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Original article

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 alpha-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 alpha-glucosidase activators.

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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 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.

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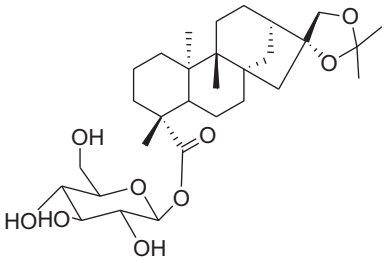


Fig. 1. Structure of Pulicarside 1.

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 μM. Figs. 1–3 show 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. Fig. 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. Fig. 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 us 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-

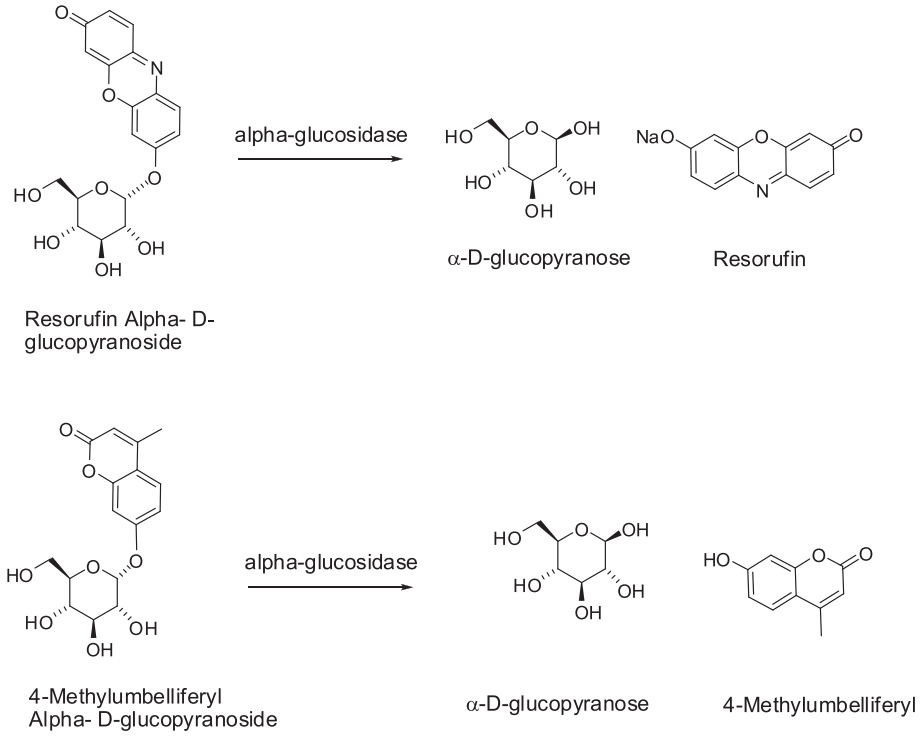


Fig. 2. Hydrolytic reactions of the red and blue dyes.

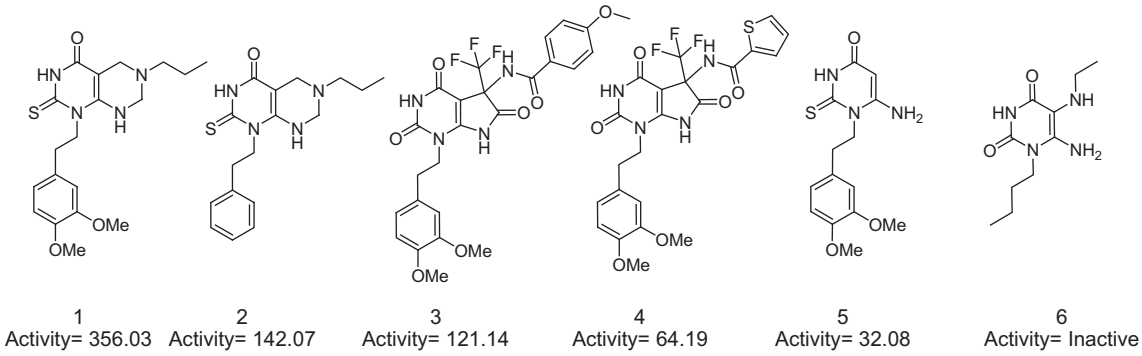


Fig. 3. Activity of the lead compound and analogues in the primary screen. The activity here disclosed corresponds with the percentage of signal increase observed after adding 100 μM of activator compound to the hydrolysis reaction using resorufin α-D-glucopyranoside (red dye) as hydrolytic substrate and spleen homogenate as reaction conditions.

4(1H)-one derivative **9**. Last, a primary amine **10** and formaldehyde were added to a solution of amine **9** in ethanol and refluxed for 3 h to produce target 2-thioxo-2,3,5,6,7,8-hexahydropyrimido[4,5-d]pyrimidin-4(1H)-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. Schemes 2 and 3 describe their synthesis.

The synthesis of the 2-thioxo-2,3-dihydro-1H-purin-6(9H)-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 iodinized and dehydrated in the presence of phosphorus oxychloride to produce intermediate **22**. Sonogashira 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 AC_{50} (concentration necessary to obtain 50% of maximal activation) should be analyzed. To generate a proper AC_{50} the maximum possible activation must be reached at the highest concentration, but at the tested concentrations (twelve points between 650 pM and 100 μM) 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 1–4 show the SAR of our compounds.

Fig. 7 shows the concentration–% response curves of our best compounds in several assays. In addition, Fig. 6 disclosed the red dye-tissue homogenate AC_{50} 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 2–4.

In order to further evaluate our best activators, we measured their specific ability to activate the hydrolysis of 4MU-α-glu. Fig. 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. Fig. 9 shows that none of our compounds improved the hydrolysis of glycogen.

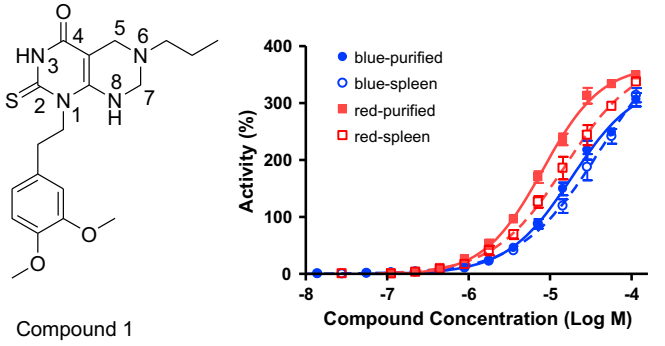


Fig. 4. Activation of substrate hydrolysis by lead compound using two different substrates (resorufin α-D-glucopyranoside = red dye and 4-methylumbelliferyl-α-D-glucopyranoside = blue dye) and two different conditions (spleen homogenated and purified enzyme).

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 Fig. 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.

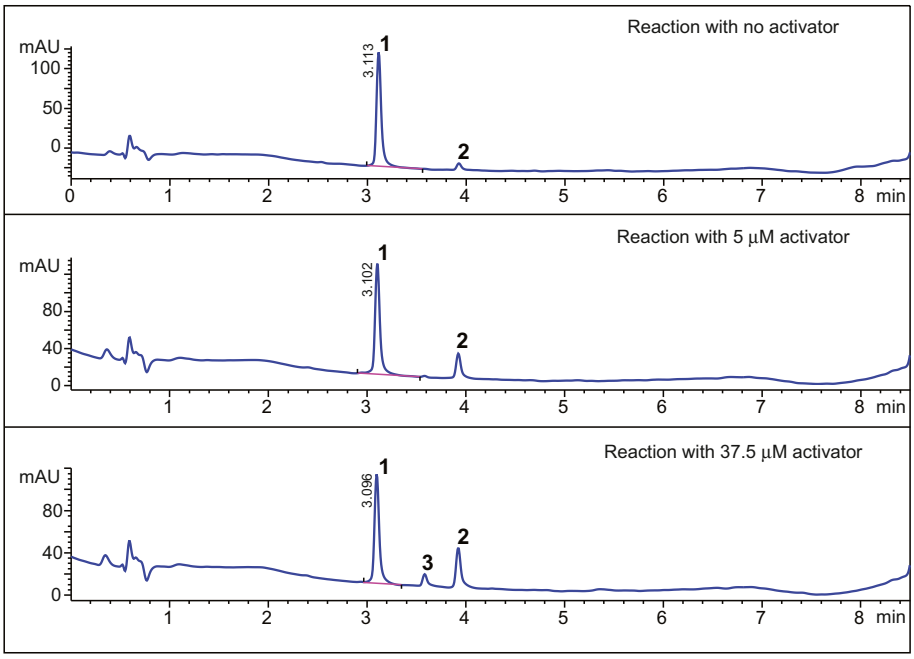


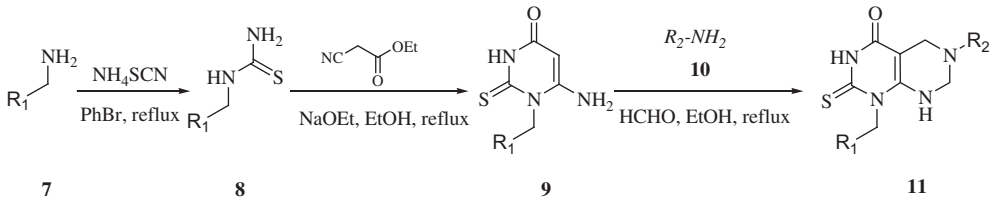
Fig. 5. Evaluation of the hydrolysis of 4-methylumbelliferyl-α-D-glucopyranoside (blue dye) in the presence of several concentrations of compound **1** by direct measurement using LC–MS. Peak 1: 4MU-α-glu, peak 2: 4MU, peak 3: compound **1**.

