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Erratum

Design, synthesis and binding properties of novel and selective 5-HT_3 and 5-HT_4 receptor ligands☆,☆☆,☆☆☆

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Abstract – This work reports the synthesis and the binding tests on the 5-HT_3 and 5-HT_4 receptors of new thienopyrimidopiperazine and piperazinylacylaminodimethylthiophene derivatives, in order to identify potent and selective ligands for each receptor. The 3-amino-2-(4-benzyl-1-piperazinyl)-5,6-dimethyl-thieno[2,3-*d*]pyrimidin-4(3*H*)-one derivative **28** showed the highest affinity and selectivity for the 5-HT_3 over the 5-HT_4 receptor (5-HT_3 $K_i = 3.92$ nM, 5-HT_4 not active), whereas the 2-[4-[4-(2-pyrimidinyl)-1-piperazinyl]butanoylamino]-4,5-dimethyl-3-thiophenecarboxylic acid ethyl ester (**41**) showed the highest affinity and selectivity for the 5-HT_4 over the 5-HT_3 receptor (5-HT_4 $K_i = 81.3$ nM, 5-HT_3 not active). Conformational analyses were carried out on the compounds of the piperazinylacylaminodimethylthiophene series (**39**–**42**) taking compound **41** as the template. © 2001 Éditions scientifiques et médicales Elsevier SAS

5-HT_3 and 5-HT_4 receptors / ligands / arylpiperazines / conformational analysis

1. Introduction

Serotonin modulates the activity of both the central nervous system and peripheral tissues; as far as we know, it acts on 14 receptor subtypes and plays a role in a wide range of physiological and pathophysiological processes. Considerable attention has been paid to the identification of agents, which act selectively on each of these receptor subtypes.

For many years, we have been studying high-affinity, selective 5-HT_{1A} receptor subtype ligands [1–3] and molecular modelling studies have been also reported [4–6].

Recently our interest has been extended to 5-HT_3 and 5-HT_4 receptors, too.

Of serotonin receptors, the 5-HT_3 is the only one belonging to the ligand-gated ion channel receptor family; 5-HT_3 antagonists are used as anti-emetic agents to prevent the vomiting associated with chemotherapy or radiation-induced emesis, but there are numerous potential therapeutic applications such as: pain, psychosis, memory impairment, depression, anxiety, schizophrenia and drug abuse. Less is known about selective and potent 5-HT_3R agonists and their therapeutic potential. The key pharmacophoric elements of 5-HT_3R antagonists generally include an aromatic moiety, a carbonyl dipole (or a bioisosteric equivalent function) and a basic amino group, as in the case of drugs such as ondansetron, granisetron or tropisetron [7].

The 5-HT_4 receptor, positively coupled to adenylate cyclase, first identified in 1988 and recently cloned [8], is localised in gastrointestinal, atrial and urinary blad-

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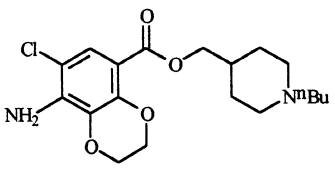
der tissue, as well as in the central nervous system. Therapeutic applications, such as the treatment of irritable bowel syndrome, atrium arrhythmia and urinary incontinence are proposed for the antagonists to the 5-HT_4 receptor, while the use of agonists (as prokinetic drugs) is suggested in the treatment of gastrointestinal motility disorders such as gastro-esophageal reflux, functional dyspepsia and constipation.

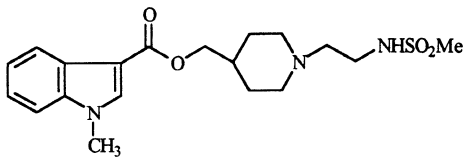
Despite being so different, the same structural elements for the 5-HT_3 receptor characterise the 5-HT_4 pharmacophore, as shown by QSAR studies on 5-HT_4 ligands like, for example, SB 204070, GR 113808 (*figure 1*) and others [7]. This explains the poor selectivity for the 5-HT_3 and 5-HT_4 receptors of many ligands, which often show affinity for other serotonin and monoamine receptors, too.

This work reports the synthesis, binding tests and molecular modelling of new derivatives **A** and **B** (*figure 1*), bearing the three key pharmacophoric elements, in order to identify potent and selective ligands for each receptor.

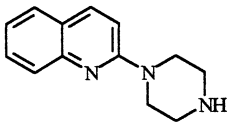
The thienopyrimidothioalkylpiperazine system was chosen according to the good affinity for the 5-HT_3 receptor ($IC_{50} = 20$ nM) showed by compound **A** ($R_1 = R_2 = CH_3$; $R_3 = 2$-pyrimidinyl, $X = NNH_2$, $Y = S(CH_2)_3$ previously reported by us as a high-affinity and selective 5-HT_{1A} ligand [1]. The structural modifications on this compound, chosen as the lead, were: (i) the introduction of a tetramethylene chain instead of two methyls in the R_1 and R_2 positions; (ii) the introduction of the orthomethoxyphenyl and the benzyl groups in the R_3 position instead of the 2-pyrimidinyl and (iii) the replacement of the NNH_2 group with the NH group or S atom, together with the shortening and the elimination of the thioalkyl chain.



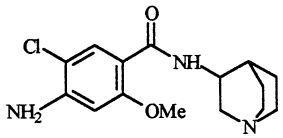
SB 204070



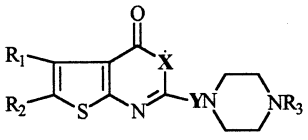
GR 113808



Quipazine

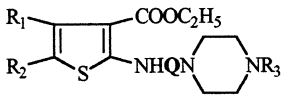


Zacopride



A

R_1, R_2 = CH_3, CH_3; -$(CH_2)_4$-;
R_3 = $C_6H_4OCH_3$(o), $CH_2C_6H_5$, 2-pyrimidinyl;
X = NNH_2, NH, S;
Y = /, CH_2, $S(CH_2)_2$.



B

R_1, R_2 = CH_3, CH_3; -$(CH_2)_4$-; H, C_6H_5; CH_3, $COOC_2H_5$;
R_3 = $C_6H_4OCH_3$(o), $CH_2C_6H_5$, 2-pyrimidinyl;
Q = CS, $CO(CH_2)_{1\text{-}4}$.

Figure 1. Ligands for the 5-HT_3 and 5-HT_4 receptors and the new synthesized compounds **A** and **B**.

