	20.8 ± 2.7
17e	H		26.3 ± 2.0
17f	H		10.5 ± 3.3
17g	H		0.54 ± 0.04
17h	H		0.63 ± 0.12
17i	H		0.24 ± 0.04
17j	H		1.7 ± 0.1
17k	H		5.6 ± 1.3
17l	H		39.7 ± 9.9

Table 1 (*continued*)

Compound	R_1	R_2	IC_{50}[a](μM)
17m	H		15.5 ± 0.3
17n	H		0.58 ± 0.02
17o	H		0.21 ± 0.03
17p	H		0.040 ± 0.005
7DX			32.2 ± 5.1

[a] Values are mean, $n = 3$.

15d & **15h**, the results showed that the inclusion of a phenyl ring had improved the TP inhibition by 1.5, 4.1, 4.4 and 3.3 times respectively. A similar phenyl effect was also observed for compounds **17a** & **17j**, **17b** & **17g** and **17c** & **17h**. The changes in TP inhibition in these cases were even greater; registering an enhancement in TP inhibition of 51.3, 51.1 and 70.5 times respectively. Therefore, it is very clear from the results that the presence of the phenyl ring is important for the inhibitory activity of this type of compounds, and it seems to suggest that there is a potential hydrophobic interaction site close to positions 7 and 8. Based on the comparison above, it showed that position 8 was possibly closer to the hydrophobic site.

In addition to the requirement of a phenyl ring at position 8, the substituents on position 4 of this phenyl ring also appeared to be an important contributory factor to the inhibitory activity. On the one hand, the fact that inhibitory activity of **17l** < **17m** < **17j** suggested that increasing the hydrophobicity of substituents on the phenyl ring would improve the potency. This is consistent with the Craig plot where the methoxy group (OCH_3), the ethoxy group (OCH_2CH_3), and the methyl group (CH_3) are known to possess similar electron donating properties while the hydrophobicity of methoxy group < ethoxy group < methyl group. However, such requirement for increase in hydrophobicity appeared to have a limit. The fact that **17k** was about 3.3 times less potent than **17j** demonstrated that when the bulkiness got too large, the inhibitory activity would not increase accordingly. On the other hand, the fact that **17n** was 18.1 times more potent than **17f** suggested that greater electron withdrawing effect would lead to more active compounds, since the fluoro group (F) and the nitro group (NO_2) possess similar hydrophobicity constant while the electron withdrawing property of fluoro group was weaker than that of nitro group. Therefore, it could be deduced that the more hydrophobic and electron withdrawing substituents inserted on para position of the phenyl ring, the compounds would become more potent. This conclusion was consistent with the observation that potency of **17i** > **17g**, **17h** > **17f** > **17e**.

Based on the above SAR derived from the results of the 50 compounds tested, two more compounds, namely, **17o** and **17p** were synthesized to validate the structural requirements for TP inhibition. The SAR results appeared to suggest that substituents located in the top right hand quadrant of the Craig plot (+σ, +π) would give good TP inhibitory activity. Therefore, two more compounds bearing the iodo group (I) and the pentafluorosulfur group (SF_5) at position 4 of the phenyl ring (**17o** and **17p** respectively) were synthesized as per the method described above and their TP inhibitory action was evaluated. **17o** inhibited TP at an IC_{50} value of 0.21 μM while **17p** exhibited 50% inhibition at a concentration of

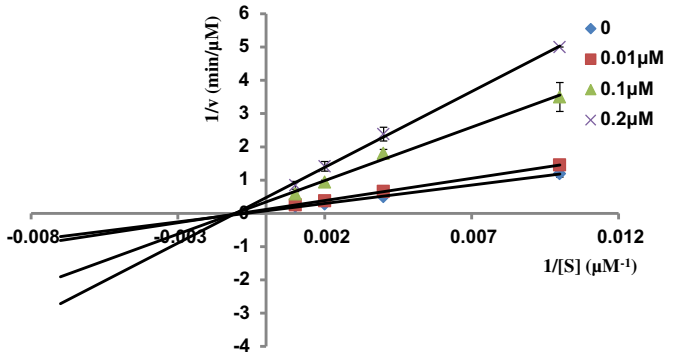


Fig. 2. Lineweaver–Burk plots of thymidine phosphorylase inhibition by compound **17p** prepared at different concentrations. All data points are means of three experiments.

0.04 μM. Therefore, **17p** was found to be around 800 times more potent than **7DX** under the evaluating conditions used for the bioassay.

2.3. Enzyme inhibition kinetic studies

Some reported TP inhibitors have been investigated for their action modes [31,32]. With the identification of **17p** as the most active compound, it was of interest to determine whether **17p** would interact with TP in any way that would be different from 7DX. Therefore, based on a reported method [32], further investigation was carried out to determine the enzyme inhibition kinetics of **17p** using four different concentrations of the substrate thymidine and four different concentrations of **17p** (0, 0.01 μM, 0.1 μM, and 0.2 μM). It was demonstrated on the Lineweaver–Burk plot that the lines generated by **17p** at different concentrations had different gradients and they converged at a point on the negative side of the *x* axis. This observation pointed to the fact that **17p** exhibited non-competitive inhibition kinetics on TP with respect to thymidine being the substrate (Fig. 2). This was further supported by the fact that the V_{max} values decreased from 9.9 μM/min to 2.6 μM/min in the presence of **17p**, while the K_m values did not change significantly as the inhibitor concentration increased from 0 to 0.2 μM. (K_m and V_{max} values were calculated from the Eadie–Hofstee plots) This characteristic V_{max} and K_m values of the enzyme is typical in the presence of an inhibitor that exhibits non-competitive inhibition kinetics.