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.

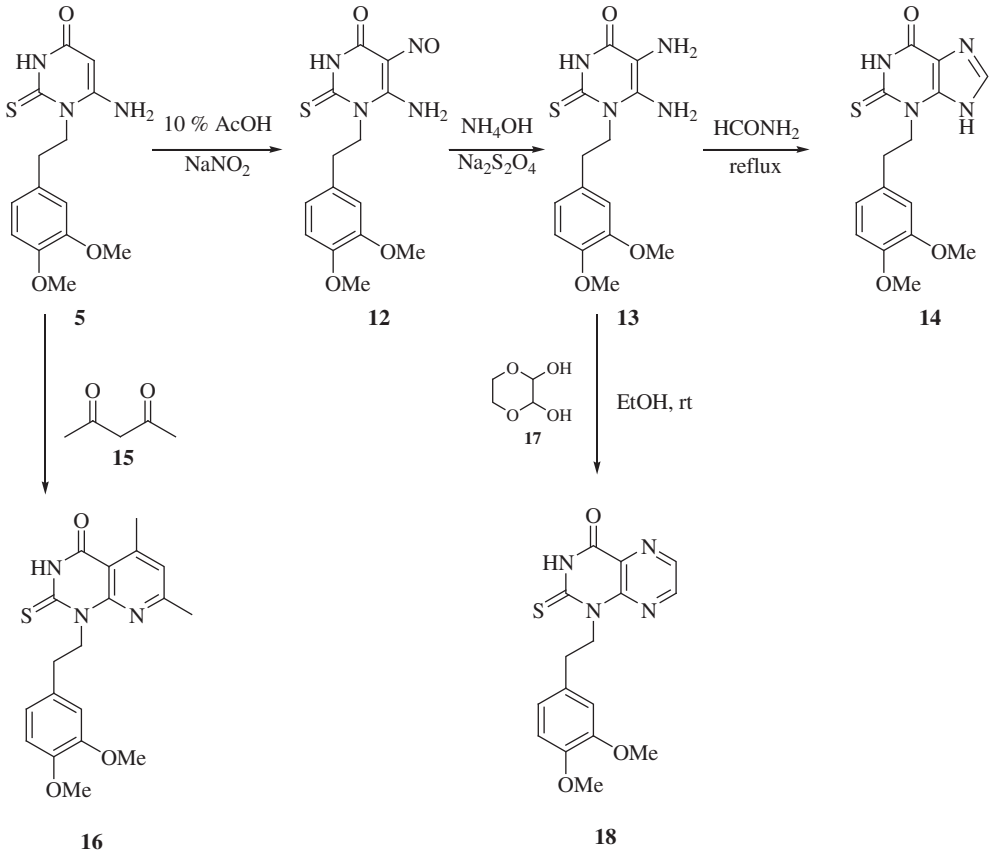
SAR structural changes show that all the core modifications synthesized failed to further activate hydrolysis of our fluorogenic substrate and even replacement of the thiocarbonyl functional group by a carbonyl group diminished activity (Table 1, compounds **32** and **1**). Replacement of the pendant 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**, produces 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. Figs. 6 and 7 present the red dye-tissue homogenate AC_{50} 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 AC_{50} of the series. Our best compound **49** had an AC_{50} of 1.00 µM 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 Fig. 8, and enzyme specific because the series does not activate the hydrolysis of substrates for other glycosidase enzymes, such us alpha-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, Fig. 9 shows that these compounds do not activate the hydrolysis of the natural substrate, glycogen. We speculate that the conformation adopt by GAA for cleaving the polymeric natural substrate may be different than the one adopted in the presence of a small substrate such us resorufin α-D-glucopyranoside or 4MU-α-glu. Other lysosomal enzymes such as beta-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



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

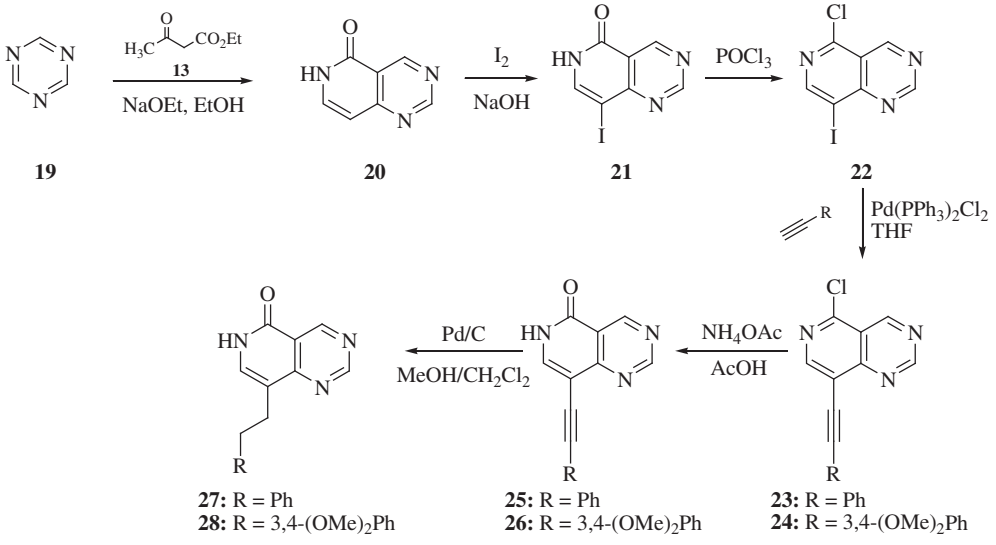


Scheme 2. Modifications at the molecular core.

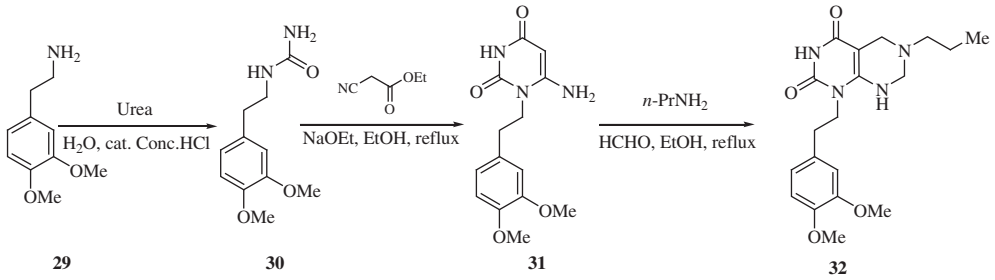
working on the development of a new HTS-adaptable assay that is able to measure the glycogen hydrolysis by tissue homogenate GAA. In addition, the glycogen-based assay involves the cleavage of both 1,4 and 1,6 glycosidic bonds, and it is possible that this series selectively activates only 1,4 bond cleavage 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



Scheme 3. Additional template modifications.



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

polymeric natural substrate may be different than the one adopted in the presence of a small substrate such us 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). ^{1}H NMR spectra were recorded with a Bruker 400 MHz spectrometer from solutions in $CDCl_3$ and DMSO-d_6. Chemical shifts in ^{1}H NMR spectra are reported in parts per million (ppm, δ) downfield from the internal standard Me_4Si (TMS, $\delta = 0$ ppm). Molecular weight confirmation was performed using an Agilent Time-Of-Flight Mass Spectrometer (TOF, Agilent Technologies, Santa Clara, CA). A 3 min gradient from 4 to 100% acetonitrile (0.1% formic acid) in water (0.1% formic acid) was used with a 4 min run time at a flow rate of 1 mL/min. A Zorbax SB-C18 column (3.5 μm, 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 CH_2Cl_2) 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_2CH_2O)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	t-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 CH_2Cl_2) to afford pyrimidinone **9**. The products were characterized by ^{1}H NMR and MS analysis.

*5.1.2.1. Compound **5** (R = 3,4-di(OMe)benzyl).* Yield = 31%; ^{1}H 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, NH_2), 4.85 (s, 1H, C(5)-H), 4.48 (br s, 2H, N–CH_2), 3.73 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 2.84 (t, J = 8 Hz, 2H, N–CH_2–**CH_2**); HRMS (ESI) m/z calculated for $[C_{14}H_{17}N_3O_3S + H]^+$ 307.0991, found 307.0993.

*5.1.2.2. Compound **9b** (R = 2,3-di(OMe)benzyl).* Yield = 30%; ^{1}H 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 NH_2), 4.89 (s, 1H, C(5)-H), 4.54 (br s, 2H, N–CH_2), 3.78 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 2.91 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**); MS (ESI) m/z 308 $[C_{14}H_{17}N_3O_3S + H]^+$.

*5.1.2.3. Compound **9c** (R = 2,4-di(OMe)benzyl).* Yield = 30%; ^{1}H 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 NH_2), 4.89 (s, 1H, C(5)-H), 4.54 (br s, 2H, N–CH_2), 3.78 (s, 3H, OCH_3), 3.73 (s, 1H, OCH3), 2.91 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**); MS (ESI) m/z 308 $[C_{14}H_{17}N_3O_3S + H]^+$.

*5.1.2.4. Compound **9d** (R = 2,5-di(OMe)benzyl).* Yield = 32%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.83 (s, 1H, NH), 7.01 (t, J = 8.0 Hz,

Table 1
Analogues with modifications at the core template.

