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Original article

# Design, synthesis and evaluation of novel diaryl pyrrolopyrimidine and pyrrolothiazine derivatives as inhibitors of tumor necrosis factor stimulated gene-14 (TSG-14) production

Khalid M.H. Hilmy [a,*], Dalia H. Soliman [b], Esmat B.A. Shahin [c], Hala S. El-Deeb [a], Salah M. El-Kousy [a]

[a] Department of Chemistry, Faculty of Science, Monufyia University, Shebin El-Kom, Egypt
[b] Pharmaceutical Chemistry Department, Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt
[c] Department of Biochemistry, Faculty of Medicine (Girls), Al-Azhar University, Cairo, Egypt

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ABSTRACT

A novel series of pyrrolothiazines **2**–**4** and pyrrolopyrimidines **5**–**7** have been synthesized. The structures of these compounds were established by spectroscopic and element microanalytical data. The newly synthesized compounds were evaluated as inhibitors of TSG-14. The most effective results were obtained by the *S*-sec-butyl derivatives **6e** (80%) and the *N*-ethyl derivatives **4e** (70%).



## 1. Introduction

TNF-stimulated gene 14/Pentraxinprotein 3 (***TSG-14/PTX3***) is a gene inducible by tumor necrosis factor-alpha (TNF-α), interleukine-1β (IL-β) and lipopolysaccharide (LPS) in fibroblasts, macrophages and endothelial cells [1–4]. TSG-14 gene encodes a 42-kd secreted glycoprotein that belongs to long pentraxin family of proteins 381 amino acids, it also shares homology with the short pentraxin family of acute-phase proteins such as C-reactive protein (CRP) and serum amyloid P component (SAP) [1,2]. TSG-14 gene is one of the TNF-α stimulated gene (TSGs); such genes are known as inflammatory genes that stimulate the inflammatory response [5]. This altered cytokine-induced gene expression contributes to the etiology of inflammatory diseases. Previous studies have suggested a role for TSG-14 in the inflammatory process, it binds to the C1q component of the complement cascade [6] high levels of TSG-14 were detected in the serum of LPS-injected humans and mice [3,7], and in the joint fluid of rheumatoid arthritis patients [8]. In addition, circulating TSG-14 levels increase in several pathological conditions, such as autoimmune, infectious, and degenerative disturbances [9–11]. Interestingly, expression of the TSG-14 gene is controlled by TNF-α [3,12,13] and in a positive feedback, TSG-14 appears to enhance the production of TNF-α when exposed to LPS [1]. Consequently, TSG-14 could be considered as a potential target for the development of new anti-inflammatory therapies. The validation of the use of small molecules as inhibitors of TNF-α induced kinases and their role in altering the levels of inflammatory-related proteins has encouraged us to investigate the inhibitory effect of orally available small molecules on TSG-14 [14]. This could be considered as a chemical–genetic interaction that benefits from the advantages of orally available small molecule inhibitors, over the currently used biopharmaceuticals [15–18]. Moreover, to date there is not any reported data of small molecule inhibitors of TSG-14, only the biochemicals, cycloheximide, the most common laboratory reagent used to inhibit protein synthesis [19,20] and the antioxidant pyrrolidine dithiocarbamate [19,21].

Here in, we report the synthesis and evaluation of a new series of pyrrolo[2,3-*d*]pyrimidine and pyrrolo[2,3-*d*]thiazine derivatives as inhibitors of TSG-14. The design of the new compounds was based on their structural similarity to purines and pyrimidines that

* Corresponding author. P.O. Box 73, Souhbra Misr, Cairo, Egypt.
*E-mail address:* hilmykhaled@yahoo.com (K.M.H. Hilmy).

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play key roles in cellular processes. One important class of pyrimidine is 2-thiopyrimidine (2-TP) and its derivatives [22], which have attracted substantial interest of synthetic-biochemists [23,24].

## 2. Results and discussion

### 2.1. Chemistry

A representative procedure for this synthesis is shown in Scheme 1. Compounds **1a**–**e** was considered the starting key materials using synthetic sequence reported in previous work [25]. The reaction of **1a**–**e** with carbon disulfide in pyridine afforded the corresponding pyrrolothiazine-2-thione derivatives **2a**–**e**. Subsequent alkylation of pyrrolothiazin-2-thione **2a**–**e** with α-halo and β-halo alkyl halide gave the corresponding *S*-alkylated pyrrolothiazine-4-imine derivatives **3a**–**e** and **4a**–**e**. Upon refluxing compounds **2a**–**e** with 5% potassium hydroxide solution, 4-iminopyrrolopyrimidine derivatives **5a**–**e** were directly obtained. Alternatively, 3-ethylpyrrolo- pyrimidines **6a**–**e** and 3-amino pyrrolopyrimidines **7a**–**e** were obtained by stirring of **2a**–**e** with ethylamine or hydrazine hydrate, independently, for seven days. The synthesized compounds were purified by standard procedures like column chromatography and crystallization. All the assigned structures were confirmed by spectroscopic and element microanalytical data. (c.f. experimental and Scheme 1).

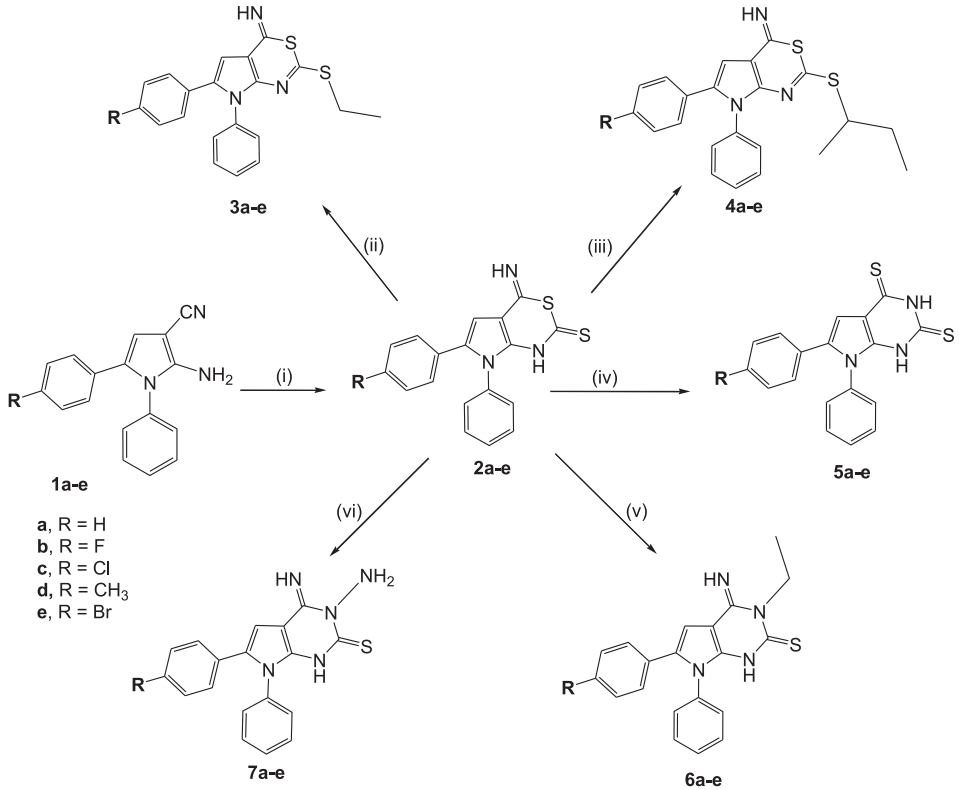
**Table 1**
TSG-14 inhibition values for some of the synthesized compounds.

