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Original article

Discovery and kinetic evaluation of 6-substituted 4-benzylthio-1,3,5-triazin-2(1*H*)-ones as inhibitors of cathepsin B

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ABSTRACT

Cathepsin B is a lysosomal cysteine protease that has various physiological and pathophysiological functions. We present here the discovery of 6-substituted 4-benzylthio-1,3,5-triazin-2(1*H*)-ones as inhibitors of cathepsin B, starting from screening of a library of variously 2,4,6-trisubstituted 1,3,5-triazines and 1,3,5-triazin-2(1*H*)-ones on three different human cathepsins. The synthesis and enzymatic evaluation of a focused library of new 1,3,5-triazin-2(1*H*)-ones is also described. The detailed kinetics analyses have shown that these compounds can act as reversible, partial mixed-type inhibitors of cathepsin B, with K_i and K_i' values in the low micromolar range. The inhibitory activities of selected compounds were also assessed against two related cysteine proteases, cathepsin H and cathepsin L, to estimate their selectivity; these compounds have a selective profile for catB and catL over catH.

1. Introduction

Cathepsin B (catB) (EC 3.4.22.1) is a lysosomal cysteine protease that belongs to the papain family (C1) of clan CA of the cysteine proteases [1]. It is unique among cathepsins as it exists in two conformations, which are responsible for endopeptidase and dipeptidyl carboxypeptidase activities [2]. In the exopeptidase conformation access of the extended substrates into the active site of catB is limited by a 21 amino acid insertion termed the occluding loop (Ile105-Thr125) [3] that is held on to the body of the enzyme by two salt bridges (Asp22-His110 and Asp224-Arg116). Additionally, the loop provides two His residues (His110 and His111) that bind the substrate's C-terminal carboxylate, enabling the exopeptidase activity [4] that has a pH optimum around 5 [5], suitable for lysosomal compartments. However, catB can also act as an endopeptidase since the occluding loop is flexible and can move away from the active-site cleft [6]. It appears that the conformation of the loop is pH dependent [3], with a prevalence of endopeptidase activity at neutral pH (typical of the environment of the membrane-bound or extracellular catB), suggesting an extralysosomal as well as an extracellular role for cathepsin B endopeptidase activity. The endopeptidase conformation of the occluding loop can also be stabilized with inhibitors such as cystatin C [7] and chagasin [8].

As well as participating in protein turnover in lysosomes [2], catB has some more specific physiological functions, such as bone resorption [9], liberation of thyroid hormones from thyroglobulin [10], antigen processing [11], and skin wound healing [12]. Alterations in catB expression, protein levels, activity and localization are associated with several disease states. Notably, catB has been shown to be critically involved in tumor metastasis, angiogenesis and progression [13]. These wide-ranging functions indicate that catB is a promising druggable target in cancers, rheumatoid arthritis and other important diseases.

Inhibitors of catB include endogenous inhibitors, such as the cystatin superfamily of proteins, low molecular weight natural inhibitors, and synthetic inhibitors. The majority of synthetic inhibitors that have been described to date are peptidyl compounds containing an electrophilic functionality, which reacts reversibly or irreversibly with the catalytic cysteine in the active site of catB [14]. So far, none of these compounds have reached the clinical practice, mostly due to poor bioavailability, off-target side effects and high toxicity [15]. Therefore, due to the great number of possible

Abbreviations: Z, benzyloxycarbonyl; AMC, 7-amido-4-methylcoumarin; catB, cathepsin B; EI, enzyme–inhibitor complex; ES, enzyme–substrate complex; ESI, enzyme–substrate–inhibitor complex.

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therapeutic applications, inhibitors of catB still have potential in drug design and development; thus a search for reversible, selective, and low molecular weight inhibitors should remain of great interest in the future.

Recently, Mott and coworkers [16] described triazine nitriles as promising reversible inhibitors of the catB-like protease from the parasite *Trypanosoma brucei* (TbcatB). As it has been claimed that the triazine nitrile scaffold is privileged in its nature toward cysteine proteases [16,17], we screened our in-house library of variously 2,4,6-trisubstituted-1,3,5-triazines (**1**–**23**) that were recently synthesized [18] against three different human cathepsins: B, H and L (Supplementary data, Tables S1, S2).

All of these 1,3,5-triazines **1**–**23** were tested for relative inhibition of catB, catH and catL according to the protocol described in Materials and methods. As seen from Supplementary Tables S1 and S2, all of these compounds were practically inactive against all cathepsins at 50 μM, except for compound **23**, which had a 6-substituted 4-benzylthio-1,3,5-triazin-2(1*H*)-one core. Compound **23** showed high inhibitory activity against catB and catL, whereas it showed no inhibition of catH. As compound **23** is unique in the series (it has an additional hydrophobic substituent on position 6 of the 1,3,5-triazine scaffold) and because of our research interest in the field of catB inhibitors [19], we synthesized new focused library of similar compounds in order to explore and determine the structural requirements for catB inhibition. Additionally, detailed kinetic analysis was performed for all catB inhibitors.

2. Chemistry

The 6-substituted 4-benzylthio-1,3,5-triazin-2(1*H*)-ones **23**–**47** were prepared similarly as in the previously described general synthetic route (Scheme 1) [20]. Briefly, 4-benzylthio-6-mercapto-1,3,5-triazin-2(1*H*)-one **22** was prepared from the appropriate amidine *S*-(benzyl)isothiourea hydrochloride and ethoxycarbonyl isothiocyanate, in the presence of 2 M NaOH in toluene at room temperature. Furthermore, product **22** (Scheme 1) was readily alkylated using various alkyl chlorides or bromides in a basic EtOH solution at room temperature, which provided the 6-benzylthio (**23**–**38**), 6-alkylthio (**39**–**43**), and 6-cycloalkylmethylthio derivatives (**44**–**47**) in very good yields (71%–93%). Purification of these derivatives was achieved by crystallization from CH_3CN or EtOH.

3. Results and discussion

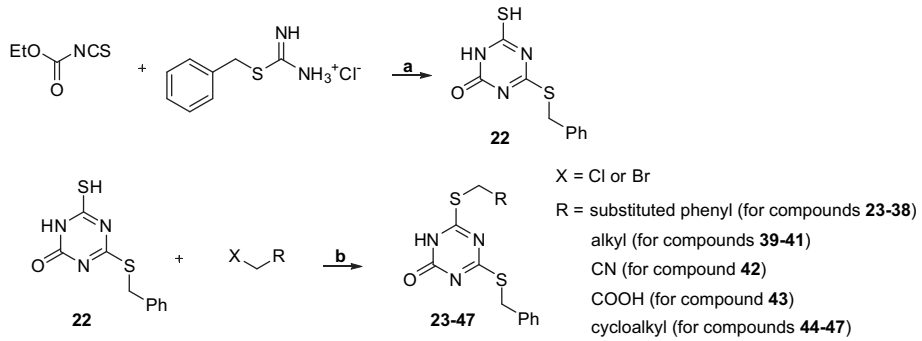
3.1. Cathepsin B inhibitory activities

The ease of the chemistry in the second reaction step allowed the evaluation of more than 20 different moieties attached to position 6 of the 4-benzylthio-1,3,5-triazin-2(1*H*)-one core. These newly synthesized compounds were evaluated for inhibition of catB, and to estimate their selectivity some selected compounds additionally for inhibition of catH and catL. These data are presented in Tables 1 and 2, and the assay results clearly indicate that these compounds have a selective profile for catB and catL over catH.

From comparisons of the inhibitory activities of newly synthesized compounds **23**–**38** in Table 1 with the compounds from Table S1 (e.g. compounds **8**–**10**, **12**, **15**) and Table S2 (e.g. compounds **19**–**22**), it can be postulated that the additional hydrophobic benzylthio residue on position 6 of the 1,3,5-triazine scaffold is essential for effective inhibition. When examining the influence of the different substituents on the phenyl ring of the 6-benzylthio moiety (Table 1, compounds **23**–**38**), it appears that the electronic nature and the position of the substituent do not have important roles in the mode or potency of catB inhibition. For instance, the inhibitory activities of compounds with an electron donating methoxy group on the phenyl ring (**23**–**25**) were comparable with compounds that have an electron withdrawing nitro group (**29**–**31**). Placement of a chlorine atom on the phenyl ring resulted in the most potent analogs (**32**–**34**), suggesting the need for a large hydrophobic substituent on this part of the molecule.

