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Original article

Inhibitory effects of 5-benzylidene barbiturate derivatives on mushroom tyrosinase and their antibacterial activities

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ABSTRACT

A series of novel 5-benzylidene barbiturate and thiobarbiturate derivatives were synthesized and evaluated as tyrosinase inhibitors and antibacterial agents. The results demonstrated that some compounds had more potent inhibitory activities than the parent compound 4-hydroxybenzaldehyde ($IC_{50} = 1.22$ mM). Particularly, compounds **1a** and **2a** were found to be the most potent inhibitors with IC_{50} value of 13.98 μM and 14.49 μM, respectively. The inhibition mechanism study revealed that these compounds were irreversible inhibitors. The circular dichroism spectra indicated that these compounds induced conformational changes of mushroom tyrosinase upon binding. In addition, these compounds exhibited selectively antibacterial activity against *Staphylococcus aureus*. All these results suggested that further development of such compounds may be of interest.

1. Introduction

Tyrosinase (EC1.14.18.1), a multifunctional copper-containing oxygenase, is widely distributed in nature. It catalyzes the hydroxylation of a monophenol and the conversion of *o*-diphenols to the corresponding *o*-quinones [1]. It is responsible for melanization in animals and browning in plants and fungi. The unfavorable darkening from enzymatic oxidation of phenols generally results in a loss of nutritional value and has been of great concern. It has recently been discovered that various dermatological disorders, such as age spots and freckle, were also caused by the accumulation of an excessive level of epidermal pigmentation. Therefore, tyrosinase inhibitors have become increasingly important in food industry as well as in the medicinal and cosmetic products [2,3].

Many efforts have been spent in the search for effective and safe tyrosinase inhibitors, and a large number of naturally occurring and synthetic tyrosinase inhibitors have already been reported. But some of their individual activities are either not potent enough to be considered of practical use or not compatible with safety regulations for food and cosmetic additives. Therefore, it is still necessary to search and develop novel tyrosinase inhibitors with potent activities and lower side effects.

In the last decades, benzaldehyde derivatives especially hydroxylated analogues have been extensively investigated [4] and structure–activity relationship (SAR) analysis indicated that electron-donating substituents at 4-position of benzaldehyde are necessary for potent inhibitory activities [5]. Recently, *N*-hydroxy-*N*′-phenylthiourea, *N*-hydroxy-*N*′-phenylurea and thiosemicarbazide derivatives were reported to exhibit potential inhibitory activity for tyrosinase and oxygen, nitrogen and sulfur atom of those compounds were able to complex the two copper ions in the active site of tyrosinase [6].

More recently, barbiturate and thiobarbiturate derivatives bearing urea and thiourea moiety, respectively, attracted considerable attention owing to their various biological effects such as inhibiting collagenase-3 (MMP-3) [7], matrix metalloproteinases [8], recombinant cytochrome P450 enzymes [9], methionine aminopeptidase-1 (MetAP-1) [10], and their broad spectrum pharmacological properties including hypnotic [11], sedative [12] and antibacterial [10]. In addition, it was well known that tyrosinase contained two copper ions in the active center and a lipophilic long-narrow gorge near to the active center, so we hypothesized that barbiturates or thiobarbiturates could complex the binuclear copper active site of tyrosinase, and interact with the hydrophobic enzyme pocket, leading to increasing inhibitor–enzyme binding affinity and improving inhibitory effects on mushroom tyrosinase.

Based on the above information, in the present investigation, a series of novel 5-benzylidene barbiturate and thiobarbiturate derivatives bearing hydrophobic alkyl substituents were designed

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and synthesized by the condensation of 4-substituted benzaldehydes with barbituric acid or thiobarbituric acid. Their inhibitory effects on the diphenolase activity of mushroom tyrosinase and their antibacterial activities against *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* (*S. epidermidis*), *Staphylococcus albus* (*S. albus*), *Bacillus cereus* (*B. cereus*), *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) were also investigated. To the best of our knowledge, this is the first time to report the inhibitory effects on the diphenolase activity of mushroom tyrosinase of 5-benzylidene barbiturate and thiobarbiturate derivatives.

2. Chemistry

The synthesis of 5-benzylidene barbiturate and thiobarbiturate derivatives is summarized in Scheme 1. Mono-tosylates were prepared by an excess of tosyl chloride and the corresponding alcohols in catalytic amounts of NaOH as described previously [13–15], and subsequently de-protected and simultaneously reacted with 4-hydroxybenzaldehyde in dimethylformamide (DMF) with potassium carbonate at 80°C for 16 h [16,17] to afford alkylated benzylaldehydes **c**–**f**. The condensation of 4-hydroxybenzaldehyde and alkylation benzaldehydes **b**–**f** with barbituric acid (or thiobarbituric acid) via well-known Knoevenagel condensation in ethanol [18,19] gave the 5-benzylidene barbiturate and thiobarbiturate derivatives **1** and **2** in good yield, and then followed by reduction with $NaBH_4$ in methanol to afford compounds **3** and **4**, respectively [20]. All the synthesized 5-benzylidene barbiturate and thiobarbiturate derivatives were characterized by spectroscopic data.

3. Biology

3.1. Inhibitory effects on the diphenolase activity of mushroom tyrosinase

The inhibition activities of our synthetic 5-benzylidene barbiturate and thiobarbiturate derivatives on mushroom tyrosinase were investigated by usual procedure [21] and compared with those of 4-hydroxybenzaldehyde [22] and 4-methoxycinnamic acid [23]. Figs. 1 and 2 showed that the remaining enzyme activities rapidly decreased with the increasing concentration of compounds **1a** and **2a**. The IC_{50} values of all compounds investigated are summarized in Table 1. As shown in Table 1, parent compounds

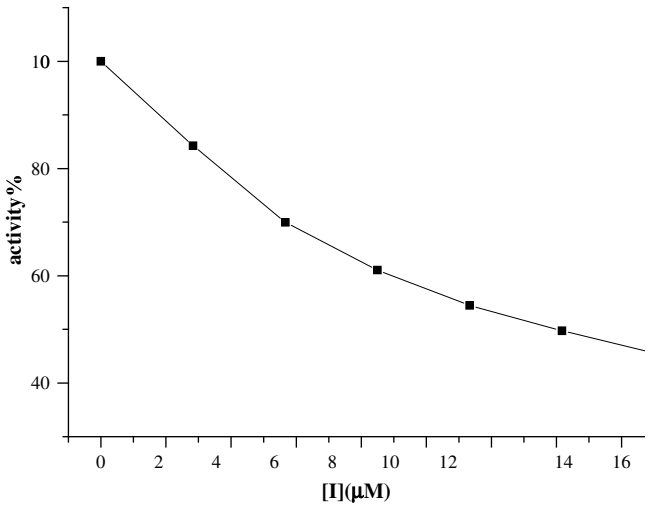
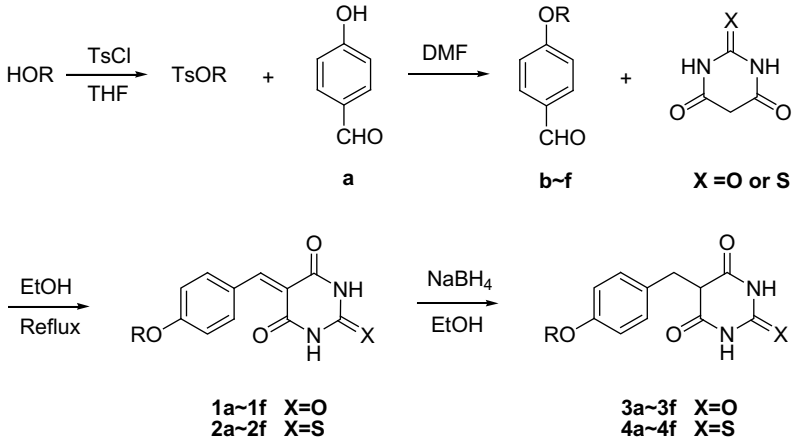


Fig. 1. Effect of compound **1a** on the diphenolase activity of mushroom tyrosinase for the catalysis of L-DOPA at 25 °C.

barbituric acid and thiobarbituric acid almost showed no activities at concentration of 200 μM, and 4-hydroxybenzaldehyde exhibited weak inhibitory activities with IC_{50} value of 1.22 mM. As predicted, most of the 5-benzylidene barbiturate and thiobarbiturate derivatives displayed more potent tyrosinase inhibitory activities than parent compounds barbituric acid, thiobarbituric acid and 4-hydroxybenzylaldehyde.

