Published in final edited form as:

Eur J Med Chem. 2010 May ; 45(5): 1880–1897. doi:10.1016/j.ejmech.2010.01.027.

Evaluation of 2-Thioxo-2,3,5,6,7,8-hexahydropyrimido[4,5-d]pyrimidin-4(1H)-one analogues as GAA Activators

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Abstract

Pompe disease is a lysosomal storage disease (LSD) caused by a deficiency in the lysosomal enzyme acid α -glucosidase. In several LSDs, enzyme inhibitors have been used as small molecule chaperones to facilitate and increase the translocation of mutant protein from the endoplasmic reticulum to the lysosome. Enzyme activators with chaperone activity would be even more desirable as they would not inhibit the enzyme after translocation and might potentiate the activity of the enzyme that is successfully translocated. Herein we report our initial findings of a new series of acid α -glucosidase activators.

Keywords

Pompe disease; Lysosomal Storage Disorder; acid alpha-glucosidase; activation of GAA

1. Introduction

Pompe disease is an autosomal recessive disorder caused by the deficiency or dysfunction of the lysosomal enzyme acid alpha-glucosidase (GAA,[1] EC 3.2.1.20/3). Epidemiological studies have estimated its frequency to be 1 in every 40,000 births [1,2]. The function of GAA is to hydrolyze terminal α -1,4- and α -1,6-glucosidic linkages of glycogen in the lysosome. Mutations in this enzyme result in lysosomal enlargement due to glycogen accumulation. The accumulation is especially severe in cardiac and skeletal muscle, affecting breathing and mobility [1]. The only FDA treatment currently approved for this disease is enzyme

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Supporting Information Available: Full concentration-% response curves of every final tested compound can be found in PubChem.

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replacement therapy (Myozyme), which is recombinant GAA produced in a Chinese hamster ovary cell line [3]. Although Myozyme has been proven to be clinically efficacious, the development of infusion related reactions is common, and the majority of the patients (89%) test positive for IgG antibodies to acid alpha-glucosidase [4]. These findings reinforce the need to develop new treatments for Pompe disease.

More than 100 different GAA mutations induce Pompe disease symptoms [5-6]. Many of these mutant proteins retain enzymatic activity *in vitro*, but are not transported to the lysosome. These proteins accumulate in the endoplasmic reticulum (ER), presumably due to an inability to fold properly or acquire the necessary shape to be recognized for transport to the lysosome [7-10].

A general LSD strategy has been to search for small molecule chaperones that are able to bind to mutant enzymes and assist with the folding and transport process. These molecules have the ability to improve the trafficking of the mutant protein between the ER and the lysosome, resulting in lysosome size restoration and phenotypic correction [11]. Paradoxically, all the small molecule chaperones reported in the literature are inhibitors of the enzyme, with the majority being iminosugars. One of these, 1-deoxynojirimycin (DNJ), is currently in phase II clinical trials for the treatment of Pompe disease [12-13]. Iminosugars are problematic due to poor selectivity and their small therapeutic window between improving translocation and inhibiting enzyme activity [11,14]. Enzyme activators with chaperone capacity would be more desirable. This type of molecule would not only improve the translocation of the enzyme, but would also potentiate the activity of the lysosomal protein, having a doubly beneficial effect. To our knowledge, Pulicarside 1 is the only compound described as being an activator of GAA [15]. Pulicarside 1 is able to enhance the hydrolysis of p-nitrophenyl- α -glucopyranoside by GAA in a dose dependent manner. To date, no data regarding its chaperone activity, or its capacity to enhance the hydrolysis of glycogen, has been published.

A core goal of the NIH Chemical Genomics Center is to use relevant, high-throughput biological assays to identify and develop small molecule probes of biomedical interest, especially in the area of rare and neglected diseases. As part of that effort, we have developed several new screening methodologies to identify novel non-iminosugar series with activity in LSD assays. We have focused testing enzymes in as native a context as possible, including testing the hydrolytic capacity of GAA in tissue homogenate [16]. Many isolated glycosidases require allosteric activation to be functional [17-18], so we wanted to avoid using purified enzyme preparations, which depend upon the use of detergents to induce the active conformation and functionality of the enzyme. We have observed that it is common to find compounds that can inhibit isolated enzymes but are inactive in cellular lysates. This is likely due to enzymatic conformational differences between detergent-induced conformation and cell lysate conformation and non-specific protein binding. Another limitation of reconstituted assays is an inability to detect enzyme activators, presumably because the detergent used in reconstituted assays activates the enzyme in a non-physiological way. One way to overcome these problems is to screen the enzyme directly from tissue homogenate using a probe specific for GAA activity, resorufin α -D-glucopyranoside [16]. Upon hydrolysis, the red dye resorufin is liberated, producing a fluorescent emission at 590 nm when excited at 530 nm. In addition, as a control for autofluorescence, we also used a second substrate, 4-methylumbelliferyl α -D-glucopyranoside (4MU- α -glu), which liberates the blue dye 4-methylumbelliferyl (4MU) at an emission wavelength of 440 nm when excited at 370 nm.

2. Results

In a quantitative high-throughput screen [16] of 199,177 compounds, we found a compound, 1-(3,4-dimethoxyphenethyl)-6-propyl-2-thioxo-2,3,5,6,7,8-hexahydropyrimido[4,5-d]pyrimidin-4(1H)-one **1**, able to activate one and a half fold the hydrolysis of the red dye in

tissue homogenate at 10 μ M. Figure 3 shows the activity of this compound and other analogues in the confirmation assay. In addition, we measured the activity of the lead compound **1** with the blue dye and with a purified enzyme preparation. The results show that the activation of the red dye hydrolysis can also be observed using purified enzyme. Figure 4 also shows that compound **1** activates the hydrolysis of 4MU in a dose-dependent manner, both in purified enzyme and tissue homogenate. Elimination of the dimethoxy substituents of the phenethyl functional group reduces the activity of the scaffold. Moreover, complete elimination of the hexahydropyrimide ring almost erases the activity and its exchange by a pyrrolopyrimidin core diminishes it.

Compound **1** is not auto-fluorescent under the assay conditions and the hydrolysis reaction can be monitored using LC-MS. Figure 5 shows the concentration-dependent increase in product (peak 2).

With these data in hand, we decided to embark on structure-activity relationship studies. Scheme 1 shows the general methodology used for the synthesis of analogues with modifications at positions 1 and 6 of the lead compound. Thus, primary amine **7** was refluxed with ammonium thiocyanate in a suitable solvent such as bromobenzene to yield the corresponding thiourea **8**. This compound was then reacted with ethyl cyanoacetate in the presence of sodium ethoxide to produce the 2-thioxo-2,3-dihydropyrimidin-4(1*H*)-one derivative **9**. Last, a primary amine **10** and formaldehyde were added to a solution of amine **9** in ethanol and refluxed for three hours to produce target 2-thioxo-2,3,5,6,7,8-hexahydropyrimido[4,5-*d*]pyrimidin-4(1*H*)-one **11**.

We also synthesized a number of analogues with modifications in the molecular core of the molecule. We were especially eager to test the necessity of the thiocarbonyl functional group within the template. In addition, we produced a number of compounds with diverse modifications in the hexahydropyrimide ring with the aim of testing the possibility of introducing aromaticity and the necessity of maintaining the hydrogen donor. Scheme 2 and 3 describe their synthesis.

The synthesis of the 2-thioxo-2,3-dihydro-1*H*-purin-6(9*H*)-one **14** analogue started with the nitrosilation of the previously described amine **5**, followed by a reduction of intermediate **12** with Sodium dithionite to produce compound **13**. Cyclization in the presence of formamide yields the final purine **14**. For the synthesis of compound **18**, intermediate **13** was reacted with 1,4-dioxane-2,3-diol **17** in ethanol. Last, compound **16** was obtained refluxing compound **5** with pentane-2,4-dione **15**.

Regarding the pyrido[4,3-*d*]pyrimidine series, triazine and ethyl acetoacetate reacted in the presence of a sodium ethoxide to yield the main core **20**. This compound was iodinated and dehydrated in the presence of phosphorus oxychloride to produce intermediate **22**. Sonagashira coupling, hydrolysis of the chloride in the presence of ammonium acetate and acetylene reduction with palladium in carbon gave the final products.

Another modification evaluated was the replacement of the thiocarbonyl functional group by a carbonyl substituent. Scheme 4 shows that the synthesis of this compound, **32**, was accomplished in a similar fashion than previous compounds replacing the thiourea moiety by a urea.

For evaluating activity improvements, both the efficacy (percentage of activatory response compared to base line) and AC₅₀ (concentration necessary to obtain 50% of maximal activation) should be analyzed.

To generate a proper AC₅₀ the maximum possible activation must be reached at the highest concentration, but at the tested concentrations (twelve points between 650 pM and 100 μ M) we were not always able to reach a response plateau. Due to solubility problems, we did not evaluate the activity at even higher concentrations, so we have decided to report here the maximum efficacy reach with the compound as initial evaluation for tracking SAR. Full concentration-% response curves of every compound can be found in PubChem.

Tables 1 to 4 show the SAR of our compounds.

Figure 7 shows the concentration-% response curves of our best compounds in several assays. In addition, Figure 6 disclosed the red dye-tissue homogenate AC₅₀ obtained with the same compounds as well as their corresponding Log P and total Polar Surface Area calculated using ChemDraw program. Corresponding efficacy values can be seen in Tables 2, 3 and 4.

In order to further evaluate our best activators, we measured their specific ability to activate the hydrolysis of 4MU- α -glu. Figure 8 shows how compounds **1**, **64**, **71**, **48**, and **49** increase the production of 4MU in a dose dependent manner when they are in the presence of recombinant human GAA but not in the presence of alpha galactosidase A.

In addition, we evaluated the effect of our lead compounds on the hydrolysis of the natural substrate glycogen. Figure 9 shows that none of our compounds improved the hydrolysis of glycogen.

3. Discussion

The aim of this project was to identify small molecule activators of GAA, and to characterize their potential to overcome the GAA deficiency in Pompe disease. Several new assays were developed for high-throughput screening to address the perceived shortcomings of the traditional purified enzyme assay. First, a red-shifted fluorogenic substrate was developed to avoid auto-fluorescent false positives. Second, the protocol was adjusted to use tissue homogenate instead of purified enzyme with the hope of assaying enzyme activity in a more relevant context, and to eliminate compounds that might bind proteins non-specifically. These conditions led to the identification of compound **1**. As shown in Figure 4, this compound has an extraordinary capacity for activation, being able to increase hydrolysis rates several times over. Compound **1** is not auto-fluorescent under the assay conditions and is able to activate the hydrolysis of both blue and red dyes. Moreover, activation of substrate hydrolysis was confirmed by LC-MS, demonstrating that compound **1** is able to increase the amount of 4MU produced in a concentration-dependent manner.

SAR structural changes show that all the core modifications synthesized failed to further activate hydrolysis of our fluorogenic substrate and even replacement of the thiocarbonyl functional group by a carbonyl group diminished activity (table 1, compounds **32** and **1**). Replacement of the pendant aromatic ring at position 1 with various alkyl chains, table 3, also abolished activity. Several heteroaromatic rings pendant to position 1 were poorly active or greatly reduced the activity of the lead compound. Shortening the linker from two carbons to one, table 3 compounds **1** and **64**, produce a small increment in efficacy. Moreover, adding one more methylene unit to the length of the alkyl linker, table 3 compounds **2** and **65**, also increased efficacy. Optimization of the substitution pattern for the phenethyl group at position 1, table 2, showed that electron donating groups in the meta- or para- positions demonstrated increased efficacy, while chloro substitution provided better results in the ortho- followed by the meta- position. Similarly, electron donating di-substituted compounds tend to provide better efficacy when substituted in the 3,4-positions. In contrast, bis-halogenated compounds were most efficacious when substituted at the 2,6- or the 2,3-positions. We also studied the influence

of substituents at position 6. Table 4 shows that propyl **1**, isobutyl **70** and benzyl **72** were the most efficacious compounds.

Overall, the data shows a quite narrow SAR in which most modifications yielded diminished activation of hydrolysis. Figure 6 and 7 present the red dye-tissue homogenate AC₅₀ and the titration curves of our most potent compounds. It can be seen that while maintaining the high efficacy of our initial lead we were able to improve over 16 times the AC₅₀ of the series. Our best compound **49** had an AC₅₀ of 1.00 μ M when it was tested in red dye-tissue homogenate, and 355 nM when it was tested in red-dye isolate enzyme conditions. In addition, it can be seen that the most active molecules have very reasonable calculated log P and tPSA, and in general increments of log P and reduction of tPSA correlated with improvements in activity. This activity is GAA-dependent, as shown in Figure 8, and enzyme specific because the series does not activate the hydrolysis of substrates for other glycosidase enzymes, such as α -Galactosidase A or Glucocerebrosidase (data not shown).

