

Graphical Abstract

Trisubstituted barbiturates and thiobarbiturates: synthesis and biological evaluation as xanthine oxidase inhibitors, antioxidants, antibacterial and anti-proliferative agents

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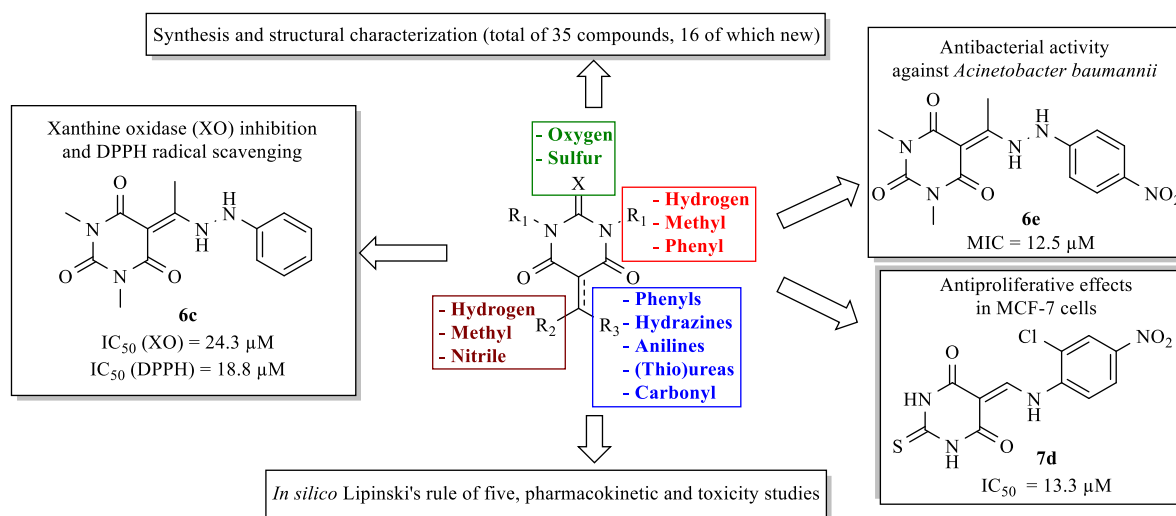
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ABSTRACT

Barbituric and thiobarbituric acid derivatives have become progressively attractive to medicinal chemists due to their wide range of biological activities. Herein, different series of 1,3,5-trisubstituted barbiturates and thiobarbiturates were prepared in moderate to excellent yields and their activity as xanthine oxidase inhibitors, antioxidants, antibacterial agents and as anti-proliferative compounds was evaluated *in vitro*. Interesting bioactive barbiturates were found namely, 1,3-dimethyl-5-[1-(2-phenylhydrazinyl)ethylidene]pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (**6c**) and 1,3-dimethyl-5-[1-[2-(4-nitrophenyl)hydrazinyl]ethylidene]pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (**6e**), which showed concomitant xanthine oxidase inhibitory effect (IC₅₀ values of 24.3 and 27.9 μM, respectively), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (IC₅₀ values of 18.8 and 23.8 μM, respectively). In addition, 5-[1-(2-phenylhydrazinyl)ethylidene]pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (**6d**) also revealed DPPH radical scavenger effect, with an IC₅₀ value of 20.4 μM. Moreover, relevant cytotoxicity against MCF-7 cells (IC₅₀ = 13.3 μM) was observed with 5-[[2-(4-chloro-4-nitrophenyl)amino]methylene]-2-thioxodihydropyrimidine-4,6(1*H*,5*H*)-dione (**7d**). Finally, different 5-hydrazinylethylidenepyrimidines revealed antibacterial activity against *Acinetobacter baumannii* (MIC values between 12.5 to 25.0 μM) which paves the way for developing new treatments for infections caused by this Gram-negative coccobacillus bacterium, known to be an opportunistic pathogen in humans with high relevance

in multidrug-resistant nosocomial infections. The most promising bioactive barbiturates were studied *in silico* with emphasis on compliance with the Lipinski's rule of five as well as several pharmacokinetics and toxicity parameters.

Keywords: barbiturates; xanthine oxidase inhibition; antioxidant; antibacterial; cytotoxicity.

1. Introduction

Barbituric acid (BA) is a relevant pyrimidine derivative that was synthesized for the first time in 1864 by cyclization of urea and malonic acid [1]. Until the commercialization of benzodiazepines in the 1960s, barbiturates were almost the only group of drugs used as sedative-hypnotics [2, 3]. More recently, this class of compounds has become increasingly attractive to medicinal chemists due to the discovery that they bear a wide range of biological activities. More specifically, several of these compounds are enzyme inhibitors [collagenase-3, matrix metalloproteinases (MMPs) [4], xanthine oxidase (XO) [5] and methionine aminopeptidase-1] as well as antibacterial, anticancer, antiangiogenic, immunomodulatory, antifungal and antioxidant agents [6-9]. These activities are associated to several structural changes at positions *N1*, *N3*, *C2* and *C5*, the latter being the most studied position. Within these structural modifications, various cyclization reactions at *C5* and *C6* of BA, thiobarbituric acid (TBA) and their derivatives (BADs and TBADs, respectively) [10-19] as well as at *C5* with spiro formation [20-24] have been described. Interestingly, some of these transformations involve a multicomponent reaction (MCR) [11, 14, 16]. However, in most cases, the cyclization path is preceded by the formation of substituted methyl [10, 18], arylidene [12, 15, 19-24], or methylene [13, 17, 19] derivatives at *C5* as key isolated or potential precursors of these modified pyrimidines.

Among the most important *C5* functionalized BAs and TBAs are the 5-benzylidene or 5-methylene derivatives, usually produced usually by their reaction with benzaldehydes or triethyl orthoformate, respectively. Out of these, a large variety of 5-arylidene BADs and TBADs have been biologically evaluated and several compounds of this class was found to possess XO inhibitory effect [5] and antimicrobial activity against a number of Gram-positive and Gram-negative bacteria, as well as against fungal strains [25]. Methylene BADs and TBADs are most often used as intermediates for the synthesis of other derivatives [26, 27]. Within these, hydrazone BADs have the ability to inhibit fungal growth in the μM range [28]. In addition, through the introduction of urea, thiourea, guanidine, hydrazine or hydroxylamino groups to BADs and TBADs, new bioactive compounds can be obtained [13, 17, 29].

XO is an important and versatile molybdo-flavoprotein responsible for the oxidation of purine substrates, catalyzing the conversion of hypoxanthine into xanthine and xanthine into uric acid, with subsequent reduction of oxygen, forming reactive oxygen species (ROS) which include the superoxide anion radical. An

increase in this enzyme's activity, as well as in its metabolites levels (uric acid and ROS), are associated with many pathological conditions, including gout and oxidative damage of tissues [30-32]. Therefore, the discovery of new XO inhibitors, preferably with antioxidant capacity, is an important goal in medicinal chemistry [33].

Infections caused by microorganisms, especially those involving multidrug-resistant strains, place public health at serious risk. *Acinetobacter baumannii* is a bacterial species increasingly associated with nosocomial infections. Risk factors for an infection with this bacterium include prolonged hospitalization and recent surgical procedures. In this context, the identification of new antibacterial agents against *A. baumannii*, with new mechanisms of action, is an object of major efforts by the scientific community [34].

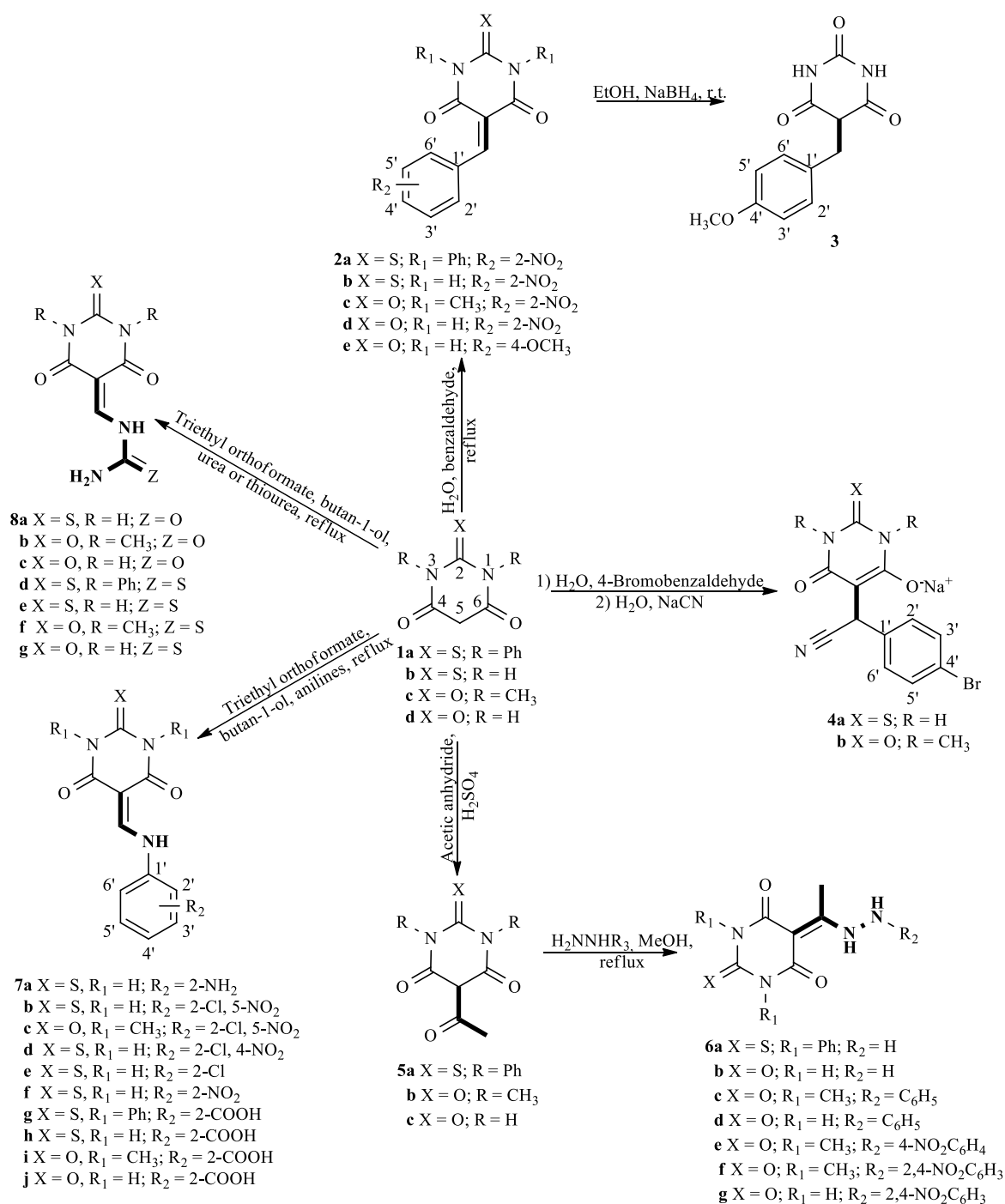
Taken together, the basic BA and TBA scaffolds may play a critical role in both XO inhibitory and promoting antibacterial effects. Therefore, in search for new active BADs and TBADs with these activities, herein we describe the synthesis and *in vitro* biological evaluation of 35 (16 new) 1,3,5-trisubstituted barbiturates and thiobarbiturates where position 5 was conveniently substituted with methylene, benzylidene and methyl derivatives. All BADs and TBADs were evaluated as XO inhibitors and their antioxidant activity was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method. In addition, their antibacterial activity against several Gram-positive and Gram-negative strains and cytotoxicity towards normal human dermal fibroblasts (NHDF) and a mammalian cancer cell line (MCF-7) were assessed. Structure-activity relationships were also discussed and relevant pharmacokinetic and toxicity properties were calculated *in silico* to evaluate their potential interest for *in vivo* applications and/or in the development of compounds with improved pharmacological properties.

2. Results and discussion

2.1. Chemistry

Thirty-five BADs and TBADs were synthesized as shown in Scheme 1, in moderate to excellent yields, i.e. 41-97% (Experimental section and Table S1). The majority of 5-substituted BAD and TBAD families described herein are known precursors of fused heterocycles reported in the literature [10, 12, 13, 15, 17-24].

From this set of compounds and to the best of our knowledge, two 5-phenylcyanomethylpyrimidines (**4a-b**), one 5-hydrazinylethylidenepyrimidine (**6a**), seven 5-phenylaminomethylenepyrimidines (**7a-g**) and six 1-(pyrimidine-5-ylidene)ureas and thioureas (**8a-c, e-g**) are described for the first time. All starting materials were commercially obtained, except 1,3-diphenylthiobarbituric acid (**1a**) which was synthesized from phenylisothiocyanate, aniline, malonic acid and acetyl chloride, in two steps [35, 36]. Almost all prepared 5-substituted BADs and TBADs were easily isolated in a high degree of purity by simple filtration, as confirmed by nuclear magnetic resonance (NMR) spectra. In general, the BADs yields were higher than their congener TBADs.



Scheme 1. Chemical synthesis of BADs and TBADs **2-8**.

A full spectroscopic characterization of all 5-substituted BADs and TBADs **2-8** was made, including ^1H - and ^{13}C -NMR shift assignments, which were established whenever possible with the aid of distortionless enhancement by polarization transfer (DEPT), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) experiments. The most representative ^1H - and ^{13}C -NMR chemical shifts of 5-substituted BADs and TBADs **2-8** are presented in Tables S2 and S3, corresponding to the structural moiety depicted in bold, in Scheme 1.

5-Benzylidenepyrimidines **2a-e** were synthesised by a Knoevenagel condensation reaction in excellent yields (90-97%), based on the method reported by Deb *et al.* [37] in which the reaction temperature was changed from 70 °C to reflux. Then, the C-5 double bond of 5-benzylidenepyrimidine **2e** was reduced at room temperature by NaBH_4 in ethanol according to the method described by Yan *et al.* [38] to give compound **3**, in 41% yield. The change of the yellow arylidene color to white, due to a conjugation extension reduction, together with the absence of the ^1H -NMR singlet at 8.25 ppm (**2e** unsaturated methine CH) and the observation of a triplet and doublet signals corresponding to the methine and methylene in adjacent carbons at 3.82 and 3.19 ppm, respectively, observed in the ^1H -NMR spectra of **3**, are the most important evidences of a successful reduction reaction. Additionally, the cyanomethyl derivatives **4a-b** were obtained, in 67-77% yield, from the respective 5-(4-bromobenzylidene)pyrimidines followed by the addition of sodium cyanide [39]. Once again the reduction was evidenced by the change in color of the solid to white and by observation of deshielded methylene CH ^1H -NMR singlet signals at 5.24-5.35 ppm for compounds **4a-b**.