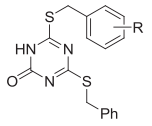
To further assess the importance for catB inhibition of the planar phenyl ring of the 6-benzylthio substituent, this ring was replaced with the methyl, ethyl, propyl, cyano, carboxyl, cyclopropyl, cyclobutyl, cyclopentyl, and cyclohexyl groups (compounds **39**–**47**). Of the analogs tested, compounds **41** and **45**–**47** retained comparable potencies of catB inhibition to those of the 6-benzylthio substituted derivatives **23**–**38**. This thus shows that the nature of the hydrophobic residue does not have a decisive role in catB inhibition. Although compounds **39**, **40** and **42**–**44** did inhibit catB as mixed-type inhibitors (see below), their inhibition was significantly attenuated by up to two orders of magnitude, indicating that short alkylthio, cyclopropylthio, $-SCH_2CN$, and $-SCH_2COOH$ fragments are not well tolerated on position 6 of the 4-benzylthio-1,3,5-triazin-2(1*H*)-one core. Only compounds **41** and **45**–**47**, which have larger hydrophobic substituents, can inhibit catB with a K_i value in the low micromolar range.

It is not very surprising that the compounds do not exhibit selectivity between catB and catL since these two enzymes are structurally very similar [21]. However, this does not represent crucial obstacle in their further optimization and research, because recent studies have shown that these two cysteine proteases have more important contributions to the proteolytic events during tumor progression than catH [22]. Moreover, the latest trends in the field of protease inhibitors implicate that the inhibition of multiple proteases is likely to prove more effective than targeting an individual protease within the protease network [23].



Scheme 1. General synthesis of compounds **22**–**47**. Reagents and conditions: (a) 2 M NaOH, toluene, H_2O, 25 °C, 1 h; (b) 2 M NaOH, EtOH, 25 °C, 2 h.

Table 1
Inhibitory activities and K_i values of compounds with the substituted 6-benzylthio fragment on the 4-benzylthio-1,3,5-triazin-2(1*H*)-one core against cathepsins B, H and L.



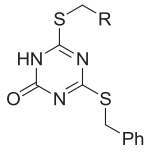
Compound	R	Cathepsin B inhibition			Cathepsin H inhibition (%)[c, d]	Cathepsin L inhibition (%)[c, d]
		K_i (μM)[a]	K_i' (μM)[a]	β[b]		
23	*o*-OMe	15.6 ± 4.7	4.4 ± 0.2	0.22 ± 0.03	1.06	61.33
24	*m*-OMe	13.0 ± 0.3	21.9 ± 4.9	0.42 ± 0.12	n.d.	n.d.
25	*p*-OMe	11.1 ± 0.5	10.3 ± 3.7	0.20 ± 0.06	n.d.	n.d.
26	*o*-F	9.5 ± 0.5	9.0 ± 1.4	0.36 ± 0.14	n.d.	n.d.
27	*m*-F	10.2 ± 0.1	7.4 ± 1.4	0.22 ± 0.03	4.18	7.68
28	*p*-F	16.6 ± 4.2	13.8 ± 1.1	0.33 ± 0.04	n.d.	n.d.
29	*o*-NO_2	11.9 ± 2.4	6.0 ± 0.3	0.24 ± 0.03	n.d.	n.d.
30	*m*-NO_2	11.3 ± 0.8	3.3 ± 0.2	0.15 ± 0.01	3.92	81.17
31	*p*-NO_2	10.0 ± 1.1	4.0 ± 0.04	0.17 ± 0.01	n.d.	n.d.
32	*o*-Cl	7.2 ± 3.2	3.6 ± 0.4	0.23 ± 0.04	n.d.	n.d.
33	*m*-Cl	5.4 ± 1.8	4.5 ± 2.1	0.28 ± 0.02	n.d.	n.d.
34	*p*-Cl	4.4 ± 0.3	2.5 ± 0.6	0.25 ± 0.01	6.91	85.23
35	*o*-CN	40.7 ± 7.3	10.5 ± 3.1	0.04 ± 0.02	n.d.	n.d.
36	*m*-CN	46.7 ± 0.4	9.1 ± 0.2	0.02 ± 0.01	n.d.	n.d.
37	*p*-CN	24.3 ± 2.1	10.8 ± 0.4	0.07 ± 0.00	1.55	44.94
38	*o*-F, *p*-CN	23.0 ± 5.8	9.7 ± 0.2	0.07 ± 0.01	n.d.	n.d.

n.d. Not determined.
[a] K_i values are an average of two independent determinations (performed in duplicate), with SDs given in the table.
[b] β is the factor by which the product formation changes when the inhibitor binds to the enzyme–substrate (ES) complex.
[c] Relative inhibition measurements represent averages of two independent determinations.
[d] Inhibitory activities on catH and catL were determined at 50 μM of each compound (performed in duplicate).

3.2. Enzyme kinetics

The kinetic parameters describing the inhibition of compounds **23**–**47** upon catB endopeptidase activity were determined using the catB-specific substrate Z-Arg-Arg-AMC, according to a protocol described in Materials and methods. Compounds **23**–**38** and compound **47** (Tables 1,2) inhibited catB in a reversible way and in the low micromolar range as partial mixed-type inhibitors. Interestingly, the same mechanism of action of catB inhibition was observed for chiral cyclometallated complexes derived from *N*,*N*-

Table 2
Inhibitory activities and K_i values of the compounds with the 6-alkylthio and 6-cycloalkylmethylthio substituents on the 4-benzylthio-1,3,5-triazin-2(1*H*)-one core against cathepsins B, H and L.



Compound	R	Cathepsin B inhibition			Cathepsin H inhibition (%)[c,d]	Cathepsin L inhibition (%)[c,d]
		K_i (μM)[a]	K_i' (μM)[a]	β[b]		
39	Me	220.9 ± 45.5	97.1 ± 1.8	0	−4.06	−11.45
40	Et	259.0 ± 33.2	49.4 ± 4.3	0	−4.82	−8.66
41	Pr	70.6 ± 22.5	14.8 ± 0.9	0	−1.81	9.33
42	CN	207.7 ± 33.2	51.6 ± 0.3	0	4.94	−5.28
43	COOH	560.0 ± 77.0	351.0 ± 2.9	0	6.89	−2.36
44		211.5 ± 35.08	28.30 ± 2.01	0	10.02	42.02
45		34.13 ± 6.60	10.13 ± 1.14	0	1.57	37.39
46		23.03 ± 3.01	8.95 ± 1.05	0	10.55	9.96
47		11.9 ± 4.4	3.3 ± 0.2	0.12 ± 0.01	10.17	76.24

n.d. Not determined.
[a] K_i values are an average of two independent determinations (performed in duplicate), with SDs given in the table.
[b] β is the factor by which the product formation changes when the inhibitor binds to the ES complex.
[c] Relative inhibition measurements represent averages of two independent determinations.
[d] Inhibitory activities on catH and catL were determined at 50 μM of each compound (performed in duplicate).

dimethyl-1-phenethylamine with bridging bis(diphenylphosphine) ferrocene ligand, described by Bincoletto and colleagues [24]. In the case of mixed-type inhibition the substrate and the inhibitor bind independently and reversibly to the enzyme to produce the ES, EI and ESI complexes (Fig. 1). As for the partial mixed-type inhibition, the formed ESI complex can also produce the product, but not as effectively as the ES complex. The parameter β is used to describe the perturbation of product formation when inhibitor binds to the ES complex and a low β factor value denotes poor formation of the product from the ESI complex (Fig. 1). Representative Dixon plots for compounds **24**, **34**, **40**, and **43** are shown in Fig. 2.