3. Conclusion

The selected synthetic strategy of annulating the 1,3,5-triazine ring onto a pyrazole scaffold was proven to be quite an efficient way to synthesize the pyrazolo[1,5-*a*][1,3,5]triazines as designed. The two series of 2,4-diones and 2-thioxo-4-ones were synthesized with ease and all the final target compounds were prepared in generally good yields. The hypothesis that **10a** and **15a** might possess TP inhibitory activity comparable to **7DX** was not supported by the experimental results since both of them were obviously less active than **7DX**. However, it was found that many substituted 1,3-dihydro-pyrazolo[1,5-*a*][1,3,5]triazin-2-thioxo-4-ones were more active than **7DX**. Therefore, the bioisosteric replacement of oxygen with sulfur at position 2 of the pyrazolo[1,5-*a*][1,3,5]triazine scaffold was found to be essential for the TP inhibitory activity of this type of compounds; and proper substituents on position 7 or 8 of this scaffold would enhance the inhibitory activity. In particular, the insertion of a phenyl ring at either position 7 or 8 was enough to enhance the TP inhibition, especially when the aryl group was located at position 8; the inhibitory activity was found to increase by a larger extent. Lastly, it was also found that when substituents which had a larger hydrophobic constant and more positive Hammett constant (ie. more electron withdrawing) were introduced at position 4 of the attached phenyl ring, the IC_{50} values would decrease further. This was validated by compounds **17o** and **17p** where their inhibitory activities were around 150 times and 800 times more potent than **7DX** respectively. Further kinetic studies revealed that **17p** was a non-competitive inhibitor thus it might interact with the enzyme in a way different from 7DX, which was reported as a competitive inhibitor [33].

4. Experimental section

4.1. Chemistry

All starting materials were obtained from commercial suppliers and used without further purification. Melting points were determined on a Gallenkamp melting point apparatus without correction. NMR spectra were recorded, on a Bruker DPX-300 spectrometer, in DMSO-d_6 and using TMS as the internal standard. Chemical shifts (δ) were reported in parts per million downfield from the internal standard. The signals were quoted as s (singlet), d (doublet), t (triplet), m (multiplet). Thin-layer chromatography (TLC) was developed on aluminum-supported pre-coated silica gel plates (Merck, 60 F_{254}). Column chromatography was conducted on silica gel (230–400 mesh). ESI-MS was recorded on a Finnigan MAT LC-MS. HPLC analysis was carried out on all target compounds used in biological assays, using a Hewlett–Packard series 1050 HPLC system equipped with an HP-1050 quaternary pump, a degasser, diode array detector, an HP-1100 autosampler and a LiChrosorb reversed phase C18 (5 μm) column (4.6 × 250 mm). All the samples were prepared by dissolving them in methanol. The analysis was performed at 30 °C with a suitable mobile phase at a flow rate of 1 ml/min, and the ultraviolet detection was made at wavelength 254 nm. The separations were carried out using gradient elution. The initial mobile phase consisting of water with 0.1% acetic acid and acetonitrile with 0.1% acetic acid (95:5) was eluted for 1 min. This was gradually changed to 0:100 within 6 min and remained at 0:100 for another 1 min. The mobile phase was then gradually changed back to the starting composition within 6 min and maintained for one more minute. The injection volume was 5 μl. The purity of all target compounds was found to be more than 95% which was satisfactory for bioassay.

4.2. General procedure for the preparation of aryl substituted acetonitriles (*2*)

To 8 mmol of the correspondingly substituted bromomethylbenzene in 40 ml of ethanol was added 2 ml of 6 M potassium cyanide solution. The reaction mixture was refluxed for 1 h. On cooling, the mixture was concentrated by rotary evaporation under vacuum to remove the ethanol. The residue was washed with deionized water and recrystallized from ethanol/water to give the product.

4.2.1. (4-Iodophenyl)acetonitrile (*2a*)

Yield 56%. Mp: 50–52 °C (lit [23]: 56–58 °C). ^{1}H NMR (DMSO-d_6): δ 4.02 (s, 2H, CH_2), 7.17 (d, J = 8.4 Hz, 2H, CH), 7.76 (d, J = 8.4 Hz, 2H, CH).

4.2.2. (4-Pentafluorosulfurphenyl)acetonitrile (*2b*)

Yield 69%. Mp: 53–54 °C. ^{1}H NMR (DMSO-d_6): δ 4.21 (s, 2H, CH_2), 7.60 (d, J = 8.4 Hz, 2H, CH), 7.95 (d, J = 8.4 Hz, 2H, CH).

4.3. General procedure for the preparation of 3-dimethylamino-2-aryl substituted-acrylonitriles (**3**)

To 3 mmol of the corresponding aryl substituted acetonitrile dissolved in 5 ml of anhydrous DMF was added 4.5 mmol of dimethoxymethyldimethylamine. The reaction mixture was refluxed for 5 h. On cooling, the mixture was poured into 50 ml of cold water. The precipitate that formed was filtered to give the product.

4.3.1. 3-Dimethylamino-2-(4-iodophenyl)acrylonitrile (**3a**)

Yield 80%. Mp: 91–92 °C. ^{1}H NMR (DMSO-d_6): δ 3.20 (s, 6H, CH_3), 7.13 (d, J = 8.8 Hz, 2H, CH), 7.50 (s, 1H, CH), 7.60 (d, J = 8.8 Hz, 2H, CH).

4.3.2. 3-Dimethylamino-2-(4-pentafluorosulfurphenyl)acrylonitrile (**3b**)

Yield 85%. Mp: 144–145 °C. ^{1}H NMR (DMSO-d_6): δ 3.25 (s, 6H, CH_3), 7.48 (d, J = 8.8 Hz, 2H, CH), 7.71 (s, 1H, CH), 7.76 (d, J = 8.8 Hz, 2H, CH).

4.4. General procedure for the preparation of 4-aryl substituted-1H-pyrazol-3-ylamines (**4**)

To 5 mmol of the corresponding 3-dimethylamino-2-aryl substituted acrylonitriles in 80 ml of ethanol was added 13 ml of 80%wt hydrazine water solution (200 mmol). The reaction mixture was refluxed for 8 h. On cooling, the reaction mixture was concentrated by rotary evaporation under vacuum. The residue was extracted with ethyl acetate (20ml × 2) and purified by column chromatography (HEX/EA (1:1) was first used to remove impurities followed by flashing out the product with $CHCl_3$/MeOH (5:1)) to give the product.