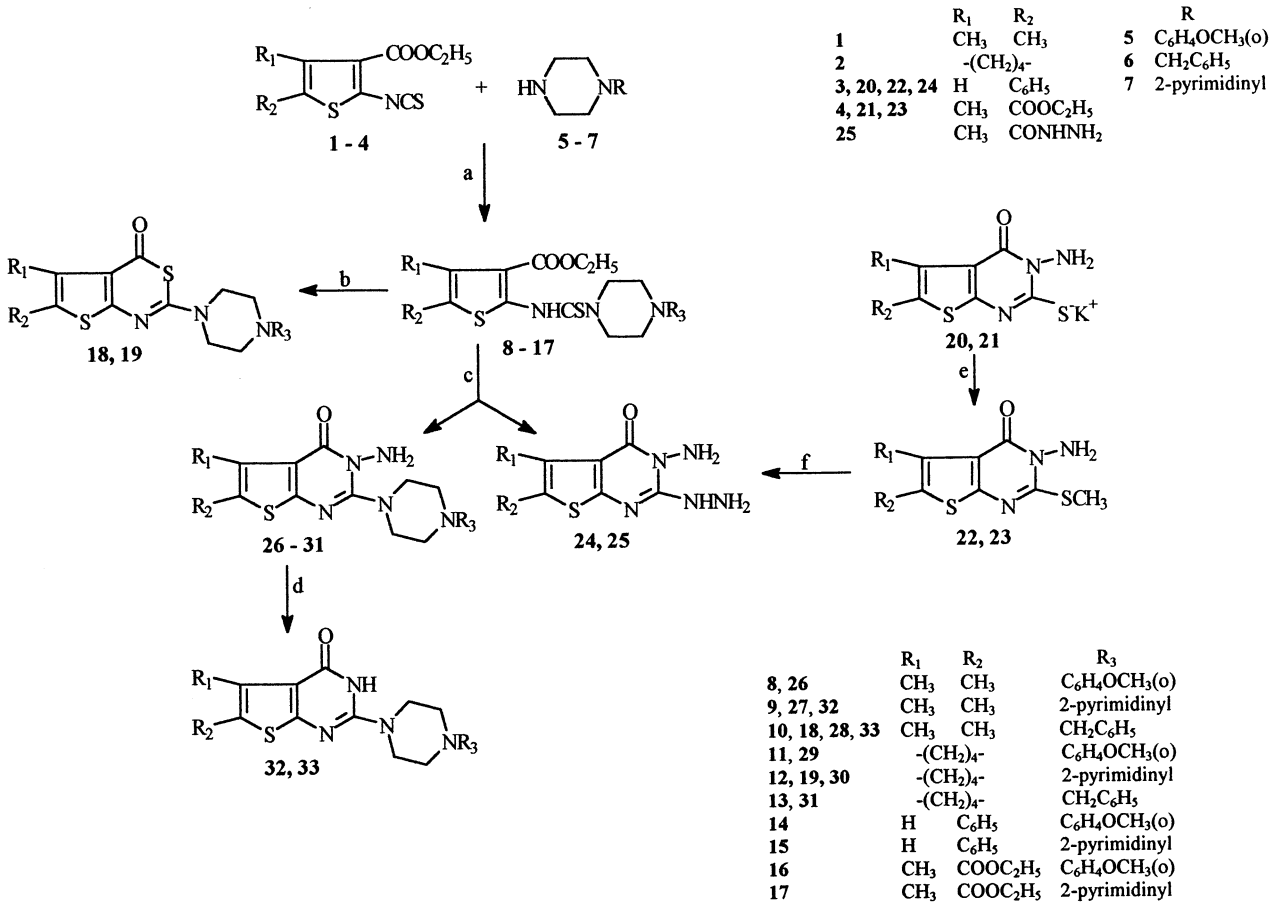


Figure 2. Synthetic procedure to compounds **18**, **19**, **24**–**33**. Reagents and conditions; (a) $CHCl_3$, reflux; (b) H_2SO_4 conc., r.t.; (c) $N_2H_4.H_2O$, EtOH, reflux; (d) HCl, $NaNO_2$, r.t.; (e) CH_3I, H_2O, r.t.; (f) $N_2H_4.H_2O$, 2-propanol, reflux.

Compounds **18**, **19**, **26**–**33** in which the piperazine nucleus is directly linked to the heterocyclic system, are related to quipazine (*figure* 1) and its derivatives, potent ligands for the 5-HT_3 receptor [9].

Type **B** compounds (*figure 1*) were prepared to investigate the influence of the structure flexibility on the affinity and selectivity for the 5-HT_4 receptor over the 5-HT_3 receptor [8]. Conformational analyses were carried out on the most active 5-HT_4 receptor ligands (see Results and discussion) using the Monte Carlo method as implemented in MACROMODEL (version 6.5) [10, 11] to obtain the main conformational and pharmacophoric requirements for receptor binding, taking compound **41** as the template and therefore assuming its conformation as the bioactive one.

2. Chemistry

Derivatives **8**–**17** were prepared by heating at reflux in chloroform, the appropriate isothiocyanates **1**–**4** [2, 12] and piperazines **5**–**7** that were commercially available (*figure 2*).

The piperazinethienothiazines **18** and **19** were obtained from compounds **10** or **12** in sulfuric acid at room temperature.

The piperazinethienopyrimidinones **26**–**31** were prepared by boiling at reflux derivatives **8**–**13** with hydrazine monohydrate. Only the hydrazine derivatives **24** and **25** were isolated by reaction of derivatives **14**–**17** with hydrazine monohydrate under the same or slightly modified conditions. Analytical and

spectral data of compounds **24** and **25** were like those of the derivatives obtained by reaction of hydrazine monohydrate and the methylthio derivatives **22** and **23**, respectively, which were in turn prepared from the monopotassium salts **20** [13] and **21** [2] and methyl iodide (analytical and spectral data are reported in Section 6.1.3).

The N3 amino group of derivatives **27** and **28** was removed by treatment with an aqueous sodium nitrite solution and 37% hydrochloric acid to give compounds **32** and **33** (*figure 2*).

The ethyl ester of 2-amino-4,5-dimethyl-3-thiophenecarboxylic acid (**34**) [14] gave the chloroderivatives **35** [15], **36**, **37**, and **38** [2] when heated at reflux with chloroacylchlorides in chloroform, which in turn produced compounds **39**–**42** by reaction with 1-(2-pyrimidinyl)piperazine dihydrochloride in boiling dimethylformamide under reflux.

Compound **43** was obtained by boiling at reflux the derivative **39** and hydrazine monohydrate in 1-propanol. Attempts to isolate other superior homologues using the same or slightly modified conditions were unsuccessful (*figure 3*).

The pyrimidinylpiperazine-thioalkylthieno [2,3-α] pyrimidinone (**47**) was prepared from the monopotassium salt **46** [1] by reaction with chloroethylpyrimidinylpiperazine (**45**). This latter compound was obtained by reaction of 1-(2-pyrimidinyl)-piperazine dihydrochloride (**44**) with 1-bromo-2-chloroethane (*figure 4*).

The proposed structures of compounds **8**–**19**, **26**–**33**, **39**–**43**, and **47** were confirmed by elemental combustion analyses (C,H,N,S), IR and ^{1}H-NMR spectra (*tables I*, *II*, *and III*).

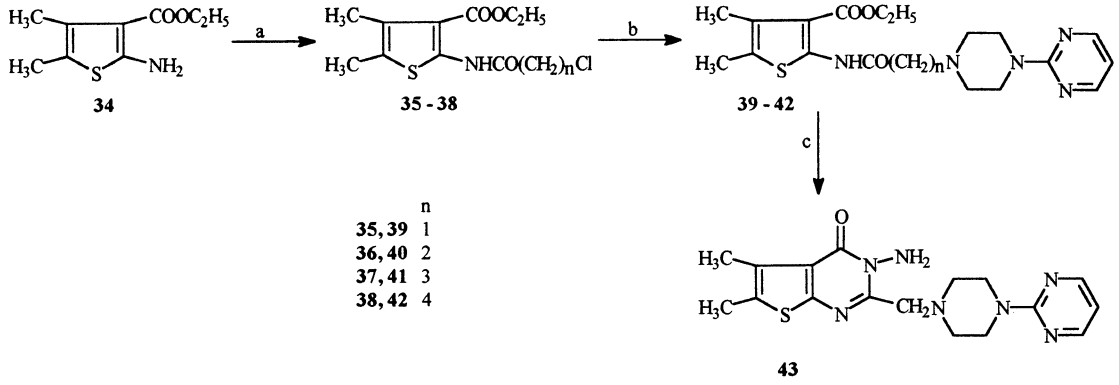


Figure 3. Synthetic procedure to compounds **35**–**43**. Reagents and conditions: (a) $ClCO(CH_2)_nCl$, $CHCl_3$, reflux; (b) 1-(2-pyrimidinyl)piperazine dihydrochloride, K_2CO_3, dimethylformamide, reflux; (c) $N_2H_4.H_2O$, 1-propanol, reflux.