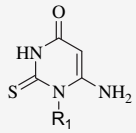
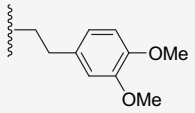
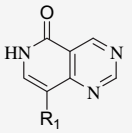
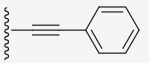
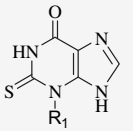
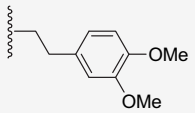
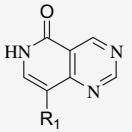
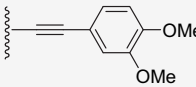
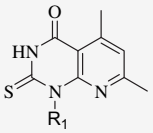
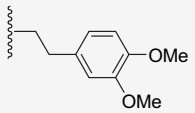
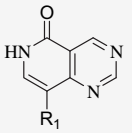
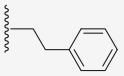
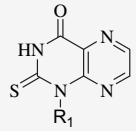
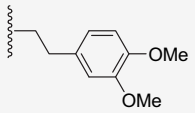
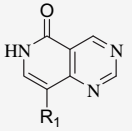
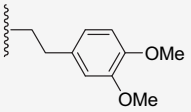
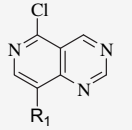
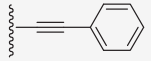
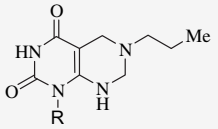
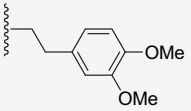
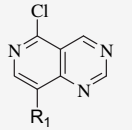
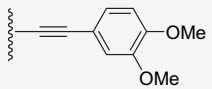
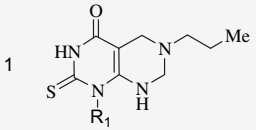
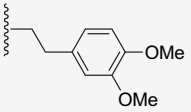
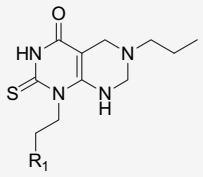
Compound number	Core structure	R1		Maximum response	Compound number	Core structure	R1		Maximum response
9a			inactive	Inactive	**25**				Inactive
14			inactive	Inactive	**26**				Inactive
16			inactive	Inactive	**27**				Inactive
18			inactive	Inactive	**28**			inactive	Inactive
23				Inactive	**32**			inactive	297.37
24				Inactive	**1**			inactive	365.03

Table 2
Analogues with modifications at the aromatic substituent.



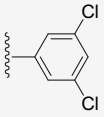
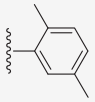
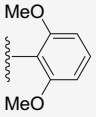
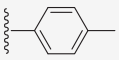
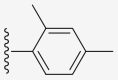
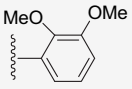
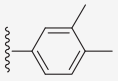
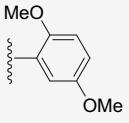
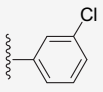
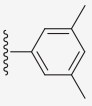
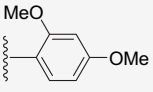
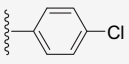
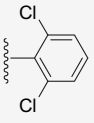
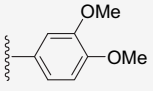
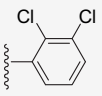
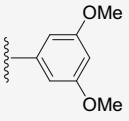
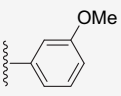
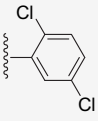
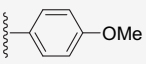
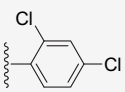
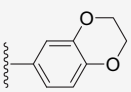
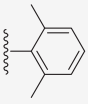
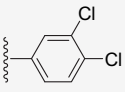
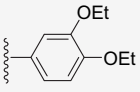
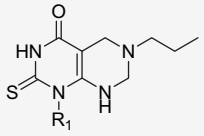
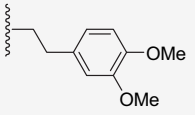
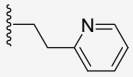
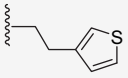
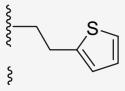
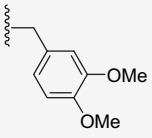
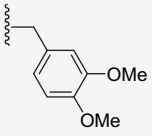
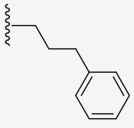
Compound number	R1	Maximum response	Compound number	R1	Maximum response	Compound number	R1	Maximum response
33		135.96	**43**		300.10	**53**		315.83
34		295.22	**44**		244.98	**54**		226.60
35		299.89	**45**		322.93	**55**		262.27 262.27
36		310.68	**46**		337.60	**56**		327.26
37		197.44	**47**		218.24	**57**		308.06
38		120.29	**48**		332.48	**1**		356.03
39		242.70	**49**		345.62	**58**		331.78
40		301.87	**50**		325.11	**2**		142.07
41		308.72	**51**		321.42	**59**		328.21
42		112.26	**52**		88.81	**60**		305.52

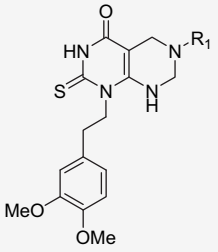
Table 3
Additional analogues with modifications in the functional group at position 1.

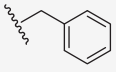


Compound number	R1	Maximum response
1	 inactive	356.03
61		30.59
62		192.02
63		221.14
64		365.30
2		142.07
65		165.72
66		Inactive
67		Inactive
68		Inactive

1H, Ar–H), 6.99–6.85 (m, 4H, Ar–H and NH_2), 4.89 (s, 1H), 4.54 (br s, 2H, N–CH_2), 3.78 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 2.91 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**); MS (ESI) m/z 308 $[C_{14}H_{17}N_3O_3S + H]^+$.

Table 4
Analogues with modifications in the functional group at position 6.



Compound number	R1	Maximum response
1		356.03
69		282.39
70		338.17
71		233.61
72		334.61
73		274.75

*5.1.2.5. Compound **9e** (R = 2,6-di(OMe)benzyl).* Yield = 34%; ^{1}H 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, NH_2), 6.60 (d, J = 8.4 Hz, 2H, Ar–H), 4.85 (s, 1H, C(5)-H), 4.47 (br s, 2H, N–CH_2), 3.73 (s, 6H, OCH_3), 2.93 (br s, 2H, N–CH_2–**CH_2**); MS (ESI) m/z 308 $[C_{14}H_{17}N_3O_3S + H]^+$.

*5.1.2.6. Compound **9f** (R = 3,5-di(OMe)benzyl).* Yield = 39%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.88 (s, 1H, NH), 7.07 (s, 2H, NH_2), 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–CH_2), 3.71 (s, 6H, OCH_3), 2.83 (t, J = 8 Hz, 2H, N–CH_2–**CH_2**); MS (ESI) m/z 308 $[C_{14}H_{17}N_3O_3S + H]^+$.

*5.1.2.7. Compound **9g** (R = 3,4-di(OEt)benzyl).* Yield = 27%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.85 (s, 1H, NH), 7.05 (s, 2H, NH_2), 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–CH_2), 4.01–3.93 (m, 4H, O–CH_2), 2.82 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 1.33–1.27 (m, 6H, O–CH_2–**CH_3**); MS (ESI) m/z 336 $[C_{16}H_{21}N_3O_3S + H]^+$.

*5.1.2.8. Compound **9h** (R = 3,4-(OCH_2CH_2O)benzyl).* Yield = 42%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.86 (s, 1H, NH), 7.07 (s, 2H, NH_2), 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–CH_2), 4.19 (s, 4H, O–**CH_2CH_2**–O), 2.77 (t, J = 8.4 Hz, 2H, N–CH_2–**CH_2**); MS (ESI) m/z 306 $[C_{14}H_{15}N_3O_3S + H]^+$.

*5.1.2.9. Compound **9i** (R = 2,4-dichlorobenzyl).* Yield = 32%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.78 (s, 1H, NH), 7.55 (s, 1H, Ar–H),

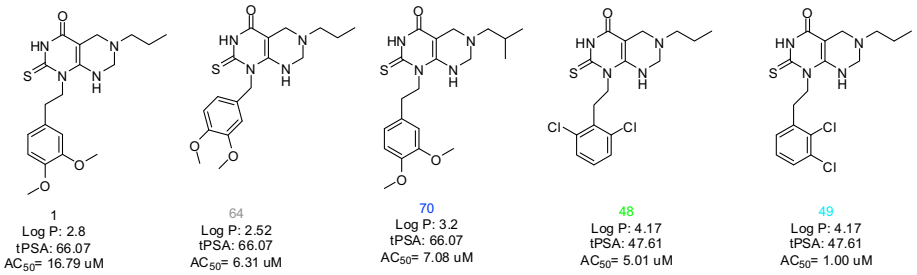


Fig. 6. Structure of best activators.

7.38 (s, 2H, Ar–H), 6.97 (s, 2H, NH_2), 4.90 (s, 1H, C(5)-H), 4.87–4.66 (m, 2H, N–CH_2), 3.03 (t, J = 7.6 Hz, 2H, N–CH_2–**CH_2**); MS (ESI) m/z 316 $[C_{12}H_{11}Cl_2N_3OS + H]^+$.

*5.1.2.10. Compound **9j** (R = 3,4-dichlorobenzyl).* Yield = 39%; ^{1}H 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, NH_2), 4.85 (s, 1H, C(5)-H), 4.47 (br s, 2H, N–CH_2), 2.92 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**); MS (ESI) m/z 316 $[C_{12}H_{11}Cl_2N_3OS + H]^+$.