| Compd. No | TSG-14 (ng/ml)[a] | % Inhibition of TSG-14 |
|---|---|---|
| **2a** | 0.10 ± 0.011 | 0 |
| **2c** | 0.098 ± 0.002 | 2 |
| **2d** | 0.10 ± 0.006 | 0 |
| **2e** | 0.056 ± 0.006 | 44 |
| **3a** | 0.10 ± 0.008 | 0 |
| **3c** | 0.099 ± 0.002 | 1 |
| **3d** | 0.1 ± 0.01 | 0 |
| **3e** | 0.099 ± 0.01 | 1 |
| **4a** | 0.05 ± 0.001 | 50 |
| **4c** | 0.098 ± 0.012 | 2 |
| **4d** | 0.098 ± 0.002 | 2 |
| **4e** | 0.03 ± 0.001 | 70 |
| **6a** | 0.035 ± 0.006 | 65 |
| **6c** | 0.039 ± 0.006 | 61 |
| **6d** | 0.040 ± 0.002 | 60 |
| **6e** | 0.02 ± 0.001 | 80 |
| **7a** | 0.077 ± 0.002 | 23 |
| **7c** | 0.1 ± 0.006 | 0 |
| **7d** | 0.1 ± 0.01 | 0 |
| **7e** | 0.067 ± 0.002 | 33 |
| Control | 0.10 ± 0.006 | – |
| LPS | 0.5 ± 0.006 | – |

[a] Conc. of LPS-stimulated TSG-14 production in rat whole blood, expressed as means ± S.E.M. ($n = 3$).

### 2.2. Elisa measurement of TSG-14/PTX3

The newly synthesized derivatives were evaluated for their ability to inhibit LPS stimulated TSG-14/PTX3. It is well documented that TSG-14 become elevated in the serum of mice, rat and human after injection with bacterial lipopolysaccharide, it is also induced by TNF-α [22,23]. Thus, the same protocol for TNF-α evaluation in whole serum rat was followed where production was determined by comparison of yield, after LPS injection, with a control to which no test compound was added [26,27]. One variation from this protocol is that serum TSG-14/PTX3 levels were determined at 4–6 h after LPS injection, which is a time point of maximal elevation of serum TSG-14, as described in the literature [22], results presented in Table 1. In the course of evaluating the TNF-α




**Scheme 1.** Synthesis of pyrrolothiazines **2**–**4** and pyrrolopyrimidines **5**–**7** series. Reagents and conditions: (i) $CS_2$, Pyridine, reflux, 3 h; (ii) $CH_3CH_2I$, NaH, DMF, stirring, 24 h; (iii) 2-Bromobutane, NaH, DMF, stirring, 24 h; (iv) 5% KOH, reflux in w.b., 2 h; (v) $CH_3CH_2NH_2$, stirring, 7 days; (vi) $NH_2NH_2.H_2O$, stirring, 7 days.

inhibitory effect of new small molecules we attempted to explore sulfur containing derivatives. The prototypical vicinal diaryl was held constant in all the prepared compounds, while varying substituents on the pyrimidine or thiazine heterocycle. The most potent compounds of the synthesized series was the 6-(4-Bromophenyl)-3-ethyl-4-iminepyrrolo-pyrimidine-2-thione **6e**, showing an 80% inhibition in the TSG-14 induction, in fact all derivatives of this series were potent inhibitors of the TSG-14. Insights into the structure activity relationship of the tested compounds showed that within the same series of compounds the 6-(4-bromophenyl) derivatives displayed the best inhibitory effect followed by the unsubstituted 6-phenyl derivatives. This can be clearly observed from the results obtained from the 2-*sec*-butylthio-4-iminepyrrolothiazines **4a** and **4e** (50% and 70%, respectively), the 3-amino-4-imine pyrrolopyrimidine-2-thione derivatives **7a** and **7e** (23% and 33%, respectively) and the 4-iminepyrrolothiazine-2-thione **2e** (44%). Moreover, it was observed that these *N*-alkylated derivatives **6a**–**e** were more potent compared to their *S*-alkylated analogs **4a**–**e** and **3a**–**e**. Furthermore, it seemed that increasing the bulk of the S-substituent, resulted in more effective compounds, as observed in the results obtained for **4e** and **2e** (70% and 44%, respectively). It could be concluded that upon comparing the activity of the *S*-alkylated derivatives to their *N*-alkylated counterparts, it was observed that the *N*-alkyl derivatives were more potent, especially for the bromo and unsubstituted derivatives **4a** and **4e**.

## 3. Conclusions

In conclusion, we found that nine of the tested compounds were potent inhibitors of TSG-14. The most potent derivatives were **6e** and **4e** with 80% and 70% inhibition, respectively. Both of them were 6-(4-bromophenyl) derivatives. Compounds **6a** and **6c** showed good inhibitory action (65% and 61%, respectively), while **4a** displayed moderate activity as it inhibited 50% of the induced gene. These new series of compounds may serve as valuable new agents that could play an important role in controlling acute and chronic inflammatory reactions through novel targets that play a crucial role in these processes.

## 4. Experimental

### 4.1. Chemistry

Melting points were determined on a Stuart melting point apparatus and are uncorrected. IR spectra (KBr) were measured on Jasco FT/IR 460 plus (Japan), $^1$H NMR and $^{13}$C NMR spectra were recorded on Gemini 300 MHz in DMSO-$d_6$ as solvent, using tetramethyl-silane (TMS) as internal reference standard. The chemical shifts values are expressed in ppm. Elemental analysis (C, H, and N) was performed by a Vario III CHN analyzer (Germany). All compounds were within ±0.4% of the theoretical values. Mass spectra were run on DI analysis Shimadzu QP-2010 plus mass spectrometer. All spectroscopic analysis were made at the Microanalytical Unit of Cairo University. The progress of the reaction and the purity of the compounds were monitored by TLC analytical silica gel plates 60 F254 eluting with $CH_2Cl_2$/$CH_3OH$ (39:1). The chemical reagents used in synthesis were purchased From Fluka, Sigma, and Aldrich.