As a whole, 5-benzylidene thiobarbiturates exhibited more potent inhibitory effects than 5-benzylidene barbiturates. Compounds **1c**, **1e** and **1f** showed inhibitory activities with IC_{50} values of 75.42, 137.29 and >200 μM, respectively, while their thiobarbiturate analogues **2c**, **2e** and **2f** displayed inhibitory activities with IC_{50} values of 28.43, 34.20 and 77.80 μM, respectively. Similarly, compounds **3a**–**e** had no inhibitory activities at the concentration of 200 μM, whereas their thiobarbiturate analogues **4a**–**e** demonstrated potent inhibitory activities with IC_{50} values in the range of 70.02–179.31 μM, respectively. These results suggested that thiobarbiturate moiety was more favorable than barbiturate moiety for their inhibitory effects on the diphenolase activity of mushroom tyrosinase.

Among all the investigated compounds, compounds **1a** and **2a** bearing a hydroxyl group at position-4 of phenyl ring were found to



a R= H; **b** R= CH_2CH_2OH; **c** R= $CH_2CH_2OCH_3$; **d** R= $CH_2CH_2OC_4H_9$
e R= $CH_2CH_2OCH_2CH_2OCH_3$; **f** R= $(CH_2)_4OCH_3$

Scheme 1. Synthesis of 5-benzylidene barbiturate and thiobarbiturate derivatives.

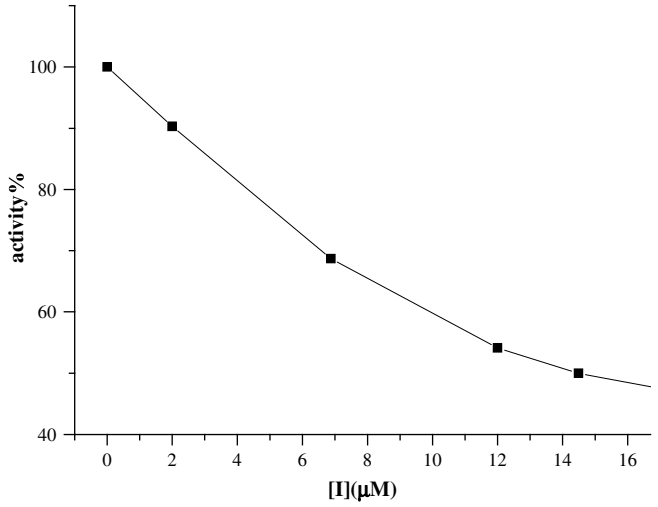


Fig. 2. Effect of compound **2a** on the diphenolase activity of mushroom tyrosinase for the catalysis of L-DOPA at 25 °C.

be the most potent inhibitors with IC_{50} values of 13.98 μM and 14.49 μM, respectively. Replacement of the hydroxyl of compounds **1a** and **2a** at position-4 of phenyl ring with 2-hydroxyethoxy (compounds **1b** and **2b**), 2-methoxyethoxy (**1c** and **2c**), 2-butoxyethoxy (**1d** and **2d**), 2-(2-methoxyethoxy) ethoxy (**1e** and **2e**) and 4-methoxybutoxy (**1f** and **2f**), respectively, led to a dramatic decline in inhibitory activities, indicating that the increase of the length of alkoxyl chain at position-4 of phenyl ring might cause stereo-hindrance for inhibitors approaching the active site, and the hydroxyl group was more favorable.

Unlike compounds **1a–f** and 2**a–f** having an arylidene substituent at position-5, their reduced congeners **3a–f** and **4a–f** (except **4b**), bearing a benzyl substituent at position-5, exhibited comparatively weaker tyrosinase inhibitory activities. This might be due to the breaking of the conjugation of the barbituric moiety to the aromatic phenyl ring, implying that the increase of molecular flexibility might be detrimental to their inhibitory activities.

3.2. Inhibitory mechanism of compounds *1a* and *2a* on mushroom tyrosinase

The inhibition mechanism of the most potent compounds **1a** and **2a** on mushroom tyrosinase for the oxidation of L-DOPA was first determined. Figs. 3 and 4 showed the relationship of enzyme activity with its concentration in the presence of different concentrations of compounds **1a** and **2a**. The results displayed that the plots of the remaining tyrosinase activity versus the concentrations of tyrosinase gave a family of parallel lines with the same slopes. These results demonstrated that the inhibition of compounds **1a** and **2a** on the tyrosinase enzyme were irreversible, suggesting that 5-benzylidene (thio)barbiturate derivatives might exhibit such tyrosinase inhibitory effects by the mode of making the enzyme inactivity forever.

3.3. The influence of compounds *1a* and *2a* on the secondary structure of mushroom tyrosinase

The influence of 5-benzylidene barbiturate and thiobarbiturate derivatives **1a** and **2a** on the secondary structure of mushroom tyrosinase was investigated by circular dichroism spectroscopy [24,25]. The results are summarized in Table 2. As shown in Table 2, the α-helix content of tyrosinase was 59.7% and the β-sheet was not

Table 1
Inhibitory effects on mushroom tyrosinase and antibiotic activities of 5-arylidene barbiturate (thiobarbiturate) derivatives.

Compound	X	R	Tyrosinase		MIC (μg/mL)
			IC_{50}[c] (μM)	Inhibition rate (%) at 200 μM	*S. aureus*
1a	O	H	13.98	100	>25
1b	O	CH_2CH_2OH	>200	22.41	>25
1c	O	$CH_2CH_2OCH_3$	75.42	100	>25
1d	O	$CH_2CH_2OCH_2CH_2CH_2CH_3$	45.45	100	>25
1e	O	$CH_2CH_2OCH_2CH_2OCH_3$	137.29	88.67	>25
1f	O	$CH_2CH_2CH_2CH_2OCH_3$	>200	12.20	>25
2a	S	H	14.49	100	3.1
2b	S	CH_2CH_2OH	>200	16.54	>25
2c	S	$CH_2CH_2OCH_3$	28.43	100	>12.5
2d	S	$CH_2CH_2OCH_2CH_2CH_2CH_3$	107.43	85.88	3.1
2e	S	$CH_2CH_2OCH_2CH_2OCH_3$	34.20	100	>12.5
2f	S	$CH_2CH_2CH_2CH_2OCH_3$	77.80	100	>25
3a	O	H	>200	47.50	>12.5
3b	O	CH_2CH_2OH	>200	5.27	>12.5
3c	O	$CH_2CH_2OCH_3$	>200	23.12	>12.5
3d	O	$CH_2CH_2OCH_2CH_2CH_2CH_3$	>200	Ni[d]	>12.5
3e	O	$CH_2CH_2OCH_2CH_2OCH_3$	>200	14.30	>12.5
3f	O	$CH_2CH_2CH_2CH_2OCH_3$	>200	1.15	>12.5
4a	S	H	70.02	100	>25
4b	S	CH_2CH_2OH	155.93	95.86	>25
4c	S	$CH_2CH_2OCH_3$	112.76	100	>12.5
4d	S	$CH_2CH_2OCH_2CH_2CH_2CH_3$	179.31	78.67	6.25
4e	S	$CH_2CH_2OCH_2CH_2OCH_3$	132.46	97.49	>12.5
4f	S	$CH_2CH_2CH_2CH_2OCH_3$	>200	9.85	>12.5
Barbituric acid			>200	5.95	–
Thiobarbituric acid			>200	8.21	–
4-Hydroxybenzaldehyde			1220[a]	16.40	–
4-Methoxycinnamic acid			410[b]	46.62	–
Levofloxacin					0.4

[a] Value in the literature [22] is 1.2 mM.
[b] Value in the literature [23] is 420 μM.
[c] Assay performed using mushroom tyrosinase. Values are means of three different experiments.
[d] Not active against tyrosinase.