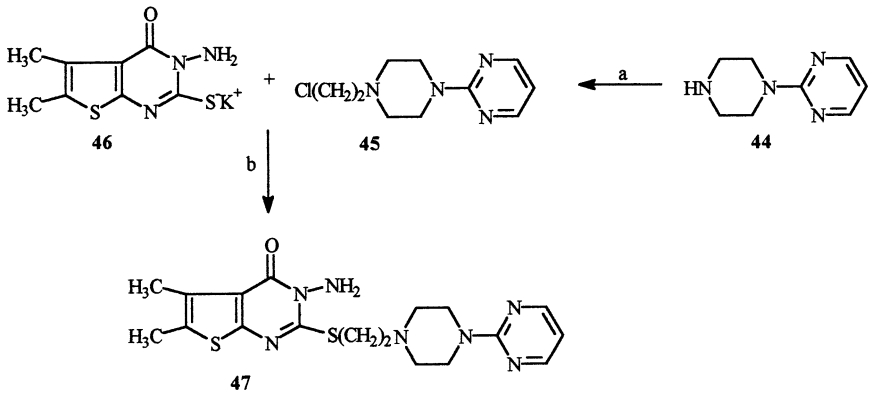
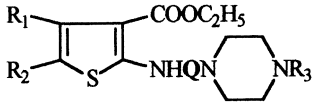


Figure 4. Synthetic procedure to compound **47**. Reagents and conditions; (a) $Cl(CH_2)_2Br$, K_2CO_3, dimethylformamide, r. t.; (b) EtOH, reflux.

Table I. Physical and chemical properties of compounds **8**–**17**, **39**–**42**.



Compound	R_1	R_2	Q	R_3	Melting point (°C)	Recryst. solvent yield (%)	I.R. KBr ν(NH)	(cm^{-1}) ν(C=O)	Formula
8	CH_3	CH_3	CS	$C_6H_4OCH_3$(o)	165–167	EtOH/dioxane, 65	/	1645	$C_{21}H_{27}N_3O_3S_2$
9	CH_3	CH_3	CS	2-$C_4H_3N_2$[a]	207–208 decomp.	EtOH/dioxane, 41	/	1650	$C_{18}H_{23}N_5O_2S_2$
10	CH_3	CH_3	CS	$CH_2C_6H_5$	111–113	EtOH, 92	/	1660	$C_{21}H_{27}N_3O_2S_2$
11	–$(CH_2)_4$–		CS	$C_6H_4OCH_3$(o)	145–146	EtOH/dioxane, 68	/	1650	$C_{23}H_{29}N_3O_3S_2$
12	–$(CH_2)_4$–		CS	2-$C_4H_3N_2$[a]	210–211	DMF, 28	/	1650	$C_{20}H_{25}N_5O_2S_2$
13	–$(CH_2)_4$–		CS	$CH_2C_6H_5$	144–146	EtOH, 33	/	1650	$C_{23}H_{29}N_3O_2S_2$
14	H	C_6H_5	CS	$C_6H_4OCH_3$(o)	187–189	DMF, 30	/	1660	$C_{25}H_{27}N_3O_3S_2$
15	H	C_6H_5	CS	2-$C_4H_3N_2$[a]	213–215	DMF, 95	/	1660	$C_{22}H_{23}N_5O_2S_2$
16	CH_3	$COOC_2H_5$	CS	$C_6H_4OCH_3$(o)	180–182	EtOH/dioxane/DMF, 95	/	1705, 1660	$C_{23}H_{29}N_3O_5S_2$
17	CH_3	$COOC_2H_5$	CS	2-$C_4H_3N_2$[a]	206–208	EtOH/dioxane/DMF, 51	/	1710, 1655	$C_{20}H_{25}N_5O_4S_2$
39	CH_3	CH_3	$COCH_2$	2-$C_4H_3N_2$[a]	191	EtOH/dioxane, 40	3225	1680, 1665	$C_{19}H_{25}N_5O_3S$
40	CH_3	CH_3	$CO(CH_2)_2$	2-$C_4H_3N_2$[a]	135–137	EtOH, 69	3245	1700, 1650	$C_{20}H_{27}N_5O_3S$
41	CH_3	CH_3	$CO(CH_2)_3$	2-$C_4H_3N_2$[a]	88–90	[b], 30	3240	1675, 1660	$C_{21}H_{29}N_5O_3S$
42	CH_3	CH_3	$CO(CH_2)_4$	2-$C_4H_3N_2$[a]	86–88	[b], 25	3270	1700, 1660	$C_{22}H_{31}N_5O_3S$

[a] 2-Pyrimidinyl ring.
[b] Purified by liquid chromatography.

3. Pharmacology

Derivatives **8**–**19**, **26**–**33**, **39**–**43** and **47** were evaluated for their affinity on the 5-HT_3 and 5-HT_4 receptors by radioligand binding assays. The results are shown in *table IV* as K_i values.

4. Results and discussion

4.1. Structure–affinity relationships

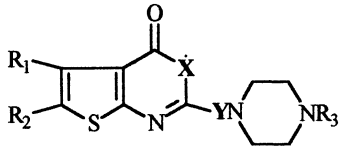
The thienothiazinone derivative **18** and some compounds of the piperazine series **26**–**33** show affinity with a great selectivity for the 5-HT_3 over the 5-HT_4 receptor. The piperazinylacylamino derivatives **39**–**42** show affinity for the 5-HT_4 receptor with a great selectivity over the 5-HT_3 receptor. The other tested compounds do not have any affinity for either receptor. This trend complies with previously reported findings: that is, those compounds where the basic nitrogen, connected to the aromatic acyl, is constrained within rigid structures, like for example the piperidine into a tropane ring system, show higher affinity for the 5-HT_3 receptor rather than the 5-HT_4 receptor; on the contrary, flexible structures where the aromatic acyl group is connected to the piperidine ring through an amide linkage have a higher affinity for the 5-HT_4 receptor rather than the 5-HT_3 receptor [8].

Compound **28** showed the highest affinity for the 5-HT_3 receptor ($K_i = 3.92$ nM) and acts as a full agonist in the Bezold-Jarisch reflex [unpublished data].

The structurally related compounds **33** and **18** showed a much lower affinity (K_i 265 and 377 nM, respectively).

These results show that, besides the three pharmacophoric elements above-mentioned, (i) the two methyls in position 5 and 6 of the thiophene nucleus (the substitution of the two methyls with a cyclic tetramethylene chain in compound **31** ($K_i = 2035$ nM) leads to a remarkable decrease in affinity); (ii) the amino group linked to the N3 of the pyrimidinone ring (see the biological results of the thienothiazinone and the thienopyrimidinone derivatives **18** and **33**) and (iii) the benzyl group bound to the N4 of the piperazine, analogously to the well known N4 benzyl derivatives showing affinity and selectivity for the 5-HT_3 receptor

Table II. Physical and chemical properties of compounds **18**, **19**, **26**–**33**, **43**, and **47**.



