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Synthesis and binding properties of novel selective 5-HT_3 receptor ligands

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Abstract—This work reports on the synthesis and affinities for the 5-HT_3 versus the 5-HT_4 receptor of new piperazinyl-substituted thienopyrimidine derivatives **20**–**45** with a view to identify potent and selective ligands for the 5-HT_3 receptor. Some of the new compounds show good affinity for the 5-HT_3 receptor and, notably, do not display any affinity for the 5-HT_4 receptor. 4-(4-Methyl-1-piperazinyl)-2-methylthio-6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidine **31** exhibits the highest affinity for the 5-HT_3 receptor ($K_i = 33$ nM) and behaves as noncompetitive antagonist.

1. Introduction

The serotonin neurotransmitter is involved in a number of different physiological functions through its interaction with 14 types of receptors.[1] These are G-protein-coupled receptors, with the exception of the 5-HT_3, which is a ligand gated-ion channel receptor. 5-HT_3 receptor antagonists, such as ondansetron, granisetron, or tropisetron,[2] are used as antiemetic drugs to prevent vomiting associated with chemotherapy or radiation-induced emesis, but literature studies[3] indicate that they could possess numerous other potential therapeutic applications in the control of pain or in the treatment of psychosis, memory impairment, depression, anxiety, schizophrenia, and drugs abuse. On the other hand, little is known about the therapeutic potential of 5-HT_3 receptor agonists although some potent and selective ligands with full agonistic properties[4] were recently reported. It has been suggested that stimulation of the 5-HT_3 receptor modulates, in the central nervous system, the release of dopamine, cholecystokinin, and acetylcholine.[5] Moreover, 5-HT_3 receptors are involved in the peripheral control of acethylcholine release of the distal colon.[6]

In recent years, we have been engaged in the preparation of piperazinyl-substituted thieno[2,3-*d*]pyrimidin-4(3*H*)-one derivatives as 5-HT_3 receptor ligands.[7] These compounds possess the three key pharmacophoric elements (an aromatic moiety, a hydrogen-bond acceptor and a basic amino group) required for interaction with the 5-HT_3 receptor and are structurally related to quipazine (Fig. 1), a potent ligand for the 5-HT_3 receptor.[8] Among these thieno[2,3-*d*]pyrimidine derivatives, 3-amino-5,6-dimethyl-2-[4-(1-phenylmethyl)-1-piperazinyl]thieno[2,3-*d*]pyrimidin-4(3*H*)-one **A** (Fig. 1) exhibited the highest affinity and selectivity for the 5-HT_3 receptor (5-HT_3: $K_i = 3.92$ nM; 5-HT_4: not active), behaving as a full agonist in the Bezold–Jarisch reflex assay.[7] This result induced us to continue research in this field with the aim to obtain more potent and selective ligands for the 5-HT_3 receptor. The present work reports on the synthesis and 5-HT_3 receptor-binding properties of a series of new derivatives **B**[9,10] (Fig. 1), which can be regarded as structural analogues of compound **A**. The new molecules maintain the thieno[2,3-*d*]pyrimidine scaffold as in **A** and exhibit the following structural variations: (i) the piperazine moiety at 4-position of the pyrimidine

Keywords: 5-HT_3 receptor; Ligands; Thieno[2,3-*d*]pyrimidine derivatives.
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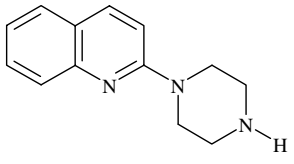
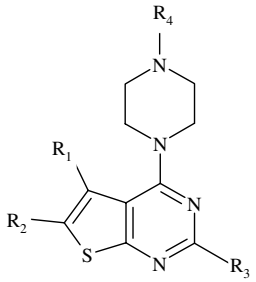
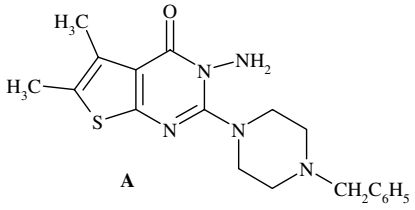


Quipazine



B



A

R_1 , R_2 = CH_3 , CH_3 ; -$(CH_2)_3$- ;
-$CH(COOC_2H_5)CH_2CH_2$- ;
-$CH(COOC_2H_5)CH_2CH_2CH_2$- .
R_3 = H , SCH_3 .
R_4 = $CH_2C_6H_5$, $C_6H_4OCH_3$(o) , C_6H_5 , pyrimidyl , CH_3 , H .

Figure 1.

nucleus in place of the carbonyl group; (ii) in some compounds, 5- and 6-positions of the bicyclic system bear methyl groups (as in **A**); in others, a trimethylene or tetramethylene chain forms a third condensed ring, which, in some cases, is substituted with an ethoxycarbonyl group; (iii) 2-position of the pyrimidine is unsubstituted or bears a methylthio group.

The groups on N-4 of the piperazine ring (e.g., benzyl, 2-methoxyphenyl, phenyl, 2-pyrimidyl, methyl, and hydrogen) were the same as in a previous work.[7] They were examined, together with the other structural modifications, with the aim to obtain more information on the structure–activity relationships in this new series.

2. Chemistry

Compounds **20**–**45** were prepared according to Scheme 1. Derivatives **3** and **4** were obtained by refluxing in acetone the corresponding β-amino esters of 4,5-disubstituted-thiophenes **1** and **2**[11] with benzoyl isothiocyanate, commercially available or prepared in situ. When refluxed in an ethanolic potassium hydroxide solution, **3** and **4** gave the respective monopotassium salts of the 5,6-disubstituted-2-thioxothieno[2,3-*d*]pyrimidinones **5** and **6**. On acidification of the potassium salts **5** and **6** with concentrated hydrochloric acid, the corresponding thioxo compounds **8** and **9** were obtained, which confirmed the structures of salts **5** and **6**. The 2-methylthio derivatives **10**–**12** were obtained by reaction of the potassium salts **5**–**7**[12] with methyl iodide in water at room temperature. The 4-chloro derivatives **14**–**18** were obtained by heating the corresponding 4-oxo derivatives **10**–**12** and **13**[13] with an excess of phosphorus oxychloride, according to a literature method.[14] The 4-(1-piperazinyl)thieno[2,3-*d*]pyrimidine derivatives **20**–**45** were synthesized by refluxing in ethanol the 4-chloro derivatives **14**–**17**, **18**,[15] and **19**[16] with piperazine or substituted piperazines. The proposed structures for **3**–**12**, **14**–**17**, and **20**–**45** were confirmed by elemental analyses, and by the IR and ^{1}H NMR data (see Experimental section).