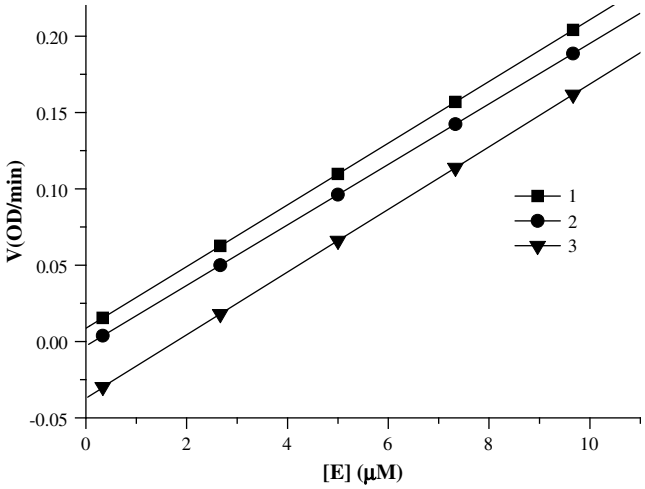


Fig. 3. The effect of concentrations of tyrosinase on its activity for the catalysis of L-DOPA at different concentration of compound **1a**. The concentrations of compound **1a** for curves 1–3 are 0, 0.5 and 2.0 μM, respectively.

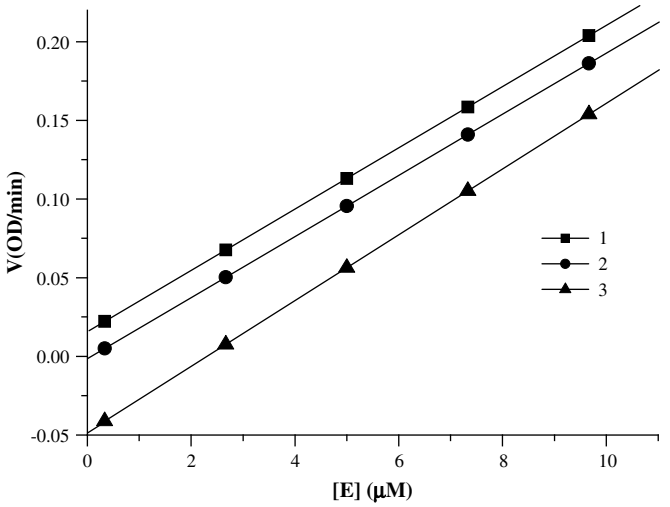


Fig. 4. The effect of concentrations of tyrosinase on its activity for the catalysis of L-DOPA at different concentration of compound **2a**. The concentrations of compound **2a** for curves 1–3 are 0, 0.5 and 2.0 μM, respectively.

found in the absence of inhibitor. While the β-sheet was observed in the presence of inhibitors without obvious regularity. On the other hand, it was observed that the content of rigidity structure (α-helix + β-sheet) of tyrosinase was decreased while that of loop structure (β-turn + random) of tyrosinase was increased accompanying the increasing of concentration of compound **1a** and **2a** from 0 to 150 μM. These results suggested that the enhanced flexibility of enzyme completely destructed the hydrogen bonds of the protein structure and produced irreversible damage on the structure of tyrosinase leading to the enzyme inactivation. Interestingly, the content of rigidity structure (α-helix + β-sheet) of tyrosinase was dramatically increased while the content of loop structure (β-turn + random) of tyrosinase was significantly decreased accompanying the increasing of concentration of compound **1a** from 150 to 200 μM. These results implied that the enhanced rigidity of enzyme might increase the ratio of misfolded tyrosinase and further prevent the formation of natural conformation of tyrosinase.

3.4. Antibacterial activities in vitro

In the present study, antibiotic activities of 5-benzylidene (2-thio)barbiturate derivatives were screened on *S. aureus*, *S. epidermidis*, *S. albus*, *B. cereus*, *E. coli* and *P. aeruginosa in vitro* by broth diffusion method [26]. All the synthesized compounds showed no antibacterial activities *in vitro* against *S. epidermidis*, *S. albus*, *B. cereus*, *E. coli* and *P. aeruginosa* at the concentration of 1 mg/mL (data not shown), whereas some thiobarbiturates exhibited potent antibacterial activities against *S. aureus*. The results are summarized in Table 1.

Previous report described that barbiturate and thiobarbiturate derivatives displayed potent antibacterial activity against *E. coli* [10]. Unfortunately, our synthesized 5-benzylidene (2-thio)-barbiturate derivatives were inactive against *E. coli* at the concentration of 1 mg/mL. Interestingly, thiobarbiturates **2a**, **2d** and **4d** exhibited selective antibacterial activities against *S. aureus* with the minimum inhibitory concentration (MIC) value of 3.1, 3.1 and 6.25 μg/mL, respectively, whereas their corresponding barbiturate derivatives **1a**, **1d** and **3d** had no antibacterial activities at the concentration of 25, 25 and 12.5 μg/mL, respectively. These results suggested that sulfur atom was more favorable than oxygen atom for their antibacterial activities. Moreover, compounds **2a** and **2d** were found to be the most potent antibacterial agents against Gram-positive *S. aureus* with the MIC value of 3.1 μg/mL, suggesting that these molecules might be served as lead compounds for further designing and developing more potent compounds.

4. Conclusion

In the present investigation, for the first time, we synthesized a series of novel 5-benzylidene barbiturate and thiobarbiturate derivatives and determined their inhibitory effects on the diphenolase activity of mushroom tyrosinase and their antibacterial activities against Gram-positive and Gram-negative bacteria. The results showed that most of compounds had potential tyrosinase inhibitory activities. Particularly, compounds **1a** and **2a** were found to be the most potent inhibitors with IC_{50} value of 13.98 μM and 14.49 μM, respectively. Preliminary SARs analysis suggested that: (1) the inhibitory effects of 5-benzylidene thiobarbiturates on tyrosinase were more potent than 5-benzylidene barbiturates; (2) the increase of molecular flexibility might be detrimental to their inhibitory activities; (3) the increase of the length of alkyl chain at hydroxyl group located in position-4 might cause stereo-hindrance for the inhibitors approaching the active site. The inhibition mechanism study revealed that these compounds were irreversible tyrosinase inhibitors. Circular dichroism spectra indicated that these compounds induced conformational changes of mushroom tyrosinase upon binding. Interestingly, thiobarbiturates **2a**, **2d** and **4d** exhibited potent and selective antibacterial effects on Gram-positive bacteria *S. aureus*. These results suggested that further development of such compounds may be of interest.

5. Experimental protocols

5.1. Reagents and general procedures

Melting points were determined on a WRS-1B digital instrument without correction. NMR spectra were recorded on a Varian Mercury-Plus 300 spectrometers in $CDCl_3$ or DMSO-d_6 at 25 °C. All chemical shifts (δ) are quoted in parts per million downfield from TMS and coupling constants (J) are given in Hertz. Mass spectra were obtained from VG ZAB-HS, LCMS-2010A or LCQ DECA XP spectrometer. Elemental analyses were performed on a Vario EI instrument and were with in ±0.4% of the theoretical values.

Table 2
The influence of compounds **1a** and **2a** on the secondary structure of tyrosinase.