Compound	R_1	R_2	X	Y	R_3	Melting point (°C)	Recryst. solvent	I.R. (KBr) ν(NH)	(cm^{-1}) ν(C=O)	Formula
18	CH_3	CH_3	S	/	$CH_2C_6H_5$	183–185	EtOH/dioxane, 57		1655	$C_{19}H_{21}N_3OS_2$
19	$-(CH_2)_4$		S	/	2-$C_4H_3N_2$[a]	219–221	EtOH/dioxane, 38		1660	$C_{18}H_{19}N_5OS_2$
26	CH_3	CH_3	NNH_2	/	$C_6H_4OCH_3$(o)	162–164	Ethyl acetate, 54	3310, 3185	1660	$C_{19}H_{23}N_5O_2S$
27	CH_3	CH_3	NNH_2	/	2-$C_4H_3N_2$ [a]	249–251	EtOH/dioxane, 46	3305, 3210	1665	$C_{16}H_{19}N_7OS$
28	CH_3	CH_3	NNH_2	/	$CH_2C_6H_5$	132–133	Cyclohexane, 30	3290, 3185	1680	$C_{19}H_{23}N_5OS$
29	$-(CH_2)_4-$		NNH_2	/	$C_6H_4OCH_3$(o)	182–184	EtOH/dioxane, 58	3300	1660	$C_{21}H_{25}N_5O_2S$
30	$-(CH_2)_4-$		NNH_2	/	2-$C_4H_3N_2$ [a]	233–235	EtOH/dioxane, 20	3290, 3200	1675	$C_{18}H_{21}N_7OS$
31	$-(CH_2)_4-$		NNH_2	/	$CH_2C_6H_5$	163–165	Ethyl acetate, 38	3290, 3195	1680	$C_{21}H_{25}N_5OS$
32	CH_3	CH_3	NH	/	2-$C_4H_3N_2$ [a]	>310	Dioxane/DMF, 33		1670	$C_{16}H_{18}N_6OS$
33	CH_3	CH_3	NH	/	$CH_2C_6H_5$	261–263	EtOH, 50		1655	$C_{19}H_{22}N_4OS$
43	CH_3	CH_3	NNH_2	CH_2	2-$C_4H_3N_2$ [a]	211	EtOH, 68	3305, 3205	1670	$C_{17}H_{21}N_7OS$
47	CH_3	CH_3	NNH_2	$S(CH_2)_2$	2-$C_4H_3N_2$ [a]	192–194	EtOH/dioxane, 31	3320, 3215	1680	$C_{18}H_{23}N_7OS_2$

[a] 2-Pyrimidinyl.

Table III. ^{1}H-NMR spectra of compounds **8**–**19**, **26**–**33**, **39**–**43** and **47**.