#### 4.1.1. General procedure for the synthesis of compounds *2a*–*e*

A mixture of 2-amino-3-cyano-1-phenyl-5-arylpyrroles **1a**–**e** [25] (10 mmol) and carbon disulfide (10 ml) was refluxed in pyridine (8 ml) for 3 h. On addition of diethyl ether gave **2a**–**e**. The precipitate was crystallized from ethanol.

##### 4.1.1.1. 6,7-Diphenyl-4-iminopyrrolo[2,3-d][1,3]thiazin-2(1H,4H,7H)-thione (*2a*).

Yield: (2.21 g, 66%); mp: 245–247 °C. IR (KBr) cm$^{-1}$: 3277 (NH), 1568 (C=S), 1635 (C=NH); MS (*m*/*z*, %): 335.00 ($M^+$, 100), 336.00 ($M^{+1}$, 19.70), 337.00 ($M^{+2}$, 12.60). $^1$H NMR ((DMSO-$d_6$) δ/ppm: 6.72 (s, 1H, Pyrrole-H), 6.95–7.48 (m, 10H, Ar–H), 9.58 (s, 1H, NH-, $D_2O$ exchangeable), 12.07 (s, 1H,imine–NH–, $D_2O$ exchangeable). Anal. calcd for $C_{18}H_{13}N_3S_2$ (335.06): C, 64.45; H, 3.91; N, 12.53; S, 19.12. Found: C, 64.17; H, 3.54; N, 12.75; S, 19.47.

##### 4.1.1.2. 6-(4-Fluorophenyl)-4-imino-7-phenylpyrrolo[2,3-d][1,3]thiazin-2 (1H,4H,7H)-thione (*2b*).

Yield: (2.75 g, 78%); mp: 248–250 °C. IR (KBr) cm$^{-1}$: 3277 (NH), 1570 (C=S), 1640 (C=N); MS (*m*/*z*, %): 353.00 ($M^+$, 100), 353.90 ($M^{+1}$, 30.63), 355.00 ($M^{+2}$, 12.18). $^1$H NMR (DMSO-$d_6$) δ/ppm: 6.76 (s, 1H, Pyrrole-H), 6.90–7.47 (m, 9H, Ar–H), 9.59 (s, 1H, NH–, $D_2O$ exchangeable), 12.07 (s, 1H, imine–NH–, $D_2O$ exchangeable). Anal. calcd for $C_{18}H_{12}FN_3S_2$ (353.05): C, 61.17; H, 3.42; F, 5.38; N, 11.89; S, 18.14. Found: C, 61.47; H, 3.12; F, 5.08; N, 11.50; S, 18.38.

##### 4.1.1.3. 6-(4-Chlorophenyl)-4-imino-7-phenylpyrrolo[2,3-d][1,3]thiazin-2 (1H,4H,7H)-thione (*2c*).

Yield: (2.65 g, 72%); mp: 240–242 °C. IR (KBr) cm$^{-1}$: 3271 (NH), 1565 (C=S), 1625 (C=NH); MS (*m*/*z*, %): 369.00 ($M^+$, $Cl^{35}$, 100), 370.00 ($M^{+1}$, 27.66), 371.00 ($M^{+2}$, $Cl^{37}$, 45.89). $^1$H NMR (DMSO-$d_6$) δ/ppm: 6.77 (s, 1H, Pyrrole-H), 6.88–7.50 (m, 9H, Ar–H), 9.59 (s, 1H, NH-, $D_2O$ exchangeable), 12.07 (s, 1H, imine–NH–, $D_2O$ exchangeable). $^{13}$C NMR (DMSO-$d_6$) δ/ppm: 97.88, 101.96, 128.52, 128.71, 129.03, 129.50, 129.67, 132.61, 134.62, 135.66, 153.76 (C=N), 170.61, 180.91(C=S). Anal. Calcd for $C_{18}H_{12}ClN_3S_2$ (369.02): C, 58.45; H, 3.27; Cl, 9.58; N, 11.36; S, 17.34. Found: C, 58.13; H, 3.57; Cl, 9.23; N, 11.04; S, 17.67.

##### 4.1.1.4. 4-Imino-7-phenyl-6-p-tolylpyrrolo[2,3-d][1,3]thiazin-2(1H,4H,7H)-thione (*2d*).

Yield: (2.72 g, 78%); mp: 238–240 °C. IR (KBr) cm$^{-1}$: 3267 (NH), 1558 (C=S), 1631 (C=NH); MS (*m*/*z*, %): 349.00 ($M^+$, 100), 350.00 ($M^{+1}$, 27.08), 351.00 ($M^{+2}$, 12.66). $^1$H NMR (DMSO-$d_6$) δ/ppm: 2.17 (s, 3H, $CH_3$), 6.75 (s, 1H, Pyrrole-H), 6.78–7.48 (m, 9H, Ar–H), 9.59 (s, 1H, NH-, $D_2O$ exchangeable), 12.08 (s, 1H, imine–NH–, $D_2O$ exchangeable). Anal. calcd for $C_{19}H_{15}N_3S_2$ (349.07): C, 65.30; H, 4.33; N, 12.02; S, 18.35. Found: C, 65.10; H, 4.66; N, 11.63; S, 18.03.

##### 4.1.1.5. 6-(4-Bromophenyl)-4-imino-7-phenylpyrrolo[2,3-d][1,3]thiazin-2 (1H,4H,7H)-thione (*2e*).

Yield: (3.06 g, 74%); mp: 265–267 °C. IR (KBr) cm$^{-1}$: 3272 (NH), 1562 (C=S), 1639 (C=NH); MS (*m*/*z*, %): 413.00 ($M^+$, $Br^{79}$, 67.2), 414.00 ($M^{+1}$, 24.66), 415.00 ($M^{+2}$, $Br^{81}$, 74.1). $^1$H NMR (DMSO-$d_6$) δ/ppm: 6.81 (s, 1H, Pyrrole-H), 6.88–7.46 (m, 9H, Ar–H), 9.62 (s, 1H, NH-, $D_2O$ exchangeable), 12.07 (s, 1H, imine–NH–, $D_2O$ exchangeable).Anal. calcd for $C_{18}H_{12}BrN_3S_2$ (413.97): C, 52.18; H, 2.92; Br, 19.28; N, 10.14; S, 15.48; found: C, 52.54; H, 2.90; Br, 19.08; N, 10.43; S, 15.15.