Furthermore, the kinetics analyses indicated that compounds **39**–**46** had slightly different mechanisms of action (mixed inhibitors instead of partial mixed inhibitors) as their counterparts with a planar phenyl ring or a cyclohexyl ring (Fig. 2). The equations to which the data were fitted and that specify the mechanism of action are disclosed in Materials and methods.

Since the fluorometric measurements with AMC and other fluorophores are often biased by inner filter effects brought about by molecules assayed, the compounds **23**–**47** were also tested for absorption in the spectral range of the AMC substrates (350–500 nm) at 50 μM in the assay buffer used for measuring catB activity. As seen in Fig. 3, the tested compounds did not show significant absorption in the spectral range of wavelengths of the AMC assay in this way excluding the possibility of inner filter effects caused by the tested compounds.

3.3. Molecular docking of compound *34* in the active site of catB

It has been previously established [25–28] that the S1′, S2′ and S2, S3 subsites of catB are likely to bind hydrophobic residues. We predicted that hydrophobic moieties on positions 4 and 6 of 1,3,5-triazin-2(1*H*)-one can bind to two of the hydrophobic subsites of catB. The plausible binding mode of compound **34** was assessed by molecular docking in the active site of catB (PDB entry: 1gmy) [27] using FlexX incorporated into LeadIT 1.3 [29]. In the crystal structure the active site was defined as the area within 13 Å of the catalytic Cys29. All water molecules and the co-crystallized ligand were removed, and all OH and SH groups were defined as freely rotatable. The inhibitor occupied the S1′ and S2′ subsites, as anticipated, forming Van der Waals, hydrophobic and π-stacking interactions (Fig. 4).

4. Conclusions

Recently, research has been focused mostly on the discovery of reversible covalent inhibitors of different cathepsins that have a mildly electrophilic 'warhead', of which the nitrile-containing functionalities are receiving the most attention. These 'warheads' combine strong, but reversible binding to the target protein (through the formation of a thioimidate with the active site cysteine), with low reactivities toward other cellular nucleophiles [31]. As it is known [14,31] that covalent interactions with the cysteine residue in the active site of the enzyme account for a substantial portion of the binding energy of the inhibitor, it is very promising that inhibitors **23**–**38** and **47** presented here, which are without this electrophilic 'warhead' moiety, already show good potencies and selectivities toward catB and also toward catL. The subsequent introduction of an additional electrophilic 'warhead' on an appropriate part of these molecules will most probably lead to further increases in their potencies.

In summary, our initial screening experiment was focused on determining whether the 1,3,5-triazine scaffold truly represents a privileged scaffold toward cysteine proteases. This screening identified 6-substituted 4-benzylthio-1,3,5-triazin-2(1*H*)-ones as low micromolar and noncovalent inhibitors of catB and catL. These compounds also show selectivity when evaluated against the related cysteine protease, catH. Further studies will be focused on additional exploration of the chemical space of these compounds, to address as many noncovalent interactions with the enzyme as possible. In a second phase, we will introduce the electrophilic functionality into these compounds, thus attempting to further improve the binding affinities of these molecules.

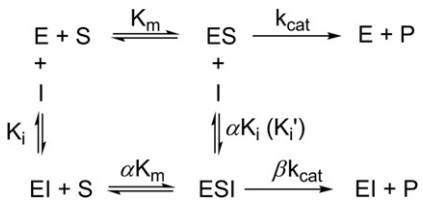


Fig. 1. Representation of the mechanism of partial mixed-type inhibition. E is used to denote the enzyme, S the substrate, K_m the Michaelis–Menten constant, k_{cat} the first-order rate constant, P the product, I the inhibitor, K_i the inhibition constant, α the K_m perturbation parameter, and β the k_{cat} perturbation parameter.

5. Experimental section

5.1. Enzymes and assay buffers

Human recombinant cathepsin B (EC 3.4.22.1) was prepared as reported [32]. Human native cathepsin H (EC 3.4.22.16) was isolated from human liver [33] and human recombinant cathepsin L (EC 3.4.22.15) was expressed in *Esherichia coli* [34]. Assay buffers for cathepsins B, H and L consisted of 100 mM phosphate buffer, pH 6.0, 100 mM phosphate buffer, pH 6.8, and 100 mM acetate buffer, pH 5.5, respectively. Each contained 0.1% PEG 8000 (Sigma–Aldrich), 5 mM DTT and 1.5 mM EDTA. Prior to the assay each enzyme was activated in the assay buffer for 5 min at 37 °C.

5.2. Relative inhibition determination

The substrates Z-Arg-Arg-AMC (Calbiochem), Arg-AMC (Biomol), Z-Phe-Arg-AMC (Bachem) were used to assess the activities of catB, catH and catL, respectively. Five μl of Z-Arg-Arg-AMC (5 μM), Arg-AMC (5 μM) or Z-Phe-Arg-AMC (1 μM) and 5 μl of the respective compound (final concentration 50 μM) were added into the wells of a black microplate. The reaction was initiated by addition of 90 μl enzyme (final concentration for catB and catH was 5 nM and for catL 0.5 nM) in the assay buffer. Formation of the fluorescent degradation products of the AMC substrates was continuously monitored at 460 nm ± 10 nm with excitation at 380 nm ± 20 nm, at 37 °C. All assay mixtures contained 5% (v/v) DMSO and 0.01% of Triton X-100, to prevent false-positive inhibition due to the formation of compound aggregates [35]. All measurements were performed in duplicate. The relative inhibition of enzyme activities was calculated according to the equation: Relative inhibition (%) $= 100 \times (1 - \nu_i/\nu_o)$, where ν_i and ν_o denote the reaction velocity in the presence and absence of inhibitor, respectively.

5.3. Determination of K_i values and the mechanism of inhibition

Inhibition constants were determined by measuring the initial rate of hydrolysis at various substrate concentrations in the absence or presence of different concentrations of inhibitors. Five μl of substrate Z-Arg-Arg-AMC at 60, 180 and 360 μM and 5 μl of respective inhibitor at 0, 10, 20, 30, 40, 50 and 70 μM were added into the wells of a black microplate. The reaction was initiated by addition of 90 μl enzyme (133 pM) in the assay buffer. Formation of