Compound	δ (DMSO-d_6)
8	1.33 (t, $J = 7$ Hz, 3H, CH_3CH_2), 2.21 (s, 6H, $2CH_3$), 3.08 (m, 4H, piperazine H), 3.81 (s, 3H, OCH_3), 4.03 (m, 4H, piperazine H), 4.32 (q, $J = 7$ Hz, 2H, CH_3CH_2), 6.87–7.01 (m, 4H, ArH), 12.16 (s, 1H, NH).
9	1.33 (t, $J = 7$ Hz, 3H, CH_3CH_2), 2.21 (s, 3H, CH_3), 2.23 (s, 3H, CH_3), 3.90 (m, 4H, piperazine H), 4.06 (m, 4H, piperazine H), 4.33 (q, $J = 7$ Hz, 2H, CH_3CH_2), 6.69 (t, $J = 4.6$ Hz, 1H, ArH), 8.41 (d, $J = 4.6$ Hz, 2H, ArH), 12.06 (s, 1H, NH).
10	1.31 (t, $J = 7$ Hz, 3H, CH_3CH_2), 2.20 (s, 6H, $2CH_3$), 2.50 (m, 4H, piperazine H), 3.54 (s, 2H, CH_2), 3.90 (m, 4H, piperazine H), 4.30 (q, $J = 7$ Hz, 2H, CH_3CH_2), 7.31 (m, 5H, ArH), 12.08 (s, 1H, NH).
11	1.32 (t, $J = 7$ Hz, 3H, CH_3CH_2), 1.72 (m, 4H, H-6,7), 2.59 (m, 2H, H-5), 2.73 (m, 2H, H-8), 3.09 (m, 4H, piperazine H), 3.82 (s, 3H, OCH_3), 4.04 (m, 4H, piperazine H), 4.31 (q, $J = 7$ Hz, 2H, CH_3CH_2), 6.91–6.98 (m, 4H, ArH), 12.20 (s, 1H, NH).
12	1.32 (t, $J = 7$ Hz, 3H, CH_3CH_2), 1.72 (m, 4H, H-6,7), 2.60 (m, 2H, H-5), 2.73 (m, 2H, H-8), 3.90 (m, 4H, piperazine H), 4.05 (m, 4H, piperazine H), 4.31 (q, $J = 7$ Hz, 2H, CH_3CH_2), 6.69 (t, $J = 4.6$ Hz, 1H, ArH), 8.41 (d, $J = 4.6$ Hz, 2H, ArH), 12.10 (s, 1H, NH).
13	1.30 (t, $J = 7$ Hz, 3H, CH_3CH_2), 1.71 (m, 4H, H-6,7), 2.57 (m, 2H, H-5), 2.70 (m, 2H, H-8), 3.32 (m, 4H, piperazine H), 3.54 (s, 2H, CH_2), 3.90 (m, 4H, piperazine H), 4.28 (q, $J = 7$ Hz, 2H, CH_3CH_2), 7.33–7.35 (m, 5H, ArH), 12.10 (s, 1H, NH).
14	1.36 (t, $J = 7$ Hz, 3H, CH_3CH_2), 3.12 (m, 4H, piperazine H), 3.82 (s, 3H, OCH_3), 4.08 (m, 4H, piperazine H), 4.37 (q, $J = 7$ Hz, 2H, CH_3CH_2), 6.88–7.68 (m, 6H, thiophene H and ArH), 11.93 (s, 1H, NH).
15	1.18 (t, $J = 7.4$ Hz, 3H, CH_3CH_2), 3.12 (m, 4H, piperazine H), 3.94 (m, 4H, piperazine H), 4.40 (q, $J = 7.4$ Hz, 2H, CH_3CH_2), 6.71 (t, $J = 4.8$ Hz, 1H, ArH), 7.30–7.68 (m, 6H, thiophene H and ArH), 8.43 (d, $J = 4.8$ Hz, 2H, ArH), 11.84 (s, 1H, NH).
16	1.29 (t, $J = 7$ Hz, 3H, CH_3CH_2), 1.35 (t, $J = 7$ Hz, 3H, CH_3CH_2), 2.70 (s, 3H, CH_3), 3.12 (m, 4H, piperazine H), 3.82 (s, 3H, OCH_3), 4.07 (m, 4H, piperazine H), 4.26 (q, $J = 7$ Hz, 2H, CH_3CH_2), 4.37 (q, $J = 7$ Hz, 2H, CH_3CH_2), 6.88–6.98 (m, 4H, ArH), 12.43 (s, 1H, NH).
17	1.29 (t, $J = 7$ Hz, 3H, CH_3CH_2), 1.35 (t, $J = 7$ Hz, 3H, CH_3CH_2), 2.70 (s, 3H, CH_3), 3.93 (m, 4H, piperazine H), 3.82 (s, 3H, OCH_3), 4.08 (m, 4H, piperazine H), 4.26 (q, $J = 7$ Hz, 2H, CH_3CH_2), 4.37 (q, $J = 7$ Hz, 2H, CH_3CH_2), 6.70 (t, $J = 4.8$ Hz, 1H, ArH), 8.42 (t, $J = 4.8$ Hz, 2H, ArH), 12.34 (s, 1H, NH).
18	2.24 (s, 6H, $2CH_3$), 2.49 (m, 4H, piperazine H), 3.52 (s, 2H, CH_2), 3.65 (m, 4H, piperazine H), 7.32–7.34 (m, 5H, ArH).
19	1.76 (m, 4H, H-6,7), 2.65 (m, 2, H-5), 2.77 (m, 2H, H-8), 3.81 (m, 4H, piperazine H), 3.89 (m, 4H, piperazine H), 6.72 (t, $J = 4.6$ Hz, 1H, ArH), 8.43 (d, $J = 4.6$ Hz, 2H, ArH).
26	2.33 (s, 3H, CH_3), 2.38 (s, 3H, CH_3), 3.12 (m, 4H, piperazine H), 3.61 (m, 4H, piperazine H), 3.82 (s, 2H, OCH_3), 5.68 (s, 2H, NH_2), 6.95 (m, 4H, ArH).
27	2.30 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 3.53 (m, 4H, piperazine H), 3.86 (m, 4H, piperazine H), 5.68 (s, 2H, NH_2), 6.67 (t, $J = 4.6$ Hz, 1H, ArH), 8.39 (d, $J = 4.6$ Hz, 2H, ArH).
28	2.31 (s, 3H, CH_3), 2.36 (s, 3H, CH_3), 2.54 (m, 4H, piperazine H), 3.46 (m, 4H, piperazine H), 3.55 (s, 2H, CH_2), 5.62 (s, 2H, NH_2), 7.34 (m, 5H, ArH).
29	1.77 (m, 4H, H-6,7), 2.67 (m, 2H, H-5), 2.83 (m, 2H, H-8), 3.10 (m, 4H, piperazine H), 3.59 (m, 4H, piperazine H), 3.80 (s, 3H, OCH_3), 5.66 (s, 2H, NH_2), 6.89–6.96 (m, 4H, ArH).
30	1.77 (m, 4H, H-6,7), 2.67 (m, 2H, H-5), 2.83 (m, 2H, H-8), 3.55 (m, 4H, piperazine H), 3.87 (m, 4H, piperazine H), 5.69 (s, 2H, NH_2), 6.67 (t, $J = 4.8$ Hz, 1H, ArH), 8.40 (d, $J = 4.6$ Hz, 2H, ArH).
31	1.76 (m, 4H, H-6,7), 2.50 (m, 4H, piperazine H), 2.65 (m, 2H, H-5), 2.82 (m, 2H, H-8), 3.44 (m, 4H, piperazine H), 3.52 (s, 2H, CH_2), 5.60 (s, 2H, NH_2), 7.29–7.34 (m, 5H, ArH).
32	2.25 (s, 3H, CH_3), 2.30 (s, 3H, CH_3), 3.67 (m, 4H, piperazine H), 3.81 (m, 4H, piperazine H), 6.67 (t, $J = 4.6$ Hz, 1H, ArH), 8.39 (d, $J = 4.6$ Hz, 2H, ArH).
33	2.26 (s, 3H, CH_3), 2.30 (s, 3H, CH_3), 3.10 (m, 4H, piperazine H), 4.33 (m, 6H, piperazine H and CH_2), 7.47–7.59 (m, 5H, ArH).
39	1.30 (t, $J = 7$ Hz, 3H, CH_3CH_2), 2.21 (s, 3H, CH_3), 2.24 (s, 3H, CH_3), 2.59 (m, 4H, piperazine H), 3.31 (s, 2H, CH_2), 3.83 (m, 4H, piperazine H), 4.30 (q, $J = 7$ Hz, 2H, CH_3CH_2), 6.65 (t, $J = 4.6$ Hz, 1H, ArH), 8.37 (d, $J = 4.6$ Hz, 2H, ArH), 12.14 (s, 1H, NH).
40	1.31 (t, $J = 7$ Hz, 3H, CH_3CH_2), 2.21 (s, 3H, CH_3), 2.24 (s, 3H, CH_3), 2.53 (m, 4H, piperazine H), 2.69 (m, 4H, CH_2CH_2), 3.84 (m, 4H, piperazine H), 4.27 (q, $J = 7$ Hz, 2H, CH_3CH_2), 6.63 (t, $J = 4.6$ Hz, 1H, ArH), 8.37 (d, $J = 4.6$ Hz, 2H, ArH), 11.75 (s, 1H, NH).
41	1.31 (t, $J = 7.2$ Hz, 3H, CH_3CH_2), 1.80 (m, 2H, $CH_2CH_2CH_2$), 2.18 (s, 3H, CH_3), 2.20 (s, 3H, CH_3), 2.36 (m, 6H, CH_2N and piperazine H), 2.52 (t, $J = 6.8$, 2H, $COCH_2$), 3.65 (m, 4H, piperazine H), 4.29 (q, $J = 7.2$ Hz, 2H, CH_3CH_2), 6.60 (t, $J = 4.6$ Hz, 1H, ArH), 8.33 (d, $J = 4.6$ Hz, 2H, ArH), 10.97 (s, 1H, NH, exchanges with D_2O).
42	1.31 (t, $J = 7$ Hz, 3H, CH_3CH_2), 1.52 (m, 2H, CH_2), 1.64 (m, 2H, CH_2), 2.19 (s, 3H, CH_3), 2.22 (s, 3H, CH_3), 2.28–2.56 (m, 8H, CH_2N, piperazine H and $COCH_2$), 3.69 (m, 4H, piperazine H), 4.28 (q, $J = 7$ Hz, 2H, CH_3CH_2), 6.61 (t, $J = 4.6$ Hz, 1H, ArH), 8.34 (d, $J = 4.6$ Hz, 2H, ArH), 10.96 (s, 1H, NH).
43	2.36 (s, 3H, CH_3), 2.41 (s, 3H, CH_3), 2.57 (m, 4H, piperazine H), 3.75 (m, 6H, CH_2N and piperazine H), 6.18 (s, 2H, NH_2, exchanges with D_2O), 6.62 (t, $J = 4.6$ Hz, 1H, ArH), 8.35 (d, $J = 4.6$ Hz, 2H, ArH).
47	2.28 (s, 3H, CH_3), 2.32 (s, 3H, CH_3), 2.45 (m, 4H, piperazine H), 2.60 (t, $J = 6.8$ Hz, 2H, CH_2N), 3.13 (t, $J = 6.8$ Hz, SCH_2), 3.68 (m, 4H, piperazine H), 5.64 (s, 2H, NH_2), 6.56 (t, $J = 4.6$ Hz, 1H, ArH), 8.30 (*d*, $J = 4.6$ Hz, 2H, ArH).

Table IV. Affinity of compounds **8**–**19**, **26**–**33**, **39**–**43**, **47** and of two reference compounds on 5-HT_3 and 5-HT_4 serotonin receptors.

	K_i (nM) ($\pm$ S.D.)[a]	
Compound	5-HT_3 rat cortex [^{3}H] Zacopride	5-HT_4 guinea pig striatum [^{3}H] GR 113808
8	N.A.[b]	N.A.[b]
9	N.A.[b]	N.A.[b]
10	N.A.[b]	N.A.[b]
11	N.A.[b]	N.A.[b]
12	N.A.[b]	N.A.[b]
13	N.A.[b]	N.A.[b]
14	N.A.[b]	N.A.[b]
15	N.A.[b]	N.A.[b]
16	N.A.[b]	N.A.[b]
17	N.A.[b]	N.A.[b]
18	377 ± 60	N.A.[b]
19	N.A.[b]	N.A.[b]
26	N.A.[b]	N.A.[b]
27	N.A.[b]	N.A.[b]
28	3.92 ± 0.88	N.A.[b]
29	N.A.[b]	N.A.[b]
30	N.A.[b]	N.A.[b]
31	2035 ± 240	N.A.[b]
32	N.A.[b]	N.A.[b]
33	265 ± 89	N.A.[b]
39	N.A.[b]	332 ± 27
40	N.A.[b]	564 ± 36
41	N.A.[b]	81.3 ± 9
42	N.A.[b]	118 ± 30
43	N.A.[b]	N.A.[b]
47	3106 ± 261	N.A.[b]
Quipazine	0.80 ± 0.1	
Serotonin	354 ± 97	79.3 ± 10

[a] K_i values were derived from the IC_{50} values, determined by dose-inhibition curves with seven concentrations of the displacers each performed in triplicate, according to the method of Cheng and Prusoff [20].
[b] $<50\%$ inhibition at 10^{-5} M.