#### 4.1.2. General methods of preparation of *3a*–*e*

A mixture of **2a**–**e** (14 mmol), ethyl iodide (3310 mg, 21.2 mmol) and anhydrous NaH (100 mg, 41 mmol) in DMF (30 ml) was stirred for 24 h. Then the mixture was poured onto ice. The precipitate was then filtered and crystallized out from methanol.

##### 4.1.2.1. 2-(Ethylthio)-6,7-diphenylpyrrolo[2,3-d][1,3]thiazin-4(7H)-imine (*3a*).

Yield: (3.55 g, 70%); mp: 135–137 °C. IR (KBr) cm$^{-1}$: 1631 (C=NH), 3293 (NH); MS (*m*/*z*, %): 363.00 ($M^+$, 17.81), 364.00 ($M^{+1}$, 4.75), 365 ($M^{+2}$, 1.63). $^1$H NMR (DMSO-$d_6$) δ/ppm: 1.32 (t, 3H, $CH_3$), 3.15 (q, 2H, $CH_2$), 6.70 (s, 1H, H-Pyrrole), 7.00–7.46 (m, 10H, Ar–H), 13.66 (s, 1H, imine–NH–, $D_2O$ exchangeable). Anal. calcd for $C_{20}H_{17}N_3S_2$ (363.08): C, 66.08; H, 4.71; N, 11.56; S, 17.64. Found: C, 65.88; H, 4.40; N, 11.26; S, 17.32.

*4.1.2.2. 2-(Ethylthio)-6-(4-fluorophenyl)-7-phenylpyrrolo[2,3-d][1,3] thiazin-4(7H)-imine (**3b**).* Yield: (4.53 g, 85%); mp: 140–142 °C. IR (KBr) $cm^{-1}$: 1632 (C=NH), 3291 (NH); MS (*m*/*z*, %): 381.00 ($M^+$, 21.92), 382.00 ($M^{+1}$, 7.65), 383.00 ($M^{+2}$, 2.55). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 1.31 (t, 3H, $CH_3$), 3.11 (q, 2H, $CH_2$), 6.80 (s, 1H, H-Pyrrole), 7.05–7.54 (m, 9H, Ar–H), 13.69 (s, 1H, imine–NH–, $D_2O$ exchangeable).Anal. calcd for $C_{20}H_{16}FN_3S_2$ (381.08): C, 62.97; H, 4.23; F, 4.98; N, 11.01; S, 16.81. found: C, 62.58; H, 4.55; F, 4.65; N, 11.33; S, 16.45.

*4.1.2.3. 6-(4-Chlorophenyl)-2-(ethylthio)-7-phenylpyrrolo [2, 3-d] [1,3]thiazin-4(7H)-imine (**3c**).* Yield: (4.44 g, 80%); MP: 162–164 °C. IR (KBr) $cm^{-1}$: 1628 (C=NH), 3299 (NH); MS (*m*/*z*, %): 397.00 ($M^+$, $Cl^{35}$, 4.82), 398.00 ($M^{+1}$, 4.10), 399.00 ($M^{+2}$, $Cl^{37}$, 2.06). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 1.34 (t, 3H, $CH_3$), 3.13 (q, 2H, $CH_2$), 6.79 (s, 1H, H-Pyrrole), 7.13–7.53 (m, 9H, Ar–H), 13.67 (s, 1H, imine–NH–, $D_2O$ exchangeable). Anal. calcd for $C_{20}H_{16}ClN_3S_2$ (397.05): C, 60.36; H, 4.05; Cl, 8.91; N, 10.56; S, 16.12. Found: C, 60.03; H, 4.44; Cl, 8.55; N, 10.23; S, 16.47.

*4.1.2.4. 2-(Ethylthio)-7-phenyl-6-p-tolylpyrrolo[2,3-d][1,3]thiazin-4(7H)-imine (**3d**).* Yield: (4.06 g, 77%); mp: 160–162 °C. IR (KBr) $cm^{-1}$: 1634 (C=NH), 3287 (NH); MP (*m*/*z*, %): 377.00 ($M^+$, 31.03), 378.00 ($M^{+1}$, 17.04), 379.00 ($M^{+2}$, 6.80). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 1.36 (t, 3H, $CH_3$), 2.28 (s, 3H, $CH_3$), 3.13 (q, 2H, $CH_2$), 6.73 (s, 1H, H-Pyrrole), 7.02–7.50 (m, 9H, Ar–H), 13.66 (s, 1H, imine–NH–, $D_2O$ exchangeable). $^{13}C$ NMR (DMSO-$d_6$) δ/ppm: 14.84($CH_2\underline{C}H_3$), 22.77 ($S\underline{C}H_2CH_3$), 24.74 ($CH_3$), 98.80, 101.96, 127.83, 128.01, 128.12,128.44, 128.99, 135.68, 137.72, 139.03, 151.52($\underline{C}$=N), 162.69. Anal. calcd for $C_{21}H_{19}N_3S_2$ (377.10): C, 66.81; H, 5.07; N, 11.13; S, 16.99. found: C, 66.46; H, 4.78; N, 10.93; S, 16.66.

*4.1.2.5. 6-(4-Bromophenyl)-2-(ethylthio)-7-phenylpyrrolo[2,3-d][1,3] thiazin-4(7H)-imine (**3e**).* Yield: (4.63 g, 75%); mp: 165–167 °C. IR (KBr) $cm^{-1}$: 1634 (C=NH), 3301 (NH); MS (*m*/*z*, %): 441.00 ($M^+$, $Br^{79}$, 42.03), 442.00 ($M^{+1}$, 21.04), 443.00 ($M^{+2}$, $Br^{81}$, 7.80). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 1.32 (t, 3H, $CH_3$), 3.11 (q, 2H, $CH_2$), 6.84 (s, 1H, H-Pyrrole), 6.91–7.51 (m, 9H, Ar–H), 13.67 (s, 1H, imine–NH–, $D_2O$ exchangeable). Anal. calcd for $C_{20}H_{16}BrN_3S_2$ (442.40): C, 54.30; H, 3.65; Br, 18.06; N, 9.50; S, 14.50. Found: C, 54.01; H, 3.33; Br, 17.71; N, 9.18; S, 14.15.

*4.1.3. General method for preparation of **4a–e***

A mixture of **2a–e** (14 mmol), 2-Bromobutane (2900 mg, 2.12 mmol) and anhydrous NaH (100 mg, 41 mmol) in DMF (30 ml) was stirred for 24 h. Then the mixture was poured onto ice. The precipitate was then filtered and crystallized from ethanol.