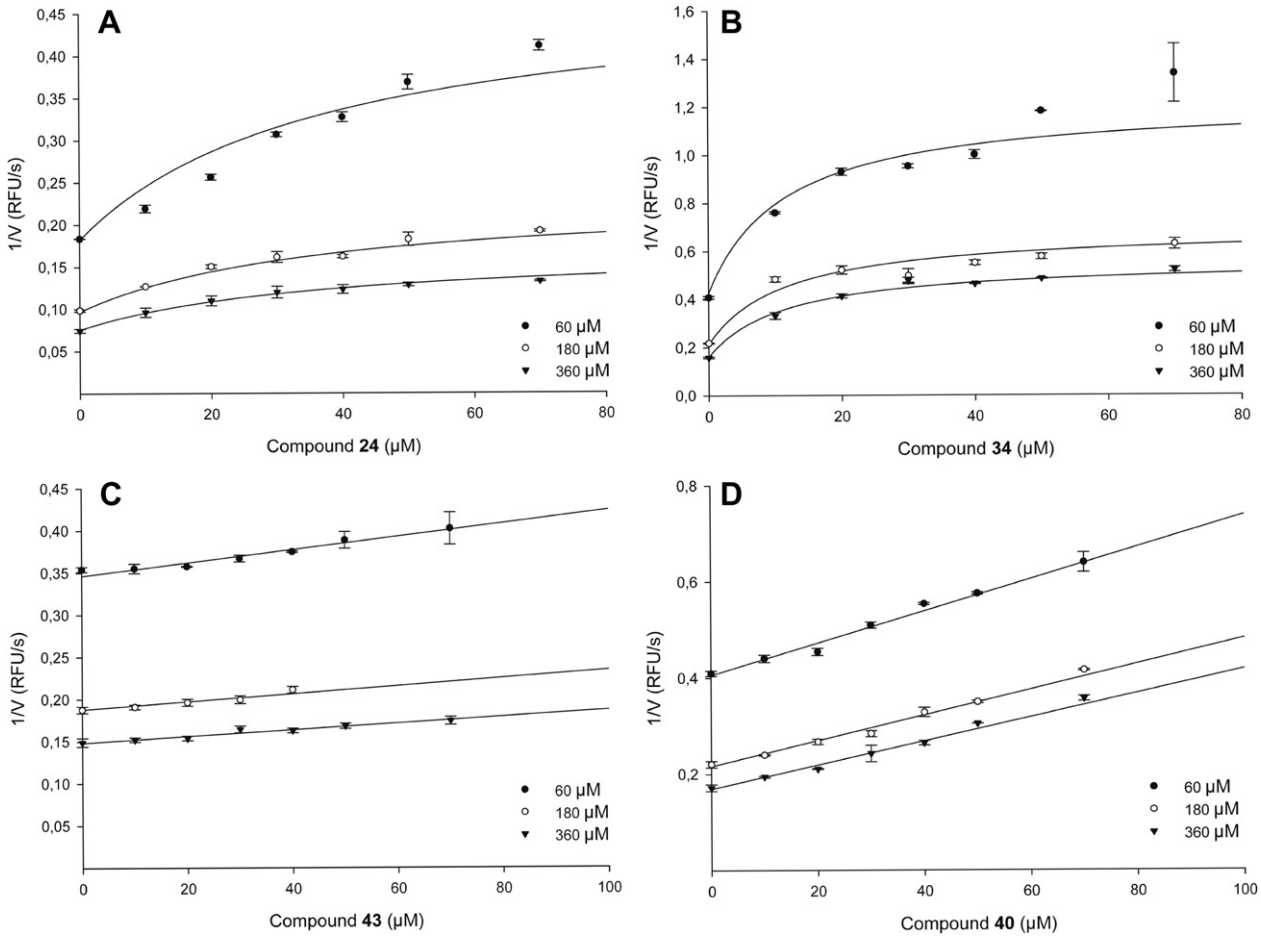


Fig. 2. Effect of compounds **24**, **34**, **40**, **43** on catB activity. Compounds **24** (A) and **34** (B) are reversible, partial mixed-type inhibitors, whereas compounds **40** (C) and **43** (D) are reversible, mixed-type inhibitors. Five μl of substrate at 60, 180 and 360 μM and 5 μl of the respective inhibitor at 0, 10, 20, 30, 40, 50 and 70 μM were added into the wells of a black microplate. The reaction was initiated by addition of 90 μl enzyme (133 pM). Formation of the fluorescent degradation product AMC was continuously monitored at 460 nm ± 10 nm with excitation at 380 nm ± 20 nm at 37 °C. All measurements were performed in duplicate and the Dixon plots presented here are representative of at least two independent experiments.

the fluorescent degradation product AMC was continuously monitored at 460 nm ± 10 nm with excitation at 380 nm ± 20 nm, at 37 °C. All assay mixtures contained 5% (*v*/v) DMSO. All measurements were performed in duplicate. The resulting data were analyzed by non-linear regression using the software SigmaPlot® 11, Enzyme Kinetics Module™ 1.3 and were fitted to the equation: $v = V_{max}/((K_m/S) \times (1 + I/K_i) + (1 + I/(\alpha \times K_i)))$, depicting

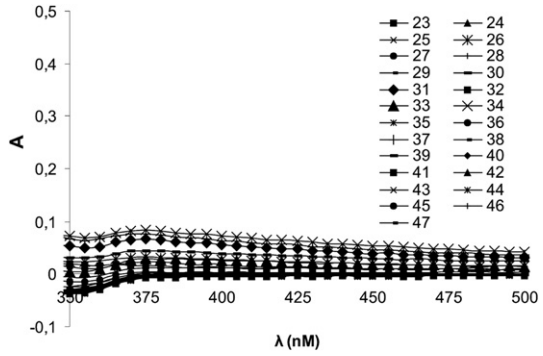


Fig. 3. Absorption spectra of compounds **23**–**47**. Data presented in the figure were obtained by deduction of absorbance values acquired in the presence of DMSO which was used as control.

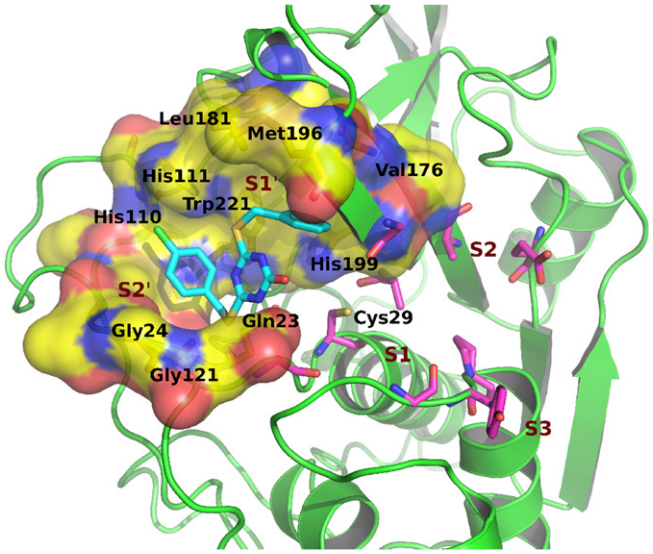


Fig. 4. Compound **34** (blue) modeled into the catB active site. Subsites S1′ and S2′ are shown as semi-transparent surfaces. All of the pocket-forming amino-acid residues are shown in yellow (S1′, S2′) and magenta (S1, S2 and S3) sticks, and are labeled. The figure was prepared using PyMol [30]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the mixed type of inhibition; or equation $\nu = V_{max} \times ((1+\beta \times I/(\alpha \times K_i))/(1 + I/(\alpha \times K_i)))/(1+(K_m/S) \times (1 + I/K_i)/(1 + I/(\alpha \times K_i)))$, depicting the partial mixed-type inhibition, where ν denotes reaction velocity, V_{max} the maximal reaction velocity, K_m the Michaelis–Menten constant, S the substrate, I the inhibitor, K_i the inhibition constant, α the K_m perturbation parameter, and β the V_{max} (k_{cat}) perturbation parameter.

5.4. Measurements of absorption spectra

All compounds (**23**–**47**) were tested for absorption in the spectral range of the AMC substrates. Five μl of compound in DMSO and 95 μl of assay buffer (used for measuring catB activity) were added to the wells of a black microplate. The concentration of each compound was 50 μM. The absorbance spectra were recorded in the range from 350 to 500 nm in steps of 5 nm.

5.5. Materials and methods

5.5.1. Chemistry

Reagents and solvents were obtained from commercial sources (Fluka, Sigma–Aldrich, Acros Organics, Alfa Aesar, Fluorochem). Solvents were distilled before use, while the other chemicals were used as received. Analytical TLC was performed on Merck silica gel ($60F_{254}$) pre-coated plates (0.25 mm). The compounds were visualized under UV light and/or stained with the relevant reagent. Column chromatography was performed on Merck silica gel 60 (mesh 70–230), using the indicated solvents. Yields refer to the purified products, and they were not optimized. All of the melting points were determined on a Reichert hot-stage apparatus, and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker Avance 400 DPX spectrometer at 302 K, and are reported in ppm using tetramethylsilane or solvent as an internal standard (DMSO-d_6 at 2.50 ppm, $CDCl_3$ at 7.26 ppm). The coupling constants (J) are given in Hz, and the splitting patterns are designated as: s, singlet; bs, broad singlet; d, doublet; dd, double doublet; t, triplet; dt, double triplet; td, triple doublet; and m, multiplet. ^{13}C NMR spectra were recorded on a Bruker Avance 400 DPX spectrometer at 302 K, and are reported in ppm using solvent as an internal standard (DMSO-d_6 at 39.43 ppm). Mass spectra data and high-resolution mass measurements were performed on a VG-Analytical Autospec Q mass spectrometer. All computational procedures used for molecular docking were performed on workstation, which has 4 dual core AMD Opteron 2.0 GHz processors, 16 GB of RAM, 4320 GB hard drives in RAID10 array and Nvidia GeForce 7900 graphic card and is running 64bit Fedora 7. The purity of all assayed compounds, as determined by HPLC, was >95%. HPLC experiments were performed on an Agilent Eclipse C18 column (4.6 × 50 mm, 5 μm) with a flow rate of 1.0 mL/min, detection at 254 nm, and an eluent system of: A = H_2O with 0.1% TFA; B = MeOH. The following gradient was applied: 0–3 min, 40% B; 3–18 min, 40% B → 80% B; 18–23 min, 80% B; 23–30 min, 80% B → 40% B; run time = 30 min; T = 25 °C.