The 5-acetylpyrimidines **5a-c** were synthesized using the process described by Jursic *et al.* [40] in good to very good yields (45-83%). Then, their hydrazine derivatives **6a-g** were easily prepared after reaction with hydrazine and different phenylhydrazines, in refluxing methanol, using the method described by the same authors [40], also in good to very good yields (50-92%).

The 5-phenylaminomethylenepyrimidines **7a-j** and 1-(pyrimidin-5-ylidene)ureas/thioureas **8a-g** were obtained in moderate to very good yields (52-98%) by reaction of triethyl orthoformate with urea or thiourea and different anilines, respectively, in refluxing butan-1-ol, by a MCR based on the method described by Rauf *et al.* [27]. In this procedure, the solvent was exchanged from butan-2-ol to butan-1-ol, which allowed the

increase of the reflux temperature, resulting in higher yields of products. As representative examples, the yields of 5-phenylaminomethylenepyrimidines **7h** and **7j** were changed from 82 and 72% to 97 and 84%, respectively, when the reaction temperature increased.

Detailed ^1H - and ^{13}C -NMR analysis suggested that the enamine tautomer form **A** was present in all pyrimidine derivatives **6-8**, in $\text{DMSO-}d_6$. The imine form (hydrazone) **B** appears to be absent (Figure 1), contrary to what was reported for 5-hydrazinylethylidenepyrimidines [28]. Specifically, the NH ^1H -NMR signal between 11.10 and 13.57 ppm correlated to the 5-C signal in HMBC experiments, which makes unequivocal evidence of the exclusive existence of tautomer **A** in $\text{DMSO-}d_6$.

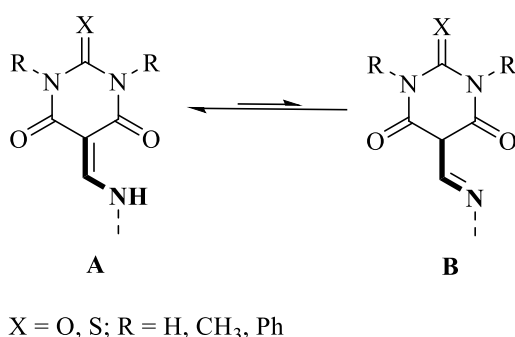


Figure 1. Enamine **A** and imine **B** tautomeric forms.

2.2. Xanthine oxidase inhibitory activity

The evaluation of the XO inhibitory activity of BADs and TBADs **2-8** was made using a spectrophotometric method, under aerobic conditions, with xanthine as the substrate, and following uric acid formation by measuring the absorbance at 295 nm, as described in the literature [41, 42]. The commercial drug allopurinol was used as the positive control. The study started with a screening at 30 μM concentration (Table S4) and after this preliminary evaluation, concentration-response studies were performed for the BADs and TBADs which presented an inhibitory effect of 40% or higher, in order to determine their half maximal inhibitory concentration (IC_{50}) values.

In general, the majority of BADs and TBADs was able to inhibit XO activity (Table S4), with the most active compounds being the 5-benzylidenepyrimidines **2c** and **2d** and the 5-hydrazinylethylidenepyrimidines

6c and **6e** (Figure 2), with an IC_{50} of 26.1, 31.5, 24.3 and 27.9 μM , respectively (Table 1). For the positive control (allopurinol) and under the assay conditions employed, an IC_{50} value of 3.17 μM was calculated, which is in agreement with the reported values [43]. Through the analysis of all these results, some important structural features for the XO inhibition could be inferred. Generally, BADs showed better XO inhibitory effect in comparison to their congeners TBADs, and bulkier substituents at C5 and smaller groups at N1 and N3 (Scheme 1) also seemed to favour this activity. Moreover, the 5-hydrazinylethylidenepyrimidine **6c**, substituted with a methyl group at N1 and N3, clearly presented better activity than its hydrogenated analogue **6d**. Additionally, the presence of a 2,4-dinitro system (**6f**) seemed to reduce this activity when compared to the congeners 5-hydrazinylphenyl **6c** and 4-nitrophenyl **6e** (Figure 2).

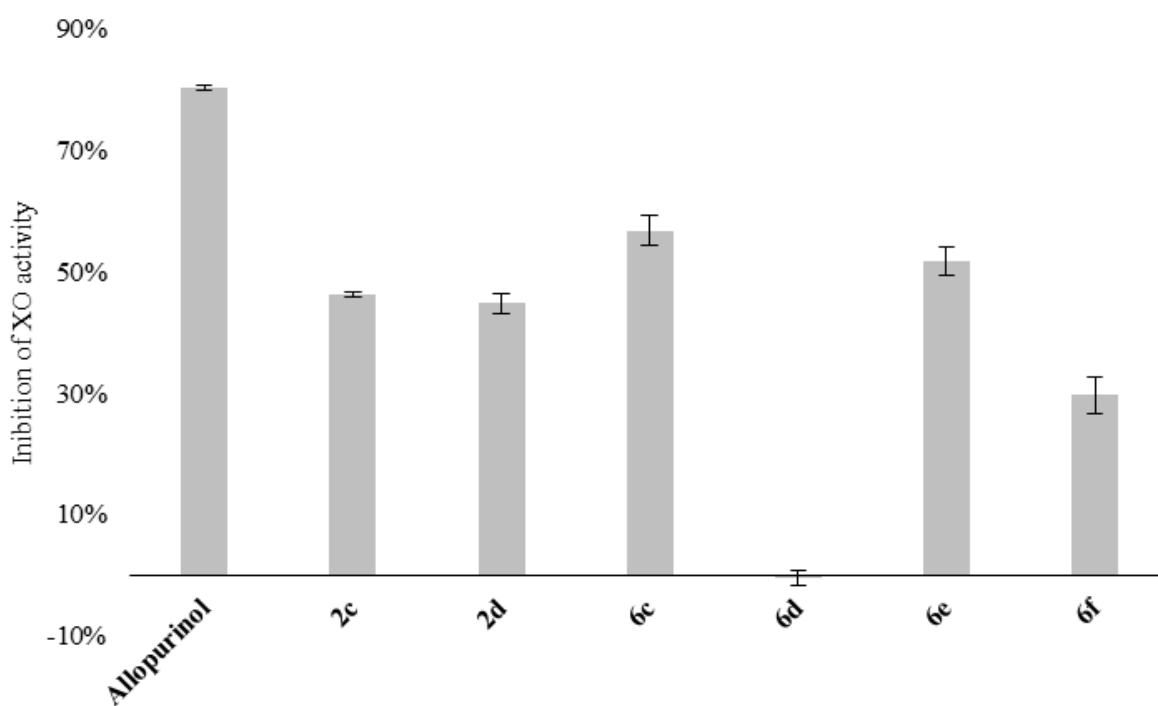


Figure 2. *In vitro* XO inhibitory activity of compounds **2c**, **2d**, **6c-f** and allopurinol. Results are expressed as average values \pm standard error of the mean (SEM). A $p < 0.05$ versus negative control in the statistical significance analysis (Student's *t*-test) was observed for all compounds except for **6d**.

Table 1. *In vitro* IC_{50} values for XO inhibition and DPPH radical scavenging activity of compounds **2c**, **2d**, **6c-e** and respective references allopurinol and Trolox.

Compound	XO (μM) ^a	DPPH (μM) ^a	
		20 min	60 min
2c	26.1	nd ^b	nd ^b
2d	31.5	nd ^b	nd ^b
6c	24.3	22.6	18.8
6d	nd ^b	23.9	20.4
6e	27.9	23.9	23.8
Allopurinol	3.2	nd ^b	nd ^b
Trolox	nd ^b	33.8	35.9

^aIC₅₀ values represent as mean of three determinations.

^b nd: not determined

2.3. Antioxidant activity as DPPH radical scavenger

The DPPH radical scavenging activity of BADs and TBADs **2-8** was evaluated using a spectrophotometric method by measuring the absorbance of this radical at 517 nm, based on the available literature [41, 42] and using Trolox as the reference compound. A screening was first performed at the concentration of 30 μM , after 20 and 60 minutes of incubation (Table S4). IC₅₀ values were further determined for BADs and TBADs which presented a DPPH radical scavenging activity higher than 50% (Table 1).

Most of the tested compounds presented no appreciable DPPH radical scavenging capacity (Table S4). However, 5-hydrazinylethylidenepyrimidines **6c-e** were clear exceptions (Figure 3) and the determined IC₅₀ values were 22.6, 23.9 and 23.9 μM , respectively, at 20 minutes, and 18.8, 20.4 and 23.8 μM , at 60 minutes (Table 1). Particularly, under these experimental conditions, the radical scavenging ability of these 5-hydrazinylethylidenepyrimidines was superior to the one observed with Trolox, whose calculated IC₅₀ values (33.8 and 35.9 μM after 20 and 60 minutes, respectively) are in agreement with the literature [44]. Concerning the structural features relevant for this activity, the hydrazinyl derivatives globally presented higher DPPH radical scavenging capacity when compared with most of the other compounds. More specifically, the aromatic 5-hydrazinylethylidenepyrimidines **6c-e** can be relevant starting points to develop improved antioxidant compounds acting as radical scavengers. In addition, it is interesting to note that the 5-hydrazinylphenyl **6c-d** and 5-hydrazinyl-(4-nitrophenyl) **6e** derivatives had better activity than their hydrogenated **6a-b** or 2,4-dinitrophenyl **6f-g** counterparts. In this context, it is worth mentioning that the

antioxidant action of other different organic hydrazines has already been described [45-47]. Finally, the 1-(pyrimidin-5-ylidene)ureas **8d** and **8f** exhibited a moderate radical scavenging activity (Figure 3).

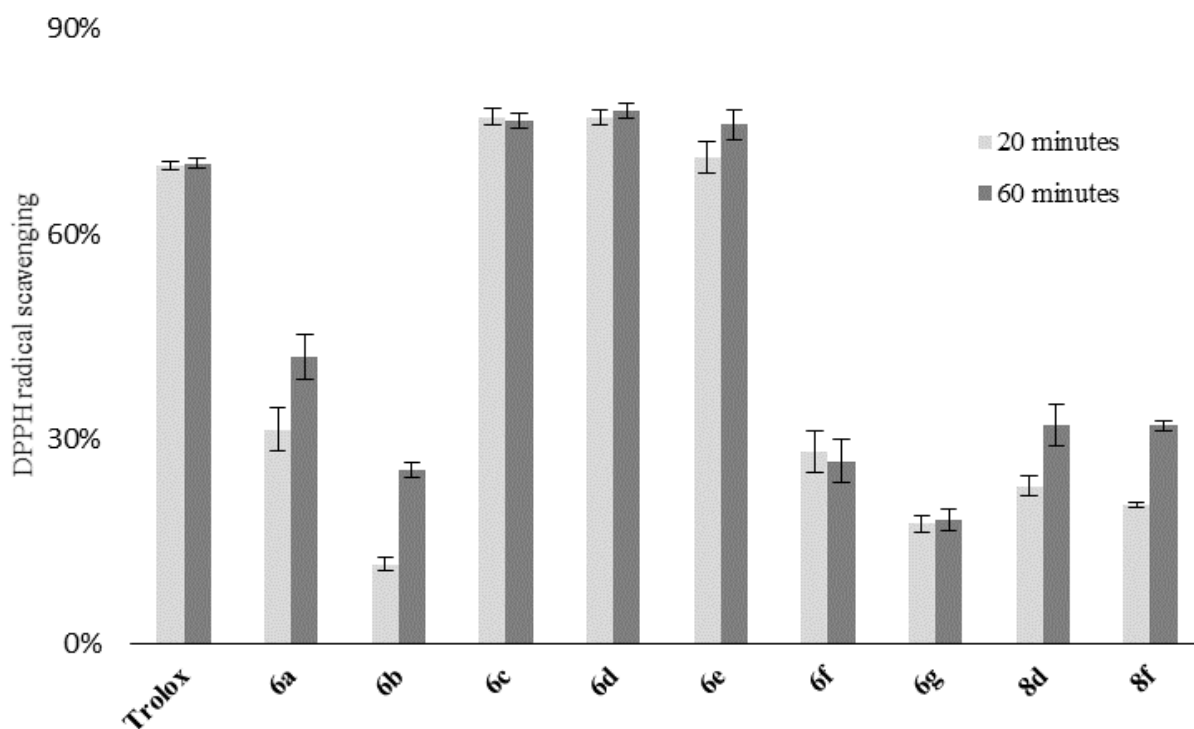


Figure 3. *In vitro* DPPH radical scavenging activity for 5-hydrazinylethylidenepyrimidines **6a-g**, 1-(pyrimidin-5-ylidene)ureas **8d**, **8f** and Trolox (positive control). Results are expressed as average values \pm SEM. A $p < 0.05$ versus negative control in the statistical significance analysis (Student's *t*-test) was observed for all compounds.

Taken together, it is important to note the relevant XO inhibition and the high DPPH radical scavenging activity of the 5-hydrazinylethylidenepyrimidines **6c** and **6e**. As the new XO inhibitors should have a remarkable radical scavenging capacity, these results seem promising [43].

2.4. Antibacterial activity

The antibacterial potential of all synthesized BADs and TBADs **2-8** was studied in *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Acinetobacter baumannii* strains, using tetracycline as the reference compound (Table S5).

Regarding the antibacterial activity of the compounds under study, most of the assayed BADs and TBADs did not show activity against the tested Gram-positive and Gram-negative bacterial strains, with minimum inhibitory concentrations (MIC) higher than 200 μM . These results are in agreement with the previous reports referring to several 5-functionalized BADs and TBADs which were inactive against different strains of bacteria and fungi [28, 48-50]. However, pyrimidines **6c-d** (MIC 25.0 μM) and **6e** (MIC 12.5 μM) exhibited a relevant and selective antibacterial activity against *A. baumannii*. In fact, the presence of a *p*-nitrophenyl moiety bound to the hydrazine group of 5-hydrazinylethylidenepyrimidines (Scheme 1) seems to play an important role, since an increase in this antibacterial activity was observed in relation to the unsubstituted phenyl analogs **6c-d**. In addition, the presence of more than one nitro group in the phenyl group (2,4-dinitrophenyl derivatives **6f-g**) decreased the activity of these 5-hydrazinylethylidenepyrimidines. Hence, a suitable aromatic substituent at R₃ of these pyrimidines can favour this activity. Furthermore, it is important to note that compound **6d**, with unsubstituted pyrimidinone nitrogens, had antibacterial activity against *A. baumannii* but was devoid of XO inhibitory effects.

The emergence of infections and outbreaks associated with strains of *A. baumannii* resistant to antibiotics, or even extensively drug-resistant and pandrug-resistant, has highlighted the lack of available drugs to treat infections by this bacterium. In fact, carbapenem-resistant *A. baumannii* is marked as one of the critical pathogens in the World Health Organization priority list for research and development of new antibiotics [51]. Therefore, the activity of compounds **6c-e** against *A. baumannii* makes them promising new starting points for the development of antibacterial agents against this high-profile pathogen.