3. Pharmacology

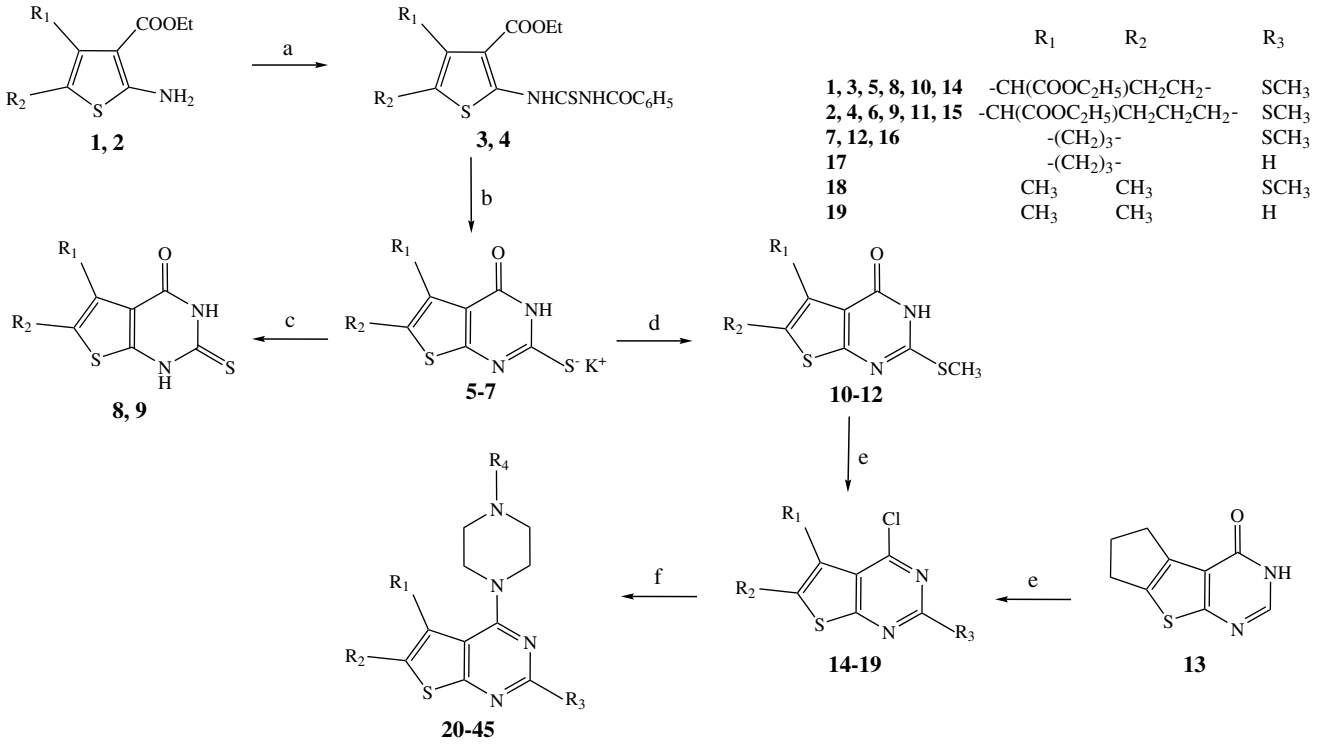
The title compounds **20**–**45** were tested in in vitro binding assays to evaluate their affinity for the 5-HT_3 and the 5-HT_4 receptors, using [^{3}H]LY 278584 or [^{3}H]GR113808 as radioligand, respectively. Binding data, reported in Table 1, are expressed as K_i values.

Moreover, in order to evaluate the putative agonistic or antagonistic properties of new molecules, two of them (**31** and **33**) were tested in an in vitro functional assay on isolated guinea pig distal colon.[17] Results are reported in Figure 2 and Table 2.

4. Results and discussion

The results of the binding tests, presented in Table 1, demonstrate that many of the title compounds (**20**, **24**–**27**, **31**–**33**, **38**, **39**, and **44**) possess good affinities for the 5-HT_3 receptor and high selectivity over the 5-HT_4 receptor. In fact, none of the new derivatives displays measurable affinity for the latter receptor.

In this new series of derivatives the presence of the third unsubstituted cyclopentane ring fused with the thieno[2,3-*d*]pyrimidine system leads to the compounds with the best affinities (**31** and **33**, $K_i = 33$ and 70 nM, respectively); the affinity is lower when two methyl groups or a condensed ethoxycarbonyl cyclopentane are present at 5- and 6-positions of the thieno[2,3-*d*]pyrimidine system (**20**, **24**, **26**, **38**, and **39**, $K_i = 723$, 216, 114, 202, and 249 nM, respectively).



Scheme 1. Reagents and conditions: (a) $SCNCOC_6H_5$, acetone, reflux; (b) KOH, EtOH, reflux; (c) HCl, H_2O, rt; (d) CH_3I, H_2O, rt; (e) $POCl_3$, 150 °C; (f) unsubstituted or substituted piperazine, EtOH, K_2CO_3, reflux.

The best substituents on N-4 of the piperazine ring for the affinity at the 5-HT_3 receptor are a hydrogen and a methyl group (**24**, **26**, **31**, **33**, **38**, and **39**, $K_i = 216$, 114, 33, 70, 202, and 249 nM, respectively), while the benzylpiperazine-substituted derivatives **20** and **27** display decreased affinities ($K_i = 723$ and 421 nM, respectively). The corresponding analogues **34** and **40**, which bear an ethoxycarbonyl group on a fused cyclopentane and a cyclohexane ring at positions R_1 and R_2 of the thiophene nucleus, do not exhibit any measurable affinity for this receptor.

The introduction on compounds **31** and **33** of an ethoxycarbonyl group on the cyclopentane ring, as in **38** and **39**, is detrimental for affinity ($K_i = 202$ and 249 nM, respectively).

When the cyclopentane ring in **38** and **39** is enlarged to a cyclohexane ring, as in **44** and **45**, a notable drop in affinity ($K_i = 1791$ nM and not active, respectively) is seen.

Substitution on N-4 of the piperazine ring with a 2-methoxyphenyl (**21**, **28**, **35**, and **41**), a phenyl (**22**, **29**, **36**, and **42**) or a 2-pyrimidyl nucleus (**23**, **30**, **37**, and **43**) leads to compounds without affinity for the 5-HT_3 receptor; this behavior was earlier reported for 3-amino-5,6-dimethylthieno[2,3-*d*]pyrimidin-4(3*H*)-one analogues[7] in which the piperazine nucleus was at 2-position of the pyrimidine system.

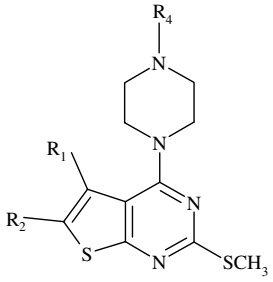
Removal of the methylthio group at 2-position of the pyrimidine nucleus in **24** and **31** leads to **25** and **32**, which display lower affinities ($K_i = 341$ and 221 nM, respectively). Thus, the presence of the methylthio group seems to be important for the affinity, but its mode of interaction with the binding site is not clear.

This new series of thieno[2,3-*d*]pyrimidine derivatives possesses the key elements of the three-component pharmacophoric model proposed for the interaction of 5-HT_3 ligands structurally related to quipazine with the 5-HT_3 receptor-binding site:[18] a charge-assisted hydrogen bond (N-4 of the piperazine nucleus), a hydrogen-bond interaction (the nitrogen of the pyrimidine system), and an aromatic interaction (the thiophene nucleus). The location of the piperazine nucleus in a different position in comparison with quipazine and our previous derivatives[7] could explain the lower affinities of these new compounds.

In this new series of derivatives, compounds with the best affinities are characterized by an unsubstituted piperazine or a methyl-substituted piperazine, a benzyl-substituted piperazine leading to compounds with lower affinity. This trend is peculiar for this series of derivatives because, in the quipazine-like ligands,[19] the introduction of a benzyl moiety leads to compounds with higher affinity for the 5-HT_3 receptor with respect to the unsubstituted piperazine derivatives.