[9], may act as determinants for selectivity. The N4 orthomethoxyphenyl and the N4 pyrimidine derivatives **26** and **27** do not show an affinity for either the $5HT_3$ or the $5HT_4$ receptors.

The structural feature which differentiates the most active and selective 5-HT_4 ligands **39**–**42** between them is the length of the amidomethylenic chain linking the thiophene to the piperazinylpyrimidine system. The derivative with higher affinity and selectivity (also inactive to either 5-HT_{1A} or α_1A receptors) for the 5-HT_4 receptor is the 2-[4-[4-(2-pyrimidinyl)-l-piperazinyl]-butanoylamino] - 4,5 - dimethyl - 3 - thiophenecarboxylic acid ethyl ester **41** ($K_i = 81.3$ nM), followed by the pentanoylamino derivative **42** ($K_i = 118$ nM), ethanoylamino **39** ($K_i = 332$ nM) and propanoylamino **40** ($K_i = 564$ nM).

Further research is currently in progress to improve the affinity of these *chemotypes* for the 5-HT_3 and 5-HT_4 receptors.

4.2. Conformational analysis

The spatial disposition of the above listed potential pharmacophoric functionalities as adopted by compound **41** ($n = 3$), showing the highest level of receptor affinity was first investigated and then considered to characterise the 'active conformation(s)' by which the molecule might interact at the receptor site both in its neutral (*figure 5*) or charged state (*figure 6*). The latter, which most likely is the bioactive form, is assumed to be due to the protonation of the piperazine N1. Charged forms as bioactives are well documented for biomolecules with ionizable basic groups. Evidence resulting from the computational studies reported below might further support this view. Assuming the piperazine ring of the molecule as charged on the N1 atom at the pH of the biological experiment, a planar geometry allowing the formation of a seven terms *pseudo-cycle* was identified in the most active compounds **41** and **42**; the *pseudo-cycle* includes the protonated piperazine nitrogen and the opposite amide oxygen atom in the side chain as *head–tail* respectively, and might act as a *conformational selector* for favourable/unfavourable conformations in the molecule binding to the receptor.

All the most active 5-HT_4R ligands (**41** and **42**) show one energetically favourable planar low energy conformation (15 conformers within 1 kcal mol^{-1}) with $2.4 \pm 0.5°$ as the dihedral angle (τ) defined by the S1–C2–Nam–Cam atom sequence. The less active derivatives **39** and **40** show a more distorted geometry as measured by a dihedral angle (τ) of $8.00 \pm 0.6°$.

The above planar conformation allows the *anti* C4 carbonyl-amide NH groups in the side chain of compounds **39**–**42** to strongly hydrogen bond (2.000 ± 0.1 Å) forming a six atoms *pseudo-cycle* which contributes to make the molecule planar. A second hydrogen bond (1.72 ± 0.06 Å) between the protonated piperazine nitrogen and the opposite amide oxygen atom in the side chain of compound **41** ($n = 3$) constrains the molecule to form a seven terms pseudo-cycle which favours the chair conformation of the piperazine ring (*figure 6*). This fact might contribute to the optimal spatial arrangement of

the pyrimidine ring thus emphasising the possible role of heteroaromatic interactions in the ligand-binding process.

Compound **42** ($n = 4$) showed the additional seven terms hydrogen bond only when calculations were carried out in vacuo, thus suggesting that the steric hindrance might mainly affect the activity (exceeding molecular *included* volume) [16] depicting the ligand receptor interaction in a hydrophobic environment and allowing the protonated piperazine nitrogen and the amide oxygen to interact, without the 'breaking off' due to the solvation effects.

X-rays studies are currently in progress to complement the computational results on a possible low energy boat conformation as detected for the protonated form of compound **42** ($n = 4$). Provided that false minima might come out from the calculation itself, it may be further speculated that compound **42** should reach the receptor in an unfavourable boat conformation which precludes it from interacting at all with the receptor binding site.

The additional *pseudocycle*, formed by six terms instead of seven as for compound **41**, negatively affects the activity of compound **40** ($n = 2$) making its structure too rigid and therefore unfavourably locating the pyrimidine ring and precluding the ligand from key interactions in the ligand–receptor binding (*figure 7*).

The better activity of compound **39** ($n = 1$) when compared to compound **40** ($n = 2$) might be due to a more pronounced rotational freedom of the side chain lacking the additional hydrogen bond. It may allow the compound to reach a bioactive conformation close to that of compound **41** ($n = 3$), however, the shorter chain might preclude this ligand from substantial key interactions.

Considering the molecules in their neutral form (in accordance with a suggestion during the review process) the overall view doesn't change. Likewise, their protonated counterparts all the examined compounds **39**–**42** are characterised by a six atoms *pseudocycle* formed by the hydrogen bonding between the sp^3 oxygen of the ester and the amide NH in the side chain (2.000 ± 0.1 Å), as reported above. The most active compounds **41**, **42** and the *less inactive* compound **39** show a common 'U-shape' conformation (15 conformers within 2 kcal mol^{-1}) with a differently distorted piperazine ring and stabilised by thiophene-phenyl π cloud interactions. This conformation is stable in aqueous conditions and might allow the proper location of the potential pharmacophoric groups. The strong intramolecular interaction between the two aromatic rings should preclude

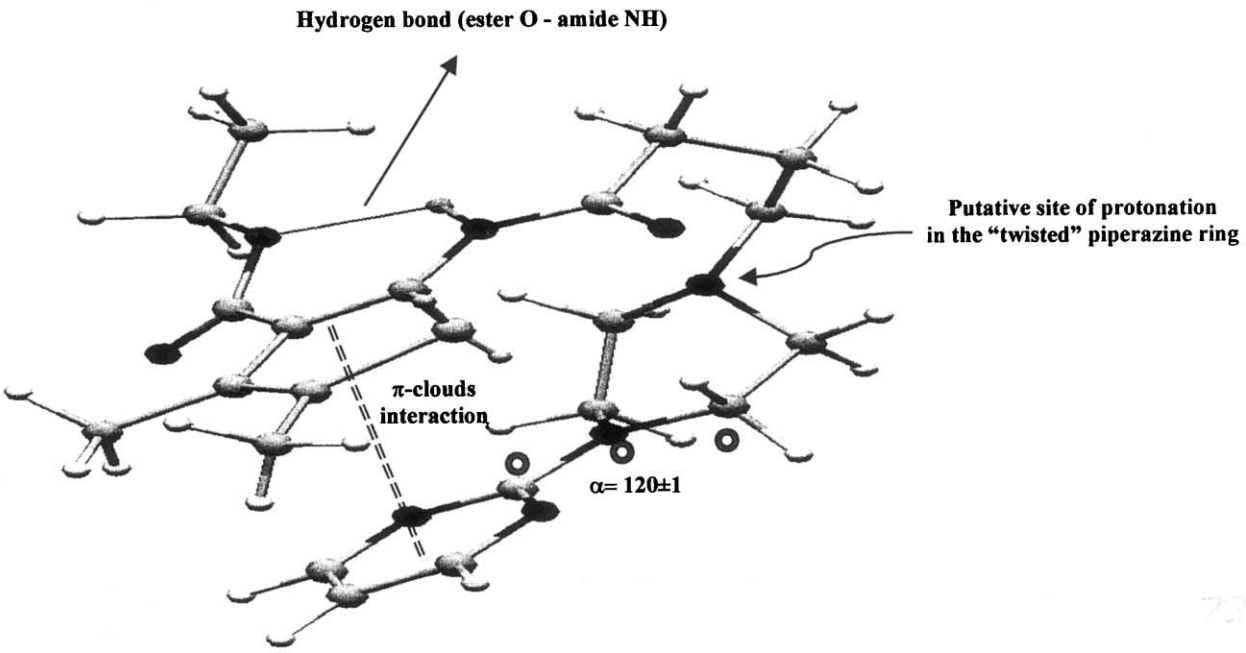


Figure 5. *U-shape* conformation of compound **41** *in vacuo* (see text for explanation). The piperazine ring is in a twisted boat conformation.