*4.1.3.1. 2-(Sec-butylthio)-6,7-diphenylpyrrolo[2,3-d][1,3]thiazin-4(7H)-imine (**4a**).* Yield: (4.38 g, 80%); mp: 150–152 °C. IR (KBr) $cm^{-1}$: 1650 (C=NH), 3287 (NH); MS (*m*/*z*, %): 391.00 ($M^+$, 100), 392.00 ($M^{+1}$, 27.96), 393.00 ($M^{+2}$,12.56). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 0.95 (t, 3H, $CH_3$), 1.38 (d, 3H, $CH_3$), 3.23 (m, 1H, CH), 4.14 (m, 2H, $CH_2$), 6.76 (s, 1H, H-pyrrole), 6.87–7.48 (m, 10H, Ar–H), 13.66 (s, 1H, imine–NH–$D_2O$ exchangeable). Anal. calcd for $C_{22}H_{21}N_3S_2$ (391.12): C, 67.49; H, 5.41; N, 10.73; S, 16.38. Found: C, 67.28; H, 5.03; N, 10.40; S, 16.08.

*4.1.3.2. 2-(Sec-butylthio)-6-(4-fluorophenyl)-7-phenylpyrrolo[2,3-d] [1,3] thiazin-4(7H)-imine (**4b**).* Yield: (4.86 g, 85%); m.p.180–182 °C. IR (KBr) $cm^{-1}$: 1648 (C=NH), 3291 (NH). *m*/*z*, %: 409.00 ($M^+$, 100), 410.00 ($M^{+1}$, 27.49), 411.00 ($M^{+2}$, 12.45). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 0.95 (t, 3H, $CH_3$), 1.37 (d, 3H, $CH_3$), 4.12 (m, 2H, $CH_2$), 3.21 (m, 1H, CH), 6.75 (s, 1H, H-pyrrole), 6.85–7.49 (m, 9H, Ar–H), 13.64 (s, 1H, imine–NH–, $D_2O$ exchangeable). Anal. calcd for $C_{22}H_{20}FN_3S_2$ (409.11): C, 64.52; H, 4.92; F, 4.64; N, 10.26; S, 15.66; Found: C, 64.13; H, 4.59; F, 4.33; N, 10.59; S, 15.98.

*4.1.3.3. 2-(Sec-butylthio)-6-(4-chlorophenyl)-7-phenylpyrrolo[2,3-d] [1,3] thiazin-4(7H)-imine (**4c**).* Yield: (5.05 g, 85%); mp: 160–162 °C .IR (KBr) $cm^{-1}$: 1643 (C=NH), 3286 (NH); MS (*m*/*z*, %): 425.00 ($M^+$, $Cl^{35}$, 100), 426.00 ($M^{+1}$, 29.79), 427.00 ($M^{+2}$,$Cl^{37}$, 44.90). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 0.96 (t, 3H, $CH_3$), 1.39 (d, 3H, $CH_3$), 4.13 (m, 2H, $CH_2$), 3.26 (m, 1H, CH), 6.77 (s, 1H, H-pyrrole), 6.86–7.46 (m, 9H, Ar–H), 13.66 (s, 1H, imine–NH–, $D_2O$ exchangeable). Anal. calcd for $C_{22}H_{20}ClN_3S_2$ (426.01): C, 62.03; H, 4.73; Cl, 8.32; N, 9.86; S, 15.05; . Found: C, 62.36; H, 4.40; Cl, 8.02; N, 9.50; S, 15.35.

*4.1.3.4. 2-(Sec-butylthio)-7-phenyl-6-p-tolylpyrrolo[2,3-d][1,3]thiazin-4(7H)-imine (**4d**).* Yield: (4.65 g, 82%); mp: 160–162 °C IR (KBr) $cm^{-1}$: 1639 (C=NH), 3286 (NH); (*m*/*z*, %): 405.00 ($M^+$, 66.62), 406.00 ($M^{+1}$, 20.68), 407.00 ($M^{+2}$, 8.35). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 0.97 (t, 3H, $CH_3$), 1.39 (d, 3H, $CH_3$), 2.32 (s, 3H, $CH_3$), 4.15 (m, 2H, $CH_2$), 3.20 (m, 1H, CH), 6.78 (s, 1H, H-pyrrole), 6.88–7.49 (m, 9H, Ar–H), 13.65 (s, 1H, imine–NH–, $D_2O$ exchangeable). Anal. calcd for $C_{23}H_{23}N_3S_2$ (405.59): C, 68.11; H, 5.72; N, 10.36; S, 15.81. Found: C, 68.44; H, 5.36; N, 10.06; S, 15.47.

*4.1.3.5. 6-(4-Bromophenyl)-7-phenyl-2-(Sec-butylthio)pyrrolo[2,3-d] [1,3] thiazin- 4(7H)-imine (**4e**).* Yield: (5.58 g, 85%); mp: 155–157 °C. IR (KBr) ($cm^{-1}$): 1655 (C=NH), 3302 (NH); MS (*m*/*z*, %): 469.00 ($M^+$, $Br^{79}$, 15.07), 470.00 ($M^{+1}$, 7.83), 471.00 ($M^{+2}$, $Br^{81}$, 16.51). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 0.94 (t, 3H, $CH_3$), 1.37 (d, 3H, $CH_3$), 1.51 (m, 2H, $CH_2$), 4.17 (m, 1H, CH), 6.80 (s, 1H, H-pyrrole), 6.91–7.50 (m, 9H, Ar–H), 12.63 (s, 1H, imine–NH–, $D_2O$ exchangeable). Anal. calcd for $C_{22}H_{20}BrN_3S_2$ (469.03) : C, 56.17; H, 4.29; Br, 16.98; N, 8.93; S, 13.63. Found: C, 56.51; H, 4.50; Br, 16.64; N, 8.60; S, 13.31.

*4.1.4. General procedure for the synthesis of compounds **5a–e***

The compounds **2a–e** (10 mmol) was refluxed with aqueous 5% KOH (5 ml) on a steam bath for 2 h, and then cooled to room temperature; the precipitate was collected and washed with water. The filtrate and washings were acidified with glacial acetic acid to precipitate the pyrrolopyrimidine dithione **5a–e**. The precipitate was crystallized from ethyl acetate.