*5.1.2.11. Compound **9k** (R = 2,6-dichlorobenzyl).* Yield = 32%; ^{1}H 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, NH_2), 4.85 (s, 1H, C(5)-H), 4.47 (br s, 2H, N–CH_2), 2.92 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**); MS (ESI) m/z 316 $[C_{12}H_{11}Cl_2N_3OS + H]^+$.

*5.1.2.12. Compound **9l** (R = 2,6-dimethylbenzyl).* Yield = 30%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.91 (s, 1H, NH), 6.99–6.98 (m, 3H, Ar–H and NH_2), 6.82 (s, 2H, Ar–H), 4.94 (s, 1H, C(5)-H), 4.47 (br s, 2H, N–CH_2), 2.92 (t, J = 7.2 Hz, 2H, N–CH_2–**CH_2**), 2.38 (s, 6H, CH_3); MS (ESI) m/z 276 $[C_{14}H_{17}N_3OS + H]^+$.

*5.1.2.13. Compound **9m** (R = 2,3-dimethylbenzyl).* Yield = 32%; ^{1}H 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 NH_2), 4.89 (s, 1H, C(5)-H), 4.49 (br s, 2H, N–CH_2), 2.83 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.30 (s, 3H, CH_3), 2.21 (s, 3H, CH_3); MS (ESI) m/z 276 $[C_{14}H_{17}N_3OS + H]^+$.

*5.1.2.14. Compound **9n** (R = 3,4-dimethylbenzyl).* Yield = 30%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.84 (s, 1H, NH), 7.12–7.05 (m, 5H, Ar–H and NH_2), 4.87 (s, 1H, C(5)-H), 4.47 (br s, 2H, N–CH_2), 2.83 (t, J = 8 Hz, 2H, N–CH_2–**CH_2**), 2.18 (s, 3H, CH_3), 2.17 (s, 3H, CH_3); MS (ESI) m/z 276 $[C_{14}H_{17}N_3OS + H]^+$.

*5.1.2.15. Compound **9o** (R = 2,4-dimethylbenzyl).* Yield = 45%; ^{1}H 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 NH_2), 4.89 (s, 1H, C(5)-H), 4.49 (br s, 2H, N–CH_2), 2.83 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.30 (s, 3H, CH_3), 2.21 (s, 3H, CH_3); MS (ESI) m/z 276 $[C_{14}H_{17}N_3OS + H]^+$.

*5.1.2.16. Compound **9p** (R = 2,3-dichlorobenzyl).* Yield = 29%; ^{1}H 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, NH_2), 4.88 (s, 1H, C(5)-H), 4.22–4.19 (m, 2H, N–CH_2), 3.10 (t, J = 7.6 Hz, 2H, N–CH_2–**CH_2**); MS (ESI) m/z 316 $[C_{12}H_{11}Cl_2N_3OS + H]^+$.

*5.1.2.17. Compound **9q** (R = 3,5-dimethylbenzyl).* Yield = 50%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.83 (s, 1H, NH), 7.03 (s, 2H, Ar–H),

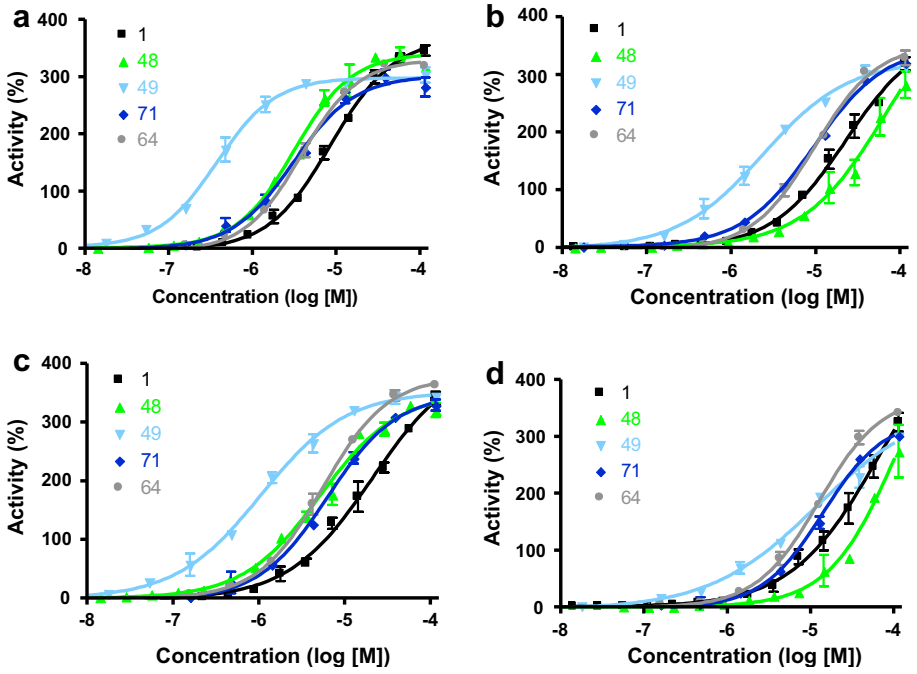


Fig. 7. Titration curves of best activators. a. Red purified enzyme; b. blue purified enzyme; c. red spleen; d. blue spleen.

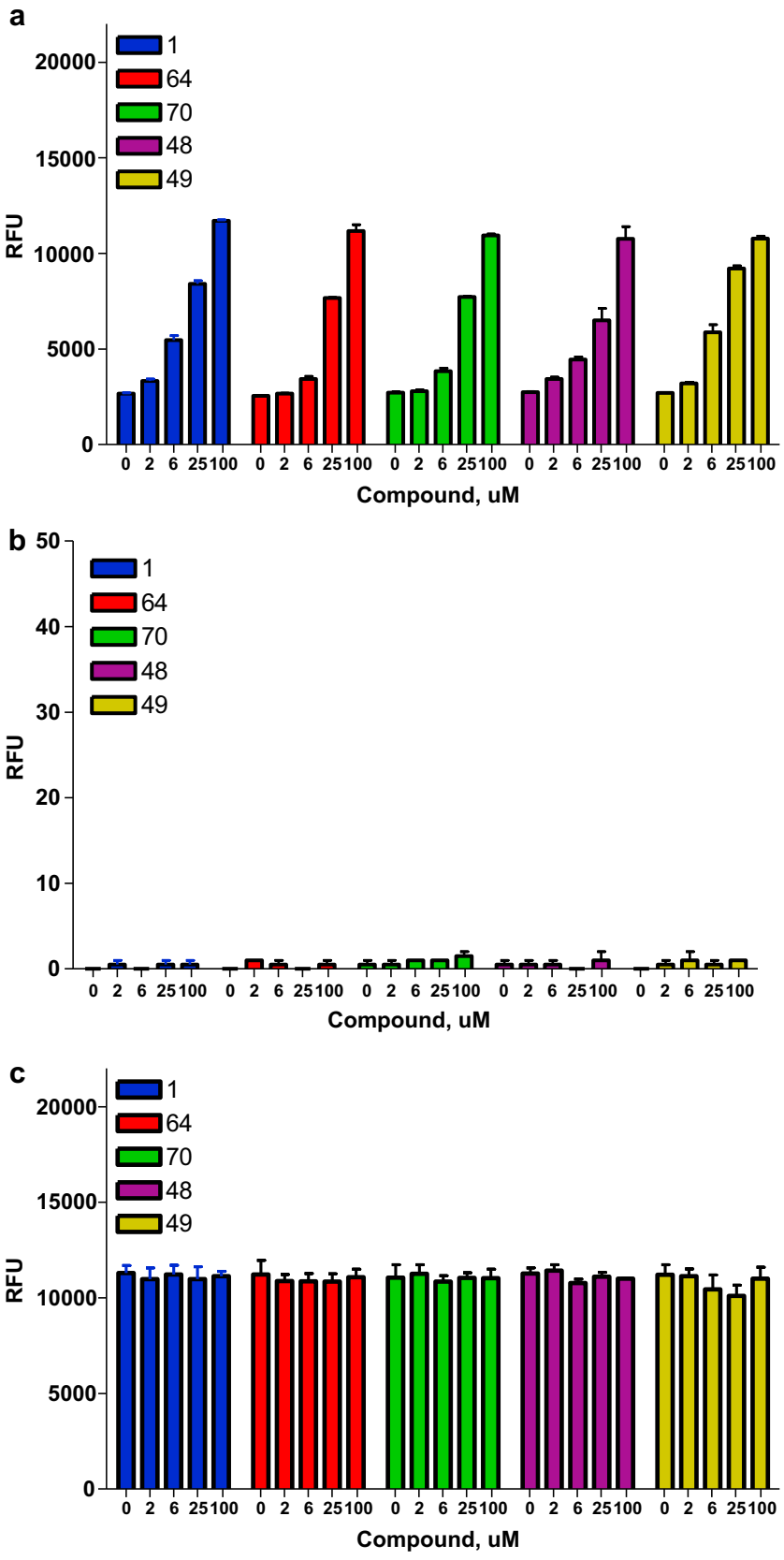


Fig. 8. a. Concentration-dependent effect of most active compounds on the hydrolysis of 4-methylumbelliferyl-α-D-glucopyranoside (4MU-α-glu) in the presence of purified GAA. b. Concentration-dependent effect of most active compounds on the hydrolysis of 4-methylumbelliferyl-α-D-glucopyranoside (4MU-α-glu) 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.