The presence of two methyl groups at 5- and 6-positions of the thieno[2,3-*d*]pyrimidine system in this new series of derivatives furnishes compounds with good affinity, which is increased when a third cyclopentane ring is fused with the thieno[2,3-*d*]pyrimidine system. On

Table 1. Binding properties of compounds **20**–**45** on 5-HT_3 and 5-HT_4 serotonin receptors



Compd	R_1	R_2	R_4	K_i (nM) (±SD)	
				5-HT_3 [^{3}H]LY 278584 Rat cortex	5-HT_4 [^{3}H]GR 113808 Guinea pig striatum
20	CH_3	CH_3	$CH_2C_6H_5$	723 ± 109	N.A.[b]
21	CH_3	CH_3	$C_6H_4OCH_3$(o)	N.A.[b]	N.A.[b]
22	CH_3	CH_3	C_6H_5	N.A.[b]	N.A.[b]
23	CH_3	CH_3	2-Pyrimidyl	N.A.[b]	N.A.[b]
24	CH_3	CH_3	CH_3	216 ± 22	N.A.[b]
25[a]	CH_3	CH_3	CH_3	341 ± 45.74	N.A.[b]
26	CH_3	CH_3	H	114 ± 16	N.A.[b]
27	$-(CH_2)_3-$		$CH_2C_6H_5$	421 ± 102	N.A.[b]
28	$-(CH_2)_3-$		$C_6H_4OCH_3$(o)	N.A.[b]	N.A.[b]
29	$-(CH_2)_3-$		C_6H_5	N.A.[b]	N.A.[b]
30	$-(CH_2)_3-$		2-Pyrimidyl	N.A.[b]	N.A.[b]
31	$-(CH_2)_3-$		CH_3	33 ± 5	N.A.[b]
32[a]	$-(CH_2)_3-$		CH_3	221 ± 27.67	N.A.[b]
33	$-(CH_2)_3-$		H	70 ± 7	N.A.[b]
34	$-CH(COOC_2H_5)CH_2CH_2-$		$CH_2C_6H_5$	N.A.[b]	N.A.[b]
35	$-CH(COOC_2H_5)CH_2CH_2-$		$C_6H_4OCH_3$(o)	N.A.[b]	N.A.[b]
36	$-CH(COOC_2H_5)CH_2CH_2-$		C_6H_5	N.A.[b]	N.A.[b]
37	$-CH(COOC_2H_5)CH_2CH_2-$		2-Pyrimidyl	N.A.[b]	N.A.[b]
38	$-CH(COOC_2H_5)CH_2CH_2-$		CH_3	202 ± 32	N.A.[b]
39	$-CH(COOC_2H_5)CH_2CH_2-$		H	249 ± 82	N.A.[b]
40	$-CH(COOC_2H_5)CH_2CH_2CH_2-$		$CH_2C_6H_5$	N.A.[b]	N.A.[b]
41	$-CH(COOC_2H_5)CH_2CH_2CH_2-$		$C_6H_4OCH_3$(o)	N.A.[b]	N.A.[b]
42	$-CH(COOC_2H_5)CH_2CH_2CH_2-$		C_6H_5	N.A.[b]	N.A.[b]
43	$-CH(COOC_2H_5)CH_2CH_2CH_2-$		2-Pyrimidyl	N.A.[b]	N.A.[b]
44	$-CH(COOC_2H_5)CH_2CH_2CH_2-$		CH_3	1791 ± 187	N.A.[b]
45	$-CH(COOC_2H_5)CH_2CH_2CH_2-$		H	N.A.[b]	N.A.[b]
	Serotonin			354 ± 97	79 ± 10

[a] The methylthio group is replaced by a hydrogen.
[b] <50% inhibition at 10^{-5} M.

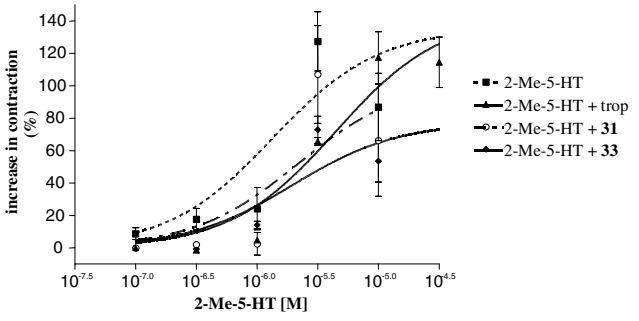


Figure 2. The effect of 10 nM of tropisetron (trop), compound **31** and **33** on the contraction increasing action of selective 5-HT_3 agonist 2-Me-5-HT in the isolated guinea pig colon in vitro. The effect of 2-Me-5-HT was expressed as the percent of contraction increase compared to basal colon activity ($n = 6$).

Table 2. Change of effective concentration 50 (EC_{50}) and maximum effect (E_{max}) in contraction increasing effect of 2-Me-5-HT in the presence of 10 nM of tropisetron, compound **31** and **33** in the guinea pig colon in vitro

	EC_{50} (M) ± SEM[a]	E_{max} (%) ± SEM[a]
2-Me-5-HT	$1.2\times10^{-6} \pm 1.6\times10^{-7}$	145.4 ± 22.0
2-Me-5-HT + trop	$4.7\times10^{-6} \pm 1.1\times10^{-6}$[b]	145.8 ± 11.3[c]
2-Me-5-HT + **31**	$1.4\times10^{-6} \pm 2.4\times10^{-7}$[c]	85.7 ± 32.3[d]
2-Me-5-HT + **33**	$2.0\times10^{-6} \pm 3.8\times10^{-7}$[c]	79.5 ± 17.0[d]

Level of significance is indicated next to the values compared to the 2-Me-5-HT values.
[a] Standard error mean.
[b] $p < 0.01$.
[c] Not significant.
[d] $p < 0.05$.

the other hand, the enlargement of the fused ring to six atoms as well as the introduction of an ethoxycarbonyl group are detrimental for affinity at 5-HT_3 receptor, leading to very poor ligands or inactive compounds.

Compounds **31** and **33**, which showed the highest affinities for the 5-HT_3 receptor, were also tested in vitro to evaluate their functional activities. Tropisetron (trop), a well-known 5-HT_3 antagonist, was used as reference compound. In the isolated guinea pig colon tropisetron (10 nM) shifted the dose response curve of the selective 5-HT_3 agonist 2-methyl-5-hydroxytryptamine (2-Me-5-HT) to the right, indicating its competitive antagonist properties (Fig. 2, Table 2). Compounds **31** and **33** (10 nM), however, did not cause any shift in the dose response curve, but elicited a decrease in maximum effect of 2-Me-5-HT, suggesting their character as noncompetitive antagonists. This result would suggest the possibility of an interaction of these compounds with a postulated modulatory binding site at the 5-HT_3 receptor.[20]

In conclusion, a new series of thieno[2,3-*d*]pyrimidine derivatives as 5-HT_3 receptor ligands has been prepared. Some compounds, namely **31** and **33**, showed good affinity for the 5-HT_3 receptor coupled to a complete lack of affinity for the 5-HT_4 receptor. Moreover, results of in vitro functional assays indicate that **31** and **33**, unlike tropisetron, could be reported as noncompetitive antagonists. Further studies to better clarify the pharmacological profile of **31** and **33** are in progress.

5. Experimental section

Melting points were determined in open capillary tubes on a Gallenkamp M.p. apparatus and are uncorrected. Elemental analyses for C, H, N, and S were performed on a Fisons-Carlo Erba EA1108 Elemental Analyzer and were within 0.4% of the theoretical values. The IR spectra were recorded in KBr disks on a Perkin Elmer 1600 Series FT-IR spectrometer. ^{1}H NMR spectra were recorded in DMSO-d_6 solution at 200 MHz on a Varian Inova-Unity 200 spectrometer; chemical shifts (δ) are reported in ppm, with TMS as internal standard; coupling constants (J) are in Hertz. Signal multiplicities are denoted by s (singlet), d (doublet), t (triplet), q (quartet), br s (broad singlet), or m (multiplet). The purity of compounds was checked by TLC on Merck silica gel 60 F-254 plates. All commercial chemicals were purchased from Aldrich, Fluka, Merck, and Carlo Erba and were used without further purification.