Unfortunately, this series' activity is also substrate dependent. Although the data demonstrate that the series can activate the hydrolysis of both red and blue dyes, Figure 9 shows that these compounds do not activate the hydrolysis of the natural substrate, glycogen. We speculate that the conformation adopted by GAA for cleaving the polymeric natural substrate may be different than the one adopted in the presence of a small substrate such as resorufin α -D-glucopyranoside or 4MU- α -glu. Other lysosomal enzymes such as β -glucosidase, require the binding of an allosteric modulator [17]. It is therefore possible that the isolated enzyme assay is not an adequate model to test for the hydrolysis of glycogen *in situ*. This demonstrates the need for the development of assays that would be able to predict the efficacy and activity of new molecules under more physiologically relevant conditions. Currently, our group is working on the development of a new HTS-adaptable assay that is able to measure the glycogen hydrolysis by tissue homogenate GAA. In addition, the glycogen-based assay involves the cleavage of both 1,4 and 1,6 glycosidic bonds, and it is possible that this series selectively activates only 1,4 bond cleavage and thus this assay's results may be misleading. More importantly, we expect this series to directly interact with the protein, and as such may still possess chaperone activity; this will require further testing.

4. Conclusions

In conclusion, we have developed the SAR of an initial lead compound **1** with modifications in all the main areas of the molecule using a tissue-homogenate assay. Activation of hydrolysis by this compound is enzyme and substrate dependent. It is not obvious what the reason for this substrate selectivity is but we speculate that the conformation adopted by GAA for cleaving the polymeric natural substrate may be different than the one adopted in the presence of a small substrate such as resorufin α -D-glucopyranoside or 4MU- α -glu. We are in the process of testing the chaperone capacity of our molecules [28-29]. Independently of the inhibitory or activatory activity of a series, improving the binding might result in an increment in the capacity of the molecule to stabilize folding. Further results of that work will be presented in a separate paper.

5. Experimental Section

5.1 Chemistry

The reagents and solvents were used as commercial anhydrous grade without further purification. The column chromatography was carried out over silica gel (100–200 mesh). ¹H NMR spectra were recorded with a Bruker 400 MHz spectrometer from solutions in CDCl₃ and DMSO-*d*₆. Chemical shifts in ¹H NMR spectra are reported in parts per million (ppm, δ) downfield from the internal standard Me₄Si (TMS, δ = 0 ppm). Molecular weight confirmation was performed using an Agilent Time-Of-Flight Mass Spectrometer (TOF, Agilent

Technologies, Santa Clara, CA). A 3 minute gradient from 4 to 100% acetonitrile (0.1% formic acid) in water (0.1% formic acid) was used with a 4 minute run time at a flow rate of 1 mL/min. A Zorbax SB-C18 column (3.5 micron, 2.1 × 30 mm) was used at a temperature of 50° C. Confirmation of molecular formula was confirmed using electrospray ionization in the positive mode with the Agilent Masshunter software (version B.02).

5.1.1 General Procedure for the Preparation of Thiourea 8 [19]—Ammonium thiocyanate (27.58 mmol) was added to a stirred solution of amine **7** (27.58 mmol) in bromobenzene (10 mL) at room temperature. The reaction mixture was heated at reflux temperature for 6 h. The reaction mixture was cooled to room temperature and concentrated under reduced pressure. The crude residue was purified by silica-gel column chromatography (2–10% MeOH in CH₂Cl₂) to afford thiourea **8**. The products were characterized by MS analysis and subjected to the next step without further purification.

Compound number	Identity of R on Table 2	Yield (%)	MS ESI, m/z [M + H] ⁺
8a	3,4-Di(OMe)benzyl	33	241
8b	2,3-Di(OMe)benzyl	26	241
8c	2,4-Di(OMe)benzyl	23	241
8d	2,5-Di(OMe)benzyl	20	241
8e	2,6-Di(OMe)benzyl	21	241
8f	3,5-Di(OMe)benzyl	24	241
8g	3,4-Di(OEt)benzyl	22	269
8h	3,4-(OCH ₂ CH ₂ O)benzyl	20	238
8i	2,4-Dichlorobenzyl	20	248
8j	3,4-Dichlorobenzyl	23	248
8k	2,6-Dichlorobenzyl	23	248
8l	2,6-Dimethylbenzyl	18	209
8m	2,3-Dimethylbenzyl	22	209
8n	3,4-Dimethylbenzyl	29	209
8o	2,4-Dimethylbenzyl	36	209
8p	2,3-Dichlorobenzyl	30	250
8q	3,5-Dimethylbenzyl	25	209
8r	2-Chlorobenzyl	17	215
8s	3-Chlorobenzyl	22	215
8t	4-Chlorobenzyl	17	215
8u	2-Methylbenzyl	20	195
8v	3-Methylbenzyl	20	195
8w	4-Methylbenzyl	20	195
8x	2-Methoxybenzyl	21	211
8y	3-Methoxybenzyl	20	211
8z	4-Methoxybenzyl	17	211
8aa	Methyl	20	119
8ab	Isopropyl	21	147
8ac	<i>t</i> -Butyl	29	161
8ad	Phenethyl	20	195
8ae	2-Thiophene	24	187
8af	2-Pyridyl	Crude	182

5.1.2 General Procedure for the Preparation of 2-Thioxo-2,3-dihydropyrimidin-4 (1H)-one Derivative 9 [20]—Thiourea **8** (2.08 mmol) and ethyl cyanoacetate (2.28 mmol) were added to a solution of sodium (2.08 mmol) in ethanol (5 mL). The reaction mixture was heated at reflux for 4 h. The reaction mixture was cooled to room temperature and concentrated under reduced pressure. The crude residue was purified by silica-gel column chromatography (1–3% MeOH in CH₂Cl₂) to afford pyrimidinone **9**. The products were characterized by ¹H NMR and MS analysis.

Compound **5** (R = 3,4-Di(OMe)benzyl): Yield = 31%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.86 (s, 1H, NH), 7.06 (s, 2H, Ar-H), 6.97 (s, 1H, Ar-H), 6.88–6.82 (m, 2H, NH₂), 4.85 (s, 1H, C(5)-H), 4.48 (br s, 2H, N-CH₂), 3.73 (s, 3H, OCH₃), 3.70 (s, 3H, OCH₃), 2.84 (t, *J* = 8 Hz, 2H, N-CH₂-CH₂); HRMS (ESI) *m/z* calculated for [C₁₄H₁₇N₃O₃S + H]⁺ 307.0991, found 307.0993.

Compound **9b** (R = 2,3-Di(OMe)benzyl): Yield = 30%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.83 (s, 1H, NH), 7.01 (t, *J* = 8.0 Hz, 1H, Ar-H), 6.99–6.85 (m, 4H, Ar-H and NH₂), 4.89 (s, 1H, C(5)-H), 4.54 (br s, 2H, N-CH₂), 3.78 (s, 3H, OCH₃), 3.73 (s, 3H, OCH₃), 2.91 (t, *J* = 8.0 Hz, 2H, N-CH₂-CH₂); MS (ESI) *m/z* 308 [C₁₄H₁₇N₃O₃S + H]⁺.

Compound **9c** (R = 2,4-Di(OMe)benzyl): Yield = 30%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.83 (s, 1H, NH), 7.01 (t, *J* = 8.0 Hz, 1H, Ar-H), 6.99–6.85 (m, 4H, Ar-H and NH₂), 4.89 (s, 1H, C(5)-H), 4.54 (br s, 2H, N-CH₂), 3.78 (s, 3H, OCH₃), 3.73 (s, 1H, OCH₃), 2.91 (t, *J* = 8.0 Hz, 2H, N-CH₂-CH₂); MS (ESI) *m/z* 308 [C₁₄H₁₇N₃O₃S + H]⁺.

Compound **9d** (R = 2,5-Di(OMe)benzyl): Yield = 32%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.83 (s, 1H, NH), 7.01 (t, *J* = 8.0 Hz, 1H, Ar-H), 6.99–6.85 (m, 4H, Ar-H and NH₂), 4.89 (s, 1H), 4.54 (br s, 2H, N-CH₂), 3.78 (s, 3H, OCH₃), 3.73 (s, 3H, OCH₃), 2.91 (t, *J* = 8.0 Hz, 2H, N-CH₂-CH₂); MS (ESI) *m/z* 308 [C₁₄H₁₇N₃O₃S + H]⁺.

Compound **9e** (R = 2,6-Di(OMe)benzyl): Yield = 34%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.68 (s, 1H, NH), 7.16 (d, *J* = 8.4 Hz, 1H, Ar-H), 6.74 (s, 2H, NH₂), 6.60 (d, *J* = 8.4 Hz, 2H, Ar-H), 4.85 (s, 1H, C(5)-H), 4.47 (br s, 2H, N-CH₂), 3.73 (s, 6H, OCH₃), 2.93 (br s, 2H, N-CH₂-CH₂); MS (ESI) *m/z* 308 [C₁₄H₁₇N₃O₃S + H]⁺.

Compound **9f** (R = 3,5-Di(OMe)benzyl): Yield = 39%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.88 (s, 1H, NH), 7.07 (s, 2H, NH₂), 6.53 (d, *J* = 2.4 Hz, 2H, Ar-H), 6.35 (t, *J* = 2 Hz, 1H, Ar-H), 4.86 (s, 1H, C(5)-H), 4.55 (br s, 2H, N-CH₂), 3.71 (s, 6H, OCH₃), 2.83 (t, *J* = 8 Hz, 2H, N-CH₂-CH₂); MS (ESI) *m/z* 308 [C₁₄H₁₇N₃O₃S + H]⁺.

Compound **9g** (R = 3,4-Di(OEt)benzyl): Yield = 27%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.85 (s, 1H, NH), 7.05 (s, 2H, NH₂), 6.97 (s, 1H, Ar-H), 6.86 (d, *J* = 8.0 Hz, 1H, Ar-H), 6.81 (dd, *J* = 8.0, 1.2 Hz, 1H, Ar-H), 4.85 (s, 1H, C(5)-H), 4.50 (br s, 2H, N-CH₂), 4.01–3.93 (m, 4H, O-CH₂), 2.82 (t, *J* = 8.0 Hz, 2H, N-CH₂-CH₂), 1.33–1.27 (m, 6H, O-CH₂-CH₃); MS (ESI) *m/z* 336 [C₁₆H₂₁N₃O₃S + H]⁺.

Compound **9h** (R = 3,4-(OCH₂CH₂O)benzyl): Yield = 42%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.86 (s, 1H, NH), 7.07 (s, 2H, NH₂), 6.88 (s, 1H, Ar-H), 6.81–6.76 (m, 2H, Ar-H), 4.85 (s, 1H, C(5)-H), 4.38 (br s, 2H, N-CH₂), 4.19 (s, 4H, O-CH₂-CH₂-O), 2.77 (t, *J* = 8.4 Hz, 2H, N-CH₂-CH₂); MS (ESI) *m/z* 306 [C₁₄H₁₅N₃O₃S + H]⁺.

Compound **9i** (R = 2,4-Dichlorobenzyl): Yield = 32%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.78 (s, 1H, NH), 7.55 (s, 1H, Ar-H), 7.38 (s, 2H, Ar-H), 6.97 (s, 2H, NH₂), 4.90 (s, 1H, C(5)-H), 4.87–4.66 (m, 2H, N-CH₂), 3.03 (t, *J* = 7.6 Hz, 2H, N-CH₂-CH₂); MS (ESI) *m/z* 316 [C₁₂H₁₁Cl₂N₃OS + H]⁺.

Compound 9j (R = 3,4-Dichlorobenzyl): Yield = 39%; ^1H NMR (400 MHz, DMSO- d_6) δ 11.90 (s, 1H, NH), 7.64 (s, 1H, Ar-H), 7.58 (d, J = 8.0 Hz, 1H, Ar-H), 7.33 (d, J = 8.0 Hz, 1H, Ar-H), 7.12 (s, 2H, NH₂), 4.85 (s, 1H, C(5)-H), 4.47 (br s, 2H, N-CH₂), 2.92 (t, J = 8.0 Hz, 2H, N-CH₂-CH₂); MS (ESI) m/z 316 [$\text{C}_{12}\text{H}_{11}\text{Cl}_2\text{N}_3\text{OS} + \text{H}$]⁺.