Compound	Concentration (μM)	α-Helix (%)	β-Sheet (%)	α-Helix + β-sheet (%)	β-Turn (%)	Random (%)	β-Turn + random (%)
1a	0	59.7	0.0	59.7	16.2	24.1	40.3
	50	37.7	8.9	46.6	20.2	33.3	53.5
	100	42.3	3.4	45.7	21.4	32.8	54.2
	150	36.2	7.2	43.4	19.4	37.2	56.6
	200	83.3	0.0	83.0	0.0	16.7	16.7
2a	50	48.7	0.0	48.7	20.3	31.1	51.4
	100	39.3	5.2	44.5	22.4	33.0	55.4
	150	45.1	0.0	45.1	20.5	34.5	55.0
	200	79.4	0.0	79.4	0.0	20.6	20.6

Infrared (IR) spectra were measured on VECTOR 22 spectrometer using a potassium bromide (KBr) disk, scanning from 400 to 4000 cm^{-1}. All reactions were monitored by TLC (Merck Kieselgel 60 F_{254}) and spots were visualized with UV light or iodine. All commercially available reagents and solvents were used without further purification. Mushroom tyrosinase (specific activity of the enzyme is 6680 U/mg) and L-DOPA (L-3,4-dihydroxyphenylalanine) were purchased from Sigma Chemical Co.

5.2. General procedures for the synthesis of 5-benzylidene barbiturate and thiobarbiturate derivatives

5.2.1. 4-(2-Hydroxyethoxy)benzaldehyde (*b*)

A solution of 2-chloroethanol (0.03 mol) in 10 mL of *n*-BuOH was added over a period of 1 h to a boiling solution of 4-hydroxybenzaldehyde (0.01 mol) and NaOH (0.011 mol) in a mixture of 100 mL of *n*-BuOH and 15 mL of water. The reaction mixture was refluxed for an additional 12 h, the solvent was evaporated in vacuo, and the residue was purified by silica gel column chromatography (PE/EtOAc 2:1) to afford compound **b** (1.35 g, 81%) as yellow liquid. IR (KBr, cm^{-1}) ν 3370, 1684, 1600, 1509, 1309, 1164, 1112, 1034, 839; ^1H NMR ($CDCl_3$, 300 MHz) δ 9.89 (s, 1H, CHO), 7.79 (d, $J = 8.3$ Hz, 2H, ArH), 7.32 (d, $J = 8.4$ Hz, 2H, ArH), 4.75 (t, 2H, CH_2), 4.42 (t, 2H, CH_2); ESI-MS *m*/*z* (%): 167 (68) $(M + 1)^+$.

5.2.2. General procedure for the synthesis of *c*–*f*

The various alcohols (3 mmol) were dissolved in 15 mL of THF, and a solution of NaOH (360 mg, 9 mmol) in H_2O (10 mL) was added. The mixture was cooled to 0 °C, and then a solution of 4-toluenesulfonyl chloride (1.2 g, 6.3 mmol) in THF (15 mL) was added dropwise for a period of 2 h. The mixture was stirred for 5 h at 20 °C. The white precipitate, which was formed in organic layer, was dissolved by adding 20 mL of 2 M NaOH solution. The organic phase was separated, and the residue was extracted with CH_2Cl_2 (30 mL × 2). The combined organic phase was washed with water (150 mL × 3), and dried over Na_2SO_4. The solvent was removed, and the residue was purified on silica column to afford the mono-tosylate product.

Solid 4-hydroxybenzaldehyde (1.22 g, 10 mmol) was added to a mixture of K_2CO_3 (4.14 g, 30 mmol) in dry DMF (20 mL) at 80 °C. The mixture was stirred at this temperature for 1 h before addition of the mono-tosylate product. The reaction mixture was refluxed for 16 h and cooled to room temperature, then pulled into 100 mL cool water, and extracted with CH_2Cl_2 (30 mL × 3). The organic phase was washed generously with water to remove DMF and dried over $MgSO_4$. The solvent was removed and the crude product was purified on a silica column using petroleum ether/ethyl acetate.

5.2.3. 4-(2-Methoxyethoxy)benzaldehyde (*c*)

Compound **c** was obtained as yellow liquid (1.47 g, 82%). IR (KBr, cm^{-1}) ν: 2925, 1690, 1600, 1508, 1258, 1159, 1124, 833; ^1H NMR ($CDCl_3$, 300 MHz) δ: 9.90 (s, 1H, CHO), 7.79 (d, $J = 8.4$ Hz, 2H, ArH), 7.02 (d, $J = 8.4$ Hz, 2H, ArH), 4.19 (t, $J = 5.1$ Hz, 2H, CH_2), 3.79 (t, $J = 4.5$ Hz, 2H, CH_2), 3.47 (s, 3H, CH_3); ESI-MS *m*/*z* (%): 180 (50) (M^+).

5.2.4. 4-(2-Butoxyethoxy)benzaldehyde (*d*)

Compound **d** was obtained as yellow liquid (1.64 g, 74%). IR (KBr, cm^{-1}) ν: 2958, 1693, 1601, 1509, 1259, 1161, 1126, 833; ^1H NMR ($CDCl_3$, 300 MHz) δ: 9.88 (s, 1H, CHO), 7.82 (d, $J = 8.3$ Hz, 2H, ArH), 7.02 (d, $J = 8.4$ Hz, 2H, ArH), 4.20 (t, 2H, CH_2), 3.80 (t, 2H, CH_2), 3.54 (t, 2H, CH_2), 1.63–1.55 (m, 2H, CH_2), 1.43–1.32 (m, 2H, CH_2), 0.93 (t, 3H, CH_3); ESI-MS *m*/*z* (%): 222 (53) (M^+).

5.2.5. 4-(2-(2-Methoxyethoxy)ethoxy)benzaldehyde (*e*)

Compound **e** was obtained as yellow solid (1.63 g, 73%). Mp 44–45 °C; IR (KBr, cm^{-1}) ν: 2891, 1689, 1601, 1509, 1129, 1104, 1034, 836; ^1H NMR ($CDCl_3$, 300 MHz) δ: 9.89 (s, 1H, CHO), 7.81 (d, $J = 8.7$ Hz, 2H, ArH), 7.02 (d, $J = 8.6$ Hz, 2H, ArH), 4.23 (t, $J = 5.0$ Hz, 2H, CH_2), 3.90 (t, $J = 4.7$ Hz, 2H, CH_2), 3.73 (dd, $J = 2.0$ Hz, 4.8 Hz, 2H, CH_2), 3.59 (dd, 2H, $J = 2.0$ Hz, 4.8 Hz, CH_2), 3.34 (s, 3H, CH_3); ESI-MS *m*/*z* (%): 225 (82) $(M + 1)^+$.

5.2.6. 4-(4-Methoxybutoxy)benzaldehyde (*f*)

Compound **f** was obtained as yellow liquid (1.44 g, 69%). IR (KBr, cm^{-1}) ν: 2950, 1691, 1600, 1509, 1253, 1160, 1125, 834; ^1H NMR ($CDCl_3$, 300 MHz) δ: 9.90 (s, 1H, CHO), 7.76 (d, $J = 8.4$ Hz, 2H, ArH), 7.01 (d, $J = 8.4$ Hz, 2H, ArH), 4.15 (t, $J = 4.5$ Hz, 2H, CH_2), 3.76 (t, $J = 6.3$ Hz, CH_2, 2H,), 3.37 (s, 3H, OCH_3), 1.75–1.72 (m, 2H, CH_2), 1.66–1.64 (m, 2H, CH_2); ESI-MS *m*/*z* (%): 209 (35) $(M + 1)^+$.

5.2.7. General procedure for the synthesis of *1a*–*f* and *2a*–*f*

To a solution of barbituric acid or thiobarbituric acid (1 mmol) in hot ethanol (20 mL), aromatic aldehyde (1 mmol) was added. The reaction mixture was heated 2 h at 80 °C and cooled to room temperature. The precipitated solid was filtered, washed with ethanol and dried under vacuum. The crude product was recrystallized from *N*,*N*-dimethylformamide and water.