2.5. Cytotoxicity studies in human cells

Considering the relevant results observed in the XO inhibition, DPPH radical scavenging and antibacterial studies, the cytotoxicity of BADs and TBADs **2-8** was evaluated in normal human dermal fibroblasts (NHDF). Additionally, a mammalian breast cancer cell line (MCF-7) was included in this study to evaluate the anti-proliferative potential and selectivity of these compounds. The well-established 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay [52] was used in which the soluble yellow MTT is converted into purple and insoluble formazan crystals by active mitochondrial lactate dehydrogenases of living cells [53]. Initially, both cell lines were exposed to BADs and TBADs **2-8** at the concentration of 30 μM , during a period of 72 h. Untreated cells were used as the negative control and cells treated with 5-fluorouracil (5-FU) as the positive control.

The results (Table S4) showed that most of the compounds were not markedly cytotoxic for NHDF cells at 30 μM . Additionally, the IC_{50} of 5-hydrazinylethylidenepyrimidine **6c** was determined as a representative example of pyrimidines **6c-e**, and a value of 82.0 μM was obtained. Considering the potential future use of this type of compounds in the treatment of *A. baumannii* infections, this can be an interesting result as it demonstrates a possible selectivity of these agents for bacterial *versus* normal human cells.

On the other hand, a more marked reduction of MCF-7 cells proliferation was observed (Figure 4 and Table S4). The phenylaminomethylpyrimidines **7** seemed to have higher cytotoxicity than the other groups of compounds. Specifically, it is clear that the 5-hydrazinylethylidenepyrimidine **6d** and the 5-phenylaminomethylenepyrimidines **7d-e,g-h** have a relevant anti-proliferative effect at 30 μM . In this last group, the presence of a chloro or a carboxyl group at position 2 of the aromatic ring (Scheme 1) seems to improve this activity when compared to the compound containing the nitro (**7f**) group in the same position. For the 5-phenylaminomethylenepyrimidine **7d**, the most cytotoxic compound in MCF-7 cells at 30 μM , the IC_{50} value was 13.3 μM . Considering its selectivity for cancer cells *versus* NHDFs, this structure can be a future starting point to develop this class of compounds as potential novel anticancer agents.

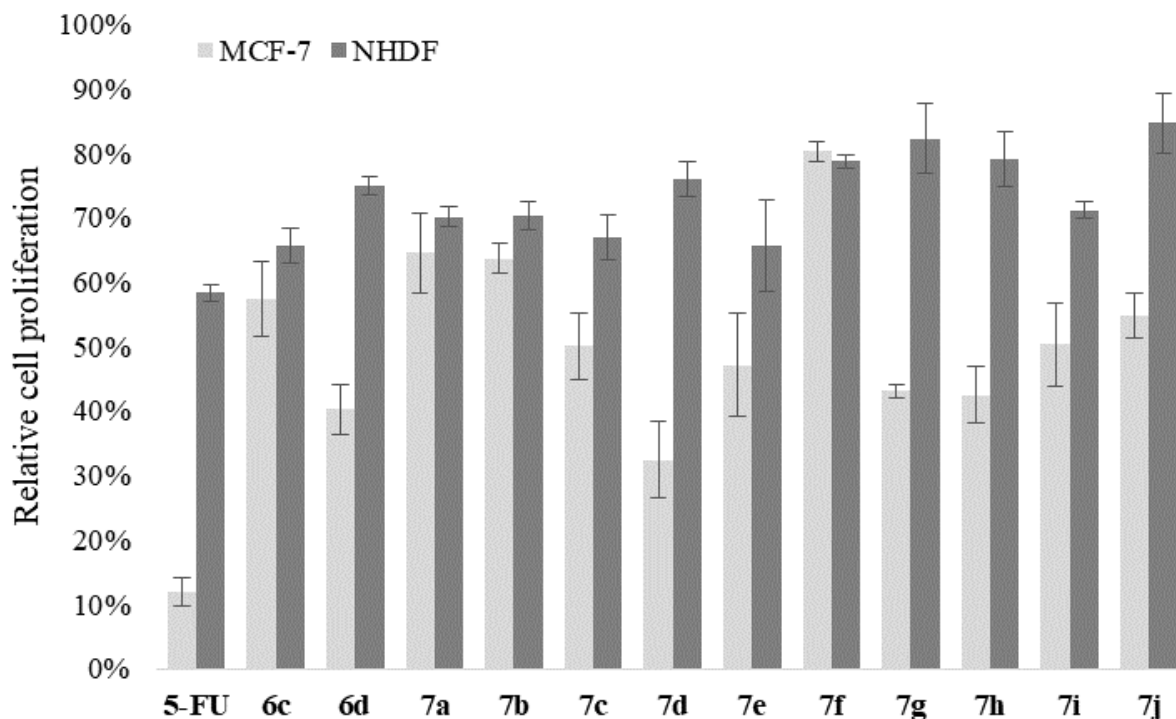


Figure 4. *In vitro* cytotoxic effect of compounds **6c**, **6d**, **7a-j** and 5-fluorouracil (5-FU) in normal human dermal fibroblasts (NHDF) and in mammalian breast cancer cells (MCF-7). Results are expressed as average values \pm SEM. A $p < 0.05$ versus negative control in the statistical significance analysis (Student's *t*-test) was observed for all compounds.

2.6. *In Silico* studies

Poor pharmacokinetics and/or unacceptable side effects are the main reasons why the majority of drugs evaluated in clinical trials do not reach the market [54]. Thus, computational approaches to predict the absorption, distribution, metabolism, excretion and toxicity (ADMET) may be a decisive tool to minimize these risks. The PkCSM online software (available from <http://bleoberis.bioc.cam.ac.uk/pkcsm/prediction>) is a recent freely accessible web platform that allows rapid evaluation of pharmacokinetic and toxicity properties and was herein used for the *in silico* evaluation of the drug-likeness, in terms of the Lipinski's rule of five (Table S6), and to predict ADMET properties (Table S7) for the six most promising pyrimidines (**2c,d**, **6c-e** and **7d**) tested *in vitro*.

Four parameters are taken into consideration in the Lipinski's rule of five, including an *n*-octanol-water partition coefficient ($\log P$) no higher than 5, a molecular weight (MW) lower than 500 Da, no more than 10 hydrogen bond acceptors (n-HBA) and no more than 5 hydrogen bond donors (n-HBD) [54]. Generally, an orally active drug has no more than one violation [55]. According to Table S6, all these pyrimidines are compatible with the parameters of the Lipinski's rule of five, suggesting that these compounds have favourable properties to accomplish the drug-likeness criteria.

The pkCSM online software was also used to predict pharmacokinetic and toxicity properties of pyrimidines **2c-d**, **6c-e** and **7d** (Table S7). The pyrimidines **2c,d**, **6c-e** and **7d** entirely fulfil the Lipinski's rule of five and a good intestinal absorption was predicted. However, for compounds **2d** and **6d-e** a poor Caco-2 permeability was estimated. In addition, despite the fact that pyrimidines **6e** and **7d** can be potential P-glycoprotein substrates, it was predicted that all studied compounds should not be potential P-glycoprotein I/II inhibitors. Therefore, a low probability of pharmacokinetic interactions involving this protein is expected. A similar situation should occur in the case of the excretory protein organic cation transporter 2 (OCT2). In the distribution field, a generic tendency for a low probability for these compounds to access the central nervous system was estimated. Concerning metabolism, in which the cytochrome P450 isoforms have a key role, only the 5-phenylaminomethylenepyrimidine **7d** was predicted to be a potential substrate of the important isoform CYP3A4. In general, it is expected that pyrimidines **2c,d**, **6c-e** and **7d** should accomplish most of the pharmacokinetic and toxicity criteria studied, however, the estimated maximum tolerated dose in humans and potential genotoxicity (Ames toxicity) for several compounds (Table S7) appeared to be the potential major problems. In fact, a mutagenic potential for the nitroaromatic derivatives **2c-d**, **6e** and **7d** is expected and therefore this point should be experimentally studied and the use of non-mutagenic bioisosteres should be considered.

3. Conclusion

A series of 1,3-trisubstituted barbiturates and thiobarbiturates substituted at the position 5 with benzylidene, benzyl, phenylcyanomethyl, acetyl, hydrazinylethylidene, phenylhydrazinylethylidene, phenylaminomethylene ylideneureas and the corresponding thioureas were synthesized, in moderate to

excellent yields. In addition, the activity of these compounds as xanthine oxidase inhibitors, antioxidants as well as antibacterial and anti-proliferative agents was also evaluated. Some of the synthesized barbiturates were found to have potential for health-related applications, namely 1,3-dimethyl-5-[1-(2-phenylhydrazinyl)ethylidene]pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (**6c**) and 1,3-dimethyl-5-[1-[2-(4-nitrophenyl)hydrazinyl]ethylidene]pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (**6e**) which showed moderate inhibition of xanthine oxidase (IC₅₀ values of 24.3 and 27.9 μM, respectively), and potent radical 2,2-diphenyl-1-picrylhydrazyl scavenging activity (IC₅₀ values of 18.8 and 23.8 μM, respectively). Additionally, 5-[1-(2-phenylhydrazinyl)ethylidene]pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (**6d**) was also highlighted as a relevant starting point for developing new radical scavengers. Interestingly, a selective antibacterial effect of 5-hydrazinylethylidenepyrimidines **6c-e** against *Acinetobacter baumannii* (12.5 to 25.0 μM MIC values) was observed with concomitant low cytotoxicity in normal human dermal fibroblasts. On the other hand, compound 5-[[2-(2-chloro-4-nitrophenyl)amino]methylene]-2-thioxodihydropyrimidine-4,6(1*H*,5*H*)-dione (**7d**) presented a relevant and selective anti-proliferative effect against MCF-7 cells (IC₅₀ = 13.3 μM). It should be noted that the presence of a conjugated carbon-carbon double bond in some of the studied barbituric and thiobarbituric acid derivatives may affect the observed biological activity due to possibility of the compounds to act as Michael acceptors [56-58]. According to *in silico* studies, all selected pyrimidines **2c**, **2d**, **6c-e** and **7d** fulfil the Lipinski's rule of five as well as most of the pharmacokinetic and toxicity criteria determined for the different ADMET parameters. Therefore, a novel compound class with members bearing xanthine oxidase inhibitory activity, antioxidant effects as well as anti-proliferative action and the ability to inhibit the growth of *Acinetobacter baumannii* was revealed. However, future studies should be performed to improve not only the potency and selectivity of this family of compounds but also its pharmacokinetics.

4. Experimental section

4.1. Chemistry

All reagents were purchased from commercial suppliers and used without further purification. Reactions were monitored by thin-layer chromatography (TLC) on Merck-Nagel 60 G/UV₂₅₄ (0.2 mm) plates which

were visualized by ultra-violet (UV) detection. Melting points were recorded on a Büchi B-540 melting point apparatus and are uncorrected. Infrared (IR) spectra were obtained with Thermo Fisher Scientific Nicolet iS10: smart iTR infrared spectrophotometer equipped with a diamond ATR crystal using OMNIC 8.2 software. The sample spectra were collected at room temperature in the 4000-400 cm^{-1} range by averaging 32 scans at a spectral resolution of 4 cm^{-1} . ^1H - (400.13 MHz) and ^{13}C - (100.62 MHz) NMR spectra were performed on a Bruker Avance III 400 MHz spectrometer and were processed with the software MestReNova 11.0.3 (trial). Chemical shifts were referenced to the residual solvent signal (DMSO- d_6 : $\delta_{\text{H}} = 2.50$ and $\delta_{\text{C}} = 39.52$, or CDCl_3 : $\delta_{\text{H}} = 7.26$ and $\delta_{\text{C}} = 77.16$). The chemical shift (δ) values are given in parts per million (ppm), and the coupling constants (J) are given in Hertz (Hz). The multiplicity of the signals is reported as s (singlet), d (doublet), dd (doublet of doublets), dt (doublet of triplets), t (triplet), td (triplet of doublets) and m (multiplet). High resolution mass spectrometry (ESI-HRMS) was carried out on a Microanalysis QSTAR XL spectrometer by the microanalysis service (Salamanca, Spain). The ^1H NMR and ^{13}C NMR spectra of all compounds are presented on Supplementary Material.

4.1.1. 1,3-Diphenylthiourea [35]. To a stirred solution of phenylisothiocyanate (1.00 mmol; 135 mg) in dichloromethane (0.5 mL) at room temperature, was added dropwise a solution of aniline (1.00 mmol; 93 mg) in dichloromethane (0.5 mL). The reaction was followed by TLC (dichloromethane) and completed in 3 hours. The resulting suspension was filtered and washed with diethyl ether; Yield 95%; white crystals; mp 142-144 °C (lit.[59] 140-142 °C); IR ν_{max} (cm^{-1}) 3202, 3053, 1598, 1526, 1491, 1449, 1342, 1313, 1288, 1242, 1171, 1069, 1021, 1003, 933, 756, 694, 642, 629, 610; ^1H NMR (400 MHz, DMSO- d_6) δ (ppm) 9.79 (s, 2H, 2 \times NH), 7.48 (d, $J = 7.4$ Hz, 4H, 2' and 6'-Ar CH), 7.33 (t, $J = 8.2$ and 7.6 Hz, 4H, 3' and 5'-Ar CH), 7.12 (t, $J = 7.4$ Hz, 2H, 4'-Ar CH); ^{13}C NMR (101 MHz, DMSO- d_6) δ (ppm) 179.63 (2- CS), 139.46 (1'-Ar C), 128.45 (Ar CH), 124.44 (4'-Ar CH), 123.66 (Ar CH).

4.1.2. 1,3-Diphenylthiobarbituric acid (1a) [36]. A stirred solution of 1,3-diphenylthiourea (1.00 mmol; 228 mg), malonic acid (1.30 mmol; 135 mg) and acetyl chloride (3.00 mmol; 235 mg; 214 μL) was heated at 60 °C for 30 minutes. The solid product obtained was ground into finer powder, filtered, washed with water and recrystallized from acetic acid; Yield 95%; needle-like yellow crystals; mp 252-253 °C (lit [36] 258-259 °C); IR ν_{max} (cm^{-1}) 3053, 2895, 1727, 1707, 1594, 1490, 1454, 1381, 1338, 1260, 1212, 1165, 1169, 1037,

1003, 927, 747, 696, 687, 667; ^1H NMR (400 MHz, CDCl_3) δ (ppm) 7.55 (m, 6H, ArCH), 7.21 (d, $J = 7.3$ Hz, 4H, 6' and 2'-ArCH), 4.10 (s, 2H, 5-CH₂); ^{13}C NMR (101 MHz, CDCl_3) δ (ppm) 181.64 (2-CS), 163.32 (4 and 5-CO), 138.75 (ArC), 129.68 (ArCH), 129.20 (ArCH), 128.64 (ArCH), 41.23 (5-CH₂).