Compound 9k (R = 2,6-Dichlorobenzyl): Yield = 32%; ^1H NMR (400 MHz, DMSO- d_6) δ 11.90 (s, 1H, NH), 7.64 (s, 1H, Ar-H), 7.58 (d, J = 8.0 Hz, 1H, Ar-H), 7.33 (d, J = 8.0 Hz, 1H, Ar-H), 7.12 (s, 2H, NH₂), 4.85 (s, 1H, C(5)-H), 4.47 (br s, 2H, N-CH₂), 2.92 (t, J = 8.0 Hz, 2H, N-CH₂-CH₂); MS (ESI) m/z 316 [$\text{C}_{12}\text{H}_{11}\text{Cl}_2\text{N}_3\text{OS} + \text{H}$]⁺.

Compound 9l (R = 2,6-Dimethylbenzyl): Yield = 30%; ^1H NMR (400 MHz, DMSO- d_6) δ 11.91 (s, 1H, NH), 6.99–6.98 (m, 3H, Ar-H and NH₂), 6.82 (s, 2H, Ar-H), 4.94 (s, 1H, C(5)-H), 4.47 (br s, 2H, N-CH₂), 2.92 (t, J = 7.2 Hz, 2H, N-CH₂-CH₂), 2.38 (s, 6H, CH₃); MS (ESI) m/z 276 [$\text{C}_{14}\text{H}_{17}\text{N}_3\text{OS} + \text{H}$]⁺.

Compound 9m (R = 2,3-Dimethylbenzyl): Yield = 32%; ^1H NMR (400 MHz, DMSO- d_6) δ 11.86 (s, 1H, NH), 7.16 (d, J = 7.6 Hz, 1H, Ar-H), 6.98–6.92 (m, 4H, Ar-H and NH₂), 4.89 (s, 1H, C(5)-H), 4.49 (br s, 2H, N-CH₂), 2.83 (t, J = 8.0 Hz, 2H, N-CH₂-CH₂), 2.30 (s, 3H, CH₃), 2.21 (s, 3H, CH₃); MS (ESI) m/z 276 [$\text{C}_{14}\text{H}_{17}\text{N}_3\text{OS} + \text{H}$]⁺.

Compound 9n (R = 3,4-Dimethylbenzyl): Yield = 30%; ^1H NMR (400 MHz, DMSO- d_6) δ 11.84 (s, 1H, NH), 7.12–7.05 (m, 5H, Ar-H and NH₂), 4.87 (s, 1H, C(5)-H), 4.47 (br s, 2H, N-CH₂), 2.83 (t, J = 8 Hz, 2H, N-CH₂-CH₂), 2.18 (s, 3H, CH₃), 2.17 (s, 3H, CH₃); MS (ESI) m/z 276 [$\text{C}_{14}\text{H}_{17}\text{N}_3\text{OS} + \text{H}$]⁺.

Compound 9o (R = 2,4-Dimethylbenzyl): Yield = 45%; ^1H NMR (400 MHz, DMSO- d_6) δ 11.86 (s, 1H, NH), 7.16 (d, J = 7.6 Hz, 1H, Ar-H), 6.98–6.92 (m, 4H, Ar-H and NH₂), 4.89 (s, 1H, C(5)-H), 4.49 (br s, 2H, N-CH₂), 2.83 (t, J = 8.0 Hz, 2H, N-CH₂-CH₂), 2.30 (s, 3H, CH₃), 2.21 (s, 3H, CH₃); MS (ESI) m/z 276 [$\text{C}_{14}\text{H}_{17}\text{N}_3\text{OS} + \text{H}$]⁺.

Compound 9p (R = 2,3-Dichlorobenzyl): Yield = 29%; ^1H NMR (400 MHz, DMSO- d_6) δ 11.80 (s, 1H, NH), 7.67 (d, J = 11.2 Hz, 1H, Ar-H), 7.52–7.50 (m, 1H, Ar-H), 7.32–7.28 (m, 1H, Ar-H), 6.99 (s, 2H, NH₂), 4.88 (s, 1H, C(5)-H), 4.22–4.19 (m, 2H, N-CH₂), 3.10 (t, J = 7.6 Hz, 2H, N-CH₂-CH₂); MS (ESI) m/z 316 [$\text{C}_{12}\text{H}_{11}\text{Cl}_2\text{N}_3\text{OS} + \text{H}$]⁺.

Compound 9q (R = 3,5-Dimethylbenzyl): Yield = 50%; ^1H NMR (400 MHz, DMSO- d_6) δ 11.83 (s, 1H, NH), 7.03 (s, 2H, Ar-H), 6.96 (s, 2H, NH₂), 6.85 (s, 1H, Ar-H), 4.88 (s, 1H, C(5)-H), 4.47 (br s, 2H, N-CH₂), 2.83 (t, J = 8.0 Hz, 2H, N-CH₂-CH₂), 2.24 (s, 6H, CH₃); MS (ESI) m/z 276 [$\text{C}_{14}\text{H}_{17}\text{N}_3\text{OS} + \text{H}$]⁺.

Compound 9r (R = 2-Chlorobenzyl): Yield = 43%; ^1H NMR (400 MHz, DMSO- d_6) δ 11.87 (s, 1H, NH), 7.37 (s, 4H, Ar-H), 7.09 (s, 2H, NH₂), 4.86 (s, 1H, C(5)-H), 4.49 (br s, 2H, N-CH₂), 2.90 (t, J = 8 Hz, 2H, N-CH₂-CH₂); MS (ESI) m/z 282 [$\text{C}_{12}\text{H}_{12}\text{ClN}_3\text{OS} + \text{H}$]⁺.

Compound 9s (R = 3-Chlorobenzyl): Yield = 60%; ^1H NMR (400 MHz, DMSO- d_6) δ 11.87 (s, 1H, NH), 7.37 (s, 4H, Ar-H), 7.09 (s, 2H, NH₂), 4.86 (s, 1H, C(5)-H), 4.49 (br s, 2H, N-CH₂), 2.90 (t, J = 8 Hz, 2H, N-CH₂-CH₂); MS (ESI) m/z 282 [$\text{C}_{12}\text{H}_{12}\text{ClN}_3\text{OS} + \text{H}$]⁺.

Compound 9t (R = 4-Chlorobenzyl): Yield = 25%; ^1H NMR (400 MHz, DMSO- d_6) δ 11.87 (s, 1H, NH), 7.37 (s, 4H, Ar-H), 7.09 (s, 2H, NH₂), 4.86 (s, 1H, C(5)-H), 4.49 (br s, 2H, N-CH₂), 2.90 (t, J = 8 Hz, 2H, N-CH₂-CH₂); MS (ESI) m/z 282 [$\text{C}_{12}\text{H}_{12}\text{ClN}_3\text{OS} + \text{H}$]⁺.

Compound 9u (R = 2-Methylbenzyl): Yield = 28%; ^1H NMR (400 MHz, DMSO- d_6) δ 11.87 (s, 1H, NH), 7.29–6.99 (m, 6H, Ar-H and NH₂), 4.90 (s, 1H, C(5)-H), 4.49 (br s, 2H, N-

CH₂), 2.91–2.87 (m, 2H, N-CH₂-CH₂), 2.35 (s, 3H); MS (ESI) *m/z* 262 [C₁₃H₁₅N₃OS + H]⁺.

Compound 9v (R = 3-Methylbenzyl): Yield = 29%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.87 (s, 1H, NH), 7.29–6.99 (m, 6H, Ar-H and NH₂), 4.90 (s, 1H, C(5)-H), 4.49 (br s, 2H, N-CH₂), 2.91–2.87 (m, 2H, N-CH₂-CH₂), 2.35 (s, 3H); MS (ESI) *m/z* 262 [C₁₃H₁₅N₃OS + H]⁺.

Compound 9w (R = 4-Methylbenzyl): Yield = 30%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.87 (s, 1H, NH), 7.29 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.13 (t, *J* = 8.0 Hz, 2H, Ar-H), 6.99 (s, 2H, NH₂), 4.90 (s, 1H, C(5)-H), 4.51 (br s, 2H, N-CH₂), 2.91 (t, *J* = 8.0 Hz, 2H, N-CH₂-CH₂), 2.35 (s, 3H); MS (ESI) *m/z* 261 [C₁₃H₁₅N₃OS + H]⁺.

Compound 9x (R = 2-Methoxybenzyl): Yield = 80%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.84 (s, 1H, NH), 7.22 (t, *J* = 8.0 Hz, 1H, Ar-H), 7.05 (s, 2H, NH₂), 6.95–6.91 (m, 2H, Ar-H), 6.79 (d, *J* = 8.0 Hz, 1H, Ar-H), 4.87 (s, 1H, C(5)-H), 4.50 (br s, 2H, N-CH₂), 3.73 (s, 3H, OCH₃), 2.89 (t, *J* = 8.0 Hz, 2H, N-CH₂-CH₂); MS (ESI) *m/z* 277 [C₁₃H₁₅N₃O₂S + H]⁺.

Compound 9y (R = 3-Methoxybenzyl): Yield = 59%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.84 (s, 1H, NH), 7.22 (t, *J* = 8.0 Hz, 1H, Ar-H), 7.05 (s, 2H, NH₂), 6.95–6.91 (m, 2H, Ar-H), 6.79 (d, *J* = 8.0 Hz, 1H, Ar-H), 4.87 (s, 1H, C(5)-H), 4.50 (br s, 2H, N-CH₂), 3.73 (s, 3H, OCH₃), 2.89 (t, *J* = 8.0 Hz, 2H, N-CH₂-CH₂); MS (ESI) *m/z* 277 [C₁₃H₁₅N₃O₂S + H]⁺.

Compound 9z (R = 4-Methoxybenzyl): Yield = 21%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.84 (s, 1H, NH), 7.22 (t, *J* = 8.0 Hz, 1H, Ar-H), 7.05 (s, 2H, NH₂), 6.95–6.91 (m, 2H, Ar-H), 6.79 (d, *J* = 8.0 Hz, 1H, Ar-H), 4.87 (s, 1H, C(5)-H), 4.50 (br s, 2H, N-CH₂), 3.73 (s, 3H, OCH₃), 2.89 (t, *J* = 8.0 Hz, 2H, N-CH₂-CH₂); MS (ESI) *m/z* 277 [C₁₃H₁₅N₃O₂S + H]^{plus}.

Compound 9aa (R = Methyl): Yield = 45%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.78 (s, 1H, NH), 7.02 (s, 2H, NH₂), 4.81 (s, 1H, C(5)-H), 4.15 (br s, 2H, N-CH₂), 1.65 (t, *J* = 8.0 Hz, 2H, N-CH₂-CH₂), 0.85 (t, *J* = 8.0 Hz, 3H, CH₃); MS (ESI) *m/z* 214 [C₉H₁₅N₃OS + H]⁺.

Compound 9ab (R = Isopropyl): Yield = 48%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.78 (s, 1H, NH), 6.79 (s, 2H, NH₂), 4.88 (s, 1H, C(5)-H), 4.39 (br s, 2H, N-CH₂), 1.68–1.61 (m, 1H, CH(CH₃)₂), 1.49 (t, *J* = 7.6 Hz, 2H, N-CH₂-CH₂), 0.94–0.91 (m, 6H, CH(CH₃)₂); MS (ESI) *m/z* 214 [C₉H₁₅N₃OS + H]⁺.

Compound 9ac (R = *t*-Butyl): Yield = 29%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.78 (s, 1H, NH), 6.79 (s, 2H, NH₂), 4.88 (s, 1H, C(5)-H), 4.39 (br s, 2H, N-CH₂), 1.49 (t, *J* = 7.6 Hz, 2H, N-CH₂-CH₂), 0.93 (s, 9H, C(CH₃)₃); MS (ESI) *m/z* 228 [C₁₀H₁₇N₃OS + H]⁺.

Compound 9ad (R = Phenethyl): Yield = 33%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.78 (s, 1H, NH), 7.28–7.14 (m, 5H, Ar-H), 7.05 (s, 2H, NH₂), 4.83 (s, 1H, C(5)-H), 4.33–4.32 (m, 2H, N-CH₂), 2.62 (t, *J* = 8 Hz, 2H, C₆H₅-CH₂), 1.90 (t, *J* = 8 Hz, 2H, N-CH₂-CH₂); MS (ESI) *m/z* 262 [C₁₃H₁₅N₃OS + H]⁺.

Compound 9ae (R = 2-Thiophene): Yield = 36%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.87 (s, 1H, NH), 7.70–7.64 (m, 1H, Ar-H), 7.08 (s, 2H, NH₂), 6.98–6.95 (m, 2H, Ar-H), 4.86 (s, 1H, C(5)-H), 4.85 (br s, 2H, N-CH₂), 3.13 (t, *J* = 8 Hz, 2H, N-CH₂-CH₂); MS (ESI) *m/z* 254 [C₁₀H₁₁N₃OS₂ + H]⁺.