5.6. Experimental procedures

5.6.1. Procedure for the synthesis of 4-(benzylthio)-6-mercapto-1,3,5-triazin-2(1H)-one (**22**) [25,26]

To a solution of *S*-(benzyl)isothiourea hydrochloride (203 mg, 1.00 mmol) in H_2O (3 mL), toluene (5 mL) was added and the mixture stirred vigorously. After 5 min, ethoxycarbonyl isothiocyanate (184 mg, 1.40 mmol) in toluene (2 mL) and NaOH (2 M, 4 mL) were simultaneously added over a period of 5 min. Additional NaOH (2 M, 2 mL) was added after 15 min and then the reaction mixture was stirred for 1 h at room temperature. The phases were then separated and the organic layer washed with NaOH (2 M, 6 mL). The combined alkaline phases were acidified to pH 1 with H_2SO_4, and in this way the precipitate was formed, which was then filtered off and crystallized from CH_3CN. Spectroscopic data (without ^{13}C NMR spectra) for this compound are described in Ref. [25]. ^{13}C NMR (100 MHz, DMSO-d_6): δ 33.62, 127.55, 128.53, 129.17, 136.14, 149.70, 168.15 (br s), 178.46 (br s).

5.6.2. General procedure for the synthesis of 6-alkylated derivatives 23–47

To a solution of compound **22** (1.0 mmol) in EtOH (8 mL) and NaOH (2 M, 5 mL), the corresponding benzyl halide (for compounds **23**–**38**), alkyl halide (for compounds **39**–**43**) or cycloalkylmethyl halide (for compounds **44**–**47**) (1.1 mmol) was slowly added. The reaction mixture was stirred for 2 h at room temperature. After the reaction was complete, H_2O (10 mL) was added, followed by the addition of H_2SO_4 (2 M, 5 mL). The precipitate formed was filtered off and the pure product obtained by crystallization from CH_3CN or EtOH.

5.6.2.1. 4-(Benzylthio)-6-(2-methoxybenzylthio)-1,3,5-triazin-2(1H)-one (**23**).

Crystallization from CH_3CN gave 304 mg (82%) of white crystals. Mp: 166.5–169.0 °C; R_f: 0.68 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.82 (s, 3H, C*H*$_3$), 4.32 (s, 2H, SC*H*$_2$), 4.38 (s, 2H, SC*H*$_2$), 6.89 (td, J = 7.5, 1.1 Hz, 1H, Ar–*H*), 7.02 (dd, J = 8.1, 0.9 Hz, 1H, Ar–*H*), 7.23–7.37 (m, 5H, Ar–*H*), 7.38–7.45 (m, 2H, Ar–*H*), 12.91 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 28.97, 33.54, 55.48, 110.91, 120.25, 123.74, 127.32, 128.46, 128.92, 129.19, 130.29, 136.68, 149.84, 153.15 (br s), 157.10, 173.01 (br s); HRMS (ESI) m/z calcd for $C_{18}H_{16}N_3O_2S_2$ $[M - H]^-$ 370.0684, found 370.0692; purity by HPLC: 100%, retention time: 22.71 min.

5.6.2.2. 4-(Benzylthio)-6-(3-methoxybenzylthio)-1,3,5-triazin-2(1H)-one (**24**).

Crystallization from CH_3CN gave 271 mg (73%) of white crystals. Mp: 125.5–127.5 °C; R_f: 0.58 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.73 (s, 3H, C*H*$_3$), 4.34 (s, 2H, SC*H*$_2$), 4.37 (s, 2H, SC*H*$_2$), 6.83 (ddd, J = 8.2, 2.5, 0.7 Hz, 1H, Ar–*H*), 6.94–7.01 (m, 2H, Ar–*H*), 7.19–7.36 (m, 4H, Ar–*H*), 7.36–7.42 (m, 2H, Ar–*H*), 12.98 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 33.60 (2C), 54.92, 112.90, 114.55, 121.09, 127.34, 128.46, 128.94, 129.55, 136.59, 138.07, 149.86, 152.89 (br s), 159.17, 173.12 (br s); HRMS (ESI) m/z calcd for $C_{18}H_{18}N_3O_2S_2$ $[M + H]^+$ 372.0840, found 372.0827; purity by HPLC: 100%, retention time: 21.82 min.

5.6.2.3. 4-(Benzylthio)-6-(4-methoxybenzylthio)-1,3,5-triazin-2(1H)-one (**25**).

Crystallization from EtOH gave 297 mg (80%) of white crystals (prisms). Mp: 176.0–177.0 °C; R_f: 0.75 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.73 (s, 3H, C*H*$_3$), 4.32 (s, 2H, SC*H*$_2$), 4.38 (s, 2H, SC*H*$_2$), 6.87 (dt, J = 8.7, 3.0 Hz, 2H, Ar–*H*), 7.23–7.37 (m, 5H, Ar–*H*), 7.37–7.44 (m, 2H, Ar–*H*), 7.36–7.42 (m, 2H, Ar–*H*), 12.96 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 33.21, 33.59, 55.00, 113.88, 127.34, 128.11, 128.47, 128.95, 130.21, 136.63, 149.31, 152.84 (br s), 158.54, 179.51 (br s); HRMS (ESI) m/z calcd for $C_{18}H_{18}N_3O_2S_2$ $[M + H]^+$ 372.0840, found 372.0838; purity by HPLC: 99.76%, retention time: 21.74 min.

5.6.2.4. 4-(Benzylthio)-6-(2-fluorobenzylthio)-1,3,5-triazin-2(1H)-one (**26**).

Crystallization from CH_3CN gave 316 mg (88%) of white crystals (needles). Mp: 191.0–193.0 °C; R_f: 0.63 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.37 (s, 2H, SC*H*$_2$), 4.39 (s, 2H, SC*H*$_2$), 7.13–7.43 (m, 8H, Ar–*H*), 7.50 (td, J = 7.7, 1.5 Hz, 1H, Ar–*H*), 13.03 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 27.31 and 27.33 (1C, $^3J_{F,C}$ = 4.0 Hz), 33.58, 115.19 and 115.40 (1C, $^2J_{F,C}$ = 21.3 Hz), 123.34 and 123.48 (1C, $^2J_{F,C}$ = 14.7 Hz), 124.42 and 124.45 (1C, $^3J_{F,C}$ = 4.2 Hz), 127.34, 128.45, 128.94, 129.66 and 129.74 (1C, $^3J_{F,C}$ = 8.1 Hz), 131.27 and 131.31 (1C, $^4J_{F,C}$ = 3.3 Hz), 136.48, 149.86, 152.92 (br s), 159.11 and 161.55 (1C, $^1J_{F,C}$ = 245.8 Hz), 174.32

(br s); HRMS (ESI) *m/z* calcd for $C_{17}H_{15}N_3OFS_2$ $[M + H]^+$ 360.0641, found 360.0642; purity by HPLC: 100%, retention time: 22.17 min.