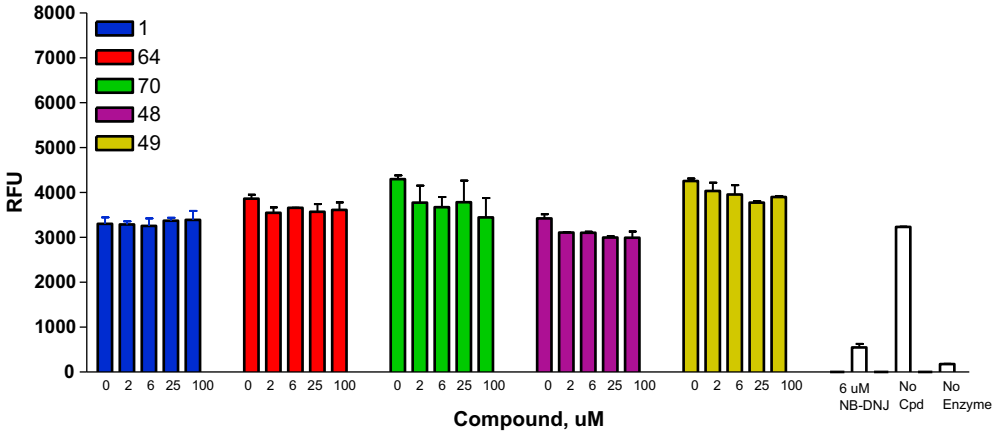


Fig. 9. Effect of lead compounds on rhGAA using glycogen as the substrate.

6.96 (s, 2H, NH_2), 6.85 (s, 1H, Ar–H), 4.88 (s, 1H, C(5)-H), 4.47 (br s, 2H, N–CH_2), 2.83 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.24 (s, 6H, CH_3); MS (ESI) m/z 276 $[C_{14}H_{17}N_3OS + H]^+$.

*5.1.2.18. Compound **9r** (R = 2-chlorobenzyl).* Yield = 43%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.87 (s, 1H, NH), 7.37 (s, 4H, Ar–H), 7.09 (s, 2H, NH_2), 4.86 (s, 1H, C(5)-H), 4.49 (br s, 2H, N–CH_2), 2.90 (t, J = 8 Hz, 2H, N–CH_2–**CH_2**); MS (ESI) m/z 282 $[C_{12}H_{12}ClN_3OS + H]^+$.

*5.1.2.19. Compound **9s** (R = 3-chlorobenzyl).* Yield = 60%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.87 (s, 1H, NH), 7.37 (s, 4H, Ar–H), 7.09 (s, 2H, NH_2), 4.86 (s, 1H, C(5)-H), 4.49 (br s, 2H, N–CH_2), 2.90 (t, J = 8 Hz, 2H, N–CH_2–**CH_2**); MS (ESI) m/z 282 $[C_{12}H_{12}ClN_3OS + H]^+$.

*5.1.2.20. Compound **9t** (R = 4-chlorobenzyl).* Yield = 25%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.87 (s, 1H, NH), 7.37 (s, 4H, Ar–H), 7.09 (s, 2H, NH_2), 4.86 (s, 1H, C(5)-H), 4.49 (br s, 2H, N–CH_2), 2.90 (t, J = 8 Hz, 2H, N–CH_2–**CH_2**); MS (ESI) m/z 282 $[C_{12}H_{12}ClN_3OS + H]^+$.

*5.1.2.21. Compound **9u** (R = 2-methylbenzyl).* Yield = 28%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.87 (s, 1H, NH), 7.29–6.99 (m, 6H, Ar–H and NH_2), 4.90 (s, 1H, C(5)-H), 4.49 (br s, 2H, N–CH_2), 2.91–2.87 (m, 2H, N–CH_2–**CH_2**), 2.35 (s, 3H); MS (ESI) m/z 262 $[C_{13}H_{15}N_3OS + H]^+$.

*5.1.2.22. Compound **9v** (R = 3-methylbenzyl).* Yield = 29%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.87 (s, 1H, NH), 7.29–6.99 (m, 6H, Ar–H and NH_2), 4.90 (s, 1H, C(5)-H), 4.49 (br s, 2H, N–CH_2), 2.91–2.87 (m, 2H, N–CH_2–**CH_2**), 2.35 (s, 3H); MS (ESI) m/z 262 $[C_{13}H_{15}N_3OS + H]^+$.

*5.1.2.23. Compound **9w** (R = 4-methylbenzyl).* Yield = 30%; ^{1}H 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, NH_2), 4.90 (s, 1H, C(5)-H), 4.51 (br s, 2H, N–CH_2), 2.91 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.35 (s, 3H); MS (ESI) m/z 261 $[C_{13}H_{15}N_3OS + H]^+$.

*5.1.2.24. Compound **9x** (R = 2-methoxybenzyl).* Yield = 80%; ^{1}H 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, NH_2), 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–CH_2), 3.73 (s, 3H, OCH_3), 2.89 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**); MS (ESI) m/z 277 $[C_{13}H_{15}N_3O_2S + H]^+$.

*5.1.2.25. Compound **9y** (R = 3-methoxybenzyl).* Yield = 59%; ^{1}H 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, NH_2), 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–CH_2), 3.73 (s, 3H, OCH_3), 2.89 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**); MS (ESI) m/z 277 $[C_{13}H_{15}N_3O_2S + H]^+$.

*5.1.2.26. Compound **9z** (R = 4-methoxybenzyl).* Yield = 21%; ^{1}H 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, NH_2), 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–CH_2), 3.73 (s, 3H, OCH_3), 2.89 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**); MS (ESI) m/z 277 $[C_{13}H_{15}N_3O_2S + H]^+$.

*5.1.2.27. Compound **9aa** (R = methyl).* Yield = 45%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.78 (s, 1H, NH), 7.02 (s, 2H, NH_2), 4.81 (s, 1H, C(5)-H), 4.15 (br s, 2H, N–CH_2), 1.65 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 0.85 (t, J = 8.0 Hz, 3H, CH_3); MS (ESI) m/z 214 $[C_9H_{15}N_3OS + H]^+$.

*5.1.2.28. Compound **9ab** (R = isopropyl).* Yield = 48%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.78 (s, 1H, NH), 6.79 (s, 2H, NH_2), 4.88 (s, 1H, C(5)-H), 4.39 (br s, 2H, N–CH_2), 1.68–1.61 (m, 1H, **CH**$(CH_3)_2$) 1.49 (t, J = 7.6 Hz, 2H, N–CH_2–**CH_2**), 0.94–0.91 (m, 6H, CH**$(CH_3)_2$**); MS (ESI) m/z 214 $[C_9H_{15}N_3OS + H]^+$.

*5.1.2.29. Compound **9ac** (R = t-butyl).* Yield = 29%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.78 (s, 1H, NH), 6.79 (s, 2H, NH_2), 4.88 (s, 1H, C(5)-H), 4.39 (br s, 2H, N–CH_2), 1.49 (t, J = 7.6 Hz, 2H, N–CH_2–**CH_2**), 0.93 (s, 9H, $C(CH_3)_3$); MS (ESI) m/z 228 $[C_{10}H_{17}N_3OS + H]^+$.

*5.1.2.30. Compound **9ad** (R = phenethyl).* Yield = 33%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.78 (s, 1H, NH), 7.28–7.14 (m, 5H, Ar–H), 7.05 (s, 2H, NH_2), 4.83 (s, 1H, C(5)-H), 4.33–4.32 (m, 2H, N–CH_2), 2.62 (t, J = 8 Hz, 2H, C_6H_5–**CH_2**), 1.90 (t, J = 8 Hz, 2H, N–CH_2–**CH_2**); MS (ESI) m/z 262 $[C_{13}H_{15}N_3OS + H]^+$.

*5.1.2.31. Compound **9ae** (R = 2-thiophene).* Yield = 36%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.87 (s, 1H, NH), 7.70–7.64 (m, 1H, Ar–H), 7.08 (s, 2H, NH_2), 6.98–6.95 (m, 2H, Ar–H), 4.86 (s, 1H, C(5)-H), 4.85 (br s, 2H, N–CH_2), 3.13 (t, J = 8 Hz, 2H, N–CH_2–**CH_2**); MS (ESI) m/z 254 $[C_{10}H_{11}N_3OS_2 + H]^+$.

*5.1.2.32. Compound **9af** (R = 2-pyridyl).* Yield = 32%; ^{1}H 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, NH_2), 4.87 (s, 1H, C(5)-H), 4.72 (br s, 2H, N–CH_2), 3.12 (t, J = 8 Hz, 2H, N–CH_2–**CH_2**); MS (ESI) m/z 249 $[C_{11}H_{12}N_4OS + H]^+$.

*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 ^{1}H NMR and MS analysis, and HRMS.

*5.1.3.1. Compound **1** (R = 3,4-di(OMe)benzyl).* Yield = 51%; ^{1}H 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–CH_2), 3.98 (s, 2H, C(7)-H_2), 3.73 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 3.36 (s, 2H, C(5)-H_2), 2.85 (t, J = 8 Hz, 2H, N–CH_2–**CH_2**), 2.33 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.44 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.86 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{19}H_{26}N_4O_3S + H]^+$ 390.1726, found 390.1733.

*5.1.3.2. Compound **2** (R = 3,5-dimethylbenzyl).* Yield = 50%; ^{1}H 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–CH_2), 3.98 (s, 2H, C(7)-H_2), 3.37 (s, 2H, C(5)-H_2), 2.82 (t, J = 8 Hz, 2H, N–CH_2–**CH_2**), 2.34 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 2.19 (s, 3H, Ar–**CH_3**), 2.17 (s, 3H, Ar–**CH_3**), 1.50–1.41 (m, 2H, CH_2**CH_2**CH_3), 0.86 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**) HRMS (ESI) m/z calculated for 330.1514 $[C_{19}H_{26}N_4OS + H]^+$, found 330.1515.