*4.1.4.1. 6,7-Diphenyl-1H-pyrrolo[2,3-d]pyrimidine-2,4(3H,7H)-dithione (**5a**).* Yield: (2.34 g, 70%); mp: 245–247 °C. IR (KBr) $cm^{-1}$: 3382 (NH), 1620 (C=S); MS (*m*/*z*, %): 335.00 ($M^+$, 100.00), 336.00 ($M^{+1}$, 19.7), 337.00 ($M^{+2}$, 9.60). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 6.79 (s, 1H, pyrrole-H), 6.85–7.46 (m, 10H, Ar–H), 9.93 (s, 1H, $NH^1$–, $D_2O$ exchangeable), 11.95 (s, 1H, $NH^3$–, $D_2O$ exchangeable). Anal. calcd for $C_{18}H_{13}N_3S_2$ (335.45): C, 64.45; H, 3.91; N, 12.53; S, 19.12. Found: C, 64.09; H, 3.60; *N*, 12.17; S, 19.45.

*4.1.4.2. 6-(4-Fluorophenyl)-7-phenyl-1H-pyrrolo[2,3-d]pyrimidine-2,4(3H,7H)-dithione (**5b**).* Yield: (2.18 g, 62%); mp: 248–250 °C. IR (KBr) $cm^{-1}$: 3381 (NH), 1622 (C=S); MS (*m*/*z*, %): 353.00 ($M^+$, 88.00), 354.00 ($M^{+1}$, 21.70), 355.00 ($M^{+2}$, 9.60). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 6.75 (s, 1H, pyrrole-H), 6.79–7.53 (m, 9H, Ar–H), 9.93 (s, 1H, $NH^1$, $D_2O$ exchangeable), 11.90 (s, 1H, $NH^3$–, $D_2O$ exchangeable). Anal. calcd for $C_{18}H_{12}FN_3S_2$ (353.05): C, 61.17; H, 3.42; F, 5.38; N, 11.89; S, 18.14. Found: C, 61.36; H, 3.10; F, 5.18; N, 11.57; S, 18.48.

*4.1.4.3. 6-(4-Chlorophenyl)-7-phenyl-1H-pyrrolo[2,3-d]pyrimidine-2,4(3H,7H)-dithione (**5c**).* Yield: (2.21 g, 60%); m.p. 240–242 °C. IR (KBr) $cm^{-1}$, 3378 (NH), 1618 (C=S); MS (*m*/*z*, %): 369.00 ($M^+$, $Cl^{35}$, 100.00), 370.00 ($M^{+1}$, 21.20), 371.00 ($M^{+2}$, $Cl^{37}$, 41.60). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 6.75 (s, 1H, H-pyrrole), 6.86–7.51 (m, 9H, Ar–H),

9.91 (s, 1H, $NH^1$–), 11.93 (s, 1H, $NH^3$–, $D_2O$ exchangeable). $^{13}C$ NMR (DMSO-$d_6$) δ/ppm: 97.88, 101.96, 128.52, 128.71, 129.03, 129.50, 129.67, 132.61, 134.62, 135.66, 153.67, 170.61, 180.91 (2C=S). Anal. calcd for $C_{18}H_{12}ClN_3S_2$ (369.90): C, 58.45; H, 3.27; Cl, 9.58; N, 11.36; S, 17.34. Found: C, 58.13; H, 2.96; Cl, 9.42; N, 11.03; S, 17.04.

*4.1.4.4. 7-Phenyl-6-p-tolyl-1H-pyrrolo[2,3-d]pyrimidine-2,4(3H,7H)-dithione (**5d**).* Yield: (2.19 g, 63%); mp: 238–240 °C. IR (KBr) ($cm^{-1}$): 3378 (NH), 1613 (C=S); MS (*m/z*, %): 349.00 ($M^+$, 100.00), 350.00 ($M^{+1}$, 23.70), 351.00 ($M^{+2}$, 12.60). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 2.33 (s, 3H, $CH_3$), 6.71 (s, 1H, pyrrole-H), 6.88–7.45 (m, 9H, Ar–H), 9.95 (s, 1H, $NH^1$–, $D_2O$ exchangeable), 11.90 (s, 1H, $NH^3$-, $D_2O$ exchangeable). Anal. calcd for $C_{19}H_{15}N_3S_2$ (349.48): C, 65.30; H, 4.33; N, 12.02; S, 18.35. Found: C, 65.62; H, 4.03; N, 12.35; S, 18.04.

*4.1.4.5. 6-(4-Bromophenyl)-7-phenyl-1H-pyrrolo[2,3-d]pyrimidine-2,4(3H,7H)-dithione (**5e**).* Yield: (2.50 g, 71%); mp: 248–250 °C. IR (KBr) ($cm^{-1}$): 3387 (NH), 1630 (C=S); MS (*m/z*, %): 414.00 ($M^+$, 88.00), 415.00 ($M^{+1}$, 21.70), 416.00 ($M^{+2}$, 9.6). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 6.74 (s, 1H, pyrrole-H), 6.79–7.63 (m, 9H, Ar–H), 9.94 (s, 1H, $NH^1$–, $D_2O$ exchangeable), 11.92 (s, 1H, $NH^3$–, $D_2O$ exchangeable). Anal. calcd for $C_{18}H_{12}BrN_3S_2$ (414.35): C, 52.18; H, 2.92; Br, 19.28; N, 10.14; S, 15.48. Found: C, 52.36; H, 3.10; Br, 19.12; N, 10.50; S, 15.15.

*4.1.5. General methods of preparation of **6a–e***

A mixture of **2a–e** (20 mmol) and excess of ethylamine, stirring the mixture for 7 days, and then the solvent was concentrated. The residue was purified by column chromatography using eluent $CH_2Cl_2$:$CH_3OH$ (39:1).

*4.1.5.1. 3-Ethyl-3,4-dihydro-4-imino-6,7-diphenyl-1H-pyrrolo[2,3-d] pyrimidine-2(7H)–thione (**6a**).* Yield: (5.39 g, 78%); mp: 210–212 °C. IR (KBr) $cm^{-1}$: 3380 (NH), 1580 (C=S), 1630 (=NH); MS (*m/z*, %): 346.00 ($M^+$, 100.00), 347.00 ($M^{+1}$, 27.08), 348.00 ($M^{+2}$, 8.94). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 1.15 (t, 3H, $CH_3$), 2.81 (q, 2H, $CH_2$), 6.76 (s, 1H, H-Pyrrole), 6.77–7.46 (m, 10H, Ar–H), 8.27 (s, 1H, NH-, $D_2O$ exchangeable), 10.35 (s, 1H, imine–NH–, $D_2O$ exchangeable). Anal. Calcd. for $C_{20}H_{18}N_4S$ (346.46): C, 69.34; H, 5.24; N, 16.17; S, 9.25. found: C, 69.02; H, 5.57; N, 16.47; S, 8.90.