5.2.8. 5-(4-Hydroxybenzylidene)pyrimidine-2,4,6(1H,3H,5H)-trione (*1a*)

Compound **1a** was obtained as yellow powder (0.20 g, 87%), mp >250 °C (mp: 299–301, lit. [18]); IR (KBr, cm^{-1}) ν: 3479, 3124, 3049, 2907, 1708, 1563, 1490, 1318, 1205, 897; ^1H NMR (DMSO-d_6, 300 MHz) δ: 11.21 (s, 1H, NH), 11.09 (s, 1H, NH), 10.77 (s, 1H, OH), 8.32 (d, $J = 8.7$ Hz, 2H, ArH), 8.18 (s, 1H, CH), 6.87 (d, $J = 8.7$ Hz, 2H, ArH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 163.89, 162.43, 158.67, 156.05, 151.66, 137.42, 133.24 (2C), 119.49, 115.52 (2C); FAB-MS *m*/*z* (%): 233 (100) $(M + 1)^+$; Anal. Calcd for $C_{11}H_8N_2O_4$: C, 56.90; H, 3.47; N, 12.06. Found: C, 56.77; H, 3.53; N, 11.95.

5.2.9. 5-(4-(2-Hydroxyethoxy)benzylidene)pyrimidine-2,4,6(1H,3H,5H)-trione (*1b*)

Compound **1b** was obtained as a yellow powder (0.22 g, 79%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3485, 3171, 3056, 2841, 1689, 1546,

1410, 1245, 1174, 1060, 908, 529; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 11.26 (s, 1H, NH), 11.13 (s, 1H, NH), 8.33 (d, J = 8.7 Hz, 2H, ArH), 7.92 (s, 1H, CH), 7.04 (d, J = 8.8 Hz, 2H, ArH), 4.13–4.10 (m, 2H, CH2), 3.75–3.72 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 164.41, 162.50, 160.13, 156.01, 152.76, 133.66, 129.96 (2C), 120.84, 115.58 (2C), 70.81, 59.96; EI-MS m/z (%): 276 (45) (M^+), 231 (23), 215 (38), 188 (42), 145 (59), 118 (100), 89 (71), 45 (64).

5.2.10. 5-(4-(2-Methoxyethoxy)benzylidene)pyrimidine-2,4,6(1H,3H,5H)-trione (**1c**)

Compound **1c** was obtained as yellow powder (0.27 g, 92%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3195, 3055, 2948, 2846, 1735, 1671, 1603, 1439, 1395, 1268, 1186, 1113, 1037, 839, 509; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 11.27 (s, 1H, NH), 11.14 (s, 1H, NH), 8.33 (d, J = 8.8 Hz, 2H, ArH), 8.22 (s, 1H, CH), 7.04 (d, J = 8.0 Hz, 2H, ArH), 4.12 (t, J = 4.2 Hz, 2H, CH_2), 3.69 (t, J = 4.5 Hz, 2H, CH_2), 3.31 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 163.87, 162.76, 160.09, 155.98, 152.85, 134.16, 128.72 (2C), 120.28, 114.57 (2C), 71.81, 68.32, 58.26; FAB-MS m/z (%): 291 (85) $(M+1)^+$; Anal. Calcd for $C_{14}H_{14}N_2O_5$: C, 57.93; H, 4.86; N, 9.65. Found: C, 57.51; H, 5. 09; N, 8.92.

5.2.11. 5-(4-(2-Butoxyethoxy)benzylidene)pyrimidine-2,4,6(1H,3H,5H)-trione (**1d**)

Compound **1d** was obtained as yellow powder (0.30 g, 89%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3206, 3082, 2945, 2855, 1742, 1680, 1578, 1440, 1248, 1200, 1124, 815, 526; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 11.26 (s, 1H, NH), 11.13 (s, 1H, NH), 8.33 (d, J = 8.3 Hz, 2H, ArH), 8.22 (s, 1H, CH), 7.05 (d, J = 8.0 Hz, 2H, ArH), 4.23–4.20 (m, 2H, CH_2), 3.74–3.70 (m, 2H, CH_2), 3.45 (t, 2H, CH_2), 1.52–1.45 (m, 2H, CH_2), 1.36–1.28 (m, 2H, CH_2), 0.88 (t, J = 6.0 Hz, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 164.12, 162.44, 159.29, 155.93, 153.02, 132.31, 129.27 (2C), 118.92, 115.47 (2C), 70.56, 69.87, 68.32, 31.69, 19.25, 14.20; FAB-MS m/z (%): 333 (65) $(M+1)^+$; Anal. Calcd for $C_{17}H_{20}N_2O_5$: C, 61.44; H, 6.07; N, 8.43. Found: C, 59.99; H, 6.14; N, 8.76.

5.2.12. 5-(4-(2-(2- Methoxyethoxy)ethoxy)benzylidene)pyrimidine-2,4,6(1H,3H,5H)-trione (**1e**)

Compound **1e** was obtained as yellow powder (0.27 g, 80%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3201, 3075, 2943, 2862, 1684, 1551, 1418, 1257, 1184, 1037, 808, 513; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 11.27 (s, 1H, NH), 11.13 (s, 1H, NH), 8.34 (d, J = 8.7 Hz, 2H, ArH), 8.22 (s, 1H, CH), 7.03 (d, J = 8.7 Hz, 2H, ArH), 4.21 (t, J = 5.4 Hz, 2H, CH_2), 3.69 (t, J = 5.4 Hz, 2H, CH_2), 3.57–3.54 (m, 2H, CH_2), 3.45–3.42 (m, 2H, CH_2), 3.21 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 164.40, 162.58, 156.03, 153.74, 152.35, 131.08, 126.17 (2C), 120.84, 115.83 (2C), 71.35, 70.01, 68.82, 68.36, 58.28; FAB-MS m/z (%): 335 (28) $(M+1)^+$; Anal. Calcd for $C_{16}H_{18}N_2O_6$: C, 57.48; H, 5.43; N, 8.38. Found: C, 57.69; H, 5.35; N, 8.45.

5.2.13. 5-(4-(4- Methoxy)benzylidene)pyrimidine-2,4,6(1H,3H,5H)-trione (**1f**)

Compound **1f** was obtained as yellow powder (0.26 g, 82%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3072, 2927, 1649, 1518, 1409, 1258, 1199, 1170, 1110, 952, 857, 510; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 11.26 (s, 1H, NH), 11.13 (s, 1H, NH), 8.38 (d, J = 8.7 Hz, 2H, ArH), 8.24 (s, 1H, CH), 7.04 (d, J = 8.6 Hz, 2H, ArH), 4.12 (t, 2H, CH_2), 3.36–3.39 (m, 2H, CH_2), 3.23 (s, 3H, CH_3), 1.75–1.80 (m, 2H, CH_2), 1.62–1.67 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 163.98, 162.31, 160.29, 155.51, 153.17, 134.42, 125.77 (2C), 119.89, 115.11 (2C), 71.95, 68.54, 58.24, 26.01, 25.96; EI-MS m/z (%): 318 (25) (M^+), 231 (40), 188 (87), 87 (100), 45 (60).

5.2.14. 5-(4-Hydroxybenzylidene)-2-thioxo-dihydropyrimidine-4,6(1H,5H)-dione (**2a**)

Compound **2a** was obtained as yellow powder (0.24 g, 97%), mp >250 °C,(mp >300 °C, lit. [19]); IR (KBr, cm^{-1}) ν: 3485, 3118, 2973, 1644, 1561, 1430, 1208, 1205, 847; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.30 (s, 1H, NH), 12.20 (s, 1H, NH), 10.94 (s, 1H, OH), 8.37 (d, J = 8.7 Hz, 2H, ArH), 8.20 (s, 1H, CH), 6.89 (d, J = 8.7 Hz, 2H, ArH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.66, 162.23, 160.09, 156.79, 149.08, 132.22, 125.47 (2C), 118.77, 115.50 (2C); FAB-MS m/z (%): 249 (100) $(M+1)^+$; Anal. Calcd for $C_{11}H_8N_2O_3S$: C, 53.22; H, 3.25; N, 11.28. Found: C, 53.07; H, 3.33; N, 11.11.