4.4.1. 4-(4-Iodophenyl)-1H-pyrazol-3-ylamine (**4a**)

Yield 29%. Mp: 168–170 °C. ^{1}H NMR (DMSO-d_6): δ 4.63 (s, 1H, CH), 5.19 (s, broad, 1H, NH), 7.33 (d, J = 6.4 Hz, 2H, CH), 7.64 (d, J = 8.4 Hz, 2H, CH), 7.77 (s, 1H, NH), 11.79 (s, 1H, NH).

4.4.2. 4-(4-Pentafluorosulfurphenyl)-1H-pyrazol-3-ylamine (**4b**)

Yield 19% as black oil. ^{1}H NMR (DMSO-d_6): δ 5.05 (s, 2H, NH_2), 7.70 (d, J = 8.4 Hz, 2H, CH), 7.79 (d, J = 8.4 Hz, 2H, CH), 7.81 (s, 1H, CH), 11.83 (s, broad, 1H, NH).

4.4.3. 3-Oxo-3-phenylpropionitrile (**6**)

To 1.99 g of 2-bromo-1-phenyl-ethanone (10 mmol) dissolved in 6 ml of ethanol was added 6 ml of 5 M potassium cyanide solution. The reaction mixture was stirred at 50 °C for 2 h and then poured into 50 ml of water. The pH was adjusted to 3 with 2 M HCl, the precipitate that formed was filtered and recrystallized from ethanol/water to give 1.1 g product. Yield 76%. Mp: 77–78 °C (lit [26]: 80 °C). ^{1}H NMR (DMSO-d_6): δ 4.76 (s, 2H, CH_2), 7.57 (t, J = 7.2 Hz, 2H, CH), 7.71 (t, J = 7.2 Hz, 1H, CH), 7.95 (d, J = 7.2 Hz, 2H, CH).

4.4.4. 5-Phenyl-1H-pyrazol-3-ylamine (**7**)

To 1.45 g of 3-oxo-3-phenyl-propionitrile (10 mmol) in 25 ml of ethanol was added 6.4 ml of 80%wt hydrazine water solution (100 mmol). The reaction mixture was refluxed for 3 h. On cooling, the precipitate that came out was filtered. The filtrate was then concentrated by rotary evaporation under vacuum to remove the ethanol. The residue was extracted with ethyl acetate (20ml × 2) and purified by column chromatography (HEX/EA (1:1) was first used to remove impurities followed by $CHCl_3$/MeOH (3:1) to flash out the product) to give 0.25 g product. Yield 16%. Mp: 122–123 °C (lit [34]: 126–127 °C). ^{1}H NMR (DMSO-d_6): δ 4.77 (s, 2H, NH_2), 5.76 (s, 1H, CH), 7.25 (t, J = 7.2 Hz, 1H, CH), 7.37 (t, J = 7.2 Hz, 2H, CH), 7.64 (d, J = 7.2 Hz, 2H, CH), 11.75 (s, 1H, NH).

4.5. General procedure for the preparation of N-ethoxycarbonyl-N′-(pyrazol-3-yl) ureas (**9**, **12**)

To a fine suspension of 1 mmol of the corresponding amine in 3 ml of anhydrous DMF was added 1 mmol of ethoxycarbonyl isocyanate. After stirring the mixture for 12 h at room temperature, 30 ml of cold water was added. The precipitated product was filtered, washed with cold water and recrystallized from acetonitrile.

4.6. General procedure for the preparation of 1,3-dihydro-pyrazolo [1,5-a][1,3,5]triazin-2,4-diones (**10**, **13**)

1 mmol of the corresponding *N*-ethoxycarbonyl-*N*′-(pyrazol-3-yl) urea was refluxed in a mixture of 4 ml of ethanol and 0.76 ml of 21%wt EtONa (2 mmol) ethanol solution for 0.5 h. The precipitate that formed was collected by filtration, then dissolved in water and acidified with HCl until pH 4. The precipitate formed was filtered and recrystallized from methanol/water.

4.6.1. 1,3-Dihydro-pyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**10a**)

Yield 64%. Mp: 310–311 °C (lit [27]: 330 °C). ESI-MS 150.5 m/z (M − 1). ^{1}H NMR (DMSO-d_6): δ 5.79 (d, J = 1.8 Hz, 1H, CH), 7.88 (d, J = 1.5 Hz, 1H, CH), 11.58 (s, 1H, NH), 11.88 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 89.6, 141.8, 145.4, 146.3, 149.4.

4.6.2. 1,3-Dihydro-7-methylpyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**10b**)

Yield 55%. Mp: 317–318 °C. ESI-MS 165.0 m/z (M − 1). ^{1}H NMR (DMSO-d_6): δ 2.19 (s, 3H, CH_3), 5.65 (s, 1H, CH), 11.49 (s, 1H, NH), 11.78 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 14.9, 90.0, 141.9, 145.1, 149.5, 155.8.

4.6.3. 1,3-Dihydro-7-tert-butylpyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**10c**)

Yield 41%. Mp: 258–259 °C. ESI-MS 207.1 m/z (M − 1). ^{1}H NMR (DMSO-d_6): δ 1.25 (s, 9H, CH_3), 5.74 (s, 1H, CH), 11.45 (s, 1H, NH), 11.81 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 29. 5, 32.4, 85.7, 140.7, 144.2, 148.3, 166.6.