5.1. 2-[(Benzoylaminothioxomethyl)amino]-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3,4-dicarboxylic acid diethyl ester (3)

A mixture of NH_4NCS (0.64 g, 8.41 mmol) and benzoyl chloride (0.82 mL, 7.06 mmol) was refluxed in anhydrous acetone (10 mL) for 5 min. A solution of amino-ester **1** (2 g, 7.06 mmol) in anhydrous acetone (25 mL) was added to the suspension and the mixture was refluxed during stirring for 1 h. After cooling, the suspension was concentrated under reduced pressure, and the precipitate was collected, washed with water, dried, and recrystallized from ethanol. Yield: 1.25 g (40%); mp 184 °C; IR (KBr, cm^{-1}) 3261, 2971, 2942, 1712, 1681, 1564, 1534, 1334, 1219, 1185, 705. ^{1}H NMR (DMSO-d_6) δ 1.16 (t, $J = 7$ Hz, 3H), 1.26 (t, $J = 7$ Hz, 3H), 2.25–2.42 (m, 1H), 2.66–3.06 (m, 3H), 3.92–4.40 (m, 5H), 7.45–8.12 (m, 5H), 11.91 (s, 1H), 14.71 (s, 1H). Anal. $C_{21}H_{22}N_2O_5S_2$.

5.2. 2-[(Benzoylaminothioxomethyl)amino]-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3,4-dicarboxylic acid diethyl ester (4)

This was prepared from the amino ester **2** by the same procedure as for **3**, and was recrystallized from ethanol. Yield: 2.07 g (64%); mp 170–171 °C; IR (KBr cm^{-1}) 3248, 2927, 1710, 1683, 1526, 1436, 1330, 1225, 1184, 1028, 708. ^{1}H NMR (DMSO-d_6) δ 1.17 (t, $J = 7.2$ Hz, 3H), 1.28 (t, $J = 7.2$ Hz, 3H), 1.55–2.18 (m, 4H), 2.61–2.85 (m, 2H), 3.90–4.48 (m, 5H), 7.50–8.18 (m, 5H), 11.90 (s, 1H), 14.74 (s, 1H). Anal. $C_{22}H_{24}N_2O_5S_2$.

5.3. Monopotassium salt of ethyl 4-oxo-2-thioxo-3,5,6,7-tetrahydro-4*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidine-5-carboxylate (5) and its thioxo derivative (8)

Benzoyl derivative **3** (1 g, 2.24 mmol) was added to a solution of KOH (0.25 g, 4.45 mmol) in absolute ethanol (13 mL) and the mixture was refluxed during stirring for 3 h. The solid was then collected while hot, washed with hot absolute ethanol and dried to obtain salt **5** (0.60 g, 80%). Potassium salt **5** (0.50 g, 1.49 mmol) was suspended in water (50 mL), acidified with concentrated hydrochloric acid, and stirred for 10 min at room temperature. The solid was collected, washed with water, dried, and recrystallized from ethanol. Yield: 0.17 g (39%); mp 260 °C (dec); IR (KBr cm^{-1}) 3430, 3068, 2898, 1718, 1668, 1548, 1376, 1202, 1157, 1021, 849. ^{1}H NMR (DMSO-d_6) δ 1.15 (t, $J = 7.2$ Hz, 3H), 2.27–2.47 (m, 1H), 2.69–3.04 (m, 3H), 3.90–4.21 (m, 3H), 12.37 (s, 1H), 13.40 (br s, 1H). Anal. $C_{12}H_{12}N_2O_3S_2$.

5.4. Monopotassium salt of ethyl 4-oxo-2-thioxo-3,4,5,6,7,8-hexahydro[1]benzothieno[2,3-*d*]pyrimidine-5-carboxylate (6) and its thioxo derivative (9)

Benzoyl derivative **4** (3 g, 6.51 mmol) was added to a solution of KOH (0.77 g, 13.72 mmol) in absolute ethanol (30 mL) and the mixture was refluxed during stirring for 6 h. After cooling, a small amount of the solvent was removed from the suspension under reduced pressure, and the solid was then collected, washed with a small amount of absolute ethanol, and dried to give salt **6** (2.22 g, 98%). A suspension of salt **6** (1 g, 2.87 mmol) in water (50 mL) was acidified with concentrated hydrochloric acid and stirred for 1 h at room temperature. The solid was collected, washed with water, dried, and recrystallized from ethanol. Yield: 0.55 g (62%); mp 234–236 °C; IR (KBr cm^{-1}) 3442, 3150, 3052, 1694, 1665, 1546, 1473, 1291, 1199, 1030, 867. ^{1}H NMR

(DMSO-d_6) δ 1.16 (t, $J = 7$ Hz, 3H), 1.59–2.08 (m, 4H), 2.60–2.81 (m, 2H), 3.85–3.97 (m, 1H), 4.05 (q, $J = 7$ Hz, 2H), 12.34 (s, 1H), 13.36 (s, 1H). Anal. $C_{13}H_{14}N_2O_3S_2$.

5.5. Ethyl 2-methylthio-4-oxo-3,5,6,7-tetrahydro-4*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidine-5-carboxylate (10)

Methyl iodide (0.74 mL, 11.83 mmol) was added under stirring to a solution of monopotassium salt **5** (1.33 g, 3.98 mmol) in water (70 mL) and the mixture was stirred at room temperature for 1 h. The solid was then filtered off, washed with water, dried, and recrystallized from ethanol. Yield: 0.75 g (61%); mp 198–200 °C; IR (KBr cm^{-1}) 3066, 2968, 2940, 2803, 1733, 1652, 1565, 1315, 1178, 1015, 643. ^{1}H NMR (DMSO-d_6) δ 1.10 (t, $J = 7.2$ Hz, 3H), 2.22–2.42 (m, 1H), 2.47 (s, 3H), 2.63–3.05 (m, 3H), 3.91–4.10 (m, 3H), 12.62 (s, 1H). Anal. $C_{13}H_{14}N_2O_3S_2$.

5.6. Ethyl 2-methylthio-4-oxo-3,4,5,6,7,8-hexahydro-[1]benzothieno[2,3-*d*]pyrimidine-5-carboxylate (11)

This was prepared from potassium salt **6** by the same procedure as for **10** and was recrystallized from ethanol. Yield: 0.67 g (52%); mp 224–226 °C; IR (KBr cm^{-1}) 3049, 2981, 1726, 1662, 1546, 1263, 1194, 1062, 1024, 857, 637. ^{1}H NMR (DMSO-d_6) δ 1.16 (t, $J = 7.2$ Hz, 3H), 1.61–2.15 (m, 4H), 2.53 (s, 3H), 2.67–2.91 (m, 2H), 3.92–4.26 (m, 3H), 12.62 (s, 1H). Anal. $C_{14}H_{16}N_2O_3S_2$.

5.7. 2-Methylthio-3,5,6,7-tetrahydro-4*H*-cyclopenta-[4,5]thieno[2,3-*d*]pyrimidin-4-one (12)

This was prepared from potassium salt **7** by the same procedure as for **10** and was recrystallized from ethanol/dioxane. Yield: 0.37 g (39%); mp 182–183 °C (dec); IR (KBr cm^{-1}) 2917, 2847, 1665, 1552, 1408, 1304, 1270, 1191, 1009, 825, 640. ^{1}H NMR (DMSO-d_6) δ 2.42–2.59 (m, 2H), 2.65 (s, 3H), 2.92–3.07 (m, 4H), 12.80 (s, 1H). Anal. $C_{10}H_{10}N_2OS_2$.

5.8. Ethyl 4-chloro-2-methylthio-6,7-dihydro-5*H*-cyclopenta-[4,5]thieno[2,3-*d*]pyrimidine-5-carboxylate (14)

Phosphorus oxychloride (2.5 mL, 26.82 mmol) was added to **10** (0.50 g, 1.61 mmol) and the suspension was heated at 150 °C for 40 min during stirring. After cooling, the suspension was poured into cold water and neutralized with 10% NaOH solution. The sticky residue was collected after 1 day, washed with water, dried, and recrystallized from cyclohexane. Yield: 0.10 g (19%); mp 77–79 °C; IR (KBr cm^{-1}) 2968, 2924, 1729, 1558, 1475, 1409, 1265, 1235, 1143, 1047, 825. ^{1}H NMR (DMSO-d_6) δ 1.16 (t, $J = 7.2$ Hz, 3H), 2.41–2.55 (m, 1H), 2.57 (s, 3H), 2.78–3.20 (m, 3H), 4.05–4.29 (m, 3H). Anal. $C_{13}H_{13}ClN_2O_2S_2$.