*5.1.3.3. Compound **33** (R = 2-methylbenzyl).* Yield = 45%; ^{1}H 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–CH_2), 3.97 (s, 2H, C(7)-H_2), 3.37 (s, 2H, C(5)-H_2), 2.89 (t, J = 7.2 Hz, 2H, N–CH_2–**CH_2**), 2.34 (m, 5H, **CH_2**CH_2CH_3 and Ar–**CH_3**), 1.45 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.86 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{18}H_{24}N_4OS + H]^+$ 344.1671, found 344.1672.

*5.1.3.4. Compound **34** (R = 3-methylbenzyl).* Yield = 45%; ^{1}H 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–CH_2), 3.99 (s, 2H, C(7)-H_2), 3.37 (s, 2H, C(5)-H_2), 2.87 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.32 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 2.28 (s, 3H, Ar–**CH_3**), 1.45 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.87 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{18}H_{24}N_4OS + H]^+$ 344.1671, found 344.1672.

*5.1.3.5. Compound **35** (R = 4-methylbenzyl).* Yield = 42%; ^{1}H 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–CH_2), 3.99 (s, 2H, C(7)-H_2), 3.37 (s, 2H, C(5)-H_2), 2.86 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.34 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 2.26 (s, 3H, Ar–**CH_3**), 1.45 (m, 2H, CH_2**CH_2**CH_3), 0.87 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{18}H_{24}N_4OS + H]^+$ 344.1671, found 344.1672.

*5.1.3.6. Compound **36** (R = 2-chlorobenzyl).* Yield = 45%; ^{1}H 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–CH_2), 3.93 (s, 2H, C(7)-H_2), 3.36 (s, 2H, C(5)-H_2), 3.05 (t, J = 7.6 Hz, 2H, N–CH_2–**CH_2**), 2.32 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.46 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.86 (t, J = 7.6 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{17}H_{21}ClN_4OS + H]^+$ 364.1125, found 364.1125.

*5.1.3.7. Compound **37** (R = 3-chlorobenzyl).* Yield = 40%; ^{1}H 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–CH_2), 3.99 (s, 2H, C(7)-H_2), 3.37 (s, 2H, C(5)-H_2), 2.93 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.34 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.44 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.87 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{17}H_{21}ClN_4OS + H]^+$ 364.1125, found 364.1128.

*5.1.3.8. Compound **38** (R = 4-chlorobenzyl).* Yield = 40%; ^{1}H 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–CH_2), 3.98 (s, 2H, C(7)-H_2), 3.37 (s, 2H, C(5)-H_2), 2.91 (t, J = 8.00 Hz, 2H, N–CH_2–**CH_2**), 2.34 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.45 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.87 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{17}H_{21}ClN_4OS + H]^+$ 364.1125, found 364.1130.

*5.1.3.9. Compound **39** (R = 2-methoxybenzyl).* Yield = 35%; ^{1}H 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–CH_2), 3.94 (s, 2H, C(7)-H_2), 3.75 (s, 3H, OCH_3), 3.36 (s, 2H, C(5)-H_2), 2.90 (t, J = 7.2 Hz, 2H, N–CH_2–**CH_2**), 2.32 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.44 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.86 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{18}H_{24}N_4O_2S + H]^+$ 360.1620, found 360.1623.

*5.1.3.10. Compound **40** (R = 3-methoxybenzyl).* Yield = 57%; ^{1}H 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–CH_2), 3.98 (s, 2H, C(7)-H_2), 3.73 (s, 3H, OCH_3), 3.37 (s, 2H, C(5)-H_2), 2.88 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.34 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.45 (m, 2H, CH_2**CH_2**CH_3), 0.86 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{18}H_{24}N_4O_2S + H]^+$ 360.1620, found 360.1621.

*5.1.3.11. Compound **41** (R = 4-methoxybenzyl).* Yield = 35%; ^{1}H 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–CH_2), 3.99 (s, 2H, C(7)-H_2), 3.71 (s, 3H, OCH_3), 3.37 (s, 2H, C(5)-H_2), 2.84 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.35 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.45 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.86 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{18}H_{24}N_4O_2S + H]^+$ 360.1620, found 360.1623.

*5.1.3.12. Compound **42** (R = 2,6-dimethylbenzyl).* Yield = 42%; ^{1}H 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–CH_2), 3.94 (s, 2H, C(7)-H_2), 3.37 (s, 2H, C(5)-H_2), 2.92 (s, 2H, N–CH_2–**CH_2**), 2.37–2.32 (m, 8H, **CH_2**CH_2CH_3 and Ar–**CH_3**), 1.45 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.85 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{19}H_{26}N_4OS + H]^+$ 358.1827, found 358.1833.

*5.1.3.13. Compound **43** (R = 2,3-dimethylbenzyl).* Yield = 33%; ^{1}H 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–CH_2), 3.97 (s, 2H, C(7)-H_2), 3.37 (s, 2H, C(5)-H_2), 2.91 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.35 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 2.24 (s, 3H, Ar–**CH_3**), 2.22 (s, 3H, Ar–**CH_3**), 1.49–1.40 (m, 2H, CH_2**CH_2**CH_3), 0.86 (t, J = 1.72 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{19}H_{26}N_4OS + H]^+$ 358.1827, found 358.1833.

*5.1.3.14. Compound **44** (R = 2,5-dimethylbenzyl).* Yield = 32%; ^{1}H 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–CH_2), 3.98 (s, 2H, C(7)-H_2), 3.37 (s, 2H, C(5)-H_2), 2.82 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.34 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 2.19 (s, 3H, Ar–**CH_3**), 2.17 (s, 3H, Ar–**CH_3**), 1.50–1.41 (m, 2H, CH_2**CH_2**CH_3), 0.86 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{19}H_{26}N_4OS + H]^+$ 358.1827, found 358.1832.

*5.1.3.15. Compound **45** (R = 2,4-dimethylbenzyl).* Yield = 32%; ^{1}H 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–CH_2), 3.97 (s, 2H, C(7)-H_2), 3.37 (s, 2H, C(5)-H_2), 2.84 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.36–2.30 (m, 5H,, **CH_2**CH_2CH_3 and Ar–**CH_3**), 2.21 (s, 3H, Ar–**CH_3**), 1.50–1.41 (m, 2H, CH_2**CH_2**CH_3), 0.86 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{19}H_{26}N_4OS + H]^+$ 358.1827, found 358.1844.

*5.1.3.16. Compound **46** (R = 3,4-dimethylbenzyl).* Yield = 32%; ^{1}H 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–CH_2), 3.98 (s, 2H, C(7)-H_2), 3.37 (s, 2H, C(5)-H_2), 2.82 (t, J = 8 Hz, 2H, N–CH_2–**CH_2**), 2.34 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 2.19 (s, 3H, Ar–**CH_3**), 2.17 (s, 3H, Ar–**CH_3**), 1.50–1.41 (m, 2H, CH_2**CH_2**CH_3), 0.86 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{19}H_{26}N_4OS + H]^+$ 358.1827, found 358.1833.

*5.1.3.17. Compound **47** (R = 3,5-dimethylbenzyl).* Yield = 50%; ^{1}H 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–CH_2), 3.97 (s, 2H, C(7)-H_2), 3.37 (s, 2H, C(5)-H_2), 2.89 (t, J = 7.2 Hz, 2H, N–CH_2–**CH_2**), 2.34 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 2.33 (s, 6H, Ar–**CH_3**), 1.45 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.86 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{19}H_{26}N_4OS + H]^+$ 358.1827, found 358.1831.

*5.1.3.18. Compound **48** (R = 2,6-dichlorobenzyl).* Yield = 40%; ^{1}H 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–CH_2), 3.90 (s, 2H, C(7)-H_2), 3.23 (m, 4H,, N–CH_2–**CH_2** and C(5)-H_2), 2.33 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.43 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.85 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{17}H_{20}Cl_2N_4OS + H]^+$ 398.0735, found 398.0741.

*5.1.3.19. Compound **49** (R = 2,3-dichlorobenzyl).* Yield = 29%; ^{1}H 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–CH_2), 3.93 (s, 2H, C(7)-H_2), 3.36 (s, 2H, C(5)-H_2), 3.11 (t, J = 7.2 Hz, 2H, N–CH_2–**CH_2**), 2.32 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.44 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.86 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{17}H_{20}Cl_2N_4OS + H]^+$ 398.0735, found 398.0741.

*5.1.3.20. Compound **50** (R = 2,5-dichlorobenzyl).* Yield = 21%; ^{1}H 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–CH_2), 3.94 (s, 2H, C(7)-H_2), 3.37 (s, 2H, C(5)-H_2), 3.07 (t, J = 7.2 Hz, 2H, N–CH_2–**CH_2**), 2.35 (t, J = 6.8 Hz, 2H, **CH_2**CH_2CH_3), 1.49–1.40 (m, 2H, CH_2**CH_2**CH_3), 0.88 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{17}H_{20}Cl_2N_4OS + H]^+$ 398.0735, found 398.0743.

*5.1.3.21. Compound **51** (R = 2,4-dichlorobenzyl).* Yield = 39%; ^{1}H 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–CH_2), 3.94 (s, 2H, C(7)-H_2), 3.36 (s, 2H, C(5)-H_2), 3.04 (t, J = 7.2 Hz, 2H, N–CH_2–**CH_2**), 2.32 (t. J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.44 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.86 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{17}H_{20}Cl_2N_4OS + H]^+$ 398.0735, found 398.0735.