5.2.15. 5-(4-(2-Hydroxyethoxy)benzylidene)-2-thioxo-dihydropyrimidine-4,6(1H,5H)-dione (**2b**)

Compound **2b** was obtained as yellow powder (0.21 g, 73%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3393, 3120, 2972, 1632, 1552, 1417, 1195, 1073, 830, 543; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.34 (s, 1H, NH), 12.25 (s, 1H, NH), 8.39 (d, J = 8.1 Hz, 2H, ArH), 8.24 (s, 1H, CH), 7.06 (d, J = 8.1 Hz, 2H, ArH), 4.13 (t, J = 2.7 Hz, 2H, CH_2), 3.74 (t, J = 3.0 Hz, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.71, 162.29, 160.13, 156.34, 154.44, 132.39, 125.39 (2C), 118.66, 114.42 (2C), 70.29, 59.32; EI-MS m/z (%): 292 (90) (M^+), 247 (100), 231 (85), 188 (30), 150 (45), 118 (25), 89 (60), 45 (73).

5.2.16. 5-(4-(2-Methoxyethoxy)benzylidene)-2-thioxo-dihydropyrimidine-4,6(1H,5H)-dione (**2c**)

Compound **2c** was obtained as yellow powder (0.27 g, 88%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3120, 2933, 1656, 1535, 1425, 1258, 1184, 815; 525; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.34 (s, 1H, NH), 12.25 (s, 1H, NH), 8.38 (d, J = 8.8 Hz, 2H, ArH), 8.24 (s, 1H, CH), 7.06 (d, J = 8.9 Hz, 2H, ArH), 4.12 (t, J = 4.2 Hz, 2H, CH_2), 3.38 (t, J = 3.9 Hz, 2H, CH_2), 3.23 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.57, 162.24, 160.09, 156.37, 154.71, 132.28, 125.48 (2C), 118.78, 115.41 (2C), 70.12, 68.10, 58.29; FAB-MS m/z (%): 307 (100) $(M+1)^+$.

5.2.17. 5-(4-(2-Butoxyethoxy)benzylidene)-2-thioxo-dihydropyrimidine-4,6(1H,5H)-dione (**2d**)

Compound **2d** was obtained as yellow powder (0.32 g, 93%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3122, 2933, 1674, 1531, 1445, 1395, 1281, 1187, 1052, 815; 523; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.29 (s, 1H, NH), 12.20 (s, 1H, NH), 8.33 (d, J = 8.9 Hz, 2H, ArH), 8.19 (s, 1H, CH), 7.02 (d, J = 8.9 Hz, 2H, ArH), 4.20–4.17 (m, 2H, CH_2), 3.68–3.65 (m, 2H, CH_2), 3.40 (t, 2H, CH_2), 1.47–1.40 (m, 2H, CH_2), 1.30–1.23 (m, 2H, CH_2); 0.83 (t, J = 5.8 Hz, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.74, 163.80, 162.65, 160.39, 156.48, 138.42, 125.77 (2C), 116.02, 115.01 (2C), 70.60, 69.80, 68.28, 31.67, 19.25, 14.18; FAB-MS m/z (%): 349 (70) $(M+1)^+$; Anal. Calcd for $C_{17}H_{20}N_2O_4S$: C, 58.60; H, 5.79; N, 8.04. Found: C, 58.33; H, 5.83; N, 8.20.

5.2.18. 5-(4-(2-(2- Methoxyethoxy)ethoxy)benzylidene)-2-thioxo-dihydropyrimidine-4,6(1H,5H)-dione (**2e**)

Compound **2e** was obtained as yellow powder (0.26 g, 75%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3201, 2933, 2892, 1680, 1529, 1418, 1257, 1184, 808, 515; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.29 (s, 1H, NH), 12.18 (s, 1H, NH), 8.37 (d, J = 8.7 Hz, 2H, ArH), 8.21 (s, 1H, CH), 7.03 (d, J = 8.7 Hz, 2H, ArH), 4.22 (t, J = 5.4 Hz, 2H, CH_2), 3.70 (t, J = 5.4 Hz, 2H, CH_2), 3.58–3.54 (m, 2H, CH_2), 3.47–3.42 (m, 2H, CH_2), 3.23 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.71, 162.25, 160.10, 157.34, 156.40, 132.33, 125.51 (2C), 118.92, 115.42 (2C), 71.31, 69.85, 68.72, 68.40, 58.08; FAB-MS m/z (%): 351 (67) $(M+1)^+$.

*5.2.19. 5-(4-(4-Methoxyethoxy)benzylidene)-2-thioxo-dihydropyrimidine-4,6(1H,5H)-dione (**2f**)*

Compound **2f** was obtained as yellow powder (0.27 g, 81%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3064, 2919, 1656, 1525, 1409, 1258, 1184, 844; 509; ^1H NMR (DMSO-d_6, 300 MHz) δ: 12.34 (s, 1H, NH), 12.25 (s, 1H, NH), 8.38 (d, $J = 8.8$ Hz, 2H, ArH), 8.24 (s, 1H, CH), 7.06 (d, $J = 8.9$ Hz, 2H, ArH), 4.15–4.10 (m, 2H, CH_2), 3.39–3.36 (m, 2H, CH_2), 3.23 (s, 3H, OCH_3), 1.80–1.73 (m, 2H, CH_2), 1.69–1.62 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.71, 163.95, 162.66, 160.38, 156.56, 138.50, 125.63 (2C), 115.85, 114.95 (2C), 71.93, 68.44, 58.28, 25.99, 25.86; FAB-MS m/z (%): 335 (55) $(M + 1)^+$; Anal. Calcd for $C_{16}H_{18}N_2O_4S$: C, 57.47; H, 5.43; N, 8.38. Found: C, 57.21; H, 5.63; N, 8.35.

*5.2.20. General procedure for the synthesis of **3a–f**, **4a–f***

5-Benzylidene barbituric acid or thiobarbituric acid (1 mmol) was dissolved in 20 mL anhydrous ethanol, and $NaBH_4$ (0.11 g, 3 mmol) was added three times. The mixture was stirred at room temperature for 2 h, and ethanol was removed under reduced pressure. To the residue was added 10 mL water and neutralized (pH 7) with 1 M HCl. The precipitated solid was filtered and the crude product was recrystallized from methanol.

*5.2.21. 5-(4-Hydroxybenzyl)pyrimidine-2,4,6(1H,3H,5H)-trione (**3a**)*

Compound **3a** was obtained as yellow powder (0.18 g, 77%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3387, 3120, 3055, 2917, 1708, 1653, 1489, 1205, 867; ^1H NMR (DMSO-d_6, 300 MHz) δ: 11.10 (s, 1H, NH), 11.09 (s, 1H, NH), 6.90 (d, $J = 8.4$ Hz, 2H, ArH), 6.77 (d, $J = 8.4$ Hz, 2H, ArH), 3.80 (t, $J = 6.0$ Hz, 1H, CH), 3.18 (t, $J = 6.0$ Hz, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 170.39 (2C), 157.95, 150.96, 130.42, 129.24 (2C), 115.62 (2C), 49.87, 33.38; FAB-MS m/z (%): 235 (25) $(M + 1)^+$.

*5.2.22. 5-(4-(2-Hydroxyethoxy)benzyl)pyrimidine-2,4,6(1H,3H,5H)-trione (**3b**)*

Compound **3b** was obtained as yellow powder (0.17 g, 61%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3392, 3112, 2970, 2801, 1704, 1635, 1510, 1387, 1296, 1239, 1076, 902, 811, 769, 538; ^1H NMR (DMSO-d_6, 300 MHz) δ: 11.12 (s, 2H, NH), 6.95 (d, $J = 7.8$ Hz, 2H, ArH), 6.79 (d, $J = 7.5$ Hz, 2H, ArH), 3.90 (t, $J = 3.9$ Hz, 2H, CH_2), 3.80 (t, $J = 6.0$ Hz, 1H, CH), 3.66 (t, $J = 3.9$ Hz, 2H, CH_2), 3.18 (t, $J = 6.0$ Hz, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 170.43 (2C), 158.15, 150.87, 131.22, 130.24 (2C), 115.11 (2C), 49.95, 33.51; EI-MS m/z (%): 278 (56) (M^+), 231 (30), 188 (85) 108 (100).