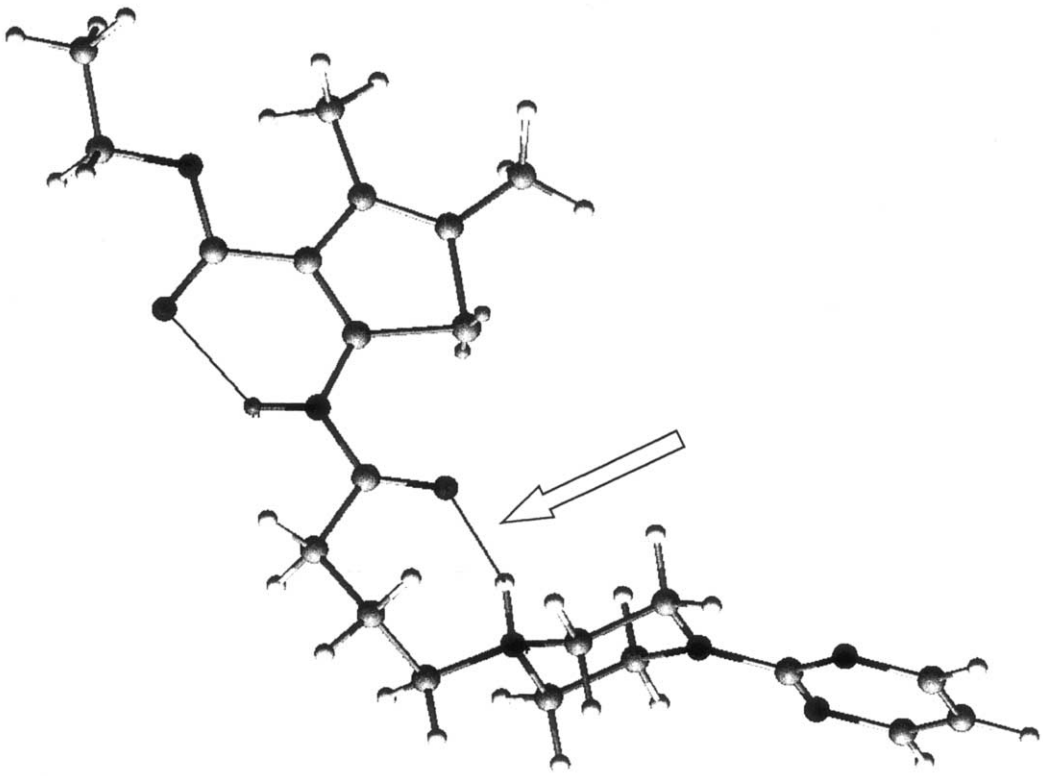


Figure 6. Ball-and-stick representation of compound **41** ($n = 3$) lowest energy conformation in the charged (protonated) form. Hydrogen bonds are displayed by dotted lines. Arrow points to the additional hydrogen bond (see text for explanation).

the molecules from establishing electronic interactions with the receptor.

Compound **40** shows only *in vacuo* the above reported 'U-shape' geometry with a strongly distorted piperazine ring. The resulting high energy unstable 'U-shape conformer', visual inspection of which clearly shows a decreased overlapping of the two aromatic rings, collapses to an unfolded planar geometry under the solvation energy effects, when the conformational search is carried out in aqueous conditions.

Comparing the two states neutral versus protonated, it can be supposed that the hydrogen bond between the protonated piperazine nitrogen (N1) and the opposite amide oxygen, therefrom the resulting additional virtual cycle, stabilises the piperazine ring in a chair conformation which affects the shape of the pendant moiety of the molecules and strongly conditions the biological activity, whereas, the π cloud interactions between the aromatic rings (thiophene–phenyl) are responsible for the stabilisation of the neutral form.

The phenyl substituted nitrogen of the piperazine ring in the neutral form, likewise the positively charged form, gives an angle of 120° degrees with the phenyl group so showing a strong sp^2 character (electron delocalization with the phenyl ring) and further supporting the N1 piperazine nitrogen as the putative site of protonation at a physiological pH of 7.4 as in the biological assay.

5. Conclusions

The synthesis and the binding tests of new thienopyrimidopiperazine and piperazinylacylamino-dimethylthiophene derivatives acting as selective ligands at the 5-HT_3 and 5-HT_4 receptors have been reported. The 3-amino-2-(4-benzyl-1-piperazinyl)-5,6-dimethyl - thieno[2,3 - *d*]pyrimidin - 4(3*H*) - one (**28**) showed higher affinity and selectivity for the 5-HT_3 over the 5-HT_4 receptor. It acts as a full agonist at the 5-HT_3 receptor in the Bezold-Jarisch reflex [unpublished data] and has been chosen by us as a lead for

further research on ligands with affinity and selectivity towards the 5-HT_3 receptor (besides being studied in terms of 3D-QSAR).

The compound with higher affinity and selectivity for the 5-HT_4 over the 5-HT_3 receptor was the 2-[4-[4-(2 - pyrimidinyl) - 1 - piperazinyl]butanoylamino] - 4,5-dimethyl-3-thiophenecarboxylic acid ethyl ester (**41**). Conformational analyses were carried out on compounds of the piperazinylacylaminodimethylthiophene series (**39**–**42**), acting as ligands at the 5-HT_4 receptor.

Basing on the computational result, it might be speculated on a dynamic process of interaction by a change in the intramolecular stabilising interaction from the *electronic* (thiophene-phenyl π cloud interactions), as in the uncharged form, to an *electrostatic* (H bond) one as it is present in the protonated form, and therefore a conformational change from a distorted *boat–twisted* conformation to an active chair conformation.

The protonation might act as a *switching on* mechanism from the silent to the active form. Compound **40** is unable to reach the optimal conformational requirements either in the neutral or in the charged form, whereas, compound **39** shows the putative optimal prerequisites when modelled as uncharged/charged, but its action is probably limited for the lack of key interactions.