*4.1.5.2. 3-Ethyl-6-(4-fluorophenyl)3,4-dihydro-4-imino-7-phenyl-1H-pyrrolo [2,3-d]pyrimidine-2(7H)–thione (**6b**).* Yield: (5.09 g, 70%); mp: 225–227 °C. IR (KBr) $cm^{-1}$: 3384 (NH), 1583 (C=S), 1630.84 (=NH); MS (*m/z*, %): 364.00 ($M^+$, 10.69), 365.00 ($M^{+1}$, 2.88), 366.00 ($M^{+2}$, 2.50). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 1.15 (t, 3H, $CH_3$), 2.83 (q, 2H, $CH_2$), 6.77 (s, 1H, H-Pyrrole), 6.79–7.45 (m, 9H, Ar–H), 8.26 (s, 1H, NH-, $D_2O$ exchangeable), 10.35 (s, 1H, imine–NH–, $D_2O$ exchangeable). Anal. calcd for $C_{20}H_{17}FN_4S$ (364.45):C, 65.91; H, 4.70; F, 5.21; N, 15.37; S, 8.80. Found: C, 65.58; H, 4.38; F, 4.90; N, 15.02; S, 9.13.

*4.1.5.3. 6-(4-Chlorophenyl)3-ethyl-3,4-dihydro-4-imino-7-phenyl-1H-pyrrolo [2,3-d]pyrimidine-2(7H)–thione (**6c**). **Y**ield* (5.32 g, 70%); mp: 280–282 °C IR (KBr) $cm^{-1}$: 3382 (NH), 1581 (C=S), 1636 (=NH); MS (*m/z*, %): 380.00 ($M^+$, $Cl^{35}$, 15.96), 381.00 ($M^{+1}$, 20.86), 382.00 ($M^{+2}$, $Cl^{37}$, 7.96). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 1.16 (t, 3H, $CH_3$), 2.81 (q, 2H, $CH_2$), 6.76 (s, 1H, H-Pyrrole), 6.79–7.45 (m, 9H, Ar–H), 8.26 (s, 1H, NH-, $D_2O$ exchangeable), 10.35 (s, 1H, =NH–, $D_2O$ exchangeable).Anal. calcd for $C_{20}H_{17}ClN_4S$ (380.90): C, 63.07; H, 4.50; Cl, 9.31; N, 14.71; S, 8.42.found: C, 63.37; H, 4.15; Cl, 8.98; N, 14.40; S, 8.23.

*4.1.5.4. 3-Ethyl-3,4-dihydro-4-imino-7-phenyl-6-p-tolyl-1H-pyrrolo [2,3-d] pyrimidine-2(7H)-thione (**6d**).* Yield: (5.04 g, 70%); mp: 198–200 °C. IR (KBr) $cm^{-1}$: 3371 (NH), 1579 (C=S), 1630.84 (=NH); MS (*m/z*, %): 360.00 ($M^+$, 8.19), 361.00 ($M^{+1}$, 9.61), 362.00 ($M^{+2}$, 8.68). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 1.15 (t, 3H, $CH_3$), 2.24 (s, 3H, $CH_3$), 2.84 (q, 2H, $CH_2$), 6.77 (s, 1H, H-Pyrrole), 6.80–7.44 (m, 9H, Ar–H), 8.27 (s, 1H, NH-, $D_2O$ exchangeable), 10.35 (s, 1H, imine–NH–, $D_2O$ exchangeable). $^{13}C$ NMR (DMSO-$d_6$) δ/ppm: 12.80 ($CH_2\underline{C}H_3$), 20.61 ($CH_3$), 34.26($N\underline{C}H_2CH_3$), 96.32, 99.50, 100.57, 127.75, 128.41, 128.47, 128.95, 135.73, 136.04, 136.87, 149.87, 162.18(C=N), 171.78(C=S). Anal. calcd for $C_{21}H_{20}N_4S$ (360.48): C, 69.97; H, 5.59; N, 15.54; S, 8.90. Found: C, 69.62; H, 5.28; N, 15.20; S, 8.55.

*4.1.5.5. 6-(4-Bromophenyl)3-ethyl-3,4-dihydro-4-imino-7-phenyl-1H-pyrrolo [2,3-d]pyrimidine-2(7H)-thione (**6e**).* Yield: (6.30 g, 75%); mp: 235–237 °C. IR (KBr) $cm^{-1}$: 3379 (NH), 1586 (C=S), 1630.84 (=NH); MS (*m/z*, %): 424.00 ($M^+$, $Br^{79}$, 20.96), 425.00 ($M^{+1}$, 9.25), 426 ($M^{+2}$, $Br^{81}$, 10.39). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 1.15 (t, 3H, $CH_3$), 2.84 (q, 2H, $CH_2$), 6.77 (s, 1H, H-Pyrrole), 6.80–7.43 (m, 9H, Ar–H), 8.26 (s, 1H, NH-, $D_2O$ exchangeable), 10.35 (s, 1H, imine–NH–, $D_2O$ exchangeable). $^{13}C$ NMR (DMSO-$d_6$) δ/ppm: 12.74 ($CH_2\underline{C}H_3$), 34.23($N\underline{C}H_2CH_3$), 96.35, 99.55, 100.59, 127.79, 128.45, 128.49, 128.85, 128.90, 135.70, 136.11, 149.88, 162.17(C=N), 171.79(C=S). Anal. calcd for $C_{20}H_{17}BrN_4S$ (425.35): C, 56.48; H, 4.03; Br, 18.79; N, 13.17; S, 7.54. Found: C, 56.28; H, 4.40; Br, 18.46; N, 13.35; S, 7.20.

*4.1.6. General methods of preparation of **7a–e***

A mixture of **2a–e** (10 mmol) and hydrazine hydrate (5.006 mg, 10 mmol) was stirred for about 7 days. Then added ice; filtered and washed with water. The precipitate was crystallized from ethanol.

*4.1.6.1. 3-Amino-6,7-diphenyl-4-imino-1,3,4,7-tetrahydro-2H-pyrrolo[2,3-d] pyrimidine-2-thione (**7a**).* Yield: (2.33 g, 70%); mp: 210–212 °C. IR (KBr) $cm^{-1}$: 3295.75 (NH), 1568 (C=S), 1635.84 (=NH), 2926 ($NH_2$); MS (*m/z*,%): 333.00 ($M^+$, 42.20), 334.00 ($M^{+1}$, 10.27) 335.00 ($M^{+2}$, 3.43). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 6.37 (s, 2H, $NH_2$, $D_2O$ exchangeable), 6.91 (s, 1H, H-Pyrrole), 6.961–7.47 (m, 10H, Ar–H), 8.30 (s, 1H, NH–, $D_2O$ exchangeable), 10.43 (s, 1H, imine–NH–, $D_2O$ exchangeable). Anal. calcd for $C_{18}H_{15}N_5S$ (333.42): C, 64.84; H, 4.53; N, 21.01; S, 9.62. Found: C, 64.45; H, 4.31; N, 21.13; S, 9.42.