4.1.3. 5-Benzylidenepyrimidines 2a-e [37]. A stirred mixture of compound **1a-d** (1.00 mmol) and benzaldehyde (1.00 mmol) in water (5 mL) was refluxed for 2 hours. After cooling, the formed product was filtered, washed with water, ethanol and diethyl ether to give the following 5-benzylidenepyrimidines:

4.1.3.1. 5-(2-Nitrobenzylidene)-1,3-diphenyl-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (2a). From 1,3-diphenylthiobarbituric acid (**1a**) and 2-nitrobenzaldehyde; Yield 90%; pale orange solid. mp 235 °C dec. (lit [60] 232 °C); IR ν_{max} (cm^{-1}) 3032, 1717, 1691, 1624, 1607, 1591, 1520, 1486, 1353, 1325, 1265, 1189, 1156, 788, 781, 752, 722, 692; ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ (ppm) 8.80 (s, 1H, 5-CCH), 8.19 (d, $J = 8.2$ Hz, 1H), 7.71 (t, $J = 7.5$ Hz, 1H), 7.59 (t, $J = 7.9$ Hz, 2H), 7.45 (t, $J = 7.6$ Hz, 2H), 7.40–7.28 (m, 6H), 7.20 (d, $J = 7.7$ Hz, 2H); ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ (ppm) 181.43 (2-CS), 160.28 (CO), 158.81 (CO), 155.03 (5-CCH), 146.20 (2'-ArC), 140.02 (ArC), 139.72 (ArC), 134.04 (5'-ArCH), 131.76 (1'-ArC), 130.40 (4'-ArCH), 130.16 (6'-ArCH), 129.05 (ArCH), 128.88 (ArCH), 128.85 (ArCH), 128.83 (ArCH), 128.28 (ArCH), 128.10 (ArCH), 124.12 (3'-ArCH), 121.30 (5-C).

4.1.3.2. 5-(2-Nitrobenzylidene)-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (2b). From thiobarbituric acid (**1b**) and 2-nitrobenzaldehyde; Yield 90%; pale yellow solid; mp 239–241 °C (lit [61] 246–250 °C); IR ν_{max} (cm^{-1}) 3255, 3156, 2876, 1718, 1692, 1626, 1540, 1513, 1439, 1351, 1288, 1203, 1141, 785, 759, 732; ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ (ppm) 12.56 (s, 1H, NH), 12.33 (s, 1H, NH), 8.63 (s, 1H, 5-CCH), 8.24 (dd, $J = 8.3$ and 1.0 Hz, 1H, 3'-ArCH), 7.80 (td, $J = 7.6$ and 1.1 Hz, 1H, 5'-ArCH), 7.72–7.66 (m, 1H, 4'-ArCH), 7.62 (dt, $J = 7.7$ and 1.2 Hz, 1H, 6'-ArCH); ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ (ppm) 179.05 (2-CS), 160.63 (CO), 159.18 (CO), 153.31 (5-CCH), 146.28 (2'-ArC), 133.77 (5'-ArCH), 131.58 (1'-ArC), 130.49 (4'-ArCH), 130.39 (6'-ArCH), 124.08 (3'-ArCH), 120.61 (5-C).

4.1.3.3. 1,3-Dimethyl-5-(2-nitrobenzylidene)pyrimidine-2,4,6(1H,3H,5H)-trione (2c). From 1,3-dimethylbarbituric acid (**1c**) and 2-nitrobenzaldehyde; Yield 97%; white solid; mp 158–159 °C (lit [62] 159–161 °C); IR ν_{max} (cm^{-1}) 3028, 2950, 1663, 1603, 1518, 1457, 1415, 1377, 1342, 1323, 1161, 1092, 1057, 787, 754, 737, 692; ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ (ppm) 8.71 (s, 1H, 5-CCH), 8.26 (dd, $J = 8.3$ and 1.2 Hz,

1H, 3'-ArCH), 7.80 (td, $J = 7.6$ and 1.3 Hz, 1H, 5'-ArCH), 7.69 (t, $J = 7.7$ Hz, 1H, 4'-ArCH), 7.53 (d, $J = 7.8$ Hz, 1H, 6'-ArCH), 3.25 (s, 3H, NCH₃), 3.06 (s, 3H, NCH₃); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 161.20 (CO), 159.96 (CO), 153.65 (5-CCH), 151.10 (2-CO), 146.15 (2'-ArC), 133.90 (5'-ArCH), 132.01 (1'-ArC), 130.09 (4'-ArCH), 130.02 (6'-ArCH), 124.06 (3'-ArCH), 120.14 (5-C), 28.44 (N-CH₃), 27.82 (N-CH₃).

4.1.3.4. 5-(2-Nitrobenzylidene)pyrimidine-2,4,6(1H,3H,5H)-trione (2d). From barbituric acid (**1d**) and 2-nitrobenzaldehyde; Yield 97%; white solid; mp 274-275 °C (lit [61] 274-276 °C); IR ν_{\max} (cm⁻¹) 3230, 3070, 2849, 1739, 1676, 1597, 1516, 1434, 1368, 1341, 1312, 1218, 848, 791, 803, 735, 710; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 11.49 (s, 1H, NH), 11.24 (s, 1H, NH), 8.60 (s, 1H, 5-CCH), 8.23 (d, $J = 7.9$ Hz, 1H, 3'-ArCH), 7.79 (t, $J = 7.4$ Hz, 1H, 5'-ArCH), 7.68 (t, $J = 7.7$ Hz, 1H, 4'-ArCH), 7.57 (d, $J = 7.7$ Hz, 1H, 6'-ArCH); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 162.38 (CO), 161.20 (CO), 152.45 (5-CCH), 150.26 (2-CO), 146.28 (2'-ArC), 133.76 (5'-ArCH), 131.72 (1'-ArC), 130.41 (4'-ArCH), 130.16 (6'-ArCH), 124.06 (3'-ArCH), 120.54 (5-C).

4.1.3.5. 5-(4-Methoxybenzylidene)pyrimidine-2,4,6(1H,3H,5H)-trione (2e). From barbituric acid (**1d**) and 4-methoxybenzaldehyde; Yield 92%; yellow solid; mp 286-289 °C (lit [37] 297-300 °C); IR ν_{\max} (cm⁻¹) 3046, 2838, 1745, 1698, 1651, 1601, 1567, 1532, 1509, 1444, 1431, 1393, 1355, 1316, 1266, 1215, 1179, 1117, 1041, 1001, 834, 790, 752, 691, 631; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 11.29 (s, 1H, NH), 11.17 (s, 1H, NH), 8.37 (d, $J = 8.9$ Hz, 2H, 2' and 6'-ArCH), 8.25 (s, 1H, 5-CCH), 7.06 (d, $J = 9.2$ Hz, 2H, 3' and 5'-ArCH), 3.87 (s, 3H, CH₃); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 163.91 (CO), 163.44 (CO), 162.18 (4'-ArCOCH₃), 154.94 (5-CCH), 150.20 (2-CO), 137.49 (2' and 6'-ArCH), 125.16 (1'-ArC), 115.56 (5-C), 113.95 (3' and 5'-ArCH), 55.70 (O-CH₃).

4.1.4. 5-(4-Methoxybenzyl)pyrimidine-2,4,6(1H,3H,5H)-trione (3) [38]. To a stirred solution of 5-(4-methoxybenzylidene)pyrimidine-2,4,6(1H,3H,5H)-trione (**2e**) (1.00 mmol) in ethanol (30 mL), were added little portions of sodium borohydride (3.00 mmol). The reaction was followed by TLC (ethyl acetate) and completed after 4 hours at room temperature. The solvent was evaporated to dryness, and water (20 mL) was added with the formation of a suspension, which was acidified with 1 M hydrochloric acid until pH 5. The formed solid was filtered and recrystallized from methanol. Yield 41%; white solid; mp 194-195 °C (lit. [63] 205-210 °C); IR ν_{\max} (cm⁻¹) 3224, 2966, 2929, 2835, 1743, 1669, 1608, 1566, 1504, 1421, 1390, 1324, 1299,

1273, 1249, 1175, 1113, 1093, 1033, 835, 797, 779, 751, 688, 668; ^1H NMR (400 MHz, DMSO- d_6) δ (ppm) 11.15 (s, 2H, NH), 6.99 (d, $J = 8.4$ Hz, 2H, 2' and 6'-Ar CH), 6.81 (d, $J = 8.5$ Hz, 2H, 3' and 5'-Ar CH), 3.82 (t, $J = 4.8$ Hz, 1H, 5- CH), 3.70 (s, 3H, CH_3), 3.19 (d, $J = 4.8$ Hz, 2H, 5- CCH_2); ^{13}C NMR (101 MHz, DMSO- d_6) δ (ppm) 170.04 (4 and 6- CO), 158.05 (4'-Ar C), 150.56 (2- CO), 130.03 (1'-Ar C), 128.93 (2' and 6'-Ar CH), 113.71 (3' and 5'-Ar CH), 54.94 (O- CH_3), 49.57 (5- CH), 32.83 (5- CHCH_2).

4.1.5. 5-Phenylcyanomethylpyrimidines 4a-b [39]. A stirred suspension of compound **1b-c** (1.00 mmol) and 4-bromobenzaldehyde (1.00 mmol) in water (5 mL) was refluxed for 2 hours. After cooling, sodium cyanide (1.00 mmol) was added and the resulting mixture was stirred at 70 °C. The reaction was followed by TLC (dichloromethane/methanol 20%) and completed after 1 hour. The reaction mixture was evaporated to dryness and the obtained solid was dissolved in an equimixture of methanol and ethyl acetate. The product formed after partial evaporation of the solvent was filtered and washed with diethyl ether to give the following 5-phenylcyanomethylpyrimidines:

4.1.5.1. Sodium 5-[(4-bromophenyl)(cyano)methyl]-6-oxo-2-thioxo-1,2,3,6-tetrahydropyrimidine-4-olate (4a). From thiobarbituric acid (**1b**); Yield 67%; white solid; mp 310 °C dec.; IR ν_{max} (cm^{-1}) 3092, 2881, 2245, 1592, 1525, 1485, 1418, 1300, 1173, 1010, 789; ^1H NMR (400 MHz, DMSO- d_6) δ (ppm) 10.72 (s, 2H, 2 \times NH), 7.47 (d, $J = 8.1$ Hz, 2H, 2 \times Ar CH), 7.31 (d, $J = 7.9$ Hz, 2 \times Ar CH , 2H), 5.24 (s, 1H, 5- CCH); ^{13}C NMR (101 MHz, DMSO- d_6) δ (ppm) 173.82 (2- CS), 161.41 (4 and 6- C), 137.61, 130.91 (2 \times Ar CH), 129.17 (2 \times Ar CH), 120.26, 119.59, 86.08 (5- C), 30.03 (5- CCH); ESI-HRMS Calcd for $[\text{M} + \text{Na}]^+$ $\text{C}_{12}\text{H}_7\text{N}_3\text{O}_2\text{Na}_2\text{SBr}$ 381.9232, found 381.9223.

4.1.5.2. Sodium 5-[(4-bromophenyl)(cyano)methyl]-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydropyrimidine-4-olate (4b) From 1,3-dimethylbarbituric acid (**1c**); Yield 77%; white solid; mp 273 °C dec.; IR ν_{max} (cm^{-1}) 3445, 2951, 2247 (CN), 1668 (C=O), 1583 (C=C), 1442, 1317, 1010, 776, ^1H NMR (400 MHz, DMSO- d_6) δ 7.45 (d, $J = 8.5$ Hz, 2H, 2 \times Ar CH), 7.31 (d, $J = 8.2$ Hz, 2H, 2 \times Ar CH), 5.35 (s, 1H, 5- CCH), 3.04 (s, 6H, 2 \times NCH_3); ^{13}C NMR (101 MHz, DMSO- d_6) δ 161.10 (4 and 6- C), 152.75 (2- CO), 138.46, 130.82 (2 \times Ar CH), 129.17 (2 \times Ar CH), 120.85, 119.37, 82.03 (5- C), 31.56 (5- CCH), 26.97 (2 \times NCH_3); ESI-HRMS Calcd for $[\text{M} + \text{Na}]^+$ $\text{C}_{14}\text{H}_{11}\text{N}_3\text{O}_3\text{Na}_2\text{Br}$ 393.9774, found 393.9760.

4.1.6. 5-Acetylpyrimidines (5a-c) [40]. A solution of compound **1a** or **1c-d** (1.00 mmol) and concentrated sulfuric acid (0.100 mmol) was refluxed in acetic anhydride (1.00 mmol) for 1 hour. The reaction was followed by TLC (dichloromethane/5% methanol). The reaction mixture was concentrated to half of the initial volume and poured onto ice. The formed solid was isolated, washed with hot water, dried and recrystallized from ethanol/water (2:1) to give the following 5-acetylpyrimidines:

4.1.6.1. 5-Acetyl-1,3-diphenyl-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (5a). From 1,3-diphenylthiobarbituric acid (**1a**); Yield 65%; brown needle crystals; mp 256-257 °C (lit [36] 258-259 °C). IR ν_{\max} (cm⁻¹) 3051, 3014, 1701, 1635, 1595, 1557, 1488, 1452, 1414, 1364, 1320, 1271, 1240, 1187, 1161, 1094, 1004, 967, 914, 828, 781, 746, 730, 639, 652, 612; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 7.48 (t, *J* = 7.4 Hz, 4H, 3' and 5'-ArCH), 7.39 (t, *J* = 7.7 Hz, 2H, 4'-ArCH), 7.30 (d, *J* = 7.7 Hz, 4H, 2' and 6'-ArCH), 2.66 (s, 3H, 5-CCCH₃); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 196.90 (5-CCCH₃), 179.97 (2-C_S), 139.68 (1'-ArC), 129.54 (2' and 6'-ArCH), 129.32 (3' and 5'-ArCH), 128.79 (4'-ArCH), 98.72 (5-C), 25.06 (5-CCCH₃).

4.1.6.2. 5-Acetyl-1,3-dimethylpyrimidine-2,4,6(1H,3H,5H)-trione (5b). From 1,3-dimethylbarbituric acid (**1c**); Yield 45%; golden solid; mp 91-92 °C (lit [64] 96.5-98.5 °C); IR ν_{\max} (cm⁻¹) 3011, 2963, 1721 (C=O), 1656 (C=O), 1557, 1494, 1455, 1364, 1337, 1274, 1221, 1164, 1017, 988, 876, 787, 754, 676; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 3.18 (s, 6H, 2 × NCH₃), 2.63 (s, 3H, 5-CCCH₃); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 194.96 (5-CCCH₃), 150.44 (2-CO), 96.36 (5-C), 28.06 (2 × N-CH₃), 24.55 (5-CCCH₃); ESI-HRMS Calcd for [M + H]⁺ C₈H₁₁N₂O₄ 199.0713, found 199.0726.