Compound 9af (R = 2-Pyridyl): Yield = 32%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.84 (s, 1H, NH), 8.49 (d, *J* = 4.4 Hz, 1H), 7.79–7.70 (m, 1H, Ar-H), 7.35–7.23 (m, 2H, Ar-H), 7.11 (s,

2H, NH₂), 4.87 (s, 1H, C(5)-H), 4.72 (br s, 2H, N-CH₂), 3.12 (t, *J* = 8 Hz, 2H, N-CH₂-CH₂); MS (ESI) *m/z* 249 [C₁₁H₁₂N₄OS + H]⁺.

5.1.3 General Procedure for the Preparation of 10 [21-23]—*n*-Propylamine (4.55 mmol) and formaldehyde (6.50 mmol) were added to a solution of amine **9** (3.25 mmol) in ethanol (20 mL). The reaction mixture was heated at reflux for 3 h, cooled to room temperature, and filtered through a sintered glass funnel to afford amine **10**. The compound was characterized by ¹H NMR and MS analysis, and HRMS.

Compound 1 (R = 3,4-Di(OMe)benzyl): Yield = 51%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.00 (s, 1H, N(3)-H), 7.37 (s, 1H, N(8)-H), 6.92–6.81 (m, 3H, Ar-H), 4.47 (br s, 2H, N-CH₂), 3.98 (s, 2H, C(7)-H₂), 3.73 (s, 3H, OCH₃), 3.70 (s, 3H, OCH₃), 3.36 (s, 2H, C(5)-H₂), 2.85 (t, *J* = 8 Hz, 2H, N-CH₂-CH₂), 2.33 (t, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.44 (q, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 0.86 (t, *J* = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) *m/z* calculated for [C₁₉H₂₆N₄O₃S + H]⁺ 390.1726, found 390.1733

Compound 2 (R = 3,5-Dimethylbenzyl): Yield = 50%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.02 (s, 1H, N(3)-H), 7.40 (s, 1H, N(8)-H), 7.10 (s, 1H, Ar-H), 7.06 (s, 2H, Ar-H), 4.44 (br s, 2H, N-CH₂), 3.98 (s, 2H, C(7)-H₂), 3.37 (s, 2H, C(5)-H₂), 2.82 (t, *J* = 8 Hz, 2H, N-CH₂-CH₂), 2.34 (t, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 2.19 (s, 3H, Ar-CH₃), 2.17 (s, 3H, Ar-CH₃), 1.50–1.41 (m, 2H, CH₂CH₂CH₃), 0.86 (t, *J* = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) *m/z* calculated for [C₁₉H₂₆N₄OS + H]⁺, found 330.1515.

Compound 33 (R = 2-Methylbenzyl): Yield = 45%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.03 (s, 1H, N(3)-H), 7.32 (s, 1H, N(8)-H), 7.27 (t, *J* = 7.2 Hz, 1H, Ar-H), 7.13 (s, 3H, Ar-H), 4.51 (br s, 2H, N-CH₂), 3.97 (s, 2H, C(7)-H₂), 3.37 (s, 2H, C(5)-H₂), 2.89 (t, *J* = 7.2 Hz, 2H, N-CH₂-CH₂), 2.34 (m, 5H, CH₂CH₂CH₃ and Ar-CH₃), 1.45 (q, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 0.86 (t, *J* = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) *m/z* calculated for [C₁₈H₂₄N₄OS + H]⁺. 344.1671, found 344.1672.

Compound 34 (R = 3-Methylbenzyl): Yield = 45%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.02 (s, 1H, N(3)-H), 7.41 (s, 1H, N(8)-H), 7.22–7.12 (m, 3H, Ar-H), 7.40 (d, *J* = 7.2 Hz, 1H, Ar-H), 4.47 (br s, 2H, N-CH₂), 3.99 (s, 2H, C(7)-H₂), 3.37 (s, 2H, C(5)-H₂), 2.87 (t, *J* = 8.0 Hz, 2H, N-CH₂-CH₂), 2.32 (t, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 2.28 (s, 3H, Ar-CH₃), 1.45 (q, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 0.87 (t, *J* = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) *m/z* calculated for [C₁₈H₂₄N₄OS + H]⁺. 344.1671, found 344.1672.

Compound 35 (R = 4-Methylbenzyl): Yield = 42%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.99 (s, 1H, N(3)-H), 7.38 (s, 1H, N(8)-H), 7.22 (d, *J* = 7.6 Hz, 2H, Ar-H), 7.12 (d, *J* = 8.0 Hz, 2H, Ar-H), 4.45 (br s, 2H, N-CH₂), 3.99 (s, 2H, C(7)-H₂), 3.37 (s, 2H, C(5)-H₂), 2.86 (t, *J* = 8.0 Hz, 2H, N-CH₂-CH₂), 2.34 (t, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 2.26 (s, 3H, Ar-CH₃), 1.45 (m, 2H, CH₂CH₂CH₃), 0.87 (t, *J* = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) *m/z* calculated for [C₁₈H₂₄N₄OS + H]⁺. 344.1671, found 344.1672.

Compound 36 (R = 2-Chlorobenzyl): Yield = 45%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.98 (s, 1H, N(3)-H), 7.41–7.25 (m, 5H, N(8)-H and Ar-H), 4.64 (s, 2H, N-CH₂), 3.93 (s, 2H, C(7)-H₂), 3.36 (s, 2H, C(5)-H₂), 3.05 (t, *J* = 7.6 Hz, 2H, N-CH₂-CH₂), 2.32 (t, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.46 (q, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 0.86 (t, *J* = 7.6 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) *m/z* calculated for [C₁₇H₂₁ClN₄OS + H]⁺ 364.1125, found 364.1125.

Compound 37 (R = 3-Chlorobenzyl): Yield = 40%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.03 (s, 1H, N(3)-H), 7.43 (s, 2H, N(8)-H and Ar-H), 7.37–7.28 (m, 3H, Ar-H), 4.47 (br s, 2H, N-CH₂), 3.99 (s, 2H, C(7)-H₂), 3.37 (s, 2H, C(5)-H₂), 2.93 (t, *J* = 8.0 Hz, 2H, N-CH₂-CH₂), 2.34

(t, $J = 7.2$ Hz, 2H, **CH₂CH₂CH₃**), 1.44 (q, $J = 7.2$ Hz, 2H, **CH₂CH₂CH₃**), 0.87 (t, $J = 7.2$ Hz, 3H, **CH₂CH₂CH₃**); HRMS (ESI) m/z calculated for $[C_{17}H_{21}ClN_4OS + H]^+$ 364.1125, found 364.1128.

Compound 38 (R = 4-Chlorobenzyl): Yield = 40%; 1H NMR (400 MHz, DMSO- d_6) δ 12.00 (s, 1H, N(3)-H), 7.42–7.34 (m, 5H, N(8)-H and Ar-H), 4.46 (br s, 2H, N-CH₂), 3.98 (s, 2H, C(7)-H₂), 3.37 (s, 2H, C(5)-H₂), 2.91 (t, $J = 8.00$ Hz, 2H, N-CH₂-**CH₂**), 2.34 (t, $J = 7.2$ Hz, 2H, **CH₂CH₂CH₃**), 1.45 (q, $J = 7.2$ Hz, 2H, **CH₂CH₂CH₃**), 0.87 (t, $J = 7.2$ Hz, 3H, **CH₂CH₂CH₃**); HRMS (ESI) m/z calculated for $[C_{17}H_{21}ClN_4OS + H]^+$ 364.1125, found 364.1130.

Compound 39 (R = 2-Methoxybenzyl): Yield = 35%; 1H NMR (400 MHz, DMSO- d_6) δ 11.91 (s, 1H, N(3)-H), 7.22–7.14 (m, 2H, N(8)-H and Ar-H), 7.07 (s, 1H, Ar-H), 6.94–6.85 (m, 2H, Ar-H), 4.57 (br s, 2H, N-CH₂), 3.94 (s, 2H, C(7)-H₂), 3.75 (s, 3H, OCH₃), 3.36 (s, 2H, C(5)-H₂), 2.90 (t, $J = 7.2$ Hz, 2H, N-CH₂-**CH₂**), 2.32 (t, $J = 7.2$ Hz, 2H, **CH₂CH₂CH₃**), 1.44 (q, $J = 7.2$ Hz, 2H, **CH₂CH₂CH₃**), 0.86 (t, $J = 7.2$ Hz, 3H, **CH₂CH₂CH₃**); HRMS (ESI) m/z calculated for $[C_{18}H_{24}N_4O_2S + H]^+$ 360.1620, found 360.1623.

Compound 40 (R = 3-Methoxybenzyl): Yield = 57%; 1H NMR (400 MHz, DMSO- d_6) δ 12.03 (s, 1H, N(3)-H), 7.41 (s, 1H, N(8)-H), 7.22 (t, $J = 7.6$ Hz, 1H, Ar-H), 6.91 (s, 2H, Ar-H), 6.80 (d, $J = 7.6$ Hz, 1H, Ar-H), 4.48 (br s, 2H, N-CH₂), 3.98 (s, 2H, C(7)-H₂), 3.73 (s, 3H, OCH₃), 3.37 (s, 2H, C(5)-H₂), 2.88 (t, $J = 8.0$ Hz, 2H, N-CH₂-**CH₂**), 2.34 (t, $J = 7.2$ Hz, 2H, **CH₂CH₂CH₃**), 1.45 (m, 2H, **CH₂CH₂CH₃**), 0.86 (t, $J = 7.2$ Hz, 3H, **CH₂CH₂CH₃**); HRMS (ESI) m/z calculated for $[C_{18}H_{24}N_4O_2S + H]^+$ 360.1620, found 360.1621.

Compound 41 (R = 4-Methoxybenzyl): Yield = 35%; 1H NMR (400 MHz, DMSO- d_6) δ 12.00 (s, 1H, N(3)-H), 7.39 (s, 1H, N(8)-H), 7.25 (d, $J = 8.4$ Hz, 2H, Ar-H), 6.89 (d, $J = 8.4$ Hz, 2H, Ar-H), 4.43 (br s, 2H, N-CH₂), 3.99 (s, 2H, C(7)-H₂), 3.71 (s, 3H, OCH₃), 3.37 (s, 2H, C(5)-H₂), 2.84 (t, $J = 8.0$ Hz, 2H, N-CH₂-**CH₂**), 2.35 (t, $J = 7.2$ Hz, 2H, **CH₂CH₂CH₃**), 1.45 (q, $J = 7.2$ Hz, 2H, **CH₂CH₂CH₃**), 0.86 (t, $J = 7.2$ Hz, 3H, **CH₂CH₂CH₃**); HRMS (ESI) m/z calculated for $[C_{18}H_{24}N_4O_2S + H]^+$ 360.1620, found 360.1623.

Compound 42 (R = 2,6-Dimethylbenzyl): Yield = 42%; 1H NMR (400 MHz, DMSO- d_6) δ 12.08 (s, 1H, N(3)-H), 7.13 (s, 1H, N(8)-H), 6.98 (s, 3H, Ar-H), 4.32 (br s, 2H, N-CH₂), 3.94 (s, 2H, C(7)-H₂), 3.37 (s, 2H, C(5)-H₂), 2.92 (s, 2H, N-CH₂-**CH₂**), 2.37–2.32 (m, 8H, **CH₂CH₂CH₃** and Ar-**CH₃**), 1.45 (q, $J = 7.2$ Hz, 2H, **CH₂CH₂CH₃**), 0.85 (t, $J = 7.2$ Hz, 3H, **CH₂CH₂CH₃**); HRMS (ESI) m/z calculated for $[C_{19}H_{26}N_4OS + H]^+$ 358.1827, found 358.1833.

Compound 43 (R = 2,3-Dimethylbenzyl): Yield = 33%; 1H NMR (400 MHz, DMSO- d_6) δ 12.03 (s, 1H, N(3)-H), 7.25 (s, 1H, N(8)-H), 7.19 (t, $J = 1.2$ Hz, 1H, Ar-H), 7.02 (s, 2H, Ar-H), 4.48 (br s, 2H, N-CH₂), 3.97 (s, 2H, C(7)-H₂), 3.37 (s, 2H, C(5)-H₂), 2.91 (t, $J = 8.0$ Hz, 2H, N-CH₂-**CH₂**), 2.35 (t, $J = 7.2$ Hz, 2H, **CH₂CH₂CH₃**), 2.24 (s, 3H, Ar-**CH₃**), 2.22 (s, 3H, Ar-**CH₃**), 1.49–1.40 (m, 2H, **CH₂CH₂CH₃**), 0.86 (t, $J = 1.72$ Hz, 3H, **CH₂CH₂CH₃**); HRMS (ESI) m/z calculated for $[C_{19}H_{26}N_4OS + H]^+$ 358.1827, found 358.1833.