5.9. Ethyl 4-chloro-2-methylthio-5,6,7,8-tetrahydro[1]-benzothieno[2,3-*d*]pyrimidine-5-carboxylate (15)

This was prepared from methylthio derivative **11** by the same procedure as for **14** and was recrystallized from ethanol. Yield: 0.40 g (72%); mp 106–108 °C; IR (KBr cm^{-1}) 2932, 1724, 1538, 1475, 1389, 1327, 1275, 1228, 1157, 1121, 839. ^{1}H NMR (DMSO-d_6) δ 1.16 (t, $J = 7.2$ Hz, 3H), 1.47–2.30 (m, 4H), 2.57 (s, 3H), 2.70–3.03 (m, 2H), 3.95 (q, $J = 7.2$ Hz, 2H), 4.19–4.30 (m, 1H). Anal. $C_{14}H_{15}ClN_2O_2S_2$.

5.10. 4-Chloro-2-methylthio-6,7-dihydro-5*H*-cyclopenta[4,5]-thieno[2,3-*d*]pyrimidine (16)

This was prepared from methylthio derivative **12** by the same procedure as for **14** and was recrystallized from ethanol. Yield: 0.32 g (78%); mp 115–116 °C (dec); IR (KBr cm^{-1}) 2920, 2853, 1554, 1468, 1412, 1301, 1251, 1182, 1141, 1015, 819. ^{1}H NMR (DMSO-d_6) δ 2.34–2.55 (m, 2H), 2.58 (s, 3H), 2.96–3.12 (m, 4H). Anal. $C_{10}H_9ClN_2S_2$.

5.11. General procedure for the synthesis of 2-methylthio-(4-substituted-1-piperazinyl)thieno[2,3-*d*]pyrimidine derivatives 20–22, 24, 25, 27–29, 31, 32, 34–36, 38, 40–42, and 44

A mixture of the appropriate chloro derivatives **14-19** (2.05 mmol) and methyl, 2-methoxyphenyl, phenyl, or benzylpiperazine (4.10 mmol) was refluxed in absolute ethanol (20 mL) during stirring for 1–2 h. After cooling, a small amount of the solvent was removed under reduced pressure, water was added to the mixture, and after 1 or 2 days the solid product was collected, washed with water, dried, and recrystallized. For compound **21**, after cooling, a solid was obtained that was collected, washed with water, dried, and recrystallized.

5.12. 5,6-Dimethyl-2-methylthio-4-[4-(1-phenylmethyl)-1-piperazinyl]thieno[2,3-*d*]pyrimidine (20)

This was recrystallized from ethanol. Yield: 0.28 g (35%); mp 99–101 °C; IR (KBr cm^{-1}) 2824, 1548, 1498, 1424, 1355, 1250, 1132, 990, 853, 745, 670. ^{1}H NMR (DMSO-d_6) δ 2.32 (s, 3H), 2.37 (s, 3H), 2.46–2.59 (s+m, 3H+4H), 3.29–3.40 (m, 4H), 3.52 (s, 2H), 7.20–7.40 (m, 5H). Anal. $C_{20}H_{24}N_4S_2$.

5.13. 5,6-Dimethyl-2-methylthio-4-[4-(2-methoxyphenyl)-1-piperazinyl]thieno[2,3-*d*]pyrimidine (21)

This was recrystallized from ethanol. Yield: 0.54 g (66%); mp 123–125 °C; IR (KBr cm^{-1}) 2816, 1546, 1498, 1451, 1359, 1232, 1128, 1023, 986, 852, 744. ^{1}H NMR (DMSO-d_6) δ 2.39 (s, 6H), 2.52 (s, 3H), 3.08–3.20 (m, 4H), 3.43–3.58 (m, 4H), 3.80 (s, 3H), 6.84–7.05 (m, 4H). Anal. $C_{20}H_{24}N_4OS_2$.

5.14. 5,6-Dimethyl-2-methylthio-4-(4-phenyl-1-piperazinyl)-thieno[2,3-*d*]pyrimidine (22)

This was recrystallized from ethanol. Yield: 0.39 g (51%); mp 138–140 °C; IR (KBr cm^{-1}) 2817, 1594, 1497, 1523, 1367, 1227, 1134, 983, 848, 761, 693. ^1H NMR (DMSO-d_6) δ 2.39 (s, 3H), 2.40 (s, 3H), 2.52 (s, 3H), 3.25–3.38 (m, 4H), 3.42–3.55 (m, 4H), 6.76–7.31 (m, 5H). Anal. $C_{19}H_{22}N_4S_2$.

5.15. 5,6-Dimethyl-4-(4-methyl-1-piperazinyl)-2-methylthiothieno[2,3-*d*]pyrimidine (24)

This was recrystallized from ethanol. Yield: 0.11 g (18%); mp 99–100 °C; IR (KBr cm^{-1}) 2921, 2845, 2792, 1499, 1449, 1409, 1357, 1252, 1136, 992, 856. ^1H NMR (DMSO-d_6) δ 2.23 (s, 3H), 2.34 (s, 3H), 2.38 (s, 3H), 2.45–2.61 (s+m, 3H+4H), 3.29–3.45 (m, 4H). Anal. $C_{14}H_{20}N_4S_2$.

5.16. 2-Methylthio-4-[4-(1-phenylmethyl)-1-piperazinyl]-6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidine (27)

This was recrystallized from ethanol. Yield: 0.36 g (44%); mp 105–107 °C; IR (KBr cm^{-1}) 2927, 2814, 2711, 1564, 1445, 1394, 1301, 1170, 911, 742, 699. ^1H NMR (DMSO-d_6) δ 2.21–2.41 (m, 2H), 2.41–2.59 (s+m, 3H+4H), 2.83–3.00 (m, 4H), 3.40–3.62 (m, 6H), 7.21–7.40 (m, 5H). Anal. $C_{21}H_{24}N_4S_2$.

5.17. 4-[4-(2-Methoxyphenyl)-1-piperazinyl]-2-methylthio-6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidine (28)

This was recrystallized from ethanol. Yield: 0.44 g (52%); mp 133–135 °C; IR (KBr cm^{-1}) 2915, 2840, 1584, 1446, 1497, 1364, 1230, 1148, 988, 826, 750. ^1H NMR (DMSO-d_6) δ 2.28–2.46 (m, 2H), 2.51 (s, 3H), 2.88–3.16 (m, 8H), 3.66–3.76 (m, 4H), 3.80 (s, 3H), 6.81–7.10 (m, 4H). Anal. $C_{21}H_{24}N_4OS_2$.

5.18. 2-Methylthio-4-(4-phenyl-1-piperazinyl)-6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidine (29)

This was recrystallized from ethanol. Yield: 0.26 g (33%); mp 145–146 °C; IR (KBr cm^{-1}) 2923, 2845, 1595, 1492, 1441, 1363, 1232, 1147, 988, 753, 688. ^1H NMR (DMSO-d_6) δ 2.28–2.46 (m, 2H), 2.51 (s, 3H), 2.88–3.10 (m, 4H), 3.22–3.33 (m, 4H), 3.62–3.76 (m, 4H), 6.72–7.35 (m, 5H). Anal. $C_{20}H_{22}N_4S_2$.