*4.1.6.2. 3-Amino-6-(4-fluorophenyl)-4-imino-7-phenyl-1,3,4,7-tetrahydro-2H-pyrrolo[2,3-d]pyrimidine-2-thione (**7b**).* Yield: (2.73 g, 78%); mp: 222–224 °C. IR (KBr) $cm^{-1}$: 3299 (NH), 1571 (C=S), 1635.84 (=NH), 2929 ($NH_2$); MS (*m/z*, %):351.00 ($M^+$, 86.03), 352.00 ($M^{+1}$, 21.24), 353.00 ($M^{+2}$, 11.11). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 6.37 (s, 2H, $NH_2$, $D_2O$ exchangeable), 6.92 (s, 1H, H-Pyrrole), 6.88–7.52 (m, 9H, Ar–H), 8.30 (s, 1H, NH-, $D_2O$ exchangeable),10.41 (s, 1H, imine–NH–, $D_2O$ exchangeable). Anal. calcd for $C_{18}H_{14}FN_5S$ (351.40): C, 61.52; H, 4.02; F, 5.41; N, 19.93; S, 9.12. Found: C, 61.23; H, 3.68; F, 5.20; N, 19.60; S, 8.84.

*4.1.6.3. 3-Amino-6-(4-chlorophenyl)-4-imino-7-phenyl-1,3,4,7-tetrahydro-2H-pyrrolo[2,3-d]pyrimidine-2-thione (**7c**).* Yield: (2.49 g, 68%); mp: 200–202 °C. IR (KBr) $cm^{-1}$: 3301 (NH), 1569 (C=S), 1635.84 (=NH), 2899 ($NH_2$); MS (*m/z*, %): 367.00 ($M^+$, $Cl^{35}$, 5.26), 368.00 ($M^{+1}$,4.68), 369.00 ($M^{+2}$, $Cl^{37}$, 100). $^1H$ NMR (DMSO-$d_6$) δ/ppm: 6.37 (s, 2H, $NH_2$, $D_2O$ exchangeable), 6.85 (s, 1H, H-Pyrrole), 6.90–7.45 (m, 9H, Ar–H), 8.32 (s, 1H, NH-, $D_2O$ exchangeable), 10.43 (s, 1H, imine–NH–, $D_2O$ exchangeable), Anal. calcd for $C_{18}H_{14}ClN_5S$ (367.86): C, 58.77; H, 3.84; Cl, 9.64; N, 19.04; S, 8.72. Found: C, 58.55; H, 3.60; Cl, 9.33; N, 18.69; S, 8.41.

*4.1.6.4. 3-Amino-4-Imino-6-(4-methylphenyl)-7-phenyl-1,3,4,7-tetrahydro-2H-pyrrolo[2,3-d]pyrimidine-2-thione (**7d**).* Yield: (2.49 g, 72%); mp: 220–222 °C. IR (KBr) $cm^{-1}$: 3289 (NH), 1558 (C=S), 1635.84 (=NH), 2926 ($NH_2$); MS (*m/z*, %): 347.00 ($M^+$, 87.22),

348.00 ($M^{+1}$, 3. 11), 349.00 ($M^{+2}$, 11). $^1$H NMR (DMSO-$d_6$) $\delta$/ppm: 2.20 (s, 3H, $CH_3$), 6.37 (s, 2H, $NH_2$, $D_2O$ exchangeable), 6.86 (s, 1H, H-Pyrrole), 7.02–7.46 (m, 9H, Ar–H), 8.26 (s, 1H, NH-, $D_2O$ exchangeable), 10.42 (s, 1H, imine–NH–, $D_2O$ exchangeable). $^{13}$C NMR (DMSO-$d_6$) $\delta$/ppm: 20.61 ($\underline{C}H_3$), 96.32, 99.50, 100.57, 127.75, 128.41, 128.47, 128.85, 128.95, 136.04, 136.87, 148.28, 149.87, 162.10 ($\underline{C}$=N), 171.78 ($\underline{C}$=S). Anal. calcd for $C_{19}H_{17}N_5S$ (347.44): C, 65.68; H, 4.93; N, 20.16; S, 9.23. Found: C, 65.35; H, 4.70; N, 19.80; S, 9.03.

*4.1.6.5. 3-Amino-6-(4-bromophenyl)-4-imino-7-phenyl-1,3,4,7-tetrahydro-2H-pyrrolo[2,3-d]pyrimidine-2-thione (**7e**).* **Y**ield: (3.08 g, 75%); mp: 280–282 °C. IR (KBr) $cm^{-1}$: 3311 (NH), 1573 (C=S), 1635.84 (=NH), 2946 ($NH_2$); MS (*m*/*z*, %): 411.00 ($M^+$, $Br^{79}$, 98.34), 412.00 ($M^{+1}$, 25.39), 413.00 ($M^{+2}$, $Br^{81}$, 100.00). $^1$H NMR (DMSO-$d_6$) $\delta$/ppm): 6.37 (s, 2H, $NH_2$, $D_2O$ exchangeable), 6.76 (s, 1H, H-Pyrrole), 6.91–7.48 (m, 9H, Ar–H), 8.31 (s, 1H, NH, $D_2O$ exchangeable), 10.40 (s, 1H, imine–NH–, $D_2O$ exchangeable). Anal. calcd for $C_{18}H_{14}BrN_5S$ (411.01): C, 52.44; H, 3.42; Br, 19.38; N, 16.99; S, 7.78. Found: C, 52.23; H, 3.11; Br, 19.2; N, 16.66; S, 7.42.

### *4.2. Elisa measurement of TSG-14*

Compounds to be tested were administered orally to the albino rats (male, weight approx. 200 g) 30 min prior to LPS (*Escherichia coli* B055/B5, 0.1 mg/kg ip) challenge. Three rats were dosed orally (30 mg/kg) with a suspension of compound in carboxy methyl-cellulose 0.5% as described in the literature [26]. Sera were assayed in duplicates with Rat Pentraxin 3 (PTX3)/TG-14 ELISA Kit, 4-6 hr after LPS injection, which is a time point of maximal elevation of serum TSG-14, as described in the literature [22]. Percent inhibition of TSG-14 production at 30 mg/kg was determined by comparison of yield with a control to which no test compound was added. Results represent means ± S.E.M. ($n = 3$).

#### *4.2.1. Principle of the assay*

This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for PTX3 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any PTX3 present is bound by the immobilized antibody.

#### *4.2.2. Sensitivity of the assay*

The minimum detectable dose of rat PTX3 is typically less than 0.02 ng/mL.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.03.068.

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