Compound 44 (R = 2,5-Dimethylbenzyl): Yield = 32%; 1H NMR (400 MHz, DMSO- d_6) δ 12.02 (s, 1H, N(3)-H), 7.40 (s, 1H, N(8)-H), 7.10 (s, 1H, Ar-H), 7.06 (s, 3H, Ar-H), 4.44 (br s, 2H, N-CH₂), 3.98 (s, 2H, C(7)-H₂), 3.37 (s, 2H, C(5)-H₂), 2.82 (t, $J = 8.0$ Hz, 2H, N-CH₂-**CH₂**), 2.34 (t, $J = 7.2$ Hz, 2H, **CH₂CH₂CH₃**), 2.19 (s, 3H, Ar-**CH₃**), 2.17 (s, 3H, Ar-**CH₃**), 1.50–1.41 (m, 2H, **CH₂CH₂CH₃**), 0.86 (t, $J = 7.2$ Hz, 3H, **CH₂CH₂CH₃**); HRMS (ESI) m/z calculated for $[C_{19}H_{26}N_4OS + H]^+$ 358.1827, found 358.1832.

Compound 45 (R = 2,4-Dimethylbenzyl): Yield = 32%; 1H NMR (400 MHz, DMSO- d_6) δ 12.06 (s, 1H, N(3)-H), 7.40 (br s, 1H, N(8)-H), 7.15 (s, 1H, Ar-H), 6.95 (s, 2H, Ar-H), 4.48

(br s, 2H, N-CH₂), 3.97 (s, 2H, C(7)-H₂), 3.37 (s, 2H, C(5)-H₂), 2.84 (t, *J* = 8.0 Hz, 2H, N-CH₂-CH₂), 2.36–2.30 (m, 5H, CH₂CH₂CH₃ and Ar-CH₃), 2.21 (s, 3H, Ar-CH₃), 1.50–1.41 (m, 2H, CH₂CH₂CH₃), 0.86 (t, *J* = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) *m/z* calculated for [C₁₉H₂₆N₄OS+H]⁺ 358.1827, found 358.1844.

Compound 46 (R = 3,4-Dimethylbenzyl): Yield = 32%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.02 (s, 1H, N(3)-H), 7.40 (s, 1H, N(8)-H), 7.10 (s, 1H, Ar-H), 7.06 (s, 3H, Ar-H), 4.44 (br s, 2H, N-CH₂), 3.98 (s, 2H, C(7)-H₂), 3.37 (s, 2H, C(5)-H₂), 2.82 (t, *J* = 8 Hz, 2H, N-CH₂-CH₂), 2.34 (t, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 2.19 (s, 3H, Ar-CH₃), 2.17 (s, 3H, Ar-CH₃), 1.50–1.41 (m, 2H, CH₂CH₂CH₃), 0.86 (t, *J* = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) *m/z* calculated for [C₁₉H₂₆N₄OS+H]⁺ 358.1827, found 358.1833.

Compound 47 (R = 3,5-Dimethylbenzyl): Yield = 50%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.03 (s, 1H, N(3)-H), 7.32 (s, 1H, N(8)-H), 7.27 (t, *J* = 7.2 Hz, 1H, Ar-H), 7.13 (s, 3H, Ar-H), 4.51 (br s, 2H, N-CH₂), 3.97 (s, 2H, C(7)-H₂), 3.37 (s, 2H, C(5)-H₂), 2.89 (t, *J* = 7.2 Hz, 2H, N-CH₂-CH₂), 2.34 (t, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 2.33 (s, 6H, Ar-CH₃), 1.45 (q, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 0.86 (t, *J* = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) *m/z* calculated for [C₁₉H₂₆N₄OS+H]⁺ 358.1827, found 358.1831.

Compound 48 (R = 2,6-Dichlorobenzyl): Yield = 40%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.79 (s, 1H, N(3)-H), 7.37 (s, 1H, N(8)-H), 7.35 (s, 1H, Ar-H), 7.24–7.20 (m, 2H, Ar-H), 4.80 (br s, 2H, N-CH₂), 3.90 (s, 2H, C(7)-H₂), 3.23 (m, 4H, N-CH₂-CH₂ and C(5)-H₂), 2.33 (t, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.43 (q, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 0.85 (t, *J* = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) *m/z* calculated for [C₁₇H₂₀Cl₂N₄OS + H]⁺ 398.0735, found 398.0741.

Compound 49 (R = 2,3-Dichlorobenzyl): Yield = 29%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.96 (s, 1H, N(3)-H), 7.52–7.50 (m, 1H, N(8)-H), 7.32–7.28 (m, 3H, Ar-H), 4.66 (br s, 2H, N-CH₂), 3.93 (s, 2H, C(7)-H₂), 3.36 (s, 2H, C(5)-H₂), 3.11 (t, *J* = 7.2 Hz, 2H, N-CH₂-CH₂), 2.32 (t, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.44 (q, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 0.86 (t, *J* = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) *m/z* calculated for [C₁₇H₂₀Cl₂N₄OS + H]⁺ 398.0735, found 398.0741.

Compound 50 (R = 2,5-Dichlorobenzyl): Yield = 21%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.96 (s, 1H, N(3)-H), 7.47 (s, 1H, N(8)-H), 7.45 (s, 1H, Ar-H), 7.34 (d, *J* = 2.4 Hz, 2H, Ar-H), 4.69 (br s, 2H, N-CH₂), 3.94 (s, 2H, C(7)-H₂), 3.37 (s, 2H, C(5)-H₂), 3.07 (t, *J* = 7.2 Hz, 2H, N-CH₂-CH₂), 2.35 (t, *J* = 6.8 Hz, 2H, CH₂CH₂CH₃), 1.49–1.40 (m, 2H, CH₂CH₂CH₃), 0.88 (t, *J* = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) *m/z* calculated for [C₁₇H₂₀Cl₂N₄OS + H]⁺ 398.0735, found 398.0743.

Compound 51 (R = 2,4-Dichlorobenzyl): Yield = 39%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.94 (s, 1H, N(3)-H), 7.55 (s, 1H, Ar-H, N(8)-H), 7.38 (s, 2H, Ar-H), 7.28 (s, 1H, Ar-H), 4.66 (br s, 2H, N-CH₂), 3.94 (s, 2H, C(7)-H₂), 3.36 (s, 2H, C(5)-H₂), 3.04 (t, *J* = 7.2 Hz, 2H, N-CH₂-CH₂), 2.32 (t, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.44 (q, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 0.86 (t, *J* = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) *m/z* calculated for [C₁₇H₂₀Cl₂N₄OS + H]⁺ 398.0735, found 398.0735.

Compound 52 (R = 3,4-Dichlorobenzyl): Yield = 50%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.04 (s, 1H, N(3)-H), 7.61–7.58 (m, 2H, N(8)-H and Ar-H), 7.44 (s, 1H, Ar-H), 7.32–7.30 (m, 1H, Ar-H), 4.48 (br s, 2H, N-CH₂), 3.99 (s, 2H, C(7)-H₂), 3.37 (s, 2H, C(5)-H₂), 2.93 (t, *J* = 8.0 Hz, 2H, N-CH₂-CH₂), 2.33 (t, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.45 (q, *J* = 7.2 Hz, 2H, CH₂CH₂CH₃), 0.87 (t, *J* = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) *m/z* calculated for [C₁₇H₂₀Cl₂N₄OS + H]⁺ 398.0735, found 398.0741.

Compound 53 (R = 3,5-Dichlorobenzyl): Yield = 40%; ^1H NMR (400 MHz, DMSO- d_6) δ 12.05 (s, 1H, N(3)-H), 7.49 (s, 1H, N(8)-H), 7.44 (s, 1H, Ar-H), 7.39 (s, 2H, Ar-H), 4.47 (br s, 2H, N-CH₂), 4.00 (s, 2H, C(7)-H₂), 3.38 (s, 2H, C(5)-H₂), 2.96 (t, J = 7.2 Hz, 2H, N-CH₂-CH₂), 2.36 (t, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.50 (m, 2H, CH₂CH₂CH₃), 0.89 (t, J = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) m/z calculated for [C₁₇H₂₀Cl₂N₄OS + H]⁺ 398.0735, found 398.0743.

Compound 54 (R = 2,6-Di(OMe)benzyl): Yield = 51%; ^1H NMR (400 MHz, DMSO- d_6) δ 11.75 (s, 1H, N(3)-H), 7.12 (t, J = 8.4 Hz, 1H, Ar-H), 6.79 (s, 1H, N(8)-H), 6.55 (d, J = 8.0 Hz, 2H, Ar-H), 4.50 (br s, 2H, N-CH₂), 3.88 (s, 2H, C(7)-H₂), 3.70 (s, 6H, OCH₃), 3.33 (s, 2H, C(5)-H₂), 2.92 (t, J = 6.0 Hz, 2H, N-CH₂-CH₂), 2.31 (t, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.43 (q, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 0.85 (t, J = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) m/z calculated for [C₁₉H₂₆N₄O₃S + H]⁺ 390.1726, found 390.1730.

Compound 55 (R = 2,3-Di(OMe)benzyl): Yield = 55%; ^1H NMR (400 MHz, DMSO- d_6) δ 12.06 (s, 1H, N(3)-H), 7.24 (s, 1H, N(8)-H), 7.08 (t, J = 8.0 Hz, 1H, Ar-H), 7.00 (d, J = 8.4 Hz, 1H, Ar-H), 6.93 (d, J = 8.4 Hz, 1H, Ar-H), 4.59 (br s, 2H, N-CH₂), 4.06 (s, 2H, C(7)-H₂), 3.85 (s, 3H, OCH₃), 3.80 (s, 3H, OCH₃), 3.45 (s, 2H, C(5)-H₂), 2.99 (t, J = 8.0 Hz, 2H, N-CH₂-CH₂), 2.41 (t, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.45 (q, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 0.94 (t, J = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) m/z calculated for [C₁₉H₂₆N₄O₃S + H]⁺ 390.1726, found 390.1734.

Compound 56 (R = 2,5-Di(OMe)benzyl): Yield = 40%; ^1H NMR (400 MHz, DMSO- d_6) δ 12.00 (s, 1H, N(3)-H), 7.37 (s, 1H, N(8)-H), 6.92–6.81 (m, 3H, Ar-H), 4.47 (br s, 2H, N-CH₂), 3.98 (s, 2H, C(7)-H₂), 3.73 (s, 3H, OCH₃), 3.70 (s, 3H, OCH₃), 3.36 (s, 3H, C(5)-H₂), 2.85 (t, J = 8 Hz, 2H, N-CH₂-CH₂), 2.33 (t, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.44 (q, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 0.86 (t, J = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) m/z calculated for [C₁₉H₂₆N₄O₃S + H]⁺ 390.1726, found 390.1730.

Compound 57 (R = 2,4-Di(OMe)benzyl): Yield = 47%; ^1H NMR (400 MHz, DMSO- d_6) δ 11.92 (s, 1H, N(3)-H), 7.04–7.02 (m, 2H, Ar-H and N(8)-H), 6.49–6.43 (m, 2H, Ar-H), 4.51 (br s, 2H, N-CH₂), 3.95 (s, 2H, C(7)-H₂), 3.73 (s, 3H, OCH₃), 3.71 (s, 3H, OCH₃), 3.35 (s, 2H, C(5)-H₂), 2.81 (t, J = 7.6 Hz, 2H, N-CH₂-CH₂), 2.31 (t, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.44 (q, J = 7.6 Hz, 2H, CH₂CH₂CH₃), 0.85 (t, J = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) m/z calculated for [C₁₉H₂₆N₄O₃S + H]⁺ 390.1726, found 390.1731.

Compound 58 (R = 3,5-Di(OMe)benzyl): Yield = 39%; ^1H NMR (400 MHz, DMSO- d_6) δ 12.03 (s, 1H, N(3)-H), 7.38 (s, 1H, N(8)-H), 6.50 (s, 2H, Ar-H), 6.36 (s, 1H, Ar-H), 4.51 (br s, 2H, N-CH₂), 3.98 (s, 2H, C(7)-H₂), 3.71 (s, 6H, OCH₃), 3.36 (s, 2H, C(5)-H₂), 2.84 (t, J = 8.0 Hz, 2H, N-CH₂-CH₂), 2.33 (t, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.49–1.40 (m, 2H, CH₂CH₂CH₃), 0.86 (t, J = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) m/z calculated for [C₁₉H₂₆N₄O₃S + H]⁺ 390.1726, found 390.1728.