4.6.4. 1,3-Dihydro-7-trifluoromethylpyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**10d**)

1 mmol of *N*-ethoxycarbonyl-*N*′-(5-trifluoromethyl-pyrazol-3-yl) urea was refluxed in a mixture of 4 ml of ethanol and 0.76 ml of 21%wt EtONa (2 mmol) ethanol solution for 0.5 h. The precipitate that formed was collected by filtration, then dissolved in 20 ml of water and acidified with HCl until pH 4. The resulting solution was extracted by ethyl acetate (20ml × 3) and the organic phase was rotary evaporated under vacuum to dryness to yield 0.16 g product. Yield 73%. Mp: 228–230 °C. ESI-MS 218.9 m/z (M − 1). ^{1}H NMR (DMSO-d_6): δ 6.21 (s, 1H, CH), 12.02 (s, broad, 2H, NH). ^{13}C NMR (DMSO-d_6): δ 86.3, 116.5, 119.2, 121.9, 124.6, 142.5, 143.8, 144.8, 145.2, 145.6, 146.0, 148.1.

4.6.5. 1,3-Dihydro-7-(4-fluorophenyl)pyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**10e**)

Yield 80%. Mp: 316–318 °C. ESI-MS 245.0 m/z (M − 1). ^{1}H NMR (DMSO-d_6): δ 6.31 (s, 1H, CH), 7.30 (t, J = 6.6 Hz, 2H, CH), 7.98 (dd, J = 6.6 Hz, J_{H-F} = 4.2 Hz, 2H, CH), 11.63 (s, 1H, NH), 12.03 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 86.02, 115.9, 128.3, 141.9, 144.3, 148.4, 154.3, 161.6, 164.0.

*4.6.6. 1,3-Dihydro-7-(4-chlorophenyl)pyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**10f**)*

Yield 64%. Mp: 296–298 °C. ESI-MS 261.2, 263.2 *m*/*z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 6.33 (s, 1H, CH), 7.53 (d, *J* = 8.8 Hz, 2H, CH), 7.95 (d, *J* = 8.8 Hz, 2H, CH), 11.64 (s, 1H, NH), 12.04 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 86.0, 127.8, 128.8, 130.4, 133.9, 141.9, 144.2, 148.3, 154.0.

*4.6.7. 1,3-Dihydro-7-(4-bromophenyl)pyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**10g**)*

Yield 58%. Mp: 310–312 °C. ESI-MS 305.2, 307.2 *m*/*z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 6.33 (s, 1H, CH), 7.53 (d, *J* = 8.8 Hz, 2H, CH), 7.95 (d, *J* = 8.8 Hz, 2H, CH), 11.64 (s, 1H, NH), 12.04 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 86.0, 122.6, 128.1, 130.8, 131.7, 141.9, 144.1, 148.3, 154.0.

*4.6.8. 1,3-Dihydro-7-(4-trifluoromethylphenyl)pyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**10h**)*

Yield 91%. Mp: 302–304 °C. ESI-MS 295.2 *m*/*z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 6.43 (s, 1H, CH), 7.83 (d, *J* = 8.4 Hz, 2H, CH), 8.16 (d, *J* = 8.4 Hz, 2H, CH), 11.70 (s, 1H, NH), 12.09 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 86.4, 122.7, 125.4, 125.6, 125.7, 126.8, 128.1, 128.8, 129.1, 129.4, 129.7, 135.5, 142.1, 144.1, 148.3, 153.6.

*4.6.9. 1,3-Dihydro-7-(4-methylphenyl)pyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**10i**)*

Yield 83%. Mp: 308–309 °C. ESI-MS 241.3 *m*/*z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 2.35 (s, 3H, CH_3), 6.26 (s, 1H, CH), 7.28 (d, *J* = 8.0 Hz, 2H, CH), 7.80 (d, *J* = 8.0 Hz, 2H, CH), 11.59 (s, 1H, NH), 11.98 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 20.9, 85.8, 126.0, 128.8, 129.4, 138.9, 141.7, 144.2, 148.4, 155.2.

*4.6.10. 1,3-Dihydro-7-(4-tert-butyl-phenyl)-pyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**10j**)*

Yield 85%. Mp: 307–309 °C. ESI-MS 283.3 *m*/*z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 1.31 (s, 9H, CH_3), 6.26 (s, 1H, CH), 7.49 (d, *J* = 8.8 Hz, 2H, CH), 7.84 (d, *J* = 8.8 Hz, 2H, CH), 11.60 (s, 1H, NH), 11.99 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 30.9, 34.4, 85.8, 125.5, 125.8, 128.7, 141.6, 144.2, 148.3, 151.9, 155.0.

*4.6.11. 1,3-Dihydro-7-(4-methoxyphenyl)pyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**10k**)*

Yield 78%. Mp: 260–262 °C. ESI-MS 257.2 *m*/*z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 3.81 (s, 3H, CH_3), 6.22 (s, 1H, CH), 7.02 (d, *J* = 8.8 Hz, 2H, CH), 7.85 (d, *J* = 8.8 Hz, 2H, CH), 11.56 (s, 1H, NH), 11.96 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 55.1, 85.5, 114.1, 124.0, 127.5, 141.6, 144.1, 148.3, 154.9, 160.1.

*4.6.12. 1,3-Dihydro-7-phenylpyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**10l**)*

Yield 89%. Mp: 308–310 °C (lit [29]: 327–329 °C). ESI-MS 227.0 *m*/*z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 6.31 (s, 1H, CH), 7.39–7.53 (m, 3H, CH), 7.93 (d, *J* = 6.8 Hz, CH, 2H), 11.63 (s, 1H, NH), 12.02 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 86.0, 126.1, 128.8, 129.3, 131.5, 141.8, 144.2, 148.3, 155.1.

*4.6.13. 1,3-Dihydro-8-methylpyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**13a**)*

Yield 51%. Mp: 318–319 °C. ESI-MS 164.5 *m*/*z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 1.96 (s, 3H, CH_3), 7.65 (s, 1H, CH), 11.47 (s, 1H, NH), 11.81 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 6.52, 97.1, 137.2, 144.2, 146.5, 148.6.

*4.6.14. 1,3-Dihydro-8-chloropyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**13b**)*

Yield 53%. Mp: 299–300 °C. ESI-MS 185.0, 186.9 *m*/*z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 7.94 (s, 1H, CH), 11.72 (s, 1H, NH), 12.33 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 91.2, 138.4, 144.4, 144.8, 149.4.