4.1.6.3. 5-Acetylpyrimidine-2,4,6(1H,3H,5H)-trione (5c). From barbituric acid (**1d**); Yield 83%; brown solid; mp 305-307 °C (lit [65] 296-300 °C); IR ν_{\max} (cm⁻¹) 3280, 3202, 3115, 3029, 2776, 1779, 1732, 1688, 1625, 1515, 1463, 1380, 1249, 1207, 1114, 1061, 1028, 966, 800, 741, 650; ¹H NMR (400 MHz DMSO-*d*₆) δ (ppm) 11.78 (s, 1H, NH), 11.04 (s, 1H, NH), 2.58 (s, 3H, 5-CCCH₃); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 195.35 (5-CCCH₃), 149.52 (2-CO), 95.89 (5-C), 24.29 (5-CCCH₃).

4.1.7. 5-Hydrazinylethylidenepyrimidines (6a-g) [40]. A stirred suspension of 5-acetylpyrimidine **5a-c** (1.00 mmol) and the appropriate hydrazine (1.00 mmol) in methanol (20 mL) was refluxed overnight. The formed solid was filtrated, washed with hot water and methanol to give the following 5-hydrazinylethylidenepyrimidines:

4.1.7.1. 5-(1-Hydrazinylethylidene)-1,3-diphenyl-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (6a).

From 5-acetyl-1,3-diphenyl-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (**5a**) and hydrazine hydrate; Yield 88%; brown solid; mp 242-243 °C; IR ν_{\max} (cm⁻¹) 3331, 3246, 3047, 1662, 1620, 1593, 1552, 1489, 1454, 1410, 1363, 1355, 1311, 1298, 1284, 1175, 1122, 1066, 1021, 977, 817, 800, 772, 752, 720, 692, 607; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 13.35 (s, 1H, 5-CCNH), 7.44-7.20 (m, 12H, ArCH and NH₂), 2.61 (s, 3H, 5-CCCH₃); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 178.47 (2-C_S), 166.18 (5-CCCH₃), 162.03 (4 and 6-CO), 140.66 (1''-ArC), 129.22 (2'' and 6''-ArCH), 128.63 (3'' and 5''-ArCH), 127.48 (4''-ArCH), 89.19 (5-C), 15.91 (5-CCCH₃); ESI-HRMS Calcd for [M + H]⁺ C₁₈H₁₇N₄O₂S 353.1067, found 353.1069.

4.1.7.2. 5-(1-Hydrazinylethylidene)pyrimidine-2,4,6(1H,3H,5H)-trione (6b). From 5-acetylpyrimidine-2,4,6(1H,3H,5H)-trione (**5c**) and hydrazine hydrate; Yield 86%; yellow solid; mp 300 °C dec.; IR ν_{\max} (cm⁻¹) 3344, 3246, 3004, 1639, 1647, 1609, 1573, 1435, 1374, 1346, 1262, 1242, 1144, 1093, 1037, 977, 923, 872, 797, 781, 755, 672; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 13.19 (s, 1H, 5-CCNH), 10.34 (s, 2H, 2 × NH), 5.55 (s, 2H, NH₂), 2.61 (s, 3H, 5-CCCH₃); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 168.35 (5-CCCH₃), 165.64 (4 and 6-CO), 149.87 (2-CO), 86.07 (5-C), 15.19 (5-CCCH₃); ESI-HRMS Calcd for [M + Na]⁺ C₆H₈N₄O₃Na 207.0489, found 207.0493.

4.1.7.3. 1,3-Dimethyl-5-(1-(2-phenylhydrazinyl)ethylidene)pyrimidine-2,4,6(1H,3H,5H)-trione (6c).

From 5-acetyl-1,3-dimethylpyrimidine-2,4,6(1H,3H,5H)-trione (**5b**) and phenylhydrazine; Yield 50%; white needle-like crystals; mp 182-184 °C; IR ν_{\max} (cm⁻¹) 3250, 3111, 2997, 2950, 1704, 1672, 1600, 1561, 1459, 1415, 1374, 1355, 1306, 1252, 1214, 1157, 1123, 1053, 1026, 971, 816, 736, 749, 690, 641; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 13.36 (s, 1H, 5-CCNH), 8.63 (s, 1H, 1'-ArCNH), 7.27 (t, *J* = 7.8 Hz, 2H, 3' and 5'-ArCH), 6.89 (t, *J* = 7.4 Hz, 1H, 4'-ArCH), 6.77 (d, *J* = 7.4 Hz, 2H, 2' and 6'-ArCH), 3.17 (s, 6H, 2 × NCH₃), 2.70 (s, 3H, 5-CCCH₃); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 174.83 (5-CCCH₃) 150.66 (2-CO), 146.43 (1'-ArC), 129.39 (3' and 5'-ArCH), 120.71 (4'-ArCH), 112.76 (2' and 6'-ArCH), 88.57 (5-C), 27.50 (2 × NCH₃), 16.28 (5-CCCH₃); ESI-HRMS Calcd for [M + H]⁺ C₁₄H₁₇N₄O₃ 289.1295, found 289.1302.

4.1.7.4. 5-[1-(2-Phenylhydrazinyl)ethylidene]pyrimidine-2,4,6(1H,3H,5H)-trione (6d).

From 5-acetylpyrimidine-2,4,6(1H,3H,5H)-trione (**5c**) and phenylhydrazine; Yield 92%; dark white solid; mp 307-310 °C; IR ν_{\max} (cm⁻¹) 3267, 3173, 3108, 3064, 3010, 2828, 1712, 1662, 1628, 1601, 1567, 1498, 1458, 1429,

1407, 1376, 1359, 1306, 1255, 1241, 1181, 1152, 1048, 832, 799, 772, 746, 691, 651; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 13.26 (s, 1H, 5-CCNH), 10.65 (s, 2H, 2 × NH), 8.54 (s, 1H, 1'-ArCNH), 7.26 (t, *J* = 8.0 Hz, 2H, 3' and 5'-ArCH), 6.89 (t, *J* = 7.8 Hz, 1H, 4'-ArCH), 6.76 (d, *J* = 7.3 Hz, 2H, 2' and 6'-ArCH), 2.67 (s, 3H, 5-CCCH₃); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 174.87 (5-CCCH₃), 149.72 (2-CO), 146.59 (1'-ArC), 129.39 (3' and 5'-ArCH), 120.66 (4'-ArCH), 112.75 (2' and 6'-ArCH), 88.20 (5-C), 15.69 (5-CCCH₃); ESI-HRMS Calcd for [M + H]⁺ C₁₂H₁₃N₄O₃ 261.0982, found 261.0982.

4.1.7.5. 1,3-Dimethyl-5-[1-[2-(4-nitrophenyl)hydrazinyl]ethylidene]pyrimidine-2,4,6(1H,3H,5H)-trione (6e). From 5-acetyl-1,3-dimethylpyrimidine-2,4,6(1H,3H,5H)-trione (**5b**) and 4-nitrophenylhydrazine; Yield 67%; brick-colored solid; mp 228-232 °C; IR ν_{max} (cm⁻¹) 3327, 3254, 2948, 2602, 2432, 2160, 1706, 1612, 1524, 1498, 1455, 1416, 1376, 1357, 1321, 1299, 1270, 1202, 1176, 1104, 971, 836, 797, 794, 690, 658; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 13.29 (s, 1H, 5-CCNH), 9.76 (s, 1H, 1'-ArCNH), 8.16 (d, *J* = 9.8 Hz, 2H, 3' and 5'-ArCH), 6.85 (d, *J* = 9.1 Hz, 2H, 2' and 6'-ArCH), 3.19 (s, 6H, 2 × NCH₃), 2.66 (s, 3H, 5-CCCH₃); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 175.81 (1'-ArC), 152.80 (5-CCCH₃), 151.07 (2-CO), 140.09 (4'-ArC), 126.51 (3' and 5'-ArCH), 111.82 (2' and 6'-ArCH), 89.98 (5-C), 28.03 (2 × N-CH₃), 16.77 (5-CCCH₃); ESI-HRMS Calcd for [M + H]⁺ C₁₄H₁₆N₅O₅ 334.1146, found 334.1148.

4.1.7.6. 5-[1-[2-(2,4-Dinitrophenyl)hydrazinyl]ethylidene]-1,3-dimethylpyrimidine-2,4,6(1H,3H,5H)-trione (6f). From 5-acetyl-1,3-dimethylpyrimidine-2,4,6(1H,3H,5H)-trione (**5b**) and 2,4-dinitrophenylhydrazine; Yield 83%; mp 245-247 °C; IR ν_{max} (cm⁻¹) 3362, 3093, 2952, 1705, 1650, 1615, 1594, 1558, 1538, 1509, 1455, 1360, 1332, 1315, 1275, 1237, 1207, 1157, 1142, 1114, 1060, 972, 923, 850, 817, 754, 744, 715, 651, 620; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 13.25 (s, 1H, 5-CCNH), 10.64 (s, 1H, 1'-ArCNH), 8.89 (d, *J* = 2.7 Hz, 1H, 3'-ArCH), 8.38 (dd, *J* = 9.4 and 2.7 Hz, 1H, 5'-ArCH), 7.17 (d, *J* = 9.5 Hz, 1H, 6'-ArCH), 3.20 (s, 6H, 2 × NCH₃), 2.65 (s, 3H, 5-CCCH₃); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 176.01 (5-CCCH₃), 150.59 (2-CO), 146.98 (1'-ArC), 137.80 (4'-ArC), 130.38 (2'-ArC), 130.37 (5'-ArCH), 122.88 (3'-ArCH), 115.37 (6'-ArCH), 90.30 (5-C), 27.65 (2 × N-CH₃), 16.73 (5-CCCH₃); ESI-HRMS Calcd for [M + Na]⁺ C₁₂H₁₀N₆O₇Na 373.0503, found 373.0498.

4.1.7.7. 5-[1-[2-(2,4-Dinitrophenyl)hydrazinyl]ethylidene]pyrimidine-2,4,6(1H,3H,5H)-trione (6g). From 5-acetylpyrimidine-2,4,6(1H,3H,5H)-trione (**5c**) and 2,4-dinitrophenylhydrazine; Yield 87%; mp 342 °C dec.;

IR ν_{\max} (cm⁻¹) 3325, 3231, 3094, 3033, 2797, 1710, 1682, 1634, 1616, 1591, 1505, 1455, 1334, 1313, 1272, 1234, 1175, 1143, 1127, 1059, 1035, 925, 912, 805, 765, 740, 714, 661, 634; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 13.22 (s, 1H, 5-CCNH), 10.95 (s, 1H, NH), 10.67 (s, 1H, NH), 10.57 (s, 1H, 1'-ArCNH), 8.89 (d, *J* = 2.6 Hz, 1H, 3'-ArCH), 8.38 (dd, *J* = 9.5 and 2.6 Hz, 1H, 5'-ArCH), 7.20 (d, *J* = 9.5 Hz, 1H, 6'-ArCH), 2.62 (s, 3H, 5-CCCH₃); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 175.82 (5-CCCH₃), 149.63 (2-CO), 147.10 (1'-ArC), 137.79 (4'-ArC), 130.89 (2'-ArC), 130.42 (5'-ArC), 122.89 (3'-ArCH), 115.45 (6'-ArCH), 89.99 (5-C), 16.08 (5-CCCH₃); ESI-HRMS Calcd for [M + H]⁺ C₁₄H₁₅N₆O₇ 379.0997, found 379.0992.

4.1.8. 5-Phenylaminomethylenepyrimidines (7a-j). Obtained from the corresponding compounds **1a-c** and anilines by the reaction conditions A or B adapted from the literature [27]. All products were isolated by hot filtration and washed with hot ethanol and diethyl ether to give the following 5-phenylaminomethylenepyrimidines.

Reaction conditions A: A stirred mixture of compound **1b-c** (1.00 mmol) and triethyl orthoformate (1.00 mmol) was refluxed in butan-1-ol (10 mL). *o*-Phenylenediamine (1.00 mmol) was added after approximately 1 hour, when the color of the reaction mixture changed to red. The resulting mixture was refluxed for 3 hours.

Reaction conditions B: A stirred mixture of compound **1a-d** (1.00 mmol), appropriate aniline (1.00 mmol) and triethyl orthoformate (1 mL) was refluxed in butan-1-ol (10 mL) for 4 hours.

4.1.8.1. 5-[[2-Aminophenyl]amino]methylene]-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (7a). From thiobarbituric acid (**1b**) by reaction conditions A; Yield 81%; dark yellow solid; mp 247-249 °C; IR ν_{\max} (cm⁻¹) 3429, 3342, 3098, 2998, 2894, 1688, 1616, 1587, 1538, 1471, 1330, 1309, 1162, 1008, 868, 776; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 12.14 (s, 1H, NH), 11.99 (s, 1H, NH), 11.89 (d, *J* = 10.8 Hz, 1H, 1'-Ar-NH), 8.43 (d, *J* = 10.8 Hz, 1H, 5-CCH), 7.35 (d, *J* = 7.8 Hz, 1H, 6'-ArCH), 7.05 (t, *J* = 7.6 Hz, 1H, 4'-ArCH), 6.87 (d, *J* = 7.7 Hz, 1H, 3'-ArCH), 6.73 (t, *J* = 7.5 Hz, 1H, 5'-ArCH), 5.20 (s, 2H, 1'-Ar-NH₂); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 177.70 (2-CS), 164.03 (CO), 161.64 (CO), 153.24 (5-CCH), 140.40 (2'-ArC), 127.71 (4'-ArCH), 126.02 (1'-ArC), 119.71 (6'-ArCH), 118.37 (5'-ArCH), 117.62 (3'-ArCH), 93.81 (5-C); ESI-HRMS Calcd for [M + Na]⁺ C₁₁H₁₀N₄O₂NaS 285.0417, found 285.0407.

4.1.8.2. 5-[[2-Chloro-5-nitrophenyl]amino]methylene]-2-thioxodihydropyrimidine-4,6(1H,5H)-dione

(7b). From thiobarbituric acid (**1b**) and 2-chloro-5-nitroaniline by reaction conditions B; Yield 97%; dark white solid; mp 348-349 °C; IR ν_{\max} (cm⁻¹) 3074, 2891, 1686, 1620, 1592, 1576, 1517, 1458, 1345, 1317, 1301, 1161, 1053, 1003, 848, 795, 740; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 12.57 (d, *J* = 13.1 Hz, 1H, 1'-Ar-NH), 12.37 (s, 1H, NH), 12.20 (s, 1H, NH), 8.91 (d, *J* = 13.0 Hz, 1H, 5-CCH), 8.76 (d, *J* = 2.6 Hz, 1H, 6'-CH), 8.06 (dd, *J* = 8.8 and 2.6 Hz, 1H, 4'-CH), 7.89 (d, *J* = 8.8 Hz, 1H, 3'-CH); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 178.04 (2-CS), 164.56 (CO), 161.06 (CO), 152.92, 147.29, 136.10, 131.07, 129.64, 120.96, 113.70, 96.17 (5-C). ESI-HRMS Calcd for [M + H]⁺ C₁₁H₈ClN₄O₄S 326.9949, found 326.9947.