Compound 59 (R = 3,4-(OCH₂CH₂O)benzyl): Yield = 33%; ^1H NMR (400 MHz, DMSO- d_6) δ 12.02 (s, 1H, N(3)-H), 7.39 (s, 1H, N(8)-H), 6.86 (s, 1H, Ar-H), 6.78 (s, 2H, Ar-H), 4.34 (br s, 2H, N-CH₂), 4.20 (s, 4H, -OCH₂CH₂O-), 3.99 (s, 2H, C(7)-H₂), 3.37 (s, 2H, C(5)-H₂), 2.78 (t, J = 8.0 Hz, 2H, N-CH₂-CH₂), 2.34 (t, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.45 (q, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 0.86 (t, J = 7.2 Hz, 3H, CH₂CH₂CH₃). HRMS (ESI) m/z calculated for [C₁₉H₂₄N₄O₃S + H]⁺ 388.1569, found 388.1571.

Compound 60 (R = 3,4-Di(OEt)benzyl): Yield = 33%; ^1H NMR (400 MHz, DMSO- d_6) δ 12.01 (s, 1H, N(3)-H), 7.37 (s, 1H, N(8)-H), 6.91–6.79 (m, 3H, Ar-H), 4.44 (br s, 2H, N-CH₂), 4.01–3.93 (m, 6H, C(7)-H₂ and OCH₂CH₃), 3.36 (s, 2H, C(5)-H₂), 2.82 (t, J = 8.0 Hz, 2H, N-CH₂-CH₂), 2.33 (t, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.45 (t, J = 7.2 Hz, 2H, CH₂CH₂CH₃),

1.33–1.27 (m, 6H, OCH₂CH₃), 0.86 (t, J = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) m/z calculated for [C₂₁H₃₀N₄O₃S + H]⁺ 418.2039, found 418.2048.

Compound 61 (R = 2-Pyridyl): Yield = 32%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.01 (s, 1H, N(3)-H), 7.38 (s, 1H, N(8)-H), 6.92–6.81 (m, 3H, Ar-H), 4.48 (br s, 2H, N-CH₂), 3.98 (s, 2H, C(7)-H₂), 3.36 (s, 2H, C(5)-H₂), 2.85 (t, J = 8.0 Hz, 2H, N-CH₂-CH₂), 2.33 (t, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.45 (q, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 0.86 (t, J = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) m/z calculated for [C₁₆H₂₁N₅OS + H]⁺ 331.1467, found 331.1468.

Compound 62 (R = 3-Thienyl): Yield = 37%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.03 (s, 1H, N(3)-H), 7.49 (d, J = 4.4 Hz, 1H, Ar-H), 7.41 (s, 1H, N(8)-H), 7.27 (s, 1H, Ar-H), 7.10 (d, J = 4.8 Hz, 1H, Ar-H), 4.51 (s, 2H, N-CH₂), 3.98 (s, 2H, C(7)-H₂), 3.32 (s, 2H, C(5)-H₂), 2.96 (t, J = 8 Hz, 2H, N-CH₂-CH₂), 2.36 (t, J = 7.2 Hz 2H, CH₂CH₂CH₃), 1.50–1.41 (m, 2H, CH₂CH₂CH₃), 0.88 (t, J = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) m/z calculated for [C₁₅H₂₀N₄OS₂+H]⁺ 336.1079, found 336.1079.

Compound 63 (R = 2-Thienyl): Yield = 40%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.02 (s, 1H, N(3)-H), 7.44 (s, 1H, N(8)-H), 7.37–7.36 (m, 1H, Ar-H), 6.97 (s, 2H, Ar-H), 4.51 (br s, 2H, N-CH₂), 3.97 (s, 2H, C(7)-H₂), 3.37 (s, 2H, C(5)-H₂), 3.14 (t, J = 8.0 Hz, 2H, N-CH₂-CH₂), 2.34 (t, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.45 (q, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 0.86 (t, J = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) m/z calculated for [C₁₅H₂₀N₄OS₂+H]⁺ 336.1079, found 336.1078.

Compound 64 (R = 3,4-Di(OMe)phenyl): ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.08 (s, 1H, N(3)-H), 7.20 (s, 1H, N(8)-H), 6.88 (d, J = 4.4 Hz, 2H, Ar-H), 6.65 (d, J = 8.0 Hz, 1H, Ar-H), 5.51 (br s, 2H, N-CH₂), 3.92 (s, 2H, C(7)-H₂), 3.70 (s, 6H, OCH₃), 3.39 (s, 2H, C(5)-H₂), 2.32 (t, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.43 (q, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 0.84 (t, J = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) m/z calculated for [C₁₈H₂₄N₄O₃S + H]⁺ 376.1569, found 376.1573.

Compound 65 (R = Phenethyl): Yield = 33%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.94 (s, 1H, N(3)-H), 7.38 (s, 1H, N(8)-H), 7.28–7.16 (m, 5H, Ar-H), 4.33 (br s, 2H, N-CH₂), 4.00 (s, 2H, C(7)-H₂), 3.36 (s, 2H, C(5)-H₂), 2.62 (t, J = 8.0 Hz, 2H, N-CH₂-CH₂), 2.35 (t, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.94–1.88 (m, 2H, C₆H₅-CH₂), 1.45 (q, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 0.87 (t, J = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) m/z calculated for [C₁₈H₂₄N₄OS + H]⁺ 344.1671, found 344.1672.

Compound 66 (R = Isopropyl): Yield = 30%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.92 (s, 1H, N(3)-H), 7.26 (s, 1H, N(8)-H), 4.32 (br s, 2H, N-CH₂), 3.97 (s, 2H, C(7)-H₂), 3.35 (s, 2H, C(5)-H₂), 2.34 (t, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.68–1.61 (m, 1H CH(CH₃)₂), 1.49–1.40 (m, 4H, N-CH₂-CH₂ and CH₂CH₂CH₃), 0.91–0.87 (m, 9H, CH₂CH₂CH₃ and CH(CH₃)₂); HRMS (ESI) m/z calculated for [C₁₄H₂₄N₄OS + H]⁺ 296.1671, found 296.1673.

Compound 67 (R = *tert*-Butyl): Yield = 34%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.95 (s, 1H, N(3)-H), 7.07 (s, 1H, N(8)-H), 4.46 (br s, 2H, N-CH₂), 3.96 (s, 2H, C(7)-H₂), 3.33 (s, 2H, C(5)-H₂), 2.34 (t, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.51–1.42 (m, 4H, *t*-Bu-CH₂ and CH₂CH₂CH₃), 0.94 (s, 9H C(CH₃)₃), 0.86 (t, J = 7.2 Hz, 3H, CH₂CH₂CH₃); HRMS (ESI) m/z calculated for [C₁₅H₂₆N₄OS + H]⁺ 310.1827, found 310.1829.

Compound 68 (R = Methyl): Yield = 32%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.92 (s, 1H, N(3)-H), 7.35 (s, 1H, N(8)-H), 4.20 (br s, 2H, N-CH₂), 4.00 (s, 2H, C(7)-H₂), 3.36 (s, 2H, C(5)-H₂), 2.34 (t, J = 7.2 Hz, 2H, CH₂CH₂CH₃), 1.65–1.58 (m, 2H, N-CH₂-CH₂), 1.56–1.40 (m,

2H, CH₂CH₂CH₃), 0.85 (m, 6H, NCH₂CH₂CH₃ and CH₂CH₂CH₃); HRMS (ESI) *m/z* calculated for [C₁₂H₂₀N₄O₃S + H]⁺ 268.1358, found 268.1360.

5.1.4 General Procedure for the Preparation of Compounds 69-73—The amine (4.55 mmol) and formaldehyde (6.50 mmol) were added to a solution of **5** (3.25 mmol) in ethanol (20 mL). The reaction mixture was heated at reflux for 3 h, then cooled to room temperature. The resulting precipitate was filtered through a sintered glass funnel to afford compounds **69-73**. The final compounds were characterized by ¹H NMR and MS analysis.

Compound 69 (R = Methyl): Yield = 37%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.10 (s, 1H, N(3)-H), 7.50 (s, 1H, N(8)-H), 7.01–6.89 (m, 3H, Ar-H), 4.55 (br s, 2H, N-CH₂), 4.00 (s, 2H, C(7)-H₂), 3.81 (s, 3H, OCH₃), 3.78 (s, 3H, OCH₃), 3.37 (s, 2H, C(5)-H₂), 2.92 (t, *J* = 8.0 Hz, 2H, N-CH₂-CH₂), 2.34 (s, 3H, NCH₃); HRMS (ESI) *m/z* calculated for [C₁₇H₂₂N₄O₃S + H]⁺ 362.1413, found 362.1415.

Compound 70 (R = Isopropyl): Yield = 33%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.98 (s, 1H, N(3)-H), 7.35 (s, 1H, N(8)-H), 6.91–6.81 (m, 3H, Ar-H), 4.46 (br s, 2H, N-CH₂), 4.03 (s, 2H, C(7)-H₂), 3.73 (s, 3H, OCH₃), 3.71 (s, 3H, OCH₃), 3.42 (s, 2H, C(5)-H₂), 2.85 (t, *J* = 8.0 Hz, 2H, N-CH₂-CH₂), 2.72–2.66 (m, 1H, CH(CH₃)₂), 1.03 (s, 6H, CH(CH₃)₂); HRMS (ESI) *m/z* calculated for [C₁₉H₂₆N₄O₃S + H]⁺ 390.1726, found 390.1730.

Compound 71 (R = Isobutyl): Yield = 36%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.98 (s, 1H, N(3)-H), 7.35 (s, 1H, N(8)-H), 6.91–6.81 (m, 3H, Ar-H), 4.46 (br s, 2H, N-CH₂), 4.03 (s, 2H, C(7)-H₂), 3.73 (s, 3H, OCH₃), 3.71 (s, 3H, OCH₃), 3.42 (s, 2H, C(5)-H₂), 2.85 (t, *J* = 8.0 Hz, 2H, N-CH₂-CH₂), 2.72–2.66 (m, 1H, CH(CH₃)₂), 2.15 (d, *J* = 8.0 Hz, 2H, CH₂CH(CH₃)₂), 1.03 (s, 6H, CH(CH₃)₂); HRMS (ESI) *m/z* calculated for [C₂₀H₂₈N₄O₃S + H]⁺ 404.1882, found 404.1887.

Compound 72 (R = Phenyl): Yield = 39%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.09 (s, 1H, N(3)-H), 7.73 (s, 1H, N(8)-H), 7.24 (t, *J* = 8.0 Hz, 2H, Ar-H), 7.04 (d, *J* = 8.0 Hz, 2H, Ar-H), 6.87–6.77 (m, 4 H, Ar-H), 4.73 (s, 2H, C(7)-H₂), 4.44 (br s, 2H, N-CH₂), 4.12 (s, 2H, C(5)-H₂), 3.70 (s, 6H, OCH₃), 2.78 (t, *J* = 8.0 Hz, 2H, N-CH₂-CH₂); HRMS (ESI) *m/z* calculated for [C₂₂H₂₄N₄O₃S + H]⁺ 424.1569, found 424.1571.

Compound 73 (R = Benzyl): Yield = 31%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.01 (s, 1H, N(3)-H), 7.43 (s, 1H, N(8)-H), 7.34 (s, 4H, Ar-H), 7.32–7.25 (m, 1H, Ar-H), 6.93–6.83 (m, 3H, Ar-H), 4.48 (br s, 2H, N-CH₂), 4.02 (s, 2H, C(7)-H₂), 3.73 (s, 3H, OCH₃), 3.70 (s, 3H, OCH₃), 3.37 (s, 2H, C(5)-H₂), 3.31 (s, 2H, NCH₂Ph), 2.88 (t, *J* = 8.0 Hz, 2H, N-CH₂-CH₂); HRMS (ESI) *m/z* calculated for [C₂₃H₂₆N₄O₃S + H]⁺ 438.1726, found 438.1734.

5.1.5 Synthesis of 6-Amino-1-(3,4-dimethoxyphenethyl)-5-nitroso-2-thioxo-2, 3-dihydro Pyrimidin-4(1H)-one **12**—A suspension of **11** (110 mg, 0.36 mmol) in 10% aqueous acetic acid (2 mL) was heated to 75 °C. A solution of sodium nitrite (35 mg, 0.51 mmol) in water (1 mL) was added dropwise and heating was continued for 1 h. Additional sodium nitrite (35 mg, 0.51 mmol) in water (1 mL) was added dropwise and heating was continued for an additional 1 h. After this time, the reaction mixture was poured into ice/water and the resulting precipitate was filtered. The filter cake was washed with hexanes (5 mL) to afford **12** (70 mg, 58%) as a light green solid: ¹H NMR (400 MHz, DMSO-*d*₆) δ 13.34 (br s, 1H, N(3)-H), 12.73 (br s, 1H, NH₂), 9.31 (br s, 1H, NH₂), 6.91–6.79 (m, 3H, Ar-H), 4.51–4.50 (br s, 2H, N-CH₂), 3.72 (s, 3H, OCH₃), 3.70 (s, 3H, OCH₃), 2.83–2.79 (t, 2H, NCH₂CH₂); MS (ESI) *m/z* 337 [C₁₄H₁₆N₄O₄S + H]⁺.