5.19. 4-(4-Methyl-1-piperazinyl)-2-methylthio-6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidine (31)

This was recrystallized from ethanol/water. Yield: 0.17 g (24%); mp 127–129 °C; IR (KBr cm^{-1}) 2920, 2847, 2794, 1540, 1495, 1449, 1387, 1268, 1143, 992, 824. ^1H NMR (DMSO-d_6) δ 2.22 (s, 3H), 2.26–2.53 (m, 9H), 2.86–3.04 (m, 4H), 3.48–3.58 (m, 4H). Anal. $C_{15}H_{20}N_4S_2 \cdot 3/2H_2O$.

5.20. Ethyl 2-methylthio-4-[4-(1-phenylmethyl)-1-piperazinyl]-6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidine-5-carboxylate (34)

This was recrystallized from ethanol. Yield: 0.40 g (42%); mp 126–128 °C; IR (KBr cm^{-1}) 2932, 2811, 1729, 1550, 1493, 1445, 1318, 1171, 1130, 980, 733. ^1H NMR (DMSO-d_6) δ 1.13 (t, $J = 7$ Hz, 3H), 2.30–2.64 (m, 8H), 2.70–3.10 (m, 3H), 3.25–3.70 (s+m, 2H+4H), 4.03 (q, $J = 7$ Hz, 2H), 4.22–4.34 (m, 1H) 7.20–7.42 (m, 5H). Anal. $C_{24}H_{28}N_4O_2S_2$.

5.21. Ethyl 4-[4-(2-methoxyphenyl)-1-piperazinyl]-2-methylthio-6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidine-5-carboxylate (35)

This was recrystallized from ethanol. Yield: 0.66 g (67%); mp 128–130 °C; IR (KBr cm^{-1}) 2918, 2828, 1718, 1496, 1449, 1364, 1237, 1142, 1033, 990, 753. ^1H NMR (DMSO-d_6) δ 1.11 (t, $J = 7$ Hz, 3H), 2.32–2.60 (m, 4H) 2.70–3.28 (m, 7H), 3.35–3.60 (m, 2H), 3.70–3.90 (m+s, 2H+3H), 4.04 (q, $J = 7$ Hz, 2H), 4.30–4.42 (m, 1H), 6.83–7.08 (m, 4H). Anal. $C_{24}H_{28}N_4O_3S_2$.

5.22. Ethyl 2-methylthio-4-(4-phenyl-1-piperazinyl)-6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidine-5-carboxylate (36)

This was recrystallized from ethanol/water. Yield: 0.20 g (21%); mp 142–144 °C; IR (KBr cm^{-1}) 2921, 2841, 1728, 1598, 1534, 1496, 1368, 1231, 1155, 987, 757. ^1H NMR (DMSO-d_6) δ 1.05 (t, $J = 7$ Hz, 3H), 2.30–2.62 (m, 4H), 2.72–2.98 (m, 1H), 2.99–3.28 (m, 4H), 3.32–3.60 (m, 4H), 3.68–3.84 (m, 2H), 4.01 (q, $J = 7$ Hz, 2H), 4.28–4.42 (m, 1H), 6.78–7.32 (m, 5H). Anal. $C_{23}H_{26}N_4O_2S_2$.

5.23. Ethyl 4-(4-methyl-1-piperazinyl)-2-methylthio-6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidine-5-carboxylate (38)

This was recrystallized from ethanol. Yield: 0.31 g (39%); mp 134–136 °C; IR (KBr cm^{-1}) 2975, 2924, 2832, 2789, 1734, 1537, 1491, 1397, 1170, 1136, 986. ^1H NMR (DMSO-d_6) δ 1.14 (t, $J = 7$ Hz, 3H), 2.20 (s, 3H), 2.25–2.58 (m, 8H), 2.71–2.95 (m, 1H), 2.95–3.10 (m, 2H), 3.25–3.41 (m, 2H), 3.54–3.71 (m, 2H), 4.04 (q, $J = 7$ Hz, 2H), 4.23–4.39 (m, 1H). Anal. $C_{18}H_{24}N_4O_2S_2$.

5.24. Ethyl 2-methylthio-4-[4-(1-phenylmethyl)-1-piperazinyl]-5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidine-5-carboxylate (40)

This was recrystallized from ethanol. Yield: 0.80 g (81%); mp 140–141 °C; IR (KBr cm^{-1}) 2922, 2810, 1729,

1536, 1499, 1399, 1312, 1171, 1126, 984, 732. ^{1}H NMR (DMSO-d_6) δ 1.12 (t, $J = 7.2$ Hz, 3H), 1.61–1.88 (m, 3H), 2.18–2.45 (m, 3H), 2.50 (s, 3H), 2.55–2.75 (m, 2H), 2.78–2.90 (m, 2H), 2.97–3.15 (m, 2H), 3.30–3.50 (m, 2H), 3.51 (s, 2H, CH_2), 3.82–4.18 (m, 3H), 7.10–7.41 (m, 5H). Anal. $C_{25}H_{30}N_4O_2S_2$.

5.25. Ethyl 4-[4-(2-methoxyphenyl)-1-piperazinyl]-2-methylthio-5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidine-5-carboxylate (41)

This was recrystallized from ethanol. Yield: 0.71 g (70%); mp 152–154 °C; IR (KBr cm^{-1}) 2930, 2838, 1727, 1536, 1497, 1451, 1372, 1234, 1164, 999, 745. ^{1}H NMR (DMSO-d_6) δ 1.10 (t, $J = 7$ Hz, 3H), 1.63–1.98 (m, 3H), 2.20–2.35 (m, 1H), 2.54 (s, 3H), 2.80–3.05 (m, 4H), 3.12–3.31 (m, 4H), 3.45–3.65 (m, 2H), 3.79 (s, 3H), 3.87–4.18 (m, 3H), 6.85–7.05 (m, 4H). Anal. $C_{25}H_{30}N_4O_3S_2$.

5.26. Ethyl 2-methylthio-4-(4-phenyl-1-piperazinyl)-5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidine-5-carboxylate (42)

This was recrystallized from ethanol. Yield: 0.50 g (52%); mp 128–130 °C; IR (KBr cm^{-1}) 2970, 2837, 1737, 1597, 1499, 1374, 1233, 1168, 984, 760, 690. ^{1}H NMR (DMSO-d_6) δ 1.03 (t, $J = 7.2$ Hz, 3H), 1.62–1.98 (m, 3H), 2.20–2.35 (m, 1H), 2.54 (s, 3H), 2.80–2.92 (m, 2H), 3.05–3.30 (m, 4H), 3.30–3.60 (m, 4H), 3.87–4.18 (m, 3H), 6.78–7.32 (m, 5H). Anal. $C_{24}H_{28}N_4O_2S_2$.

5.27. Ethyl 4-(4-methyl-1-piperazinyl)-2-methylthio-5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidine-5-carboxylate (44)

This was recrystallized from ethanol/water. Yield: 0.33 g (40%); mp 129–130 °C; IR (KBr cm^{-1}) 2924, 2837, 1734, 1560, 1529, 1400, 1334, 1172, 988, 857, 772. ^{1}H NMR (DMSO-d_6) δ 1.13 (t, $J = 7.2$ Hz, 3H), 1.62–1.94 (m, 3H), 2.15–2.38 (m, 6H), 2.47–2.62 (m, 5H), 2.76–2.88 (m, 2H), 2.95–3.12 (m, 2H), 3.30–3.49 (m, 2H), 3.87–4.15 (m, 3H). Anal. $C_{19}H_{26}N_4O_2S_2$.