6. Experimental protocols

6.1. Chemistry

Melting points were determined in open capillary tubes on a Gallenkamp m.p. apparatus and are uncor-

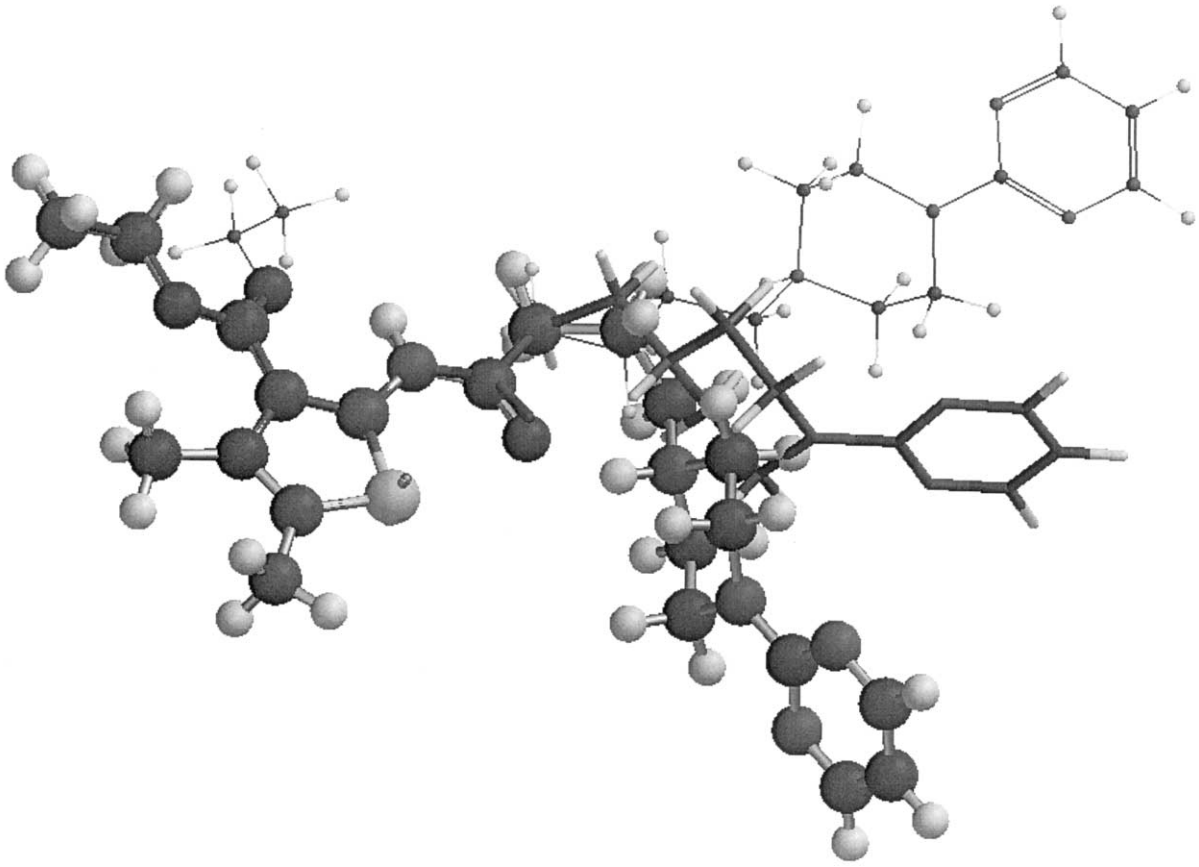


Figure 7. Superposition of compounds **40** ($n = 2$) (tube), **41** ($n = 3$) (ball-and-spoke) and **42** ($n = 4$) (ball-and-wire) lowest energy conformers in the charged (protonated) form, showing the different spatial arrangement of the piperazine ring.

rected. Elemental analyses for C, H, N, and S were obtained on an EA1108 Elemental Analyser Fisons-Carlo Erba instrument and were within 0.4% of the theoretical values. The IR spectra were recorded with FTIR Perkin–Elmer 1600 spectrometer in KBr disks. ^{1}H-NMR spectra were obtained at 200 MHz on a Varian Inova-Unity 200 spectrometer in DMSO-d_6 solution and are expressed as δ units (ppm) relative to TMS as the internal standard; coupling constants (J) are in Hertz. Signal multiplicities are presented by s (singlet), d (doublet), t (triplet), q (quartet), br s (broad singlet), and m (multiplet). The purity of the compounds was checked by thin-layer chromatography (TLC) on Merck silica gel 60 F-254 plates.

*6.1.1. General procedure for compounds **8**–**17***

To a solution of the appropriate isothiocyanate (**1**–**4**) (4.15 mmol) in chloroform (15 mL) substituted piperazine **5** or **6** (5.3 mmol) was added. A suspension of the 1-(2-pyrimidinyl)piperazine dihydrochloride (**7**) (1.25 g, 5.3 mmol) and triethylamine (2 mL) in chloroform (15 mL) was added for derivatives **9**, **12**, **15** and **17**. The mixture was heated under reflux for 6–8 h. After cooling, the solvent was removed under reduced pressure and the sticky residue was collected, washed with ethanol, dried and recrystallised by a suitable solvent. After cooling, compounds **12** and **15**, were obtained as a solid (*table I*).

*6.1.2. General procedure for compounds **18** and **19***

Compounds **10** and **12** (2 mmol), were stirred for 10 min in concentrated sulfuric acid (14 mL) and kept at room temperature (r.t.) for 2 and 6 days, respectively. The solution was than poured into cold water and neutralised with a 10% NaOH solution. The solid was collected, washed with water, dried and recrystallised by a suitable solvent (*table II*).

*6.1.3. General procedure for compounds **22** and **23***

To a suspension of the monopotassium salt **20** or **21** (9.6 mmol) in water (100 mL), methyl iodide (1.8 mL, 28.9 mmol) was added and the mixture was stirred at r.t. for 0.5–1 h. The solid was collected, washed with water, dried and recrystallised by a suitable solvent.

*6.1.3.1. 3-Amino-2-(methylthio-6-phenyl-thieno[2,3-d]-pyrimidin-4(3H)-one (**22**)*

Recrystallised from ethanol–dioxane, yield: 2 g, 72%; m.p. 213–215°C; IR (KBr) 3310 and 3205 (NH), 1685 (C=O) cm^{-1}.^{1}H-NMR (DMSO-d_6) δ 2.43 (s, 3H, SCH_3), 5.83 (s, 2H, NH_2), 7.36–7.77(m, 6H, ArH and thiophene H). Anal. ($C_{13}H_{11}N_3OS_2$) C, H, N, S.

*6.1.3.2. 5-Amino-3-methyl-6-(methylthio)-5H-thieno-[2,3-d]pyrimidin-4-one-2-carboxylic acid methyl ester (**23**)*

Recrystallised from ethanol–dioxane, yield: 1.5 g, 52%; m.p. 217–219°C; IR (KBr) 3315 and 3215 (NH), 1725 and 1650 (C=O) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ 1.30 (t, $J = 7$ Hz, 3H, CH_3CH_2), 2.42 (s, 3H, CH_3), 2.81 (s, 3H, SCH_3), 4.29 (q, $J = 7$, 2H, CH_3CH_2), 5.77 (s, 2H, NH_2). Anal. ($C_{11}H_{,3}N_3O_35_2$) C, H, N, S.

*6.1.4. General procedures for compounds **24** and **25***

A mixture of the appropriate derivative (**14**–**17**) (1.73 mmol) and hydrazine monohydrate (3.4 mL, 68 mmol) in ethanol (5 mL) was boiled under reflux for 12 h. After cooling, the solid was collected, washed with ethanol, dried and recrystallised. From derivatives **14** and **15** compound **24** was isolated, while **16** and **17** produced compound **25**. The same products were also isolated when the refluxing time was reduced (2 h) or a lower amount of hydrazine monohydrate was used.

Compounds **24** and **25** can be also obtained by reaction of methylthio derivative **22** or **23** (3.46 mmol) and hydrazine monohydrate (8 mL) boiled under reflux for 6 h in 2-propanol (15 mL), with higher yields.

*6.1.4.1. 3-Amino-2-hydrazino-6-phenyl-(1H,3H)-thieno-[2,3-d]pyrimidine-2,4-dione (**24**)*

Recrystallised from ethanol–dioxane, yield: 0.15 g, 31%; m.p. 258–260°C; IR (KBr) 3310 and 3205 (NH), 1675 (C=O) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ 4.44 (s, 2H, NH_2), 5.40