5.1.6 Synthesis of 5,6-Diamino-1-(3, 4-dimethoxyphenethyl)-2-thioxo-2,3-dihydro-pyrimidin-4(1H)-one 13.—A suspension of **12** (70 mg, 0.21 mmol) in water (4 mL) and ammonia (32% aq, 4 mL) was heated at 75 °C for 20 min. Sodium dithionite (90 mg, 0.52 mmol) was added portionwise and the reaction was heated at 75 °C for an additional 20 min. The reaction was then cooled to room temperature and stirred for 1 h. At this time, the pH of the solution was adjusted to 7 with an aqueous solution of 1 M hydrochloric acid. The resulting precipitate was collected by filtration and washed with water (5 mL) and hexanes (5 mL). The crude product was purified by silica-gel column chromatography (2% MeOH in DCM) to afford **13** (20 mg, 30%) as an off-white solid: ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.48 (br s, 1H, NH), 6.95 (br s, 1H, NH), 6.86–6.70 (m, 3H, Ar-H), 3.72 (s, 3H, OCH₃), 3.70 (s, 3H, OCH₃), 3.55–3.54 (m, 2H, -NCH₂), 2.69–2.68 (m, 2H, -NCH₂CH₂); MS (ESI) *m/z* 323 [C₁₄H₁₈N₄O₃S + H]⁺.

5.1.7 Synthesis of 3-(3,4-Dimethoxyphenethyl)-2-thioxo-2, 3-dihydro-1H-purin-6(9H)-one 14—A mixture of **13** (400 mg, 1.24 mmol) and formamide (5 mL) was heated at 165 °C for 45 min and slowly allowed to cool to room temperature. The resulting precipitate was filtered and washed with formamide (10 mL), water (10 mL), and ethanol (10 mL) and dried to afford **14** (290 mg, 70%) as a light yellow solid: ¹H NMR (400 MHz, DMSO-*d*₆) δ 13.84 (br s, 1H, N(7)-H), 12.48 (br s, 1H, N(3)-H), 8.18 (s, 1H, C(6)-H), 6.88–6.77 (m, 3H, Ar-H), 4.63–4.61 (t, 2H, -NCH₂CH₂), 3.72 (s, 3H, OCH₃), 3.70 (s, 3H, OCH₃), 2.98–2.94 (t, 2H, -NCH₂CH₂); HRMS (ESI) *m/z* calculated for [C₁₅H₁₆N₄O₃S + H]⁺ 332.0943, found 332.0944.

5.1.8 Synthesis of 1-(3,4-Dimethoxyphenethyl)-5,7-dimethyl-2-thioxo-2,3-dihydropyrido [2,3-*d*]pyrimidin-4(1H)-one 16—A mixture of **9a** (300 mg, 0.98 mmol), **15** (0.3 mL, 2.93 mmol) and trifluoroacetic acid (15 mL) was heated at 60 °C for 6 h, after which time another portion of **15** (0.3 mL, 2.93 mmol) was added and the reaction remained at reflux for 16 h. The reaction mixture was cooled to room temperature, poured into ice/water and extracted with EtOAc (2 × 25 mL). The organic layer was washed with an aqueous saturated NaHCO₃ solution, dried over Na₂SO₄, and concentrated under reduced pressure. The resulting solid was triturated with MTBE to afford **16** (120 mg, 33%) as an off-white solid: ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.60 (br s, 1H, N(3)-H), 8.18 (s, 1H, C(6)-H), 6.88–6.86 (m, 2H, Ar-H), 6.80–6.78 (d, *J* = 8.4 Hz, 1H, Ar-H), 4.90–4.86 (t, *J* = 8.0 Hz, 2H, -NCH₂CH₂), 3.74 (s, 3H, OCH₃), 3.70 (s, 3H, OCH₃), 2.92–2.88 (t, *J* = 8.0 Hz, 2H, -NCH₂CH₂), 2.67 (s, 3H, CH₃), 2.54 (s, 3H, CH₃); MS (ESI) *m/z* 372 [C₁₉H₂₁N₃O₃S + H]⁺.

5.1.9 Synthesis of 1-(3,4-Dimethoxyphenethyl)-2-thioxo-2,3-dihydropteridin-4(1H)-one 18. [24]—Diamine **13** (300 mg, 0.93 mmol) was added to the stirring solution of **10** (0.150 g, 1.25 mmol) in ethanol (20 mL). After stirring at room temperature for 3 h, the reaction was concentrated under reduced pressure. The crude residue was purified by silica-gel column chromatography (1–3% MeOH in DCM) to afford **18** (70 mg, 28%) as a light yellow solid: ¹H NMR (400 MHz, CDCl₃): δ 9.68 (br s, 1H, N(3)-H), 8.75 (s, 1H, C(7)-H), 8.66 (s, 1H, C(6)-H), 6.91–6.80 (m, 3H, Ar-H), 4.97–4.93 (t, 2H, -NCH₂CH₂), 3.92 (s, 3H, OCH₃), 3.87 (s, 3H, OCH₃), 3.07–3.03 (t, 2H, -NCH₂CH₂); HRMS (ESI) *m/z* calculated for [C₁₆H₁₆N₄O₃S + H]⁺ 344.0943, found 344.0944.

5.1.10 Synthesis of Pyrido[4,3-*d*]pyrimidin-5(6H)-one 20. [25]—Ethyl acetoacetate **13** (4.32 mL, 61.65 mmol) and 1,3,5-triazine **19** (5.00 g, 61.65 mmol) were added to the stirring solution of sodium (350 mg, 16.20 mmol) in ethanol (18 mL) at room temperature. The reaction was heated at reflux for 1 h under a nitrogen atmosphere. After this time, the reaction mixture was concentrated under reduced pressure and the residue was diluted with water (50 mL). The resulting aqueous solution was acidified with concentrated hydrochloric acid and the resulting

precipitate was filtered. The filter cake was washed with cold acetone and dried to afford compound **20** (400 mg, 4.5 %) as a brown solid: ^1H NMR (400 MHz, DMSO- d_6) δ 11.92 (s, 1H, NH), 9.40 (s, 1H, C(2)-H), 9.31 (s, 1H, C(4)-H), 7.70 (d, J = 6.8 Hz, 1H, C(7)-H), 6.55 (d, J = 7.2 Hz, 1H, C(8)-H); MS (ESI) m/z 148 [$\text{C}_7\text{H}_5\text{N}_3\text{O} + \text{H}$] $^+$.

5.1.11 Synthesis of 8-Iodopyrido[4,3-*d*]pyrimidin-5-(6*H*)-one **21. [26]**—Iodine (1.10 g, 4.34 mmol) was added to a suspension of **20** (500 mg, 3.39 mmol) in 0.4 N aqueous sodium hydroxide (17 mL) and the reaction mixture was heated at 80 °C for 18 h. The reaction mixture was cooled to room temperature and the resulting precipitate was filtered. The filter cake was washed with water (15 mL) and dried to afford **21** (550 mg, 59%) as a yellow solid: ^1H NMR (400 MHz, DMSO- d_6) δ 12.15 (s, 1H, NH), 9.41 (s, 1H, C(2)-H), 9.31 (s, 1H, C(4)-H), 8.12 (s, 1H, C(7)-H); MS (ESI) m/z 274 [$\text{C}_7\text{H}_4\text{IN}_3\text{O} + \text{H}$] $^+$.

5.1.12 Synthesis of 5-Chloro-8-iodopyrido[4,3-*d*]pyrimidine **22. [26]**—A mixture of **21** (300 mg, 1.09 mmol) and POCl₃ (9 mL) was heated at reflux for 24 h. The reaction mixture was concentrated under reduced pressure and the residue was poured into ice water. The resulting mixture was made alkaline with K₂CO₃ and extracted with CH₂Cl₂ (3 × 30 mL). The combined organic layer was dried over Na₂SO₄ and concentrated under reduced pressure. The crude residue was purified by silica-gel column chromatography (10% EtOAc in petroleum ether) to afford **22** (200 mg, 65%) as a brown solid: ^1H NMR (400 MHz, DMSO- d_6) 9.79 (s, 1H, C(2)-H), 9.67 (s, 1H, C(4)-H), 9.19 (s, 1H, C(7)-H); MS (ESI) m/z 292 [$\text{C}_7\text{H}_3\text{ClIN}_3 + \text{H}$] $^+$.

5.1.13 General Procedure for Sonagashira Coupling—A mixture of **22** (2.06 mmol) and anhydrous THF (18 mL) was purged with argon gas for 30 min. Acetylene derivative (2.48 mmol) was added to this solution followed by DIPEA (0.41 mmol), CuI (0.10 mmol) and Pd(PPh₃)₂Cl₂ (0.41 mmol). After stirring for 48 h under an argon atmosphere, the reaction mixture was concentrated under reduced pressure. The crude material was purified by silica-gel column chromatography (10%–30% EtOAc in petroleum ether) to afford compounds **23** or **24**.

5-Chloro-8-(phenylethynyl)pyrido[4,3-*d*]pyrimidine **23**: ^1H NMR (400 MHz, CDCl₃) δ 9.87 (s, 1H, C(2)-H), 9.67 (s, 1H, C(4)-H), 8.90 (s, 1H, C(7)-H), 7.70–7.69 (m, 2H, Ar-H), 7.43–7.42 (m, 3H, Ar-H); HRMS (ESI) m/z calculated for [$\text{C}_{15}\text{H}_8\text{ClN}_3 + \text{H}$] $^+$ 265.0407, found 265.0403. 300 mg (45%) yield.

5-Chloro-8-[(3,4-dimethoxyphenyl)ethynyl]pyrido[4,3-*d*]pyrimidine **24**: ^1H NMR (400 MHz, CDCl₃) δ 9.86 (s, 1H, C(2)-H), 9.67 (s, 1H, C(4)-H), 8.89 (s, 1H, C(7)-H), 7.31 (d, J = 8.4 Hz, 1H, Ar-H), 7.18 (s, 1H, Ar-H), 6.90 (d, J = 8.4 Hz, 1H, Ar-H), 3.95 (s, 3H, OCH₃), 3.94 (s, 3H, OCH₃); MS (ESI) m/z 326 [$\text{C}_{17}\text{H}_{12}\text{ClN}_3\text{O}_2 + \text{H}$] $^+$; 400 mg (47%) yield.

5.1.14 General Procedure for Hydrolysis [27]—NH₄OAc (1.89 mmol) was added to a stirring suspension of **23** or **24** (0.18 mmol) in acetic acid (2 mL). The reaction mixture was heated at 100° C for 2 h and then was concentrated under reduced pressure. The resulting residue was purified by silica-gel column chromatography (2% MeOH in CH₂Cl₂) to afford compound **25** or **26**.

8-(Phenylethynyl)pyrido[4,3-*d*]pyrimidin-5(6*H*)-one **25**: ^1H NMR (400 MHz, DMSO- d_6) δ 12.35 (s, 1H, NH), 9.46 (s, 1H, C(2)-H), 9.44 (s, 1H, C(4)-H), 8.15 (s, 1H, C(7)-H), 7.55–7.53 (m, 2H, Ar-H), 7.44–7.40 (m, 3H, Ar-H); HRMS (ESI) m/z calculated for [$\text{C}_{15}\text{H}_9\text{N}_3\text{O} + \text{H}$] $^+$ 247.0746, found 247.0746. 80 mg (42%) yield.

8-[(3,4-Dimethoxyphenyl)ethynyl]pyrido[4,3-*d*]pyrimidin-5(6*H*)-one **26**: ^1H NMR (400 MHz, DMSO- d_6) δ 12.31 (s, 1H, NH), 9.46 (s, 1H, C(2)-H), 9.44 (s, 1H, C(4)-H), 8.10 (d, J

= 7.8 Hz, 1H, C(7)-H), 7.11 (d, J = 8.4 Hz, 1H, Ar-H), 7.06 (s, 1H, Ar-H), 6.99 (d, J = 8.4 Hz, 1H, Ar-H), 3.78 (s, 6H, OCH₃); HRMS (ESI) m/z calculated for [C₁₇H₁₃N₃O₃ + H]⁺ 307.0957, found 307.0958. 80 mg (42%) yield.