*5.6.2.5. 4-(Benzylthio)-6-(3-fluorobenzylthio)-1,3,5-triazin-2(1H)-one (**27**).* Crystallization from EtOH gave 266 mg (74%) of white crystals (needles). Mp: 165.0–167.0 °C; R_f: 0.60 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.37 (s, 2H, SCH_2), 4.38 (s, 2H, SCH_2), 7.05–7.14 (m, 1H, Ar–*H*), 7.21–7.43 (m, 8H, Ar–*H*), 13.03 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 32.91 and 32.93 (1C, $^4J_{F,C}$ = 2.9 Hz), 33.56, 114.06 and 114.27 (1C, $^2J_{F,C}$ = 20.5 Hz), 115.61 and 115.83 (1C, $^2J_{F,C}$ = 22.0 Hz), 125.04 and 125.07 (1C, $^4J_{F,C}$ = 2.9 Hz), 127.37, 128.48, 128.96, 130.33 and 130.42 (1C, $^3J_{F,C}$ = 8.8 Hz), 136.54, 139.74 and 139.81 (1C, $^3J_{F,C}$ = 8.1 Hz), 149.86, 152.94 (br s), 160.71 and 163.13 (1C, $^1J_{F,C}$ = 242.1 Hz), 173.49 (br s); HRMS (ESI) *m/z* calcd for $C_{17}H_{15}N_3OFS_2$ $[M + H]^+$ 360.0641, found 360.0651; purity by HPLC: 99.62%, retention time: 21.83 min.

*5.6.2.6. 4-(Benzylthio)-6-(4-fluorobenzylthio)-1,3,5-triazin-2(1H)-one (**28**).* Crystallization from CH_3CN gave 284 mg (79%) of white crystals (needles). Mp: 213.0–214.5 °C; R_f: 0.72 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.36 (s, 2H, SCH_2), 4.39 (s, 2H, SCH_2), 7.09–7.19 (symm m, 2H, Ar–*H*), 7.23–7.36 (m, 3H, Ar–*H*), 7.37–7.49 (m, 4H, Ar–*H*), 12.97 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 32.77, 33.61, 115.12 and 115.34 (1C, $^2J_{F,C}$ = 21.3 Hz), 127.34, 128.46, 128.96, 130.92 and 131.00 (1C, $^3J_{F,C}$ = 8.1 Hz), 132.97 and 133.00 (1C, $^4J_{F,C}$ = 2.9 Hz), 136.56, 147.84, 153.14 (br s), 160.14 and 162.57 (1C, $^1J_{F,C}$ = 243.2 Hz), 175.64 (br s); HRMS (ESI) *m/z* calcd for $C_{17}H_{15}N_3OFS_2$ $[M + H]^+$ 360.0641, found 360.0628; purity by HPLC: 100%, retention time: 21.88 min.

*5.6.2.7. 4-(Benzylthio)-6-(2-nitrobenzylthio)-1,3,5-triazin-2(1H)-one (**29**).* Crystallization from CH_3CN gave 336 mg (87%) of yellow crystals. Mp: 175.0–176.5 °C; R_f: 0.62 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.37 (s, 2H, SCH_2), 4.64 (s, 2H, SCH_2), 7.23–7.36 (m, 3H, Ar–*H*), 7.37–7.44 (m, 2H, Ar–*H*), 7.53–7.62 (symm m, 1H, Ar–*H*), 7.67–7.75 (m, 2H, Ar–*H*), 8.06 (dd, *J* = 7.7, 0.8 Hz, 1H, Ar–*H*), 13.02 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 30.90, 33.55, 124.98, 127.38, 128.47, 128.97, 129.11, 132,26, 132.43, 133.97, 136.44, 147.97, 149.42, 152.83 (br s), 174.67 (br s); HRMS (ESI) *m/z* calcd for $C_{17}H_{15}N_4O_3S_2$ $[M + H]^+$ 387.0586, found 387.0574; purity by HPLC: 99.13%, retention time: 20.88 min.

*5.6.2.8. 4-(Benzylthio)-6-(3-nitrobenzylthio)-1,3,5-triazin-2(1H)-one (**30**).* Crystallization from EtOH gave 351 mg (91%) of yellow crystals (prisms). Mp: 183.5–185.0 °C; R_f: 0.70 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.36 (s, 2H, SCH_2), 4.50 (s, 2H, SCH_2), 7.24–7.41 (m, 5H, Ar–*H*), 7.62 (dd, *J* = 7.9, 7.9 Hz, 1H, Ar–*H*), 7.87 (dt, *J* = 7.7, 1.1 Hz, 1H, Ar–*H*), 8.11 (ddd, *J* = 8.2, 2.3, 0.9 Hz, 1H, Ar–*H*), 8.30 (dd, *J* = 2.3, 2.3 Hz, 1H, Ar–*H*), 13.03 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 32.65, 33.60, 122.20, 123.64, 127.37, 128.47, 128.95, 129.86, 135.68, 136.44, 139.72, 147.58, 149.87, 153.04 (br s), 175.17 (br s); HRMS (ESI) *m/z* calcd for $C_{17}H_{15}N_4O_3S_2$ $[M + H]^+$ 387.0586, found 387.0578; purity by HPLC: 100%, retention time: 20.23 min.

*5.6.2.9. 4-(Benzylthio)-6-(4-nitrobenzylthio)-1,3,5-triazin-2(1H)-one (**31**).* Crystallization from CH_3CN gave 282 mg (73%) of orange crystals. Mp: 183.0–185.0 °C; R_f: 0.54 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.36 (s, 2H, SCH_2), 4.49 (s, 2H, SCH_2), 7.23–7.41 (m, 5H, Ar–*H*), 7.67 (d, *J* = 8.7 Hz, 2H, Ar–*H*), 8.17 (d, *J* = 8.7 Hz, 2H, Ar–*H*), 13.04 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 32.78, 33.52, 123.48, 127.37, 128.46, 128.94, 130.11, 136.43, 145.33, 146.59, 149.86, 152.91 (br s), 175.88 (br s); HRMS (ESI) *m/z* calcd for $C_{17}H_{15}N_4O_3S_2$ $[M + H]^+$ 387.0586, found 387.0594; purity by HPLC: 95.31%, retention time: 20.43 min.

*5.6.2.10. 4-(Benzylthio)-6-(2-chlorobenzylthio)-1,3,5-triazin-2(1H)-one (**32**).* Crystallization from CH_3CN gave 289 mg (77%) of white crystals (needles). Mp: 158.0–160.0 °C; R_f: 0.81 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.37 (s, 2H, SCH_2), 4.46 (s, 2H, SCH_2), 7.23–7.43 (m, 7H, Ar–*H*), 7.45–7.51 (m, 1H, Ar–*H*), 7.54–7.60 (m, 1H, Ar–*H*), 13.02 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 31.82, 33.58, 127.33, 127.35, 128.46, 128.95, 129.39, 129.48, 131.42, 133.28, 133.91, 136.50, 146.40, 153.08 (br s), 175.49 (br s); HRMS (ESI) *m/z* calcd for $C_{17}H_{15}N_3OS_2Cl$ $[M + H]^+$ 376.0345, found 376.0342; purity by HPLC: 99.95%, retention time: 24.08 min.

*5.6.2.11. 4-(Benzylthio)-6-(3-chlorobenzylthio)-1,3,5-triazin-2(1H)-one (**33**).* Crystallization from CH_3CN gave 319 mg (85%) of white crystals (needles). Mp: 159.0–160.0 °C; R_f: 0.67 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.37 (s, 4H, 2× SCH_2), 7.23–7.42 (m, 8H, Ar–*H*), 7.47–7.51 (m, 1H, Ar–*H*), 12.96 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 32.87, 33.61, 127.26, 127.36, 127.64, 128.47, 128.80, 128.96, 130.26, 132.92, 136.52, 139.56, 147.66, 153.23 (br s), 175.68 (br s); HRMS (ESI) *m/z* calcd for $C_{17}H_{15}N_3OS_2Cl$ $[M + H]^+$ 376.0345, found 376.0352; purity by HPLC: 99.96%, retention time: 23.61 min.