*4.6.15. 1,3-Dihydro-8-bromopyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**13c**)*

Yield 66%. Mp: 306–308 °C. ESI-MS 228.9, 230.9 *m*/*z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 7.91 (s, 1H, CH), 11.72 (s, 1H, NH), 12.25 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 75.1, 140.0, 144.8, 146.2, 149.5.

*4.6.16. 1,3-Dihydro-8-iodopyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**13d**)*

Yield 61%. Mp: 292–294 °C. ESI-MS 276.9 *m*/*z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 7.82 (s, 1H, CH), 11.67 (s, 1H, NH), 12.01 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 143.2, 144.6, 149.6, 150.2.

*4.6.17. 1,3-Dihydro-8-phenylpyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**13e**)*

Yield 54%. Mp: 268–269 °C. ESI-MS 227.0 *m*/*z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 7.3 (t, *J* = 7.2 Hz, 1H, CH), 7.42(t, *J* = 7.2 Hz, 2H, CH), 7.53 (d, *J* = 7.2 Hz, 2H, CH), 8.13 (s, 1H, CH), 11.7 (s, 1H, NH), 11.88 (s, broad, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 104.3, 126.6, 127.1, 128.6, 129.6, 136.2, 144.1, 144.5, 148.8.

*4.6.18. 1,3-Dihydro-8-(4-fluorophenyl)pyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**13f**)*

Yield 79%. Mp: 280–282 °C. ESI-MS 245.0 *m*/*z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 7.24 (t, *J* = 6.6 Hz, 2H, CH), 7.55 (dd, *J* = 6.6 Hz, J_{H-F} = 4.2 Hz, 2H, CH), 8.10 (s, 1H, CH), 11.7 (s, 1H, NH), 11.92 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 103.5, 115.3, 115.5, 126.0, 129.2, 129.3, 136.2, 144.2, 144.5, 148.8, 159.8, 162.3.

*4.6.19. 1,3-Dihydro-8-(4-chlorophenyl)pyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**13g**)*

Yield 57%. Mp: 296–297 °C. ESI-MS 261.3, 263.1 *m*/*z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 7.45 (d, *J* = 8.4 Hz, 2H, CH), 7.55 (d, *J* = 8.4 Hz, 2H, CH), 8.14 (s, 1H, CH), 11.75 (s, 1H, NH), 11.95 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 103.2, 128.5, 128.6, 128.9, 131.1, 136.5, 144.1, 144.4, 148.8.

*4.6.20. 1,3-Dihydro-8-(4-bromophenyl)pyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**13h**)*

Yield 49%. Mp: 278–280 °C. ESI-MS 306.9 *m*/*z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 7.49 (d, *J* = 8.8 Hz, 2H, CH), 7.59 (d, *J* = 8.8 Hz, 2H, CH), 8.14 (s, 1H, CH), 11.71 (s, 1H, NH), 11.93 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 103.2, 119.6, 128.9, 129.1, 131.4, 136.5, 144.1, 144.3, 148.8.

*4.6.21. 1,3-Dihydro-8-(4-trifluoromethylphenyl)pyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**13i**)*

Yield 77%. Mp: 288–289 °C. ESI-MS 295.3 *m*/*z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 7.61–8.13 (m, 4H, CH), 8.23 (s, 1H, CH), 11.74 (s, 1H, NH), 12.05 (s, broad, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 103.0, 122.9, 125.3, 125.3, 125.3, 125.4, 125.6, 126.5, 126.8, 127.5, 134.0, 137.4, 144.1, 144.3, 148.9.

*4.6.22. 1,3-Dihydro-8-(4-methylphenyl)pyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**13j**)*

Yield 87%. Mp: 289–290 °C. ESI-MS 241.3 *m*/*z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 2.32 (s, 3H, CH_3), 7.21 (d, *J* = 8.0 Hz, 2H, CH), 7.41 (d, *J* = 8.0 Hz, 2H, CH), 8.09 (s, 1H, CH), 11.69 (s, 1H, NH), 11.81 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 20.7, 104.3, 126.6, 126.9, 129.1, 135.8, 135.9, 144.1, 144.5, 148.8.

*4.6.23. 1,3-Dihydro-8-(4-tert-butylphenyl)pyrazolo[1,5-a][1,3,5]triazin-2,4-dione (**13k**)*

Yield 74%. Mp: 301–303 °C. ESI-MS 283.0 *m*/*z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 1.31 (s, 9H, CH_3), 6.25 (s, 1H, CH), 7.48 (d, *J* = 8.4 Hz, 2H, CH), 7.84 (d, *J* = 8.4 Hz, 2H, CH), 11.59 (s, 1H, NH), 11.98 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 30.9, 34.4, 85.8, 125.5, 125.8, 128.7, 141.6, 144.2, 148.3, 151.9, 155.0.

*4.6.24. 1,3-Dihydro-8-(4-methoxyphenyl)pyrazolo[1,5-a][1,3,5] triazin-2,4-dione (**13l**)*

Yield 75%. Mp: 264–266 °C. ESI-MS 257.3 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 3.78 (s, 3H, CH_3), 6.97 (d, *J* = 8.4 Hz, 2H, CH), 7.44 (d, *J* = 8.4 Hz, 2H, CH), 8.06 (s, 1H, CH), 11.66 (s, 1H, NH), 11.81 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 55.1, 104.2, 114.1, 121.9, 128.5, 135.5, 144.2, 144.6, 148.8, 158.1.

*4.7. General method for preparation of N-ethoxycarbonyl-N′-(pyrazol-3-yl)thioureas (**14**, **16**)*

To a fine suspension of 1 mmol of the corresponding amine in 3 ml of anhydrous DMF was added 1 mmol of ethoxycarbonyl isothiocyanate. After stirring the mixture for 1 h at room temperature, 30 ml of cold water was added. The precipitated product was filtered, washed with cold water and recrystallized from ethanol.