5.1.15 General Procedure for Hydrogenation—Pd/C (10%, 50% wet, 20 mg dry weight) was added to a solution of **25** or **26** (0.32 mmol) in 1:1 MeOH and CH₂Cl₂. The reaction-mixture flask was attached to a Parr hydrogenator, evacuated, charged with hydrogen gas to a pressure of 30 psi and shaken for 4–6 h. After this time, the hydrogen was evacuated, and nitrogen was charged into the bottle. The reaction mixture was filtered through a pad of Celite and the filtrate was concentrated under reduced pressure. The crude material was purified by preparative TLC plate (5% MeOH in CH₂Cl₂) to afford **27** or **28**.

8-(Phenylethyl)pyrido[4,3-*d*]pyrimidin-5(6*H*)-one **27**: ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.74 (s, 1H, NH), 9.41 (s, 2H, C(2)-H and C(4)-H), 7.43 (d, J = 5.6 Hz, 1H, C(7)-H), 7.28–7.24 (m, 2H, Ar-H), 7.19–7.14 (m, 3H, Ar-H), 2.99–2.96 (m, 2H, -NHCH₂CH₂), 2.90–2.86 (m, 2H, -NHCH₂CH₂); HRMS (ESI) m/z calculated for [C₁₅H₁₃N₃O + H]⁺; 251.1059, found 251.1060. 20 mg (24%) yield.

8-(3,4-Dimethoxyphenethyl)pyrido[4,3-*d*]pyrimidin-5(6*H*)-one **28**: ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.74 (s, 1H, NH), 9.41 (s, 2H, C(2)-H and C(4)-H), 7.40 (s, 1H, C(7)-H), 6.81 (d, J = 8.4 Hz, 1H, Ar-H), 6.77 (s, 1H, Ar-H), 6.65 (d, J = 7.6 Hz, 1H, Ar-H), 3.70 (s, 3H, OCH₃), 3.69 (s, 3H, OCH₃), 2.96 (t, J = 8 Hz, 2H, -NHCH₂CH₂), 2.81 (t, J = 8 Hz, 2H, -NHCH₂CH₂); HRMS (ESI) m/z calculated for [C₁₇H₁₇N₃O₃ + H]⁺ 311.1270, found 311.1276. 35 mg (23%) yield.

5.1.16 Synthesis of 1-(3,4-Dimethoxyphenethyl)urea 30—Urea (2.60 g, 44.14 mmol) was added to a suspension of amine **29** (2.00 g, 11.03 mmol) in water (5 mL) and concentrated HCl (0.13 mL, 1.32 mmol). The reaction mixture was heated to reflux and stirred for 6 h at this temperature. The reaction mixture was cooled and left to stand at room temperature for 24 h. The resulting precipitate was filtered, then washed with water (10 mL) and acetone (10 mL) to afford **30** (1.10 g, 44%) as a white solid: ¹H NMR (400 MHz, DMSO-*d*₆) δ 6.84 (d, J = 8.0 Hz, 1H, Ar-H), 6.77 (s, 1H, Ar-H), 6.68 (d, J = 12 Hz, 1H, Ar-H), 5.84 (t, J = 5.2 Hz, 1H, -NHCH₂CH₂), 5.40 (s, 2H, NH₂), 3.72 (s, 3H, OCH₃), 3.69 (s, 3H, OCH₃), 3.15 (q, J = 6.8 Hz, 2H, -NHCH₂CH₂), 2.57 (t, J = 7.2 Hz, 2H, NHCH₂CH₂); MS (ESI) m/z 225 [C₁₁H₁₆N₂O₃ + H]⁺.

5.1.17 Synthesis of 6-Amino-1-(3,4-dimethoxyphenethyl)pyrimidine-2,4(1*H*,3*H*)-dione 31—Urea **30** (1.00 g, 4.46 mmol) and ethyl cyanoacetate (0.71 mL, 6.69 mmol) were added to a solution of Na (160 mg, 6.69 mmol) in EtOH (10 mL). The reaction mixture was heated to reflux and stirred for 4 h at this temperature. The reaction mixture was cooled to room temperature and the solvent was evaporated under reduced pressure. The crude residue was purified by silica-gel column chromatography (1%–3% MeOH in CH₂Cl₂) to afford **31** (400 mg, 46%) as an off-white solid: ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.29 (s, 1H, N(3)-H), 6.85–6.83 (m, 2H, Ar-H), 6.78 (s, 2H, NH₂), 6.73 (d, J = 8 Hz, 1H, Ar-H), 4.53 (s, 1H, C(5)-H), 3.92 (t, J = 7.2 Hz, 2H, -NHCH₂CH₂), 3.72 (s, 3H, OCH₃), 3.70 (s, 3H, OCH₃), 2.72 (t, J = 7.6 Hz, 2H, -NHCH₂CH₂); MS (ESI) m/z 292 [C₁₄H₁₇N₃O₄ + H]⁺.

5.1.18 Synthesis of 1-(3,4-Dimethoxyphenethyl)-6-propyl-5,6,7,8-tetrahydropyrimido [4,5-*d*]pyrimidine-2,4(1*H*,3*H*)-dione 32—Propylamine (0.19 mL, 2.40 mmol) and formaldehyde (0.27 mL, 3.42 mmol) were added to a solution of amine **31** (500 mg, 1.71 mmol) in EtOH (10 mL). The reaction mixture was heated at reflux for 3 h. The reaction mixture was cooled and concentrated under reduced pressure. The crude compound was purified by silica-gel column chromatography (1%–3% MeOH in CH₂Cl₂) to afford **32**

(180 mg, 28%) as an off-white solid: ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 10.48 (s, 1H, N(3)-H), 7.11 (s, 1H, N(8)-H), 6.84 (d, $J = 8.4$ Hz, 1H, Ar-H), 6.79 (s, 1H, Ar-H), 6.72 (d, $J = 8.0$ Hz, 1H, Ar-H), 3.93–3.89 (m, 4H, $-\text{NHCH}_2\text{CH}_2$ and C(7)-H₂), 3.71 (s, 3H, OCH₃), 3.69 (s, 3H, OCH₃), 3.32 (s, 2H, C(5)-H₂), 2.72 (t, $J = 7.6$ Hz, 2H, $\text{CH}_2\text{CH}_2\text{CH}_3$), 2.31 (t, $J = 6.8$ Hz, 2H, NHCH_2CH_2), 1.48–1.39 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_3$), 0.86 (t, $J = 7.2$ Hz, 3H, $\text{CH}_2\text{CH}_2\text{CH}_3$); HRMS (ESI) m/z calculated for $[\text{C}_{19}\text{H}_{26}\text{N}_4\text{O}_4 + \text{H}]^+$ 374.1954, found 374.1956.

5.2 Biological experiments

4-methylumbelliferyl- α -D-glucopyranoside (4MU- α -glu), N-butyldioxynojirimycin (NB-DNJ), glycogen from bovine liver, and the buffer components were purchased from Sigma-Aldrich (St. Louis, MO). Amplex Red reagent, DMEM, Opti-MEM, and other medium components were purchased from Invitrogen (Eugene, OR). Resorufin α -D-glucopyranoside (Res- α -glu) was synthesized by the Imaging Probe Development Center at the National Institutes of Health. GAA was obtained from residual solution after clinical infusions of Myozyme[®] (Genzyme). The enzyme solution was mixed with 30% glycerol and small aliquots were stored at -80°C .

The human spleen tissue was homogenized using a food blender at the maximal speed for 5 minutes, followed by 10 passes in a motor-driven 50 ml glass-Teflon homogenizer. The homogenate was centrifuged at $1000 \times g$ for 10 min. The supernatant was then filtered using a 40 μm filter and aliquots of resultant spleen homogenate were frozen at -80°C until use.

The assay buffer was composed of 50 mM citric acid, 115 mM K_2PO_4 , 110 mM KCl, 10 mM NaCl, 1 mM MgCl_2 , and 0.01% Tween-20 at pH 5. It was stored at 4°C for up to 6 months. A solution of 1 M NaOH, 1M glycine at pH 10 was used as the stop solution for the blue substrate assay. 1 M TRIS-HCl at pH 8.0 was used as the stop solution for the red substrate assay.

5.2.1 Enzyme assay in 1536-well plate format—In black 1536-well plates, 2 μl /well GAA enzyme solution was added, followed by 23 nl/well compound in DMSO solution. After 5 minute incubation at room temperature, the enzyme reaction was initiated by the addition of 2 μl /well substrate. After 45 minutes incubation at 37°C , the reaction was terminated by the addition of 2 μl /well stop solution. The assay plate was then measured in the Viewlux at a 573 nm excitation and 610 nm emission for the red substrate, and a 365 nm excitation and 440 nm emission for the blue substrate. The final concentrations of purified GAA, blue substrate, and red substrate were 5.5 nM, 75 μM , and 15 μM , respectively.

For the natural substrate assay, the above procedure was followed, but 2 μl /well of glycogen solution was used as the substrate. After 45 minutes incubation at 37°C , 2 μl /well of the Amplex Red solution was added, and the reaction was incubated 30 minutes at room temperature. The plate was then measured in the Viewlux at 573 nm excitation and 610 nm emission. The final concentrations of GAA and glycogen were 5.5 nM and 10 mg/ml, respectively.

Acknowledgments

This research was supported by the Molecular Libraries Initiative of the NIH Roadmap for Medical Research and the Intramural Research Program of the National Human Genome Research Institute, National Institutes of Health.

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Figure 1.
Structure of Pulicarside 1

Figure 2.
Hydrolytic reactions of the red and blue dyes.

Figure 3.

Activity of the lead compound and analogues in the primary screen. The activity here disclosed corresponds with the percentage of signal increase observed after adding 100 μ M of activator compound to the hydrolysis reaction using resorufin α -D-glucopyranoside (red dye) as hydrolytic substrate and spleen homogenate as reaction conditions.

Figure 4.

Activation of substrate hydrolysis by lead compound using two different substrates (resorufin α -D-glucopyranoside = red dye and 4-methylumbelliferyl α -D-glucopyranoside = blue dye) and two different conditions (spleen homogenated and purified enzyme).

Figure 5. Evaluation of the hydrolysis of 4-methylumbelliferyl α -D-glucopyranoside (blue dye) in the presence of several concentrations of compound **1** by direct measurement using LC-MS. Peak 1: 4MU- α -glu, peak 2: 4MU, peak 3: Compound **1**.

Figure 6.
Structure of best activators.

Figure 7.
Titration curves of best activators. a. red purified enzyme; b. blue purified enzyme; c. red spleen; d. blue spleen.

a.

b.

Figure 8.

- a. Concentration dependent effect of most active compounds on the hydrolysis of 4-methylumbelliferyl α -D-glucopyranoside (4MU- α -glu) in the presence of purified GAA. b. Concentration dependent effect of most active compounds on the hydrolysis of 4-methylumbelliferyl α -D-glucopyranoside (4MU- α -glu) in the absence of purified GAA. c. Concentration dependent effect of most active compounds on the hydrolysis of 4-methylumbelliferyl α -D-galactosidase (4MU- α -gal) in the presence of purified alpha-galactosidase A.

Figure 9.
Effect of lead compounds on rhGAA using glycogen as the substrate.

Scheme 1.

General methodology for the synthesis of analogues at position 1 and 6.

Scheme 2.
Modifications at the molecular core.

Scheme 3.
Additional template modifications.

Scheme 4.
Synthesis of compound **32**.

Table 1

Analogues with modifications at the core template

Compound number	Core structure
9a

Compound number	Core structure
18
23
24

Compound number	Core structure
26
27

Compound number	Core structure
28
32

Compound number	Core structure
1	<chem>O=C1NC(=S)N(R1)C2=CC(=O)N(CCN2CCN3CCCC3)C1=O</chem>

Table 2

Analogues with modifications at the aromatic substituent.

Compound number	R1
33	
34

Compound number

R1

35

36

Compound number

R1

Compound number

R1

38

Compound number

R1

MeO

Compound number

R1

40

41

Compound number

R1

Compound number

R1

Compound number

R1

Compound number

R1

45

46

Compound number

R1

Compound number

R1

48

49

Compound number

R1

Compound number

R1

51

Compound number

R1

52

Compound number

R1

Compound number

R1

54

Compound number

R1

55

Compound number

R1

Compound number

R1

57

Compound number

R1

1

58

Compound number

R1

2

Compound number

R1

59

Compound number

R1

Table 3

Additional analogues with modifications in the functional group at position 1

Compound number	R1
1
61
62
63

Compound number

R1

64

Compound number

R1

2

Compound number

R1

65

Compound number

R1

66

Compound number

R1

67

Compound number

R1

68

Table 4

Analogues with modifications in the functional group at position 6

Compound number

R1

1

R1

Compound number

Compound number

R1

70

Compound number

R1

Compound number

 R_1

72

Compound number

R1

73