4.1.8.3. 5-[[2-Chloro-5-nitrophenyl]amino]methylene]-1,3-dimethylpyrimidine-2,4,6(1H,3H,5H)-trione

(7c). From 1,3-dimethylbarbituric acid (**1c**) and 2-chloro-5-nitroaniline by reaction conditions B; Yield 92%; white solid; mp 257-259 °C; IR ν_{\max} (cm⁻¹) 3068, 2956, 1731, 1631, 1599, 1573, 1528, 1498, 1466, 1419, 1344, 1293, 1204, 1086, 1053, 1000, 882, 825, 740; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 12.52 (s, 1H, 1'-Ar-NH), 8.93 (s, 1H, 5-CCH), 8.74 (d, *J* = 2.4 Hz, 1H, 6'-Ar-CH), 8.06 (dd, *J* = 8.8 and 2.4 Hz, 1H, 4'-Ar-CH), 7.89 (d, *J* = 8.8 Hz, 1H, 3'-Ar-CH), 3.23 (s, 6H, 2 × NCH₃); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 164.67 (CO), 161.38 (CO), 152.26, 151.14 (2-CO), 147.31, 136.26, 131.02, 129.46, 120.59, 113.26, 95.00 (5-C), 27.66 (NCH₃), 27.15 (NCH₃); ESI-HRMS Calcd for [M + H]⁺ C₁₃H₁₂ClN₄O₅ 339.0490, found 339.0485.

4.1.8.4. 5-[[2-Chloro-4-nitrophenyl]amino]methylene]-2-thioxodihydropyrimidine-4,6(1H,5H)-dione

(7d). From thiobarbituric acid (**1b**) and 2-chloro-4-nitroaniline by reaction conditions B; Yield 91%; yellow solid; mp 365 °C dec.; IR ν_{\max} (cm⁻¹) 3332, 3053, 2880, 1698, 1650, 1575, 1506, 1457, 1347, 1302, 1288, 1267, 1139, 815, 744, 731; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 12.63 (d, *J* = 12.9 Hz, 1H, 1'-Ar-NH), 12.44 (s, 1H, NH), 12.27 (s, 1H, NH), 8.83 (d, *J* = 12.9 Hz, 1H, 5-CCH), 8.48 (d, *J* = 2.2 Hz, 1H, 3'-ArCH), 8.25 (dd, *J* = 9.2 and 1.9 Hz, 1H, 5'-ArCH), 8.19 (d, *J* = 9.2 Hz, 1H, 6'-ArCH); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 178.13 (2-CS), 164.48 (CO), 160.99 (CO), 151.47 (5-CCH), 144.11, 140.41, 125.45, 124.03, 123.08, 117.96, 97.31 (5-C); ESI-HRMS Calcd for [M + H]⁺ C₁₁H₈ClN₄O₄S 326.9949, found 326.9947.

4.1.8.5. 5-[[2-Chlorophenyl]amino]methylene]-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (7e). From

thiobarbituric acid (**1b**) and 2-chloroaniline by reaction conditions B; Yield 93%; pale yellow solid; mp 357-358 °C; IR ν_{\max} (cm⁻¹) 3106, 3008, 2894, 2562, 1680, 1621, 1599, 1588, 1571, 1536, 1496, 1442, 1316, 1285,

1157, 1053, 1000, 854, 802, 767; ^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ (ppm) 12.53 (d, $J = 13.4$ Hz, 1H, 1'-Ar-NH), 12.31 (s, 1H, NH), 12.14 (s, 1H, NH), 8.74 (d, $J = 13.4$ Hz, 1H, 5-CCH), 7.93 (d, $J = 8.1$ Hz, 1H, ArCH), 7.62 (dd, $J = 8.0$ and 1.1 Hz, 1H, ArCH), 7.45 (t, $J = 7.7$ Hz, 1H, ArCH), 7.29 (td, $J = 7.7$ and 1.1 Hz, 1H, ArCH); ^{13}C NMR (101 MHz, $\text{DMSO-}d_6$) δ (ppm) 177.93 (2-CS), 164.49 (CO), 161.26 (CO), 151.86 (5-CCH), 134.81, 130.00, 128.82, 127.22, 123.15, 118.18, 95.27 (5-C); ESI-HRMS Calcd for $[\text{M} + \text{H}]^+$ $\text{C}_{11}\text{H}_9\text{ClN}_3\text{O}_2\text{S}$ 282.0098, found 282.0095.

4.1.8.6. 5-[[[(2-Nitrophenyl)amino]methylene]-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (7f). From thiobarbituric acid (**1b**) and 2-nitroaniline by reaction conditions B; Yield 98%; yellow solid; mp 330 °C dec.; IR ν_{max} (cm^{-1}) 3121, 3062, 2899, 1684, 1633, 1587, 1567, 1532, 1511, 1436, 1337, 1299, 1261, 1151, 991, 856, 790, 740; ^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ (ppm) 13.33 (d, $J = 13.3$ Hz, 1H, 1'-Ar-NH), 12.32 (s, 1H, NH), 12.18 (s, 1H, NH), 8.72 (d, $J = 13.3$ Hz, 1H, 5-CCH), 8.26 (dd, $J = 8.4$ and 1.0 Hz, 1H, ArCH), 8.09 (d, $J = 8.2$ Hz, 1H, ArCH), 7.86 (td, $J = 7.9$ and 1.1 Hz, 1H, ArCH), 7.47 (td, $J = 7.8$ and 1.0 Hz, 1H, ArCH); ^{13}C NMR (101 MHz, $\text{DMSO-}d_6$) δ (ppm) 178.10 (2-CS), 163.57 (CO), 161.35 (CO), 151.86 (5-CCH), 138.03, 136.23, 133.94, 126.20, 125.98, 119.60, 96.75 (5-C); ESI-HRMS Calcd for $[\text{M} + \text{H}]^+$ $\text{C}_{11}\text{H}_9\text{N}_4\text{O}_4\text{S}$ 293.0339, found 293.0336.

4.1.8.7. 2-[[[(4,6-Dioxo-1,3-diphenyl-2-thioxotetrahydropyrimidin-5(2H)-ylidene)methyl]amino]benzoic acid (7g). From 1,3-diphenylthiobarbituric acid (**1a**) and 2-aminobenzoic acid by reaction conditions B; Yield 83%; yellow solid; mp 322-324 °C; IR ν_{max} (cm^{-1}) 3400 to 2700 (broad), 3061, 1689, 1660, 1644, 1607, 1569, 1439, 1324, 1290, 1259, 1208, 1147, 1070, 753, 723, 691; ^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ (ppm) 13.82 (s, 1H, 2'-Ar-COOH), 13.57 (d, $J = 14.2$ Hz, 1H, 1'-Ar-NH), 8.83 (d, $J = 14.1$ Hz, 1H, 5-CCH), 8.03 (dd, $J = 7.9$ and 1.8 Hz, 1H, ArCH), 7.88 (d, $J = 8.4$ Hz, 1H, ArCH), 7.73 (td, $J = 7.6$ and 1.2 Hz, 1H, ArCH), 7.52-7.43 (m, 4H, Ar-CH), 7.42-7.31 (m, 5H, ArCH), 7.29 (d, 2H, $J = 7.2$ Hz, ArCH); ^{13}C NMR (101 MHz, $\text{DMSO-}d_6$) δ (ppm) 180.74 (2-CS), 167.30 (2'-Ar-COOH), 161.92 (CO), 161.05 (CO), 152.23 (5-CCH), 140.30, 139.66, 139.33, 134.58, 131.68, 129.27, 129.09, 128.90, 128.84, 128.00, 127.88, 125.81, 119.30, 117.50, 96.12 (5-C); ESI-HRMS Calcd for $[\text{M} + \text{Na}]^+$ $\text{C}_{24}\text{H}_{17}\text{N}_3\text{O}_4\text{NaS}$ 466.0832, found 466.0823.

4.1.8.8. 2-[[[(4,6-Dioxo-2-thioxotetrahydropyrimidin-5(2H)-ylidene)methyl]amino]benzoic acid (7h). From thiobarbituric acid (**1b**) and 2-aminobenzoic acid by reaction conditions B; Yield 97%; dark white solid;

mp 365 °C dec. (lit [27] 340-344 °C); IR ν_{\max} (cm⁻¹) 3142 to 2511 (broad), 3142, 3041, 2966, 2892, 1676, 1602, 1581, 1527, 1457, 1437, 1348, 1321, 1288, 1251, 1158, 1008, 858, 757; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 13.86 (s, 1H, 2'-Ar-COOH), 13.53 (d, *J* = 14.0 Hz, 1H, 1'-Ar-NH), 12.14 (s, 1H, NH), 12.05 (s, 1H, NH), 8.67 (d, *J* = 14.0 Hz, 1H, 5-CCH), 8.03 (d, *J* = 7.7 Hz, 1H, ArCH), 7.86 (d, *J* = 8.4 Hz, 1H, ArCH), 7.70 (t, *J* = 7.5 Hz, 1H, ArCH), 7.35 (t, *J* = 7.6 Hz, 1H, ArCH); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 178.00 (2-CS), 167.61 (2'-Ar-COOH), 162.92 (CO), 161.78 (CO), 150.92 (5-CCH), 139.59, 134.56, 131.67, 125.50, 118.91, 117.30, 95.48 (5-C).

4.1.8.9. 2-[[1,3-Dimethyl-2,4,6-trioxotetrahydropyrimidin-5(2H)-ylidene)methyl]amino]benzoic acid (7i). From 1,3-dimethylbarbituric acid (**1c**) and 2-aminobenzoic acid by reaction conditions B; Yield 77%; pale yellow needle crystals; mp 314-315 °C (lit [66] 210 °C); IR ν_{\max} (cm⁻¹) 3300 to 2600 (broad), 3083, 1715, 1644, 1602, 1577, 1450, 1355, 1315, 1197, 1142, 1072, 1013, 869, 789, 752; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 13.60 (s, 1H, 2'-Ar-COOH), 13.46 (d, *J* = 14.1 Hz, 1H, 1'-Ar-NH), 8.67 (d, *J* = 13.9 Hz, 1H, 5-CCH), 8.01 (d, *J* = 7.7 Hz, 2H, ArCH), 7.79 (d, *J* = 8.4 Hz, 1H, ArCH), 7.69 (t, *J* = 7.7 Hz, 1H, ArCH), 7.32 (t, *J* = 7.5 Hz, 1H, ArCH), 3.18 (d, *J* = 3.1 Hz, 6H, 2 × NCH₃); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 167.58 (2'-Ar-COOH), 163.04 (CO), 162.06 (CO), 151.41 (5-CCH), 150.40 (2-CO), 139.81, 134.53, 131.66, 125.09, 118.69, 116.83, 94.19 (5-C), 27.64 (NCH₃), 27.03 (NCH₃).

4.1.8.10. 2-[[2,4,6-Trioxotetrahydropyrimidin-5(2H)-ylidene)methyl]amino]benzoic acid (7j). From barbituric acid (**1d**) and 2-aminobenzoic acid by reaction conditions B; Yield 84%; white solid; mp 365 °C dec. (lit [27] 310-314 °C); IR ν_{\max} (cm⁻¹) 3163 to 2514 (broad), 3136, 3084, 2815, 1729, 1673, 1614, 1593, 1573, 1503, 1458, 1439, 1420, 1350, 1324, 1290, 1255, 941, 856, 795, 758; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 13.75 (s, 1H, 2'-Ar-COOH), 13.38 (d, *J* = 13.9 Hz, 1H, 1'-Ar-NH), 10.96 (s, 1H, NH), 10.88 (s, 1H, NH), 8.62 (d, *J* = 13.8 Hz, 1H, 5-CCH), 8.02 (d, *J* = 7.6 Hz, 1H, ArCH), 7.81 (d, *J* = 8.4 Hz, 1H, ArCH), 7.69 (t, *J* = 7.9 Hz, 1H, ArCH), 7.32 (t, *J* = 7.6 Hz, 1H, ArCH); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 167.69 (2'-Ar-COOH), 165.08 (CO), 163.72 (CO), 150.71 (5-CCH), 149.85 (2-CO), 139.97, 134.57, 131.69, 124.97, 118.57, 116.87, 94.26 (5-C).

4.1.9. 1-[(Pyrimidin-5-ylidene)methyl]ureas 8a-g. A stirred mixture of compound **1a-d** (1.00 mmol), urea/thiourea (1.00 mmol) and triethyl orthoformate (1.50 mmol) was refluxed in butan-1-ol (10 mL) for 4

hours. The formed product was filtered and washed with diethyl ether to give the following 1-(pyrimidin-5-ylidene)ureas:

4.1.9.1. 1-[(4,6-Dioxo-2-thioxotetrahydropyrimidin-5(2H)-ylidene)methyl]urea (8a). From thiobarbituric acid (**1b**) and urea; Yield 77 %; reddish solid; mp 298-300 °C; IR ν_{\max} (cm⁻¹) 3406, 3147, 2889, 1730, 1693, 1643, 1529, 1375, 1259, 1149, 815, 757 ; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 12.19 (s, 1H, NH), 12.13 (1H, s, NH), 11.24 (d, *J* = 13.2 Hz, 1H, 5-CCHNH), 8.54 (d, *J* = 13.1 Hz, 1H, 5-CCH), 7.88 (s, 1H, NH₂), 7.56 (s, 1H, NH₂); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 178.46 (2-CS), 163.01 (CO), 161.73 (CO), 152.03 (NHCNH₂), 151.03 (5-CCH), 96.12 (5-C); ESI-HRMS Calcd for [M + H]⁺ C₆H₇N₄O₃S 215.0239, found 215.0237.

4.1.9.2. 1-[(1,3-Dimethyl-2,4,6-trioxotetrahydropyrimidin-5(2H)-ylidene)methyl]urea (8b). From 1,3-dimethylbarbituric acid (**1c**) and urea; Yield 52%; yellow solid; mp 260-261 °C; IR ν_{\max} (cm⁻¹) 3371, 3278, 3197, 1704, 1666, 1635, 1560, 1406, 1371, 1255, 1085; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 11.23 (d, *J* = 13.1 Hz, 1H, 5-CCHNH), 8.61 (d, *J* = 13.1 Hz, 1H, 5-CCH), 7.82 (s, 1H, NH₂), 7.47 (s, 1H, NH₂), 3.17 (d, *J* = 3.7 Hz, 6H, 2 × N-CH₃); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 163.24 (CO), 161.95 (CO), 152.17 (NHCNH₂), 151.27 (5-CCH), 150.67 (2-CO), 94.90 (5-C), 27.66 (N-CH₃), 27.02 (N-CH₃); ESI-HRMS Calcd for [M + H]⁺ C₈H₁₁N₄O₄ 227.0780, found 227.0780.