5.28. 5,6-Dimethyl-2-methylthio-4-[4-(2-pyrimidyl)-1-piperazinyl]thieno[2,3-*d*]pyrimidine (23)

A mixture of chloro derivative **18** (0.50 g, 2.04 mmol), 1-(2-pyrimidyl)piperazine dihydrochloride (0.53 g, 2.23 mmol), and potassium carbonate (0.56 g, 4.05 mmol) was refluxed in absolute ethanol (20 mL) during stirring for 40 min. After cooling, the precipitate was collected, washed with water, dried, and recrystallized from ethanol. Yield: 0.42 g (55%); mp 109–110 °C; IR (KBr cm^{-1}) 2848, 1585, 1548, 1497, 1446, 1352, 1249, 1139, 976, 860, 778. ^{1}H NMR (DMSO-d_6) δ 2.40 (s, 6H), 2.51 (s, 3H), 3.39–3.47 (m, 4H), 3.83–3.97 (m, 4H), 6.68 (t, $J = 4.8$ Hz, 1H), 8.40 (d, $J = 4.8$ Hz, 2H). Anal. $C_{17}H_{20}N_6S_2$.

5.29. 2-Methylthio-4-[4-(2-pyrimidyl)-1-piperazinyl]-6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidine (30)

It was prepared from chloro derivative **16** in the same manner as for **23**, but the refluxing time was 3 h. The compound was recrystallized from ethanol. Yield: 0.61 g (78%); mp 110–111 °C; IR (KBr cm^{-1}) 2846, 1588, 1544, 1489, 1440, 1352, 1292, 1253, 1147, 983, 794. ^{1}H NMR (DMSO-d_6) δ 2.29–2.46 (m, 2H), 2.51 (s, 3H), 2.89–3.14 (m, 4H), 3.60–3.68 (m, 4H), 3.84–3.94 (m, 4H), 6.69 (t, $J = 4.6$ Hz, 1H), 8.33 (d, $J = 4.6$ Hz, 2H). Anal. $C_{18}H_{20}N_6S_2$.

5.30. 5,6-Dimethyl-4-(4-methyl-1-piperazinyl)thieno[2,3-*d*]pyrimidine (25)

A mixture of chloro derivative **19** (0.48 g, 2.42 mmol) and 1-methylpiperazine (0.54 mL, 4.87 mmol) was refluxed during stirring for 4 h in absolute ethanol (20 mL). After cooling, water was added, and the mixture was extracted with chloroform. The combined organic layer was dried (Na_2SO_4) and evaporated under reduced pressure. After the addition of diethyl ether, the product solidified, and was recrystallized from ethanol/water. Yield: 0.33 g (52%); mp 73–75 °C; IR (KBr cm^{-1}) 2920, 2839, 2793, 1533, 1502, 1433, 1366, 1285, 1258, 1137, 977. ^{1}H NMR (DMSO-d_6) δ 2.18 (s, 3H), 2.39 (s, 3H), 2.43 (s, 3H), 2.45–2.67 (m, 4H), 3.25–3.39 (m, 4H), 8.47 (s, 1H). Anal. $C_{13}H_{18}N_4S$.

5.31. 4-(4-Methyl-1-piperazinyl)-6,7-dihydro-5*H*-cyclopenta-[4,5]thieno[2,3-*d*]pyrimidine (32)

It was obtained from chloro derivative **17** by the same procedure as for **25**. Chloro derivative **17** was prepared from **13** (0.56 g, 2.93 mmol) and phosphorus oxychloride (3 mL, 32.18 mmol), the suspension being heated at 150 °C during stirring for 2 h. After cooling, the suspension was poured into cold water and neutralized with 10% NaOH solution. The precipitate was collected, washed with water, dried, and used for the next step without further purification; yield: 0.35 g (57%).

Compound **32** was recrystallized from ethanol/water. Yield: 0.30 g (44%); mp 88–91 °C; IR (KBr cm^{-1}) 2936, 2848, 2795, 1545, 1496, 1444, 1363, 1290, 1262, 1142, 983. ^{1}H NMR (DMSO-d_6) δ 2.10–2.60 (m, 9H), 2.95–3.18 (m, 4H), 3.40–3.70 (m, 4H), 8.45 (s, 1H). Anal. $C_{14}H_{18}N_4S{\cdot}1/2H_2O$.

5.32. 5,6-Dimethyl-2-methylthio-4-(1-piperazinyl)thieno-[2,3-*d*]pyrimidine (26)

A mixture of chloro derivative **18** (0.60 g, 2.45 mmol) and piperazine (1.06 g, 12.30 mmol) was refluxed during stirring for 3 h in absolute ethanol (20 mL). After cooling, the solid was eliminated by filtration, and the solution was diluted with water and extracted with chloroform, and the combined organic layer was dried (Na_2SO_4) and evaporated under reduced pressure. After

the addition of diethyl ether to the yellow oil, a solid compound was obtained, which was recrystallized from ethanol/water. Yield: 0.22 g (27%); mp 119–120 °C; IR (KBr cm^{-1}) 3302, 2914, 2806, 1502, 1419, 1354, 1255, 1204, 1125, 990, 852. ^1H NMR (DMSO-d_6) δ 2.33 (s, 3H), 2.37 (s, 3H), 2.50 (s, 3H), 2.78–2.80 (m, 4H), 3.12–3.38 (m, 4H). Anal. $C_{13}H_{18}N_4O_2S_2$.

5.33. 2-Methylthio-4-(1-piperazinyl)-6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidine (33)

A mixture of chloro derivative **16** (0.70 g, 2.73 mmol) and piperazine (1.17 g, 13.58 mmol) was refluxed in absolute ethanol (20 mL) during stirring for 3 h. After cooling, water was added to the solution to obtain a solid product, which was recrystallized from ethanol. Yield: 0.63 g (75%); mp 98–100 °C; IR (KBr cm^{-1}) 3293, 2841, 1519, 1500, 1446, 1384, 1289, 1205, 1134, 988, 826. ^1H NMR (DMSO-d_6) δ 2.26–2.44 (m, 2H), 2.49 (s, 3H), 2.76–3.04 (m, 8H), 3.41–3.52 (m, 4H). Anal. $C_{14}H_{18}N_4S_2$.

5.34. Ethyl 2-methylthio-4-[4-(2-pyrimidyl)-1-piperazinyl]-6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidine-5-carboxylate (37)

A mixture of chloro derivative **14** (1.80 g, 5.47 mmol), 1-(2-pyrimidyl)piperazine dihydrochloride (2.60 g, 10.96 mmol), and potassium carbonate (2.55 g, 18.45 mmol) was refluxed in absolute ethanol (50 mL) during stirring for 1 h. After cooling, the solid was eliminated by filtration and water was added to the solution. The precipitated product was filtered off, washed with water, and recrystallized from ethanol/water. Yield: 1.50 g (60%); mp 138–140 °C; IR (KBr cm^{-1}) 2973, 2858, 1725, 1583, 1546, 1486, 1451, 1355, 1254, 1143, 979. ^1H NMR (DMSO-d_6) δ 1.06 (t, $J = 7.2$ Hz, 3H), 2.52 (s, 3H), 2.72–3.12 (m, 4H), 3.42–3.55 (m, 2H), 3.62–3.82 (m, 4H), 3.90–4.15 (m, 4H), 4.32–4.42 (m, 1H), 6.68 (t, $J = 4.8$ Hz, 1H), 8.41 (d, $J = 4.8$ Hz, 2H). Anal. $C_{21}H_{24}N_6O_2S_2$.