*4.8. General method for preparation of 1,3-dihydro-pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-ones (**15**, **17**)*

1 mmol of the corresponding *N*-ethoxycarbonyl-*N′*-(pyrazol-3-yl)thiourea was refluxed in a mixture of 4 ml of ethanol and 0.76 ml of 21%wt EtONa (2 mmol) ethanol solution for 0.5 h. Precipitate that came out was collected by filtration, then dissolved in water and acidified with HCl until pH 4. Precipitate that formed was filtered and recrystallized from methanol/water.

*4.8.1. 1,3-Dihydro-pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**15a**)*

Yield 54%. Mp: 284–286 °C. (lit [27]: 298–300 °C) ESI-MS 167.0 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 5.90 (d, *J* = 1.5 Hz, 1H, CH), 7.88 (d, *J* = 1.5 Hz, 1H, CH), 12.72 (s, 1H, NH), 13.46 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 90.5, 141.5, 142.6, 146.6, 174.0.

*4.8.2. 1,3-Dihydro-7-methylpyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**15b**)*

Yield 79%. Mp: 281–282 °C. ESI-MS 181.0 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 2.23 (s, 3H, CH_3), 5.77 (s, 1H, CH), 12.61 (s, 1H, NH), 13.33 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 15.0, 90.7, 141.8, 142.3, 155.8, 173.9.

*4.8.3. 1,3-Dihydro-7-tert-butylpyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**15c**)*

Yield 56%. Mp: 258–260 °C. ESI-MS 223.3 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 1.26 (s, 9H, CH_3), 5.85 (s, 1H, CH), 12.62 (s, 1H, NH), 13.41 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 29.5, 32.5, 86.5, 140.5, 141.4, 166.9, 172.8.

*4.8.4. 1,3-Dihydro-7-trifluoromethylpyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**15d**)*

1 mmol of *N*-ethoxycarbonyl-*N′*-(5-trifluoromethyl-pyrazol-3-yl) thiourea was refluxed in a mixture of 4 ml of ethanol and 0.76 ml of 21%wt EtONa (2 mmol) ethanol solution for 0.5 h. The precipitate that formed was collected by filtration, then dissolved in 20 ml of water and acidified with HCl until pH 4. The resulting solution was extracted by ethyl acetate (20ml × 3) and concentrated by rotary evaporation under vacuum. The residue was recrystallized from EA/HEX to give 75 mg product. Yield 32%. Mp: 236–237 °C. ESI-MS 234.9 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 6.27 (s, 1H, CH), 13.02 (s, broad, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 87.0, 116.4, 119.1, 121.8, 124.5, 141.0, 142.2, 144.9, 145.3, 145.7, 146.1, 173.6.

*4.8.5. 1,3-Dihydro-7-(4-fluorophenyl)pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**15e**)*

Yield 89%. Mp: 286–287 °C. ESI-MS 261.0 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 6.41 (s, 1H, CH), 7.31 (t, *J* = 8.7 Hz, 2H, CH), 8.01 (dd, *J* = 8.7 Hz, J_{H-F} = 5.7 Hz, 2H, CH), 12.76 (s, 1H, NH), 13.59 (s, broad, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 86.7, 115.9, 128.5, 141.4, 141.6, 154.4, 161.6, 164.1, 173.0.

*4.8.6. 1,3-Dihydro-7-(4-chlorophenyl)pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**15f**)*

Yield 80%. Mp: 288–290 °C. ESI-MS 277.0, 279.3 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 6.40 (s, 1H, CH), 7.53 (d, *J* = 8.4 Hz, 2H, CH), 7.97 (d, *J* = 8.4 Hz, 2H, CH), 12.71 (s, broad, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 87.9, 129.0, 130.0, 131.2, 135.2, 142.5, 142.8, 155.3, 174.1.

*4.8.7. 1,3-Dihydro-7-(4-bromophenyl)pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**15g**)*

Yield 64%. Mp: 293–294 °C. ESI-MS 320.9, 322.9 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 6.43 (s, 1H, CH), 7.68 (d, *J* = 8.4 Hz, 2H, CH), 7.91 (d, *J* = 8.4 Hz, 2H, CH), 12.78 (s, 1H, NH), 13.59 (s, broad, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 86.7, 122.8, 128.2, 130.4, 131.8, 141.3, 141.7, 154.2, 173.0.

*4.8.8. 1,3-Dihydro-7-(4-trifluoromethylphenyl)pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**15h**)*

Yield 85%. Mp: 277–278 °C. ESI-MS 311.2 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 6.53 (s, 1H, CH), 7.84 (d, *J* = 8.4 Hz, 2H, CH), 8.19 (d, *J* = 8.4 Hz, 2H, CH), 12.84 (s, 1H, NH), 13.65 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 87.1, 120.0, 122.7, 125.4, 125.7, 126.9, 128.1, 129.0, 129.3, 129.6, 129.9, 135.1, 141.4, 141.8, 153.8, 173.1.

*4.8.9. 1,3-Dihydro-7-(4-methylphenyl)pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**15i**)*

Yield 81%. Mp: 295 °C. ESI-MS 257.2 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 2.35 (s, 3H, CH_3), 6.34 (s, 1H, CH), 7.27 (d, *J* = 8.0 Hz, 2H, CH), 7.81 (d, *J* = 8.0 Hz, 2H, CH), 12.73 (s, 1H, NH), 13.53 (s, broad, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 20.9, 86.5, 126.1, 128.4, 129.4, 139.2, 141.4, 141.5, 155.4, 173.0.

*4.8.10. 1,3-Dihydro-7-(4-tert-butylphenyl)pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**15j**)*

Yield 67%. Mp: 289 °C. ESI-MS 299.1 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 1.31 (s, 9H, CH_3), 6.37 (s, 1H, CH), 7.50 (d, *J* = 8.8 Hz, 2H, CH), 7.87 (d, *J* = 8.8 Hz, 2H, CH), 12.76 (s, 1H, NH), 13.57 (s, broad, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 30.9, 34.4, 86.5, 125.5, 125.9, 128.4, 141.4, 141.4, 152.1, 155.3, 172.9.