4.1.9.3. 1-[(2,4,6-Trioxotetrahydropyrimidin-5(2H)-ylidene)methyl]urea (8c). From barbituric acid (**1d**) and urea; Yield 71%; light-brown solid; mp 248-250 °C; IR ν_{\max} (cm⁻¹) 3411, 3172, 1733, 1703, 1629, 1569, 1384, 1305, 1272, 792; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 11.10 (d, *J* = 13.1 Hz, 1H, 5-CCHNH), 11.06 (s, 1H, NH), 10.97 (s, 1H, NH), 8.51 (d, *J* = 13.1 Hz, 1H, 5-CCH), 7.83 (s, 1H, NH₂), 7.46 (s, 1H, NH₂); ¹³C NMR (101 MHz, DMSO-*d*₆) δ (ppm) 165.22 (CO), 163.57 (CO), 152.33 (NHCNH₂), 150.61 (5-CCH), 149.91 (2-CO), 95.08 (5-C); ESI-HRMS Calcd for (M⁺ + Na) C₆H₆N₄O₄Na 221.0281, found 221.0282.

4.1.9.4. 1-[(4,6-Dioxo-1,3-diphenyl-2-thioxotetrahydropyrimidin-5(2H)-ylidene)methyl]thiourea (8d). From 1,3-diphenylthiobarbituric acid (**1a**) and thiourea; Yield 85%; orange solid; mp 259-261 °C (lit 263-264 °C [67]); IR ν_{\max} (cm⁻¹) 3442, 3263, 3172, 3051, 1685, 1654, 1591, 1487, 1402, 1321, 1217, 1190, 1153, 823, 688; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 12.13 (d, *J* = 11.7 Hz, 1H, 5-CCHNH), 9.72 (s, 1H, NH₂), 9.44

(s, 1H, NH_2), 9.28 (d, $J = 11.7$ Hz, 1H, 5-C $\underline{\text{C}}\underline{\text{H}}$), 7.49-7.27 (m, 10H, Ar $\underline{\text{C}}\underline{\text{H}}$); ^{13}C NMR (101 MHz, DMSO- d_6) δ (ppm) 181.16 (NHC $\underline{\text{N}}\underline{\text{H}}_2$), 180.79 (2- $\underline{\text{C}}\underline{\text{S}}$), 162.01 ($\underline{\text{C}}\underline{\text{O}}$), 160.72 ($\underline{\text{C}}\underline{\text{O}}$), 153.88 (5-C $\underline{\text{C}}\underline{\text{H}}$), 139.94 (Ar $\underline{\text{C}}$), 139.29 (Ar $\underline{\text{C}}$), 128.96 (2 \times Ar $\underline{\text{C}}\underline{\text{H}}$), 128.91 (2 \times Ar $\underline{\text{C}}\underline{\text{H}}$), 128.80 (2 \times Ar $\underline{\text{C}}\underline{\text{H}}$), 128.75 (2 \times Ar $\underline{\text{C}}\underline{\text{H}}$), 128.00 (Ar $\underline{\text{C}}\underline{\text{H}}$), 127.94 (Ar $\underline{\text{C}}\underline{\text{H}}$), 98.49 (5- $\underline{\text{C}}$); ESI-HRMS Calcd for $[\text{M} + \text{H}]^+$ $\text{C}_{18}\text{H}_{15}\text{N}_4\text{O}_2\text{S}_2$ 383.0636, found 383.0635.

4.1.9.5. **1-[(4,6-Dioxo-2-thioxotetrahydropyrimidin-5(2H)-ylidene)methyl]thiourea (8e)**. From thiobarbituric acid (**1b**) and thiourea; Yield 74%; red solid; mp 252 °C dec.; IR ν_{max} (cm^{-1}) 3284, 3136, 1695, 1664, 1574, 1506, 1413, 1259, 1143, 1012, 997, 804; ^1H NMR (400 MHz, DMSO- d_6) δ (ppm) 12.32 (s, 1H, $\underline{\text{N}}\underline{\text{H}}$), 12.23 (s, 1H, $\underline{\text{N}}\underline{\text{H}}$), 11.94 (d, $J = 13.1$ Hz, 1H, 5-C $\underline{\text{C}}\underline{\text{H}}\underline{\text{N}}\underline{\text{H}}$), 9.70 (s, 1H, $\underline{\text{N}}\underline{\text{H}}_2$), 9.55 (s, 1H, $\underline{\text{N}}\underline{\text{H}}_2$), 9.11 (d, $J = 13.1$ Hz, 1H, 5-C $\underline{\text{C}}\underline{\text{H}}$); ^{13}C NMR (101 MHz, DMSO- d_6) δ (ppm) 181.31 (NHC $\underline{\text{N}}\underline{\text{H}}_2$), 178.48 (2- $\underline{\text{C}}\underline{\text{S}}$), 163.05 ($\underline{\text{C}}\underline{\text{O}}$), 161.50 ($\underline{\text{C}}\underline{\text{O}}$), 152.57 (5-C $\underline{\text{C}}\underline{\text{H}}$), 97.95 (5- $\underline{\text{C}}$); ESI-HRMS Calcd for $[\text{M} + \text{H}]^+$ $\text{C}_6\text{H}_7\text{N}_4\text{O}_2\text{S}_2$ 231.0010, found 231.0013.

4.1.9.6. **1-[(1,3-Dimethyl-2,4,6-trioxotetrahydropyrimidin-5(2H)-ylidene)methyl]thiourea (8f)**. From 1,3-dimethylbarbituric acid (**1c**) and thiourea; Yield 89%; yellow solid; mp 267-270 °C; IR ν_{max} (cm^{-1}) 3290, 3145, 1716, 1635, 1589, 1469, 1429, 1338, 1230, 1186, 1086, 798, 754; ^1H NMR (400 MHz, DMSO- d_6) δ (ppm) 11.95 (d, $J = 12.4$ Hz, 1H, 5-C $\underline{\text{C}}\underline{\text{H}}\underline{\text{N}}\underline{\text{H}}$), 9.59 (s, 1H, $\underline{\text{N}}\underline{\text{H}}_2$), 9.48 (s, 1H, $\underline{\text{N}}\underline{\text{H}}_2$), 9.19 (d, $J = 12.4$ Hz, 1H, 5-C $\underline{\text{C}}\underline{\text{H}}$), 3.19 (d, $J = 3.3$ Hz, 6H, 2 \times N- $\underline{\text{C}}\underline{\text{H}}_3$); ^{13}C NMR (101 MHz, DMSO- d_6) δ (ppm) 181.46 (NHC $\underline{\text{N}}\underline{\text{H}}_2$), 163.30 ($\underline{\text{C}}\underline{\text{O}}$), 161.70 ($\underline{\text{C}}\underline{\text{O}}$), 152.35 (5-C $\underline{\text{C}}\underline{\text{H}}$), 151.14 (2- $\underline{\text{C}}\underline{\text{O}}$), 95.78 (5- $\underline{\text{C}}$), 27.76 (N- $\underline{\text{C}}\underline{\text{H}}_3$), 27.17 (N- $\underline{\text{C}}\underline{\text{H}}_3$); ESI-HRMS Calcd for $[\text{M} + \text{H}]^+$ $\text{C}_8\text{H}_{11}\text{N}_4\text{O}_3\text{S}$ 243.0552, found 243.0551.

4.1.9.7. **1-[(2,4,6-Trioxotetrahydropyrimidin-5(2H)-ylidene)methyl]thiourea (8g)**. From barbituric acid (**1d**) and thiourea; Yield: 86%; light-brown solid; mp 375 °C dec.; IR ν_{max} (cm^{-1}) 3280, 3217, 3145, 3045, 2808, 1733, 1701, 1645, 1571, 1438, 1407, 1247, 1024, 783; ^1H NMR (400 MHz, DMSO- d_6) δ (ppm) 11.84 (d, $J = 12.3$ Hz, 1H, 5-C $\underline{\text{C}}\underline{\text{H}}\underline{\text{N}}\underline{\text{H}}$), 11.15 (s, 1H, $\underline{\text{N}}\underline{\text{H}}$), 11.03 (s, 1H, $\underline{\text{N}}\underline{\text{H}}$), 9.53 (s, 1H, $\underline{\text{N}}\underline{\text{H}}_2$), 9.45 (s, 1H, $\underline{\text{N}}\underline{\text{H}}_2$), 9.08 (d, $J = 12.3$ Hz, 1H, 5-C $\underline{\text{C}}\underline{\text{H}}$); ^{13}C NMR (101 MHz, DMSO- d_6) δ (ppm) 181.52 (NHC $\underline{\text{N}}\underline{\text{H}}_2$), 165.17 ($\underline{\text{C}}\underline{\text{O}}$), 163.22 ($\underline{\text{C}}\underline{\text{O}}$), 151.54 (5-C $\underline{\text{C}}\underline{\text{H}}$), 150.40 (2- $\underline{\text{C}}\underline{\text{O}}$), 96.96 (5- $\underline{\text{C}}$); ESI-HRMS Calcd for $[\text{M} + \text{H}]^+$ $\text{C}_6\text{H}_7\text{N}_4\text{O}_3\text{S}$ 215.0239, found 215.0238.

4.2. *In vitro* activity

4.2.1. *Xanthine oxidase inhibitory activity*

4.2.1.1. *Preparation of sample solutions*

All assayed compounds were dissolved in DMSO in a concentration of 10 mM for the initial screening and 50 mM for the concentration-response studies. A 10 mM xanthine stock solution was prepared in a 25 mM aqueous NaOH solution. All stock solutions were stored at 4 °C and the diluted solutions were prepared in 50 mM dihydrogen phosphate buffer (pH 7.4) before each experiment. The concentrations in each well were 30 μM for the initial screening as well as 0.1, 1, 7.5, 15, 30, 50 and 100 μM for the concentration-response studies. The final concentration of DMSO in each well was 1%, and no significant interference with the enzyme activity was observed (data not shown).

4.2.1.2. *Experimental procedure*

The xanthine oxidase (XO) activity was spectrophotometrically determined by quantifying the uric acid formation from xanthine adapting the methods of Zhao *et al.* [41] and Gupta *et al.* [68]. For each assay, 50 μL of test solution and 50 μL of a XO bovine serum suspension (0.1 U/mL) were added to each well of a 96-well plate followed by a pre-incubation at 37 °C for 5 minutes. The enzymatic reaction started after the addition of 150 μL of xanthine (0.42 mM) and further incubation at 37 °C during 10 min. The absorbances were measured at 295 nm and at every minute, with 20 seconds of a constant and automatic slow stirring before each reading. Dihydrogen phosphate buffer (50 mM, pH 7.4) was used as negative control and allopurinol as a positive control. In order to discount the absorbance of each compound at this wavelength, a blank sample containing 50 μL of test solution and 200 μL of buffer was performed. Three independent experiments, each one in triplicate, were performed. The percentage of enzyme inhibition for each compound was calculated according to the following formula:

$$\% \text{ of inhibition} = [1 - (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank of sample}}) / \text{Abs}_{\text{negative control}}] \times 100$$

The direct breakdown of uric acid by the synthesized BADs and TBADs **2-8** was spectrophotometrically studied in experimental conditions similar to the established in enzymatic inhibition assays. For each assay, 50

μL of test solution (60 μM) and 50 μL of uric acid (200 μM) were added to each well of a 96-well plate. The absorbances were measured at 295 nm after further incubation at 37 °C during 10 min. In order to discount the absorbance of each compound at this wavelength, a blank sample containing 50 μL of test solution and 50 μL of buffer was performed. Three independent experiments, each one in triplicate, were performed. No significant interference with uric acid levels was observed for any compound under study.

4.2.2. Radical scavenging activity

4.2.2.1. Preparation of sample solutions

All assayed compounds were dissolved in DMSO in a 10 mM concentration and stored at 4 °C. The diluted solutions were prepared in 99.5% ethanol before each experiment. The concentrations in each well were 30 μM for the initial screening as well as 1, 7.5, 15, 30, 50 and 100 μM for the concentration-response studies.

4.2.2.2. Experimental procedure

The radical scavenging activity was spectrophotometrically determined by the quantification of the DPPH reduction extension by the compounds under study, according to Zhao *et al.*[41] and Nicolaides *et al.* [42]. The reaction mixture contained 100 μL of the test solution and 100 μL of the DPPH solution (0.2 mM). The capacity of each compound to reduce DPPH was followed by measuring the absorbance at 517 nm after 20 and 60 minutes. The solutions remained in the absence of light between measurements. Ethanol was used as the negative control and Trolox as the positive control. To discount the absorbance of each compound at 517 nm, for each test compound a blank was performed where DPPH was replaced by ethanol. Three independent experiments were performed, each one in triplicate. The reduction capacity of DPPH was calculated according to the following formula:

$$\% \text{ Reduction} = [1 - (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{sample blank}} / \text{Abs}_{\text{negative control}})] \times 100$$

4.2.3. Antibacterial activity

4.2.3.1. Sample solutions, bacteria, stock and growth conditions

All the tested compounds were dissolved in DMSO in a concentration of 10 mM, stored at 4 °C, and diluted with culture medium before assays.

The following strains were used in the study: *Bacillus cereus* ATCC11778, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 13311 and *Acinetobacter baumannii* LMG 1025. All bacterial cultures were stocked at -80 °C in medium with 20% glycerol. The bacteria were sub-cultured on Müller Hinton Agar and incubated overnight at 37 °C prior to each antimicrobial assay.

4.2.3.2. Determination of minimum inhibitory concentration (MIC)

The antibacterial activity of the test compounds was studied by measurement of MIC by broth microdilution method according with CLSI guidelines (M7-A6). The compounds were two-fold serially diluted in a 96-well plate with Müller Hinton Broth (MHB) to a volume of 50 µL, to give a final concentration range from 200 to 1.56 µM. The maximum DMSO concentration was 2% (v/v). Bacterial suspensions were prepared from overnight culture using sterile saline solution, i.e. NaCl 0.85% (w/v), adjusted to 0.5 McFarland and diluted with MHB. Then 50 µL of suspension was added to each well to a final concentration of about 5×10^5 colony forming units/ml, and the plate was incubated at 37 °C for 24 h. Sterility, solvent and growth controls were included in the assays, also tetracycline was used as reference. The MIC was defined as the lowest concentration at which no growth was visually observed. Each test was carried out at least three independent times and modal MIC values were selected.