*5.2.23. 5-(4-(2-Methoxyethoxy)benzyl)pyrimidine-2,4,6(1H,3H,5H)-trione (**3c**)*

Compound **3c** was obtained as yellow powder (0.21 g, 71%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3192, 2938, 2846, 1744, 1615, 1580, 1387, 1296, 1186, 1076, 811, 749, 540; ^1H NMR (DMSO-d_6, 300 MHz) δ: 11.11 (s, 2H, NH), 6.97 (d, $J = 8.1$ Hz, 2H, ArH), 6.71 (d, $J = 8.1$ Hz, 2H, ArH), 4.03 (t, $J = 4.2$ Hz, 2H, CH_2), 3.81 (t, $J = 5.4$ Hz, 1H, CH), 3.66 (t, $J = 3.9$ Hz, 2H, CH_2), 3.31 (s, 3H, OCH_3), 3.16 (t, $J = 5.1$ Hz, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 171.04 (2C), 158.18, 150.67, 133.61, 130.45 (2C), 115.32 (2C), 71.68, 68.97, 58.30; FAB-MS m/z (%): 293 (100) $(M + 1)^+$.

*5.2.24. 5-(4-(2-Butoxyethoxy)benzyl)pyrimidine-2,4,6(1H,3H,5H)-trione (**3d**)*

Compound **3d** was obtained as yellow powder (0.29 g, 88%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3239, 2928, 2839, 1758, 1573, 1515, 1394, 1301, 1249, 1177, 1125, 1037, 838, 695, 546; ^1H NMR (DMSO-d_6, 300 MHz) δ: 11.13 (s, 2H, NH), 6.96 (d, $J = 8.1$ Hz, 2H, ArH), 6.80 (d, $J = 8.1$ Hz, 2H, ArH), 4.00 (t, $J = 3.9$ Hz, 2H, CH_2), 3.81 (t, $J = 3.9$ Hz, 1H, CH), 3.65 (t, $J = 3.9$ Hz, 2H, CH_2), 3.42 (t, $J = 6.6$ Hz, 2H, CH_2), 3.18 (t, $J = 3.6$ Hz, 2H, CH_2), 1.53–1.43 (m, 2H, CH_2), 1.35–1.27 (m, 2H, CH_2), 0.87 (t, $J = 7.5$ Hz, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 170.53 (2C), 156.94, 150.42, 132.61, 129.87 (2C), 115.24 (2C), 70.86, 70.17, 68.52, 48.97, 33.45, 31.71, 19.30, 14.19; EI-MS m/z (%): 334 (45) (M^+), 87 (100); Anal. Calcd for $C_{17}H_{22}N_2O_5$: C, 61.07; H, 6.63; N, 8.38. Found: C, 60.98; H, 6.29; N, 8.19.

*5.2.25. 5-(4-(2-(2-Methoxyethoxy)ethoxy)benzyl)pyrimidine-2,4,6(1H,3H,5H)trione (**3e**)*

Compound **3e** was obtained as yellow powder (0.28 g, 82%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3247, 2925, 1756, 1572, 1514, 1392, 1301, 1248, 1178, 1111, 1035, 842, 753, 694, 544; ^1H NMR (DMSO-d_6, 300 MHz) δ: 11.13 (s, 2H, NH), 6.96 (d, $J = 8.1$ Hz, 2H, ArH), 6.80 (d, $J = 8.1$ Hz, 2H, ArH), 4.01 (t, $J = 4.5$ Hz, 2H, CH_2), 3.81 (t, $J = 6.0$ Hz, 1H, CH), 3.69 (t, 2H, CH_2), 3.57–3.54 (m, 2H, CH_2), 3.45–3.42 (m, 2H, CH_2), 3.23 (s, 3H, CH_3), 3.18 (d, $J = 5.7$ Hz, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 170.40 (2C), 158.74, 150.30, 133.58, 130.87 (2C), 115.18 (2C), 71.75, 70.17, 69.91, 68.79, 58.27, 50.31, 33.57; FAB-MS m/z (%): 336 (100) (M^+).

*5.2.26. 5-(4-(4-Methoxyethoxy)benzyl)pyrimidine-2,4,6(1H,3H,5H)-trione (**3f**)*

Compound **3f** was obtained as white powder (0.28 g, 88%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3240, 2926, 2864, 2650, 1756, 1572, 1514, 1395, 1301, 1177, 1124, 1036, 841, 694, 544; ^1H NMR (DMSO-d_6, 300 MHz) δ: 11.13 (s, 1H, NH), 6.97 (d, $J = 7.0$ Hz, 2H, ArH), 6.79 (d, $J = 6.8$ Hz, 2H, ArH), 3.90–3.80 (m, 3H, CH_2, CH), 3.35–3.31 (m, 2H, CH_2), 3.31–3.28 (m, 2H, CH_2), 3.21 (s, 3H, OCH_3), 1.70–1.61 (m, 4H, $2 \times CH_2$); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 170.47 (2C), 157.94, 150.97, 130.48, 129.29 (2C), 114.67 (2C), 72.01, 68.58, 58.24, 50.03, 33.36, 26.12, 26.05; EI-MS m/z (%): 320 (35) (M^+), 233 (54), 188 (67), 87 (100), 45 (80); Anal. Calcd for $C_{16}H_{20}N_2O_5$: C, 59.99; H, 6.29; N, 8.74. Found: C, 59.30; H, 6.28; N, 8.71.

*5.2.27. 5-(4-Hydroxybenzyl)-2-thioxo-dihydropyrimidine-4,6(1H,5H)-dione (**4a**)*

Compound **4a** was obtained as yellow powder (0.18 g, 71%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3327, 3119, 2983, 1634, 1469, 1205, 877; ^1H NMR (DMSO-d_6, 300 MHz) δ: 12.10 (s, 2H, NH), 7.11 (d, $J = 8.7$ Hz, 2H, ArH), 7.00 (d, $J = 8.7$ Hz, 2H, ArH), 3.89 (t, $J = 4.5$ Hz, 1H, CH), 3.23 (t, $J = 4.5$ Hz, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.15, 173.31 (2C), 157.85, 132.43, 129.24 (2C), 115.81 (2C), 49.51, 33.38; FAB-MS m/z (%): 251 (63) $(M + 1)^+$.

*5.2.28. 5-(4-(2-Hydroxyethoxy)benzyl)-2-thioxo-dihydropyrimidine-4,6(1H,5H)-dione (**4b**)*

Compound **4b** was obtained as pale powder (0.20 g, 69%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3303, 2972, 1628, 1579, 1450, 1237, 1136, 816; ^1H NMR (DMSO-d_6, 300 MHz) δ: 12.05 (s, 2H, NH), 7.10 (d, $J = 7.8$ Hz, 2H, ArH), 6.96 (d, $J = 7.8$ Hz, 2H, ArH), 4.01 (t, $J = 2.7$ Hz, 2H, CH_2), 3.91 (t, $J = 4.5$ Hz, 1H, CH), 3.74 (t, $J = 3.0$ Hz, 2H, CH_2), 3.31 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.68, 172.09 (2C), 156.84, 132.35, 129.19 (2C), 114.92 (2C), 70.21, 59.72, 49.23, 33.40; FAB-MS m/z (%): 294 (67) (M^+); Anal. Calcd for $C_{13}H_{14}N_2O_4S$: C, 53.05; H, 4.79; N, 9.52. Found: C, 53.29; H, 5.16; N, 9.05.