*5.6.2.12. 4-(Benzylthio)-6-(4-chlorobenzylthio)-1,3,5-triazin-2(1H)-one (**34**).* Crystallization from CH_3CN gave 296 mg (79%) of white crystals. Mp: 205.0–206.0 °C; R_f: 0.80 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.35 (s, 2H, SCH_2), 4.36 (s, 2H, SCH_2), 7.23–7.37 (m, 4H, Ar–*H*), 7.38–7.45 (m, 5H, Ar–*H*), 12.97 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 32.78, 33.59, 127.35, 128.38, 128.47, 128.95, 130.78, 131.94, 136.00, 136.54, 146.95, 153.22 (br s), 175.64 (br s); HRMS (ESI) *m/z* calcd for $C_{17}H_{15}N_3OS_2Cl$ $[M + H]^+$ 376.0345, found 376.0338; purity by HPLC: 100%, retention time: 23.96 min.

*5.6.2.13. 4-(Benzylthio)-6-(2-cyanobenzylthio)-1,3,5-triazin-2(1H)-one (**35**).* Crystallization from EtOH gave 260 mg (71%) of white crystals. Mp: 157.0–158.0 °C; R_f: 0.67 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.36 (s, 2H, SCH_2), 4.54 (s, 2H, SCH_2), 7.21–7.40 (m, 5H, Ar–*H*), 7.44–7.53 (m, 1H, Ar–*H*), 7.65–7.71 (m, 2H, Ar–*H*), 7.84 (dt, *J* = 7.5, 0.9 Hz, 1H, Ar–*H*), 13.05 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 32.13, 33.58, 111.84, 117.22, 127.37, 128.31, 128.46, 128.97, 130.35, 133.03, 133.31, 136.40, 140.18, 148.00, 152.96 (br s), 176.10 (br s); HRMS (ESI) *m/z* calcd for $C_{18}H_{15}N_4OS_2$ $[M + H]^+$ 367.0687, found 367.0678; purity by HPLC: 99.60%, retention time: 18.80 min.

*5.6.2.14. 4-(Benzylthio)-6-(3-cyanobenzylthio)-1,3,5-triazin-2(1H)-one (**36**).* Crystallization from CH_3CN gave 286 mg (78%) of white crystals. Mp: 142.0–143.0 °C; R_f: 0.65 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.36 (s, 2H, SCH_2), 4.41 (s, 2H, SCH_2), 7.23–7.35 (m, 3H, Ar–*H*), 7.36–7.42 (m, 2H, Ar–*H*), 7.53 (dd, *J* = 7.7, 7.7 Hz, 1H, Ar–*H*), 7.70–7.78 (m, 2H, Ar–*H*), 7.87 (dd, *J* = 1.7, 1.7 Hz, 1H, Ar–*H*), 12.98 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 32.62, 33.55, 111.28, 118.53, 127.36, 128.46, 128.94, 129.63, 131.04, 132.50, 133.88, 136.49, 138.96, 149.86, 152.93 (br s), 176.09 (br s); HRMS (ESI) *m/z* calcd for $C_{18}H_{15}N_4OS_2$ $[M + H]^+$ 367.0687, found 367.0695; purity by HPLC: 95.14%, retention time: 18.69 min.

*5.6.2.15. 4-(Benzylthio)-6-(4-cyanobenzylthio)-1,3,5-triazin-2(1H)-one (**37**).* Crystallization from CH_3CN gave 322 mg (88%) of white crystals (needles). Mp: 182.5–184.0 °C; R_f: 0.61 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.35 (s, 2H, SCH_2), 4.44 (s, 2H, SCH_2), 7.23–7.41 (m, 5H, Ar–*H*), 7.60 (d, *J* = 8.5 Hz, 2H, Ar–*H*), 7.78 (d, *J* = 8.5 Hz, 2H, Ar–*H*), 13.00 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz,

DMSO-d_6): δ 33.06, 33.54, 109.97, 118.64, 127.38, 128.47, 128.95, 129.67, 132.29, 136.43, 143.08, 147.57, 149.98 (br s), 177.86 (br s); HRMS (ESI) *m*/*z* calcd for $C_{18}H_{15}N_4OS_2$ $[M + H]^+$ 367.0687, found 367.0698; purity by HPLC: 98.09%, retention time: 18.61 min.

*5.6.2.16. 4-(Benzylthio)-6-(2-fluoro-4-cyanobenzylthio)-1,3,5-triazin-2(1H)-one (**38**).* Crystallization from CH_3CN gave 359 mg (93%) of white crystals. Mp: 166.0–168.0 °C; R_f: 0.54 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.35 (s, 2H, SCH_2), 4.44 (s, 2H, SCH_2), 7.23–7.41 (m, 5H, Ar–*H*), 7.64–7.75 (m, 2H, Ar–*H*), 7.84 (dd, *J* = 9.9, 1.1 Hz, 1H, Ar–*H*), 12.98 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 27.07 and 27.10 (1C, $^3J_{F,C}$ = 4.0 Hz), 33.54, 111.69 and 111.79 (1C, $^3J_{F,C}$ = 9.9 Hz), 117.46 and 117.49 (1C, $^4J_{F,C}$ = 2.9 Hz), 119.08 and 119.34 (1C, $^2J_{F,C}$ = 25.3 Hz), 127.39, 128.06 and 128.31 (1C, $^2J_{F,C}$ = 25.3 Hz), 128.47, 128.64 and 128.68 (1C, $^4J_{F,C}$ = 3.6 Hz), 128.96, 132.35 and 132.40 (1C, $^3J_{F,C}$ = 4.8 Hz), 136.39, 149.89, 153.07 (br s), 158.47 and 160.94 (1C, $^3J_{F,C}$ = 249.1 Hz), 175.83 (br s); HRMS (ESI) *m*/*z* calcd for $C_{18}H_{14}N_4OS_2F$ $[M + H]^+$ 385.0593, found 385.0604; purity by HPLC: 95.07%, retention time: 19.96 min.

*5.6.2.17. 4-(Benzylthio)-6-(ethylthio)-1,3,5-triazin-2(1H)-one (**39**).* Crystallization from CH_3CN gave 201 mg (72%) of white crystals (needles). Mp: 145.0–146.0 °C; R_f: 0.62 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.27 (t, *J* = 7.3 Hz, 3H, SCH$_2$CH_3), 3.07 (q, *J* = 7.3 Hz, 2H, SCH_2CH$_3$), 4.37 (s, 2H, SCH_2), 7.23–7.37 (m, 3H, Ar–*H*), 7.38–7.42 (m, 2H, Ar–*H*), 12.92 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.23, 24.22, 33.56, 127.32, 128.47, 128.90, 136.73, 149.61, 152.75 (br s), 177.76 (br s); HRMS (ESI) *m*/*z* calcd for $C_{12}H_{14}N_3OS_2$ $[M + H]^+$ 280.0578, found 280.0571; purity by HPLC: 98.21%, retention time: 17.74 min.

*5.6.2.18. 4-(Benzylthio)-6-(propylthio)-1,3,5-triazin-2(1H)-one (**40**).* Crystallization from CH_3CN gave 208 mg (71%) of white crystals (needles). Mp: 118.0–119.0 °C; R_f: 0.67 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.93 (t, *J* = 7.4 Hz, 3H, SCH$_2$CH$_2$CH_3), 1.58–1.70 (m, 2H, SCH$_2$CH_2CH$_3$), 3.04 (t, *J* = 6.9 Hz, 2H, SCH_2CH$_2$CH$_3$), 4.37 (s, 2H, SCH_2), 7.23–7.36 (m, 3H, Ar–*H*), 7.37–7.44 (m, 2H, Ar–*H*), 12.94 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 12.98, 22.00, 31.46, 33.55, 127.30, 128.46, 128.88, 136.72, 148.65, 153.22 (br s), 175.81 (br s); HRMS (ESI) *m*/*z* calcd for $C_{13}H_{16}N_3OS_2$ $[M + H]^+$ 294.0735, found 294.0747; purity by HPLC: 98.35%, retention time: 20.06 min.