5.35. Ethyl 2-methylthio-4-(1-piperazinyl)-6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidine-5-carboxylate (39)

A mixture of chloro derivative **14** (0.70 g, 2.13 mmol) and piperazine (0.91 g, 10.56 mmol) was refluxed in absolute ethanol (10 mL) during stirring for 5 h. After cooling, the solid was eliminated by filtration, and the solution was diluted with water and extracted with chloroform. The combined organic layer was dried (Na_2SO_4) and evaporated under reduced pressure. After the addition of diethyl ether and petroleum ether to the yellow sticky product, a solid compound was obtained, which was recrystallized from ethanol/water. Yield: 0.28 g (35%); mp 76–78 °C; IR (KBr cm^{-1}) 3273, 2931, 2827, 1732, 1541, 1494, 1410, 1319, 1235, 1172, 831; ^1H NMR (DMSO-d_6) δ 1.14 (t, $J = 7.2$ Hz, 3H), 2.28–2.58 (m, 4H), 2.63–3.12 (m, 5H), 3.22–3.70 (m, 6H), 4.06 (q, $J = 7.2$ Hz, 2H), 4.25–4.35 (m, 1H). Anal. $C_{17}H_{22}N_4O_2S_2$.

5.36. Ethyl 2-methylthio-4-[4-(2-pyrimidyl)-1-piperazinyl]-5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidine-5-carboxylate (43)

A mixture of chloro derivative **15** (0.70 g, 2.04 mmol), 1-(2-pyrimidyl)piperazine dihydrochloride (0.53 g, 2.23 mmol), and potassium carbonate (0.56 g, 4.05 mmol) was refluxed in absolute ethanol (20 mL) during stirring for 6 h. After cooling, the precipitate was removed by filtration and water was added to the solution. The mixture was then extracted with chloroform, and the combined organic layer was dried (Na_2SO_4) and evaporated under reduced pressure. The sticky residue was purified by flash column chromatography (chloroform) and was solidified by the addition of diethyl ether and petroleum ether to the yellow oil. Yield: 0.31 g (32%); mp 114–116 °C; IR (KBr cm^{-1}) 2924, 1719, 1586, 1547, 1480, 1419, 1383, 1358, 1229, 1168, 977. ^1H NMR (DMSO-d_6) δ 1.03 (t, $J = 7$ Hz, 3H), 1.62–2.00 (m, 3H), 2.18–2.28 (m, 1H), 2.53 (s, 3H), 2.80–2.97 (m, 2H), 3.02–3.20 (m, 2H), 3.30–3.58 (m, 2H), 3.62–3.80 (m, 2H), 3.90–4.10 (m, 5H), 6.69 (t, $J = 4.6$ Hz, 1H), 8.33 (d, $J = 4.6$ Hz, 2H). Anal. $C_{22}H_{26}N_6O_2S_2$.

5.37. Ethyl 2-methylthio-4-(1-piperazinyl)-5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidine-5-carboxylate (45)

A mixture of chloro derivative **15** (1.30 g, 3.79 mmol) and piperazine (1.62 g, 18.81 mmol) was refluxed in absolute ethanol (20 mL) during stirring for 3 h. After cooling, the solid was eliminated by filtration and the solution was diluted with water. After 2 days, the solid compound was collected and recrystallized from ethanol/water. Yield: 0.30 g (20%); mp 225 °C (dec); IR (KBr cm^{-1}) 2929, 1735, 1502, 1405, 1368, 1328, 1268, 1157, 988, 850. ^1H NMR (DMSO-d_6) δ 1.11 (t, $J = 7$ Hz, 3H), 1.62–1.95 (m, 3H), 2.19–2.31 (m, 1H), 2.52 (s, 3H), 2.61–2.75 (m, 2H), 2.78–3.05 (m, 6H), 3.24–3.43 (m, 2H), 3.80–4.10 (m, 3H). Anal. $C_{18}H_{24}N_4O_2S_2$.

6. In vitro binding assays

Male CRL:CD(SD)BR-COBS rats (weighing about 150 g, Charles River, Italy) and male CRL:(HA) BR albino guinea pigs (weighing about 300 g, Charles River, Italy) were killed by decapitation; their brains were rapidly dissected into the appropriate areas (rat cortex for 5-HT_3 and guinea pig striatum for 5-HT_4) and stored at −80 °C until the day of assay.

The tissues were homogenized in 50 vol of ice-cold Tris HCl, 50 mM, pH 7.4 containing 0.5 mM EDTA, and 10 mM $MgSO_4$ for 5-HT_3, or Hepes HCl, 50 mM, pH 7.4, for 5-HT_4, using an Ultra Turrax TP-1810 homogenizer (2×20 s), and homogenates were

centrifuged at 50,000*g* for 10 min (Beckman Avanti J-25 refrigerated centrifuge). Each pellet was resuspended in the same volume of fresh buffer, incubated at 37 °C for 10 min, and centrifuged again at 50,000*g* for 10 min. The pellet was then washed once by resuspension in fresh buffer and centrifuged as before.

The pellet obtained was finally resuspended in the appropriate incubation buffer (Hepes HCl, 50 mM, pH 7.4, containing 10 μM pargyline for 5-HT_4, or Tris HCl, 50 mM, pH 7.4, containing 10 μM pargyline, 0.5 mM EDTA, 10 mM $MgSO_4$, 0.1% ascorbic acid, and 140 mM NaCl for 5-HT_3) just before the binding assay.

[^{3}H]LY 278584[21] (S.A. 84.0 Ci/mmol Amersham, for 5-HT_3) binding was assayed in a final incubation volume of 1 mL, consisting of 0.50 mL of tissue (16 mg/sample), 0.50 mL of the [^{3}H]ligand (4 nM), and 0.02 mL of displacing agent or solvent; nonspecific binding was measured in the presence of 1 μM quipazine. [^{3}H]GR 113808[22] (S.A. 84.0 Ci/mmol Amersham, for 5-HT_4) binding was assayed in a final incubation volume of 1.0 mL, consisting of 0.5 mL of tissue (20 mg/sample), 0.5 mL of the [^{3}H]ligand (0.1 nM), and 0.02 mL of displacing agent or solvent; nonspecific binding was measured in the presence of 10 μM serotonin. Incubation (30 min at 25 °C for 5-HT_3 or 30 min at 37 °C for 5-HT_4) was stopped by rapid filtration under vacuum through GF/B filters, which were then washed with 12 mL (4 × 3 times) of ice-cold Tris HCl, 50 mM, pH 7.4, or Hepes HCl, 50 mM, pH 7.4, using a Brandel M-48R cell harvester. Dried filters were immersed in vials containing 4 mL of Ultima Gold MV (Packard) and counted in a Wallac 1409 liquid scintillation spectrometer with a counting efficiency of about 50%. Drugs were tested in triplicate at different concentrations (from 10^{-5} to 10^{-10} M) and dose-inhibition curves were analyzed by the Allfit[23] program to obtain the concentration of unlabeled drug that caused 50% inhibition of ligand binding, K_i values were derived from the IC_{50} values.[24]

7. In vitro organ studies on isolated guinea pig colon

Animal experiments were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: IV/1813–1/2002).

The distal portion of the colon was removed from a Hartley guinea pig (400–500 g) starved 24 h before experiments. The colon was cleaned in Krebs-bicarbonate buffer (in mM: NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.7; pH = 7.4) at room temperature and cut 2 cm segments. The segments were suspended longitudinally in an organ bath containing Krebs-bicarbonate buffer warmed to 37 °C and bubbled through with 95% O_2/5% CO_2. One gram of loading tension was applied; the tissues were left to be incubated for 1 h. Isometric contractions were detected by ISOSYS Data Acquisition System (Experimetria Ltd. Hungary). The contractile action of 5-HT_3 receptor selective agonist 2-Me-5-HT (Sigma, Hungary) was investigated by cumulative way. The result was expressed as the percent of contraction increase compared to basal colon activity. Compounds **31** and **33**, and the specific 5-HT_3 receptor antagonist tropisetron (Sigma, Hungary) were added to the bath 10 min before the application of 2-Me-5-HT in concentration of 10 nM. Dose–response curves, the contraction increasing effect, the changes in EC_{50} and maximum inhibition, and the statistical analysis were calculated by Prism 2.0 software (GraphPad Software, USA).

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