*5.2.29. 5-(4-(2-Methoxyethoxy)benzyl)-2-thioxo-dihydropyrimidine-4,6(1H,5H)-dione (**4c**)*

Compound **4c** was obtained as white powder (0.22 g, 72%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3220, 2943, 2865, 1565, 1447, 1328, 1231,

1095, 810; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.04 (s, 1H, NH), 7.08 (d, J = 8.7 Hz, 2H, ArH), 6.96 (d, J = 8.7 Hz, 2H, ArH), 3.90 (m, 3H, CH_2, CH), 3.36 (t, J = 3.9 Hz, 2H, CH_2), 3.25 (m, 2H, CH_2), 3.21 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.59, 171.98 (2C), 157.38, 132.30, 125.43 (2C), 115.35 (2C), 70.21, 68.53, 48.91, 33.42; FAB-MS m/z (%): 309 (89) $(M+1)^+$.

5.2.30. 5-(4-(2-Butoxyethoxy)benzyl)-2-thioxo-dihydropyrimidine-4,6(1H,5H)-dione (**4d**)

Compound **4d** was obtained as pale powder (0.26 g, 75%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3130, 2893, 2750, 1576, 1440, 1269, 1187, 1061, 820; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.00 (s, 2H, NH), 7.03 (d, J = 8.7 Hz, 2H, ArH), 6.93 (d, J = 8.7 Hz, 2H, ArH), 3.93–3.89 (m, 3H, CH_2, CH), 3.61–3.58 (m, 2H, CH_2), 3.37 (t, 2H, CH_2), 3.24 (d, J = 6.0 Hz, 2H, CH_2), 1.47–1.40 (m, 2H, CH_2), 1.30–1.23 (m, 2H, CH_2); 0.83 (t, J = 5.8 Hz, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178. 26, 172.35 (2C), 157.14, 132.58, 129.89 (2C), 115.27 (2C), 70.96, 70.07, 68.75, 48.97, 33.37, 31.71, 19.30, 14.19; FAB-MS m/z (%): 351 (100) $(M+1)^+$.

5.2.31. 5-(4-(2-(2-Methoxyethoxy)ethoxy)benzyl)-2-thioxo-dihydropyrimidine-4,6(1H,5H)-dione (**4e**)

Compound **4e** was obtained as white powder (0.24 g, 69%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3138, 2916, 2884, 1615, 1559, 1417, 1223, 1174, 809, 520; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.01 (s, 2H, NH), 6.97 (d, J = 7.8 Hz, 2H, ArH), 6.92 (d, J = 7.8 Hz, 2H, ArH), 4.02 (t, J = 5.1 Hz, 2H, CH_2), 3.88 (t, J = 6.3 Hz, 1H, CH), 3.67 (t, J = 5.1 Hz, 2H, CH_2), 3.55–3.51 (m, 2H, CH_2), 3.43–3.38 (m, 2H, CH_2), 3.23 (s, 3H, OCH_3), 3.18 (d, J = 6.0 Hz, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.30, 172.29 (2C), 157.37, 132.30, 125.55 (2C), 115.44 (2C), 71.12, 69.67, 68.32, 67.94, 58.08, 49.19, 33.33; FAB-MS m/z (%): 353 (78) $(M+1)^+$.

5.2.32. 5-(4-(4-Methoxybutoxy)benzyl)-2-thioxo-dihydropyrimidine-4,6(1H,5H)-dione (**4f**)

Compound **4f** was obtained as white powder (0.24 g, 73%), mp >250 °C; IR (KBr, cm^{-1}) ν: 3217, 2922, 2743, 1570, 1432, 1246, 1178, 1120, 1079, 1051, 1001, 801; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.03 (s, 1H, NH), 7.03 (d, J = 8.3 Hz, 2H, ArH), 7.02 (d, J = 8.3 Hz, 2H, ArH), 3.89 (t, J = 6.3 Hz, 3H, CH_2, CH), 3.50 (m, 2H, CH_2), 3.33 (t, J = 6.3 Hz, 2H, CH_2), 3.21 (s, 3H, OCH_3), 1.66–1.72 (m, 2H, CH_2), 1.57–1.65 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.11, 173.05 (2C), 156.76, 133.32, 125.60 (2C), 115.45 (2C), 71.99, 68.67, 58.27, 49.31, 33.38, 26.01, 25.96; EI-MS m/z (%): 336 (57) (M^+), 107 (88), 87 (100), 45 (60).

5.3. Tyrosinase assay

Tyrosinase inhibition assay was performed according to the method reported by Liu et al. [21] with slight modifications. Briefly, all the synthesized compounds were screened for the diphenolase inhibitory effects of mushroom tyrosinase using L-DOPA as substrate. The reaction media (1 mL) for activity assay contained 0.5 mM L-DOPA in 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8). The final concentration of mushroom tyrosinase was 0.5 mg/mL for the *o*-diphenolase activity. The reaction was carried out at 25 °C. 5-Arylidene barbituric (thiobarbituric) acids were first dissolved in DMSO. The final concentration of DMSO in the test solution was 2.0%. Controls, without inhibitor but containing 2.0% DMSO, were routinely carried out. The enzyme reaction was monitored by measuring the change in absorbance at 475 nm of formation of the DOPA chrome for 1 min. Dose–response curves were obtained by performing assays in the presence of increasing concentrations of inhibitors. IC_{50} value, a concentration giving 50% inhibition of tyrosinase activity, was determined by interpolation of the dose–response curves. 4-Hydroxybenzaldehyde and 4-methoxycinnamic acid were used as reference compounds. Moreover, the inhibition type of the selected compounds **1a** and **2a** were assayed by a Lineweaver–Burk plot.

Circular dichroism (CD) spectra were obtained at 25 °C using a Jasco J-810 spectropolarimeter. The quartz cells had path lengths of 0.2 cm for measurements in the far-UV region (190–250 nm). For the determination of the spectra, solutions of the enzymes (39 μg/mL) were equilibrated against a 50 mM phosphate buffer (pH 6.8) containing 20 μL different concentrations of samples. The samples were dissolved in 1.0 mL methanol, and methanol had a final concentration of 2%. Each spectrum was the average of six repetitive scans and was corrected by subtracting the average spectrum of the buffer and methanol.

5.4. In vitro antibacterial activity assays

All the synthesized compounds were tested for their antibacterial activity against Gram-positive (*S. aureus*, *B. cereus*, *S. epidermidis* and *S. albus*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacteria strains. Antibacterial assays were performed by the agar dilution method according to the Clinical and Laboratory Standard Institute (CLSI) (formerly National Committee for Clinical Laboratory Standards) [26]. Twofold serial dilutions of the compounds and reference drugs were prepared in Mueller–Hinton agar (MHA). The overnight cultures (after 16–18 h of incubation at 37 °C) of all the bacteria were used for the assay and adjusted to the turbidity of a 0.5 McFarland Standard. The stock solution (1 mg/mL) of all the test chemicals was prepared by dissolving 1 mg of the test chemical in 1 mL of dimethylsulfoxide (DMSO), and DMSO was used as control for all the test compounds. Twenty milliliters of MHA and 500 mL of each test bacterial culture of 16–18 h incubation adjusted at 0.5 McFarland were mixed and poured into sterilized and labeled plates. The wells of 6 mm diameter were punched in the solidified agar plates. Test chemicals of 100 mL were added to individual wells. The loaded plates were incubated at 37 °C for 24 h. The diameter of zone of growth inhibition around each well was measured after incubation using a Vernier Caliper. The MIC value is the lowest concentration of the antimicrobial agent that prevents the development of viable growth after 16–18 h incubation. MIC values (μg/mL) were determined on Mueller–Hinton (MH) agar with medium containing dilutions of antibacterial agents by the literature method [27]. Stock solution of 1 mg/mL was prepared in DMSO and was appropriately diluted to get final concentrations of 50, 25, 12.5, 6.25, 3.1, 1.5, 0.8, 0.4 and 0.2 μg/mL. Levofloxacin (purchased from Darui Chemical Co.) was used as positive control.

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