*5.1.3.22. Compound **52** (R = 3,4-dichlorobenzyl).* Yield = 50%; ^{1}H 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–CH_2), 3.99 (s, 2H, C(7)-H_2), 3.37 (s, 2H, C(5)-H_2), 2.93 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.33 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.45 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.87 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{17}H_{20}Cl_2N_4OS + H]^+$ 398.0735, found 398.0741.

*5.1.3.23. Compound **53** (R = 3,5-dichlorobenzyl).* Yield = 40%; ^{1}H 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, 2 H, Ar–H), 4.47 (br s, 2H, N–CH_2), 4.00 (s, 2H, C(7)-H_2), 3.38 (s, 2H, C(5)-H_2), 2.96 (t, J = 7.2 Hz, 2H, N–CH_2–**CH_2**), 2.36 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.50 (m, 2H, CH_2**CH_2**CH_3), 0.89 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{17}H_{20}Cl_2N_4OS + H]^+$ 398.0735, found 398.0743.

*5.1.3.24. Compound **54** (R = 2,6-di(OMe)benzyl).* Yield = 51%; ^{1}H 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–CH_2), 3.88 (s, 2H, C(7)-H_2), 3.70 (s, 6H, OCH_3), 3.33 (s, 2H, C(5)-H_2), 2.92 (t, J = 6.0 Hz, 2H, N–CH_2–**CH_2**), 2.31 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.43 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.85 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{19}H_{26}N_4O_3S + H]^+$ 390.1726, found 390.1730.

*5.1.3.25. Compound **55** (R = 2,3-di(OMe)benzyl).* Yield = 55%; ^{1}H 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–CH_2), 4.06 (s, 2H, C(7)-H_2), 3.85 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3), 3.45 (s, 2H, C(5)-H_2), 2.99 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.41 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.45 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.94 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{19}H_{26}N_4O_3S + H]^+$ 390.1726, found 390.1734.

*5.1.3.26. Compound **56** (R = 2,5-di(OMe)benzyl).* Yield = 40%; ^{1}H 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–CH_2), 3.98 (s, 2H, C(7)-H_2), 3.73 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 3.36 (s, 3H, C(5)-H_2), 2.85 (t, J = 8 Hz, 2H, N–CH_2–**CH_2**), 2.33 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.44 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.86 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{19}H_{26}N_4O_3S + H]^+$ 390.1726, found 390.1730.

*5.1.3.27. Compound **57** (R = 2,4-di(OMe)benzyl).* Yield = 47%; ^{1}H 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–CH_2), 3.95 (s, 2H, C(7)-H_2), 3.73 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 3.35 (s, 2H, C(5)-H_2), 2.81 (t, J = 7.6 Hz, 2H, N–CH_2–**CH_2**), 2.31 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.44 (q, J = 7.6 Hz, 2H, CH_2**CH_2**CH_3), 0.85 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{19}H_{26}N_4O_3S + H]^+$ 390.1726, found 390.1731.

*5.1.3.28. Compound **58** (R = 3,5-di(OMe)benzyl).* Yield = 39%; ^{1}H 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–CH_2), 3.98 (s, 2H, C(7)-H_2), 3.71 (s, 6H, OCH_3), 3.36 (s, 2H, C(5)-H_2), 2.84 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.33 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.49–1.40 (m, 2H, CH_2**CH_2**CH_3), 0.86 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{19}H_{26}N_4O_3S + H]^+$ 390.1726, found 390.1728.

*5.1.3.29. Compound **59** (R = 3,4-(OCH_2CH_2O)benzyl).* Yield = 33%; ^{1}H 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–CH_2), 4.20 (s, 4H, –OCH_2CH_2O–), 3.99 (s, 2H, C(7)-H_2), 3.37 (s, 2H, C(5)-H_2), 2.78 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.34 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.45 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.86 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**). HRMS (ESI) m/z calculated for $[C_{19}H_{24}N_4O_3S + H]^+$ 388.1569, found 388.1571.

*5.1.3.30. Compound **60** (R = 3,4-di(OEt)benzyl).* Yield = 33%; ^{1}H 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–CH_2), 4.01–3.93 (m, 6H, C(7)-H_2 and O**CH_2**CH_3), 3.36 (s, 2H, C(5)-H_2), 2.82 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.33 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.45 (t, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 1.33–1.27 (m, 6H, OCH_2**CH_3**), 0.86 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{21}H_{30}N_4O_3S + H]^+$ 418.2039, found 418.2048.

*5.1.3.31. Compound **61** (R = 2-pyridyl).* Yield = 32%; ^{1}H 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–CH_2), 3.98 (s, 2H, C(7)-H_2), 3.36 (s, 2H, C(5)-H_2), 2.85 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.33 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.45 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.86 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{16}H_{21}N_5OS + H]^+$ 331.1467, found 331.1468.

*5.1.3.32. Compound **62** (R = 3-thienyl).* Yield = 37%; ^{1}H 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–CH_2), 3.98 (s, 2H, C(7)-H_2), 3.32 (s, 2H, C(5)-H_2), 2.96 (t, J = 8 Hz, 2H, N–CH_2–**CH_2**), 2.36 (t, J = 7.2 Hz 2H, **CH_2**CH_2CH_3), 1.50–1.41 (m, 2H, CH_2**CH_2**CH_3), 0.88 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{15}H_{20}N_4OS_2+H]^+$ 336.1079, found 336.1079.

*5.1.3.33. Compound **63** (R = 2-thienyl).* Yield = 40%; ^{1}H 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–CH_2), 3.97 (s, 2H, C(7)-H_2), 3.37 (s, 2H, C(5)-H_2), 3.14 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.34 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.45 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.86 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{15}H_{20}N_4OS_2+H]^+$ 336.1079, found 336.1078.

*5.1.3.34. Compound **64** (R = 3,4-di(OMe)phenyl).* ^{1}H 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–CH_2), 3.92 (s, 2H, C(7)-H_2), 3.70 (s, 6H, OCH_3), 3.39 (s, 2H, C(5)-H_2), 2.32 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.43 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.84 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{18}H_{24}N4O_3S + H]^+$ 376.1569, found 376.1573.

*5.1.3.35. Compound **65** (R = phenethyl).* Yield = 33%; ^{1}H 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–CH_2), 4.00 (s, 2H, C(7)-H_2), 3.36 (s, 2H, C(5)-H_2), 2.62 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.35 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.94–1.88 (m, 2H, C_6H_5–**CH_2**), 1.45 (q, J = 7.2 Hz, 2H, CH_2**CH_2**CH_3), 0.87 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{18}H_{24}N_4OS + H]^+$ 344.1671, found 344.1672.

*5.1.3.36. Compound **66** (R = isopropyl).* Yield = 30%; ^{1}H 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–CH_2), 3.97 (s, 2H, C(7)-H_2), 3.35 (s, 2H, C(5)-H_2), 2.34 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.68–1.61 (m, 1H **CH**$(CH_3)_2$), 1.49–1.40 (m, 4H, N–CH_2–**CH_2** and CH_2**CH_2**CH_3), 0.91–0.87 (m, 9H, CH_2CH_2**CH_3** and CH**$(CH_3)_2$**); HRMS (ESI) m/z calculated for $[C_{14}H_{24}N_4OS + H]^+$ 296.1671, found 296.1673.

*5.1.3.37. Compound **67** (R = t-butyl).* Yield = 34%; ^{1}H 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–CH_2), 3.96 (s, 2H, C(7)-H_2), 3.33 (s, 2H, C(5)-H_2), 2.34 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.51–1.42 (m, 4H, *t*-Bu-**CH_2** and CH_2**CH_2**CH_3), 0.94 (s, 9H C**$(CH_3)_3$**), 0.86 (t, J = 7.2 Hz, 3H, CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{15}H_{26}N_4OS + H]^+$ 310.1827, found 310.1829.

*5.1.3.38. Compound **68** (R = methyl).* Yield = 32%; ^{1}H 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–CH_2), 4.00 (s, 2H, C(7)-H_2), 3.36 (s, 2H, C(5)-H_2), 2.34 (t, J = 7.2 Hz, 2H, **CH_2**CH_2CH_3), 1.65–1.58 (m, 2H, N–CH_2–**CH_2**), 1.56–1.40 (m, 2H, CH_2**CH_2**CH_3), 0.85 (m, 6H, NCH_2CH_2**CH_3** and CH_2CH_2**CH_3**); HRMS (ESI) m/z calculated for $[C_{12}H_{20}N_4OS + H]^+$ 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 ^{1}H NMR and MS analysis.

*5.1.4.1. Compound **69** (R = methyl).* Yield = 37%; ^{1}H 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–CH_2), 4.00 (s, 2H, C(7)-H_2), 3.81 (s, 3H, OCH_3), 3.78 (s, 3H, OCH_3), 3.37 (s, 2H, C(5)-H_2), 2.92 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.34 (s, 3H, NCH_3); HRMS (ESI) m/z calculated for $[C_{17}H_{22}N_4O_3S + H]^+$ 362.1413, found 362.1415.

*5.1.4.2. Compound **70** (R = isopropyl).* Yield = 33%; ^{1}H 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–CH_2), 4.03 (s, 2H, C(7)-H_2), 3.73 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 3.42 (s, 2H, C(5)-H_2), 2.85 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.72–2.66 (m, 1H, **CH**$(CH_3)_2$), 1.03 (s, 6H, CH**$(CH_3)_2$**); HRMS (ESI) m/z calculated for $[C_{19}H_{26}N_4O_3S + H]^+$ 390.1726, found 390.1730.