*5.6.2.19. 4-(Benzylthio)-6-(butylthio)-1,3,5-triazin-2(1H)-one (**41**).* Crystallization from CH_3CN gave 261 mg (85%) of white crystals (prisms). Mp: 101.0–102.0 °C; R_f: 0.76 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.87 (t, *J* = 7.3 Hz, 3H, SCH$_2$CH$_2$CH$_2$CH_3), 1.28–1.42 (m, 2H, SCH$_2$CH$_2$CH_2CH$_3$), 1.55–1.67 (m, SCH$_2$CH_2CH$_2$CH$_3$, 2H), 3.07 (t, *J* = 7.3 Hz, 2H, SCH_2CH$_2$CH$_2$CH$_3$), 4.37 (s, 2H, SCH_2), 7.23–7.37 (m, 3H, Ar–*H*), 7.38–7.45 (m, 2H, Ar–*H*), 12.92 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 13.45, 21.22, 29.31, 30.60, 33.57, 127.31, 128.46, 128.88, 136.67, 149.85, 152.75 (br s), 177.88 (br s); HRMS (ESI) *m*/*z* calcd for $C_{14}H_{18}N_3OS_2$ $[M + H]^+$ 308.0891, found 308.0881; purity by HPLC: 99.93%, retention time: 22.10 min.

*5.6.2.20. 4-(Benzylthio)-6-(cyanomethylthio)-1,3,5-triazin-2(1H)-one (**42**).* Crystallization from EtOH gave 223 mg (77%) of white crystals. Mp: 140.5–143.0 °C; R_f: 0.55 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.18 (s, 2H, SCH_2), 4.45 (s, 2H, SCH_2), 7.25–7.38 (m, 3H, Ar–*H*), 7.38–7.45 (m, 2H, Ar–*H*), 13.20 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 12.03, 33.88, 119.36, 127.89, 128.52, 129.02, 136.45, 148.32, 153.32 (br s), 175.34 (br s); HRMS (ESI) *m*/*z* calcd for $C_{12}H_{11}N_4OS_2$ $[M + H]^+$ 291.0374, found 291.0363; purity by HPLC: 98.45%, retention time: 11.36 min.

*5.6.2.21. 4-(Benzylthio)-6-(carboxymethylthio)-1,3,5-triazin-2(1H)-one (**43**).* Crystallization from CH_3CN gave 250 mg (81%) of white crystals (needles). Mp: 193.0–195.0 °C; R_f: 0.09 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.93 (s, 2H, SCH_2), 4.38 (s, 2H, SCH_2), 7.24–7.37 (m, 3H, Ar–*H*), 7.38–7.44 (m, 2H, Ar–*H*), 12.96 (br s, 2H, N*H*CO and COO*H*); ^{13}C NMR (100 MHz, DMSO-d_6): δ 32.75, 33.61, 127.49, 128.59, 129.05, 136.52, 148.43, 153.15 (br s), 169.32, 176.00 (br s); HRMS (ESI) *m*/*z* calcd for $C_{12}H_{12}N_3O_3S_2$ $[M + H]^+$ 310.0320, found 310.0329; purity by HPLC: 100%, retention time: 6.90 min.

*5.6.2.22. 4-(Benzylthio)-6-(cyclopropylmethylthio)-1,3,5-triazin-2(1H)-one (**44**).* Crystallization from CH_3CN gave 253 mg (83%) of white crystals. Mp: 139.0–140.0 °C; R_f: 0.62 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.24–0.34 (m, 2H, cyclopropyl-*H*), 0.48–0.58 (m, 2H, cyclopropyl-*H*), 1.02–1.18 (m, 1H, cyclopropyl-*H*), 3.04 (d, *J* = 7.2 Hz, 2H, SCH_2CH), 4.37 (s, 2H, SCH_2), 7.22–7.36 (m, 3H, Ar–*H*), 7.38–7.45 (m, 2H, Ar–*H*), 12.92 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 5.63, 10.19, 33.64, 35.39, 127.31, 128.46, 128.90, 136.74, 149.88, 152.92 (br s), 176.22 (br s); HRMS (ESI) *m*/*z* calcd for $C_{14}H_{16}N_3OS_2$ $[M + H]^+$ 306.0735, found 306.0375; purity by HPLC: 100%, retention time: 20.36 min.

*5.6.2.23. 4-(Benzylthio)-6-(cyclobutylmethylthio)-1,3,5-triazin-2(1H)-one (**45**).* Crystallization from CH_3CN gave 230 mg (72%) of white crystals (prisms). Mp: 129.0–130.5 °C; R_f: 0.65 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.60–1.88 (m, 4H, cyclobutyl-*H*), 1.94–2.07 (m, 2H, cyclobutyl-*H*), 2.48–2.65 (m, 1H, cyclobutyl-*H*, overlaps with DMSO), 3.17 (d, *J* = 7.4 Hz, 2H, SCH_2CH), 4.38 (s, 2H, SCH_2), 7.23–7.43 (m, 5H, Ar–*H*), 12.93 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 17.28, 26.83, 33.56, 33.88, 35.45, 127.30, 128.45, 128.85, 136.68, 149.50, 152.70 (br s), 172.80 (br s); HRMS (ESI) *m*/*z* calcd for $C_{15}H_{18}N_3OS_2$ $[M + H]^+$ 320.0891, found 320.0888; purity by HPLC: 98.07%, retention time: 22.74 min.

*5.6.2.24. 4-(Benzylthio)-6-(cyclopentylmethylthio)-1,3,5-triazin-2(1H)-one (**46**).* Crystallization from CH_3CN gave 253 mg (76%) of white crystals. Mp: 135.0–136.5 °C; R_f: 0.67 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.17–1.30 (m, 2H, cyclopentyl-*H*), 1.40–1.82 (m, 6H, cyclopentyl-*H*), 2.03–2.22 (m, 1H, cyclopentyl-*H*), 3.08 (d, *J* = 7.2 Hz, 2H, SCH_2CH), 4.36 (s, 2H, SCH_2), 7.20–7.44 (m, 5H, Ar–*H*), 12.93 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 24.64, 31.54, 33.32, 33.36, 34.94, 127.20, 128.42, 128.83, 137.00, 149.43, 152.28 (br s), 175.62 (br s); HRMS (ESI) *m*/*z* calcd for $C_{16}H_{20}N_3OS_2$ $[M + H]^+$ 334.1048, found 334.1040; purity by HPLC: 95.89%, retention time: 24.84 min.

*5.6.2.25. 4-(Benzylthio)-6-(cyclohexylmethylthio)-1,3,5-triazin-2(1H)-one (**47**).* Crystallization from CH_3CN gave 274 mg (79%) of white crystals (needles). Mp: 144.0–145.0 °C; R_f: 0.69 (CH_2Cl_2/MeOH 5/1); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.88–1.05 (m, 2H, cyclohexyl-*H*), 1.05–1.26 (m, 3H, cyclohexyl-*H*), 1.49–1.79 (m, 6H, cyclohexyl-*H*), 3.00 (d, *J* = 6.7 Hz, 2H, SCH_2CH), 4.38 (s, 2H, SCH_2), 7.24–7.37 (m, 3H, Ar–*H*), 7.38–7.44 (m, 2H, Ar–*H*), 12.92 (br s, 1H, N*H*CO); ^{13}C NMR (100 MHz, DMSO-d_6): δ 25.33, 25.66, 31.73, 33.57, 36.25, 36.88, 127.33, 128.47, 128.87, 136.65, 149.86, 152.77 (br s), 172.40 (br s); HRMS (ESI) *m*/*z* calcd for $C_{17}H_{22}N_3OS_2$ $[M + H]^+$ 348.1204, found 348.1200; purity by HPLC: 100%, retention time: 23.65 min.

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2011.08.005.

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