*4.8.11. 1,3-Dihydro-7-(4-methoxyphenyl)pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**15k**)*

Yield 58%. Mp: 288 °C. ESI-MS 273.2 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 3.81 (s, 3H, CH_3), 6.30 (s, 1H, CH), 7.02 (d, *J* = 8.4 Hz, 2H, CH), 7.88 (d, *J* = 8.4 Hz, 2H, CH), 12.57 (s, broad, 1H, NH), 13.53 (s, broad, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 56.3, 87.6, 115.2, 124.9, 128.7, 142.7, 143.2, 156.3, 161.3, 174.0.

*4.8.12. 1,3-Dihydro-7-phenylpyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**15l**)*

Yield 69%. Mp: 285–286 °C (lit [29]: 304–305 °C). ESI-MS 243.3 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 6.41 (s, 1H, CH), 7.37–7.58 (m, 3H, CH), 7.95 (d, *J* = 6.6 Hz, 2H, CH), 12.78 (s, 1H), 13.58 (s, 1H). ^{13}C NMR (DMSO-d_6): δ 87.8, 127.3, 129.9, 130.6, 132.3, 142.5, 142.7, 156.4, 174.1.

*4.8.13. 1,3-Dihydro-8-methylpyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**17a**)*

Yield 47%. Mp: 276 °C. ESI-MS 180.6 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 2.04 (s, 3H, CH_3), 7.76 (s, 1H, CH), 12.61 (s, 1H, NH), 13.41 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 6.7, 98.4, 137.1, 141.4, 146.9, 172.9.

*4.8.14. 1,3-Dihydro-8-chloropyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**17b**)*

Yield 45%. Mp: 258–260 °C. ESI-MS *m/z* 200.9, 203.0 (M − 1). ^{1}H NMR (DMSO-d_6): δ 8.02 (s, 1H, CH), 12.80 (s, 1H, NH), 13.79 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 91.9, 138.2, 142.0, 144.8, 174.7.

*4.8.15. 1,3-Dihydro-8-bromopyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**17c**)*

Yield 56%. Mp: >360 °C. ESI-MS 245.0, 246.9 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 8.00 (s, 1H, CH), 12.78 (s, 1H, NH), 13.70 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 75.9, 139.9, 142.0, 146.7, 174.9.

*4.8.16. 1,3-Dihydro-8-iodopyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**17d**)*

Yield 60%. Mp: 199–201 °C. ESI-MS 292.7 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 7.92 (s, 1H, CH), 12.77 (s, 1H, NH), 13.50 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 42.5, 141.9, 143.1, 151.0, 174.9.

*4.8.17. 1,3-Dihydro-8-phenylpyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**17e**)*

Yield 60%. Mp: 222–224 °C (lit [14]: 251–253 °C). ESI-MS 243.3 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 7.32 (t, *J* = 7.2 Hz, 1H, CH), 7.41 (t, *J* = 7.2 Hz, 2H, CH), 7.51 (d, *J* = 7.2 Hz, 1H, CH), 8.17 (s, 1H, CH), 12.80 (s, NH, 1H). ^{13}C NMR (DMSO-d_6): δ 106.5, 128.1, 129.2, 129.5, 130.2, 137.2, 142.6, 146.2, 174.9.

*4.8.18. 1,3-Dihydro-8-(4-fluorophenyl)pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**17f**)*

Yield 68%. Mp: 310 °C. ESI-MS 261.3 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 7.25 (t, *J* = 8.7 Hz, 2H, CH), 7.55 (dd, *J* = 8.7 Hz, J_{H-F} = 5.7 Hz, 2H, CH), 8.14 (s, 1H, CH), 12.75 (s, NH, 1H). ^{13}C NMR (DMSO-d_6): δ 105.4, 116.1, 116.4, 126.66, 126.7, 131.3, 131.4, 137.6, 142.6, 146.1, 160.8, 164.1, 174.9.

*4.8.19. 1,3-Dihydro-8-(4-chlorophenyl)pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**17g**)*

Yield 90%. Mp: 262–263 °C. ESI-MS 276.7, 279.3 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 7.47 (d, *J* = 8.4 Hz, 2H, CH), 7.53 (d, *J* = 8.4 Hz, 2H, CH), 8.16 (s, 1H, CH), 12.81 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 104.2, 128.0, 128.3, 129.9, 131.7, 136.5, 141.4, 144.9, 173.9.

*4.8.20. 1,3-Dihydro-8-(4-bromophenyl)pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**17h**)*

Yield 81%. Mp: 239 °C. ESI-MS 321.1, 322.9 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 7.48 (d, *J* = 8.4 Hz, 2H, CH), 7.60 (d, *J* = 8.4 Hz, 2H, CH), 8.17 (s, 1H, CH), 12.79 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 105.3, 121.2, 129.6, 131.3, 132.3, 137.8, 142.6, 145.9, 175.0.

*4.8.21. 1,3-Dihydro-8-(4-trifluoromethylphenyl)pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**17i**)*

Yield 85%. Mp: 256–257 °C. ESI-MS 311.0 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 7.72–7.79 (m, 4H, CH), 8.24 (s, 1H, CH), 12.78 (s, 2H, NH). ^{13}C NMR (DMSO-d_6): δ 104.0, 120.3, 123.0, 125.2, 125.7, 126.7, 127.0, 127.3, 127.6, 128.4, 128.7, 133.6, 137.4, 141.5, 144.8, 174.0.

*4.8.22. 1,3-Dihydro-8-(4-methylphenyl)pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**17j**)*

Yield 94%. Mp: 241–242 °C. ESI-MS 257.0 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 2.34 (s, 3H, CH_3), 7.22 (d, *J* = 8.4 Hz, 2H, CH), 7.40 (d, *J* = 8.4 Hz, 2H, CH), 8.12 (s, 1H, CH), 12.77 (s, 1H, NH), 13.37 (s, broad, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 20.7, 105.3, 126.1, 127.9, 129.0, 135.9, 136.3, 141.5, 145.0, 173.4.