*5.1.4.3. Compound **71** (R = isobutyl).* Yield = 36%; ^{1}H 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–CH_2), 4.03 (s, 2H, C(7)-H_2), 3.73 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 3.42 (s, 2H, C(5)-H_2), 2.85 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**), 2.72–2.66 (m, 1H, **CH**$(CH_3)_2$), 2.15 (d, J = 8.0 Hz, 2H, **CH_2**$CH(CH_3)_2$) 1.03 (s, 6H, CH**$(CH_3)_2$**); HRMS (ESI) m/z calculated for $[C_{20}H_{28}N_4O_3S + H]^+$ 404.1882, found 404.1887.

*5.1.4.4. Compound **72** (R = phenyl).* Yield = 39%; ^{1}H 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)-H_2), 4.44 (br s, 2H, N–CH_2), 4.12 (s, 2H, C(5)-H_2), 3.70 (s, 6H, OCH_3), 2.78 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**); HRMS (ESI) m/z calculated for $[C_{22}H_{24}N_4O_3S + H]^+$ 424.1569, found 424.1571

*5.1.4.5. Compound **73** (R = benzyl).* Yield = 31%; ^{1}H 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–CH_2), 4.02 (s, 2H, C(7)-H_2), 3.73 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 3.37 (s, 2H, C(5)-H_2), 3.31 (s, 2H, N**CH_2**Ph), 2.88 (t, J = 8.0 Hz, 2H, N–CH_2–**CH_2**); HRMS (ESI) m/z calculated for $[C_{23}H_{26}N_4O_3S + H]^+$ 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: ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.34 (br s, 1H, N(3)-H), 12.73 (br s, 1H, NH_2), 9.31 (br s, 1H, NH_2), 6.91–6.79 (m, 3H, Ar–H), 4.51–4.50 (br s, 2H, N–CH_2), 3.72 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 2.83–2.79 (t, 2H, NCH_2**CH_2**); MS (ESI) *m/z* 337 $[C_{14}H_{16}N_4O_4S + H]^+$.

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: ^{1}H 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, OCH_3), 3.70 (s, 3H, OCH_3), 3.55–3.54 (m, 2H, –N**CH_2**), 2.69–2.68 (m, 2H, –NCH_2**CH_2**); MS (ESI) *m/z* 323 $[C_{14}H_{18}N_4O_3S + H]^+$.

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: ^{1}H 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, –N**CH_2**CH_2), 3.72 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 2.98–2.94 (t, 2H, –NCH_2**CH_2**); HRMS (ESI) *m/z* calculated for $[C_{15}H_{16}N_4O_3S + H]^+$ 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 $NaHCO_3$ solution, dried over Na_2SO_4, and concentrated under reduced pressure. The resulting solid was triturated with MTBE to afford **16** (120 mg, 33%) as an off-white solid: ^{1}H 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, –N**CH_2**CH_2), 3.74 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 2.92–2.88 (t, J = 8.0 Hz, 2H, –NCH_2**CH_2**), 2.67 (s, 3H, CH_3), 2.54 (s, 3H, CH_3); MS (ESI) *m/z* 372 $[C_{19}H_{21}N_3O_3S + H]^+$.

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: ^{1}H NMR (400 MHz, $CDCl_3$): 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, –N**CH_2**CH_2), 3.92 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 3.07–3.03 (t, 2H, –NCH_2**CH_2**); HRMS (ESI) *m/z* calculated for $[C_{16}H_{16}N_4O_3S + H]^+$ 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: ^{1}H 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 $[C_7H_5N_3O + H]^+$.

5.1.11. Synthesis of 8-iodopyrido[4,3-d]pyrimidin-5-(6H)-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: ^{1}H 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 $[C_7H_4IN_3O + H]^+$.

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 $POCl_3$ (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 K_2CO_3 and extracted with CH_2Cl_2 (3 × 30 mL). The combined organic layer was dried over Na_2SO_4 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: ^{1}H 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 $[C_7H_3ClN_3 + H]^+$.

5.1.13. General procedure for Sonogashira 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 $Pd(PPh_3)_2Cl_2$ (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**.

5.1.13.1. 5-Chloro-8-(phenylethynyl)pyrido[4,3-d]pyrimidine ***23***. ^{1}H NMR (400 MHz, $CDCl_3$) 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 $[C_{15}H_8ClN_3 + H]^+$ 265.0407, found 265.0403; 300 mg (45%) yield.

5.1.13.2. 5-Chloro-8-{(3,4-dimethoxyphenyl)ethynyl}pyrido[4,3-d]pyrimidine ***24***. ^{1}H NMR (400 MHz, $CDCl_3$) 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, OCH_3), 3.94 (s, 3H, OCH_3); MS (ESI) *m/z* 326 $[C_{17}H_{12}ClN_3O_2 + H]^+$; 400 mg (47%) yield.

5.1.14. General procedure for hydrolysis [27]

NH_4OAc (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 CH_2Cl_2) to afford compound **25** or **26**.

*5.1.14.1. 8-(Phenylethynyl)pyrido[4,3-d]pyrimidin-5(6H)-one **25**.* ^{1}H 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 $[C_{15}H_9N_3O + H]^+$ 247.0746, found 247.0746; 80 mg (42%) yield.

*5.1.14.2. 8-{(3,4-Dimethoxyphenyl)ethynyl}pyrido[4,3-d]pyrimidin-5(6H)-one **26**.* ^{1}H 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, OCH_3); HRMS (ESI) *m*/*z* calculated for $[C_{17}H_{13}N_3O_3 + H]^+$ 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 CH_2Cl_2. 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 CH_2Cl_2) to afford **27** or **28**.

*5.1.15.1. 8-(Phenylethyl)pyrido[4,3-d]pyrimidin-5(6H)-one **27**.* ^{1}H 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, –NH**CH$_2$**CH$_2$), 2.90–2.86 (m, 2H, –NHCH$_2$**CH$_2$**); HRMS (ESI) *m*/*z* calculated for $[C_{15}H_{13}N_3O + H]^+$; 251.1059, found 251.1060; 20 mg (24%) yield.

*5.1.15.2. 8-(3,4-Dimethoxyphenethyl)pyrido[4,3-d]pyrimidin-5(6H)-one **28**.* ^{1}H 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, OCH_3), 3.69 (s, 3H, OCH_3), 2.96 (t, J = 8 Hz, 2H, –NH**CH$_2$**CH$_2$), 2.81 (t, J = 8 Hz, 2H, –NHCH$_2$**CH$_2$**); HRMS (ESI) *m*/*z* calculated for $[C_{17}H_{17}N_3O_3 + H]^+$ 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: ^{1}H 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, –N**H**CH$_2$CH$_2$), 5.40 (s, 2H, NH_2), 3.72 (s, 3H, OCH_3), 3.69 (s, 3H, OCH_3), 3.15 (q, J = 6.8 Hz, 2H, –NH**CH$_2$**CH$_2$), 2.57 (t, J = 7.2 Hz, 2H, NHCH$_2$**CH$_2$**); MS (ESI) *m*/*z* 225 $[C_{11}H_{16}N_2O_3 + H]^+$.

*5.1.17. Synthesis of 6-amino-1-(3,4-dimethoxyphenethyl) pyrimidine-2,4(1H,3H)-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 CH_2Cl_2) to afford **31** (400 mg, 46%) as an off-white solid: ^{1}H 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, NH_2), 6.73 (d, J = 8 Hz, 1H, Ar–H), 4.53 (s, 1H, C(5)-H), 3.92 (t, J = 7.2 Hz, 2H, –NH**CH$_2$**CH$_2$), 3.72 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 2.72 (t, J = 7.6 Hz, 2H, –NHCH$_2$**CH$_2$**); MS (ESI) *m*/*z* 292 $[C_{14}H_{17}N_3O_4 + H]^+$.

*5.1.18. Synthesis of 1-(3,4-dimethoxyphenethyl)-6-propyl-5,6,7,8-tetrahydropyrimido [4,5-d]pyrimidine-2,4(1H,3H)-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 CH_2Cl_2) to afford **32** (180 mg, 28%) as an off-white solid: ^{1}H NMR (400 MHz, 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, –NH**CH$_2$**CH$_2$ and C(7)-H$_2$), 3.71 (s, 3H, OCH_3), 3.69 (s, 3H, OCH_3), 3.32 (s, 2H, C(5)-H$_2$), 2.72 (t, J = 7.6 Hz, 2H, **CH$_2$**CH$_2$CH$_3$), 2.31 (t, J = 6.8 Hz, 2H, NHCH$_2$**CH$_2$**), 1.48–1.39 (m, 2H, CH$_2$**CH$_2$**CH$_3$), 0.86 (t, J = 7.2 Hz, 3H, CH$_2$CH$_2$**CH$_3$**); HRMS (ESI) *m*/*z* calculated for $[C_{19}H_{26}N_4O_4 + H]^+$ 374.1954, found 374.1956.

5.2. Biological experiments

4-Methylumbelliferyl-α-D-glucopyranoside (4MU-α-glu), N-butyldeoxynojirimycin (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 min, followed by 10 passes in a motor-driven 50 mL glass-Teflon homogenizer. The homogenate was centrifuged at 1000 × *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, 1 M 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 min incubation at room temperature, the enzyme reaction was initiated by the addition of 2 μl/well substrate. After 45 min 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 min incubation at 37 °C, 2 μl/well of the Amplex Red solution was added, and the reaction was incubated 30 min 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.

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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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