4.2.4. Cytotoxicity in human cell lines

4.2.4.1. Preparation of sample solutions

All the tested compounds were dissolved in DMSO in a concentration of 10 mM and stored at 4 °C. From this solution, several solutions of the compounds in study were prepared by their adequate dilutions in the complete culture medium before each experiment. The maximum DMSO concentration in the studies was 1% (v/v), a concentration with no significant effect on cell proliferation (data not shown).

4.2.4.2. Cells cultures

The cells used in this study were human breast cancer cells (MCF-7) and normal human dermal fibroblasts (NHDF) and were obtained from American Type Culture Collection (ATCC). These cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. MCF-7 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (10,000 units/mL penicillin, 10 mg/mL streptomycin and 25 µg/mL amphotericin B). The RPMI medium supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), HEPES (10 mM), sodium pyruvate (1 mM) and 1% antibiotic/antimycotic was used to culture NHDF cells. The cells used in the experiments were used in passages 26 to 31 (MCF-7) and 6 to 11 (NHDF).

4.2.4.3. MTT assay

The cell proliferation was evaluated by quantifying the extent of the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) according to a previously described procedure [52]. Cells were seeded in 96-well plates (2×10^4 cells/mL) in the culture medium and after 48 h of adherence they were treated with the different compounds and concentrations under study for 72 h. Untreated cells were used as negative control and 5-FU as positive control. At the end of incubation, the medium was removed and replaced with fresh culture medium and MTT solution [5 mg/mL in phosphate buffer saline (NaCl 137 mM, KCl 2.7 mM, Na₂HPO₄ 10 mM and KH₂PO₄ 1.8 mM), pH 7.4] and further incubated at 37 °C during 4 h. Then, the medium-containing MTT was removed and formazan crystals were dissolved with DMSO followed by absorbance readings at 570 nm. The cytotoxicity was expressed as the relative cell proliferation in percentage in comparison with the negative control cells.

4.2.5. Statistics

The results are expressed as mean values \pm standard error of the mean (SEM) and are representative of least two independent experiments. The difference between groups was considered statistically significant at $p < 0.05$ (Student's *t*-test). The IC₅₀ values were calculated by sigmoidal fitting analysis considering a 95% confidence interval.

4.3. *In silico* simulations

The structures of tested compounds were manually drawn in ChemBioDraw 13.0 software and the SMILES notation was obtained for each molecule. To calculate the parameters of Lipinski's rule of five, pharmacokinetic (absorption, distribution, metabolism and excretion) and toxicity properties, the SMILES notation in PkCSM online software (available from <http://bleoberis.bioc.cam.ac.uk/pkcsm/prediction>, accessed at 04.04.17) was employed [54].

Acknowledgments

This work is supported by FEDER funds through the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalization in Axis I - Strengthening research, technological development and innovation (Project POCI-01-0145-FEDER-007491) and National Funds by FCT - Foundation for Science and Technology (Project UID/Multi /00709/2013). The authors also acknowledge the contribution of Dr. Adriana Santos for the revision of written English.

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Figure Captions

Figure 1. Enamine **A** and imine **B** tautomeric forms.

Figure 2. In vitro XO inhibitory activity of compounds **2c**, **2d**, **6c-f** and allopurinol. Results are expressed as average values \pm standard error of the mean (SEM). A $p < 0.05$ versus negative control in the statistical significance analysis (Student's *t*-test) was observed for all compounds except for **6d**.

Figure 3. In vitro DPPH radical scavenging activity for 5-hydrazinylethylidenepyrimidines **6a-g**, 1-(pyrimidin-5-ylidene)ureas **8d**, **8f** and Trolox (positive control). Results are expressed as average values \pm SEM. A $p < 0.05$ versus negative control in the statistical significance analysis (Student's *t*-test) was observed for all compounds.

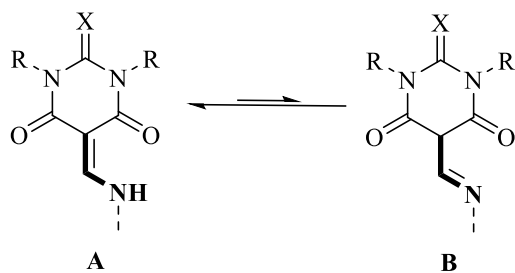
Figure 4. In vitro cytotoxic effect of compounds **6c**, **6d**, **7a-j** and 5-fluorouracil (5-FU) in normal human dermal fibroblasts (NHDF) and in mammalian breast cancer cells (MCF-7). Results are expressed as average values \pm SEM. A $p < 0.05$ versus negative control in the statistical significance analysis (Student's *t*-test) was observed for all compounds.

Table Captions

Table 1. In vitro IC₅₀ values for XO inhibition and DPPH radical scavenging activity of compounds **2c**, **2d**, **6c-e** and respective references allopurinol and Trolox.

Scheme Captions

Scheme 1. Chemical synthesis of BADs and TBADs **2-8**.



X = O, S; R = H, CH₃, Ph

Figure 1

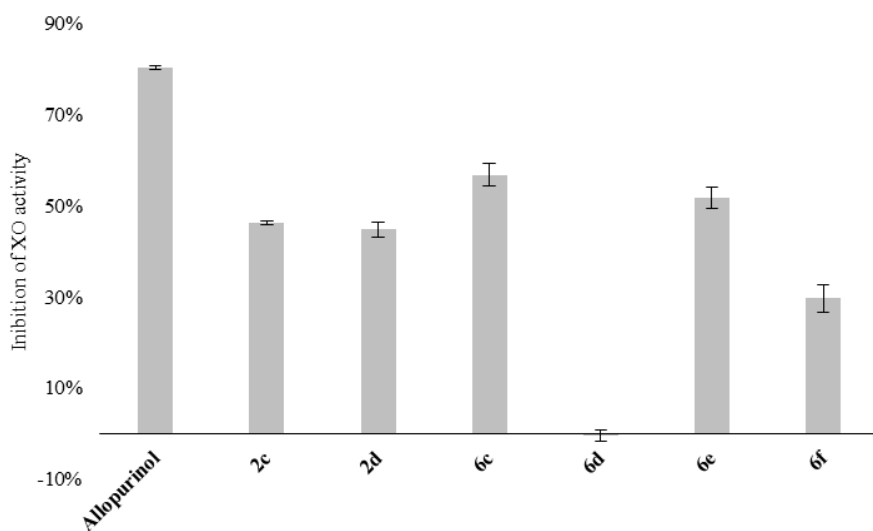


Figure 2

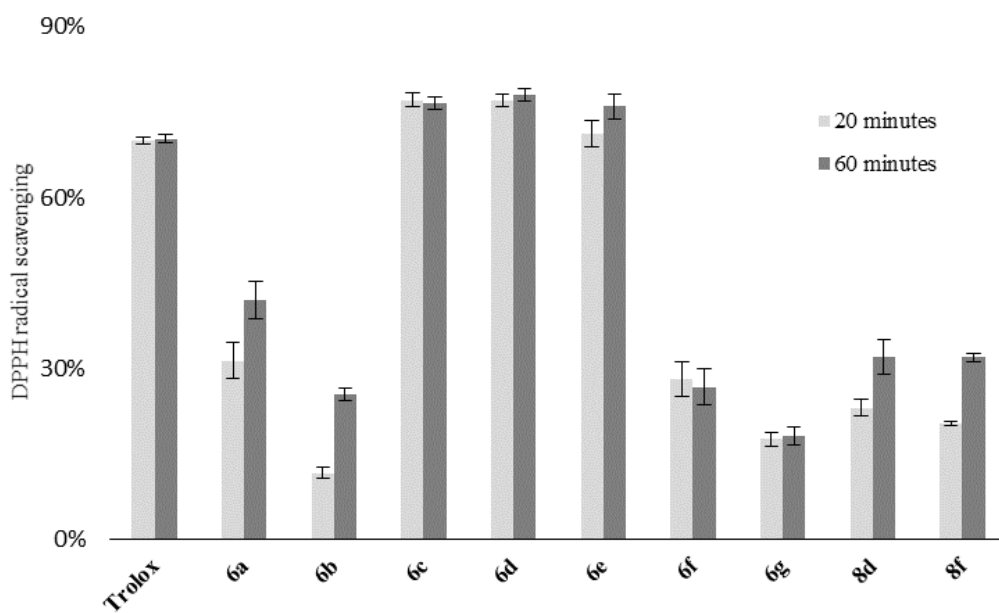


Figure 3

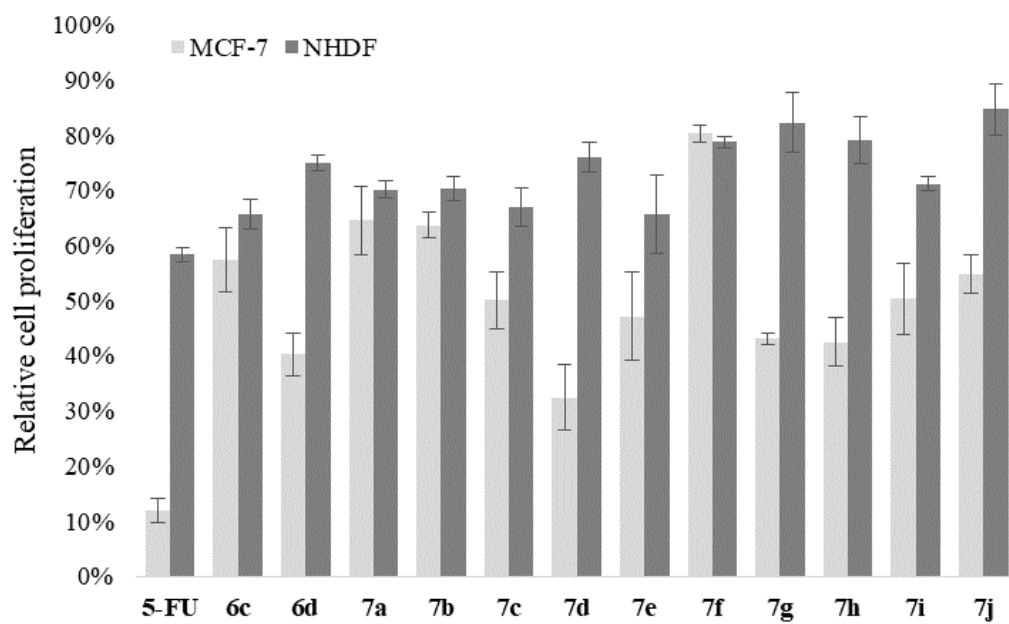


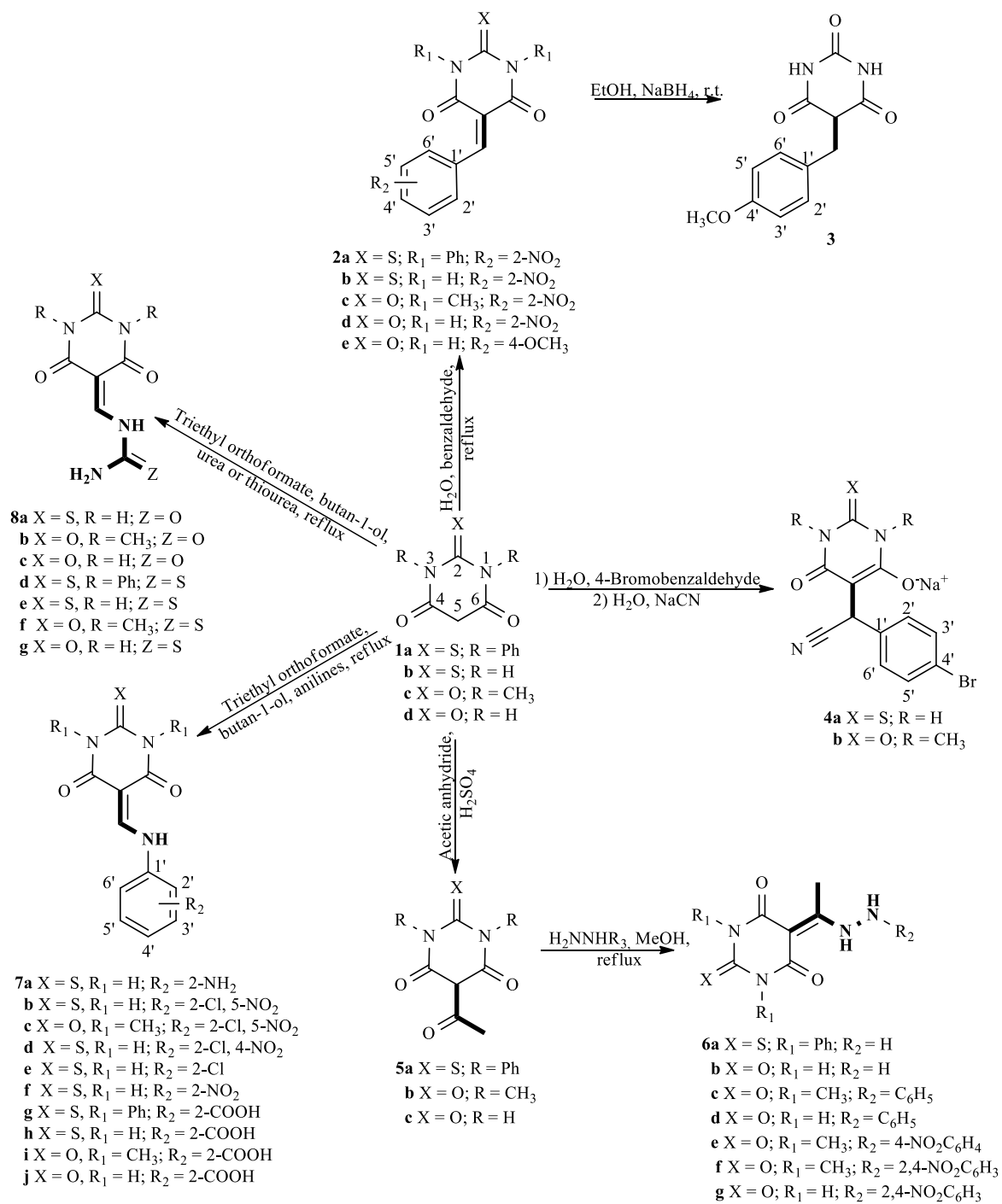
Figure 4

Table 1

Compound	XO (μM) ^a	DPPH (μM) ^a	
		20 min	60 min
2c	26.1	nd ^b	nd ^b
2d	31.5	nd ^b	nd ^b
6c	24.3	22.6	18.8
6d	nd ^b	23.9	20.4
6e	27.9	23.9	23.8
Allopurinol	3.2	nd ^b	nd ^b
Trolox	nd ^b	33.8	35.9

^aIC₅₀ values represent as mean of three determinations.

^b nd: not determined



Scheme 1