*4.8.23. 1,3-Dihydro-8-(4-tert-butylphenyl)pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**17k**)*

Yield 94%. Mp: 288–289 °C. ESI-MS 299.1 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 1.31 (s, 9H, CH_3), 6.34 (s, 1H, CH), 7.49 (d, *J* = 8.5 Hz, 2H, CH), 7.86 (d, *J* = 8.5 Hz, 2H, CH), 12.72 (s, 1H, NH), 13.54 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 30.9, 34.4, 86.5, 125.5, 125.0, 128.4, 141.4, 141.5, 152.1, 155.3, 172.9.

*4.8.24. 1,3-Dihydro-8-(4-methoxyphenyl)pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**17l**)*

Yield 85%. Mp: 227–228 °C. ESI-MS 273.2 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 3.79 (s, 3H, OCH_3), 6.98 (d, *J* = 8.5 Hz, 2H, CH), 7.45 (d, *J* = 8.5 Hz, 2H, CH), 8.10 (s, 1H, CH), 12.62 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 55.1, 105.1, 113.8, 121.3, 129.4, 135.8, 141.4, 145.0, 158.4, 173.6.

*4.8.25. 1,3-Dihydro-8-(4-ethoxyphenyl)pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**17m**)*

Yield 91%. Mp: 286–287 °C. ESI-MS 287.2 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 1.35 (t, *J* = 7.2 Hz, 3H, CH_3), 4.08 (q, *J* = 7.2 Hz, 2H, CH_2), 6.33 (s, 1H, CH), 7.01 (d, *J* = 8.8 Hz, 2H, CH), 7.86 (d, *J* = 8.8 Hz, 2H, CH), 12.72 (s, 1H, NH), 13.52 (s, broad, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 14.6, 63.1, 86.3, 114.6, 123.5, 127.7, 141.4, 141.4, 155.3, 159.6, 172.9.

*4.8.26. 1,3-Dihydro-8-(4-nitrophenyl)pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**17n**)*

Yield 81%. Mp: 261–263 °C. ESI-MS 288.1 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 7.83 (d, *J* = 8.4 Hz, 2H, CH), 8.25 (d, *J* = 8.4 Hz, 2H, CH), 8.32 (s, 1H, CH), 12.89 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 103.5, 123.5, 128.6, 136.4, 137.8, 141.4, 144.7, 145.7, 174.2.

*4.8.27. 1,3-Dihydro-8-(4-iodophenyl)pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**17o**)*

Yield 86%. Mp: 369 °C. ESI-MS 321.1, 322.9 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 7.33 (d, *J* = 7.2 Hz, 2H, CH), 7.76 (d, *J* = 7.2 Hz, 2H, CH), 8.16 (s, 1H, CH), 12.79 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 92.8, 104.3, 128.7, 130.2, 136.4, 137.1, 141.3, 144.6, 173.8.

*4.8.28. 1,3-Dihydro-8-(4-pentafluorosulfurphenyl)-pyrazolo[1,5-a][1,3,5]triazin-2-thioxo-4-one (**17p**)*

Yield 43%. Mp: 268–269 °C. ESI-MS 369.0 *m/z* (M − 1). ^{1}H NMR (DMSO-d_6): δ 7.72 (d, *J* = 8.4 Hz, 2H, CH), 7.93 (d, *J* = 8.0 Hz, 2H, CH), 8.24 (s, 1H, CH), 12.86 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 103.4, 125.7, 128.8, 133.4, 137.5, 141.4, 144.7, 151.0, 174.0.

4.9. In vitro TP enzyme assay

The recombinant *E. coli* thymidine phosphorylase (EC Number 2.4.2.4) expressed in *E. coli* was purchased from a commercial supplier (Sigma, T-2807). The enzymatic activity assay was performed at room temperature in a Hitachi U-1900 spectrophotometer using a detection wavelength of 290 nm. This method is based on the manufacturer's instruction which is a classical method described by Krenitsky [35]. For all the compounds, the percentage inhibition at a certain concentration was obtained by the following procedure:

10 μl of TP solution (1.5 U/ml in pH 7.0 phosphate buffer), 10 μl of the test compound solution in DMSO (for blank, 10 μl DMSO was added) and 200 μl of thymidine solution (5 mM in pH 7.4 phosphate buffer) were added to 780 μl of phosphate buffer (pH 7.4) in a 1.5 ml cuvette. After gentle shaking by inversion of the cuvette, the absorbance values at 4 min, 8 min, 12 min, 16 min and 20 min were recorded successively. These absorbance values were plotted against time and linear regression was performed to obtain the

slope of the line plotted which was taken to be the velocity of enzyme catalyzed reaction. The percentage inhibition at this concentration was calculated by dividing the difference between the enzymatic velocity of the blank and the enzymatic velocity in the presence of the inhibitor (or test compound) by the enzymatic velocity of the blank.

To determine the IC_{50} value of a certain compound, percentage inhibitions of at least 7 different concentrations that span over the estimated IC_{50} values were determined by the method mentioned above and plotted against logarithmic concentration using OriginPro 8 SR0 v8.0724 (B724). The IC_{50} value was determined as the concentration of the inhibitor that caused 50% inhibition. Results were means of three experiments.

4.10. TP enzyme kinetic studies

To investigate the inhibitory effect of the most potent compound **17p** against TP at varying thymidine concentrations, velocities of the enzyme catalyzed reactions at different concentrations of thymidine (100 μM, 250 μM, 500 μM and 1000 μM) were determined using the same procedure as described previously. **17p** was used at concentrations of 0 μM, 0.01 μM, 0.1 μM, and 0.2 μM. Lineweaver–Burk plots of TP inhibition by **17p** were then generated using Microsoft Office Excel 2007 based on data obtained from triplicate experiments to determine the inhibition type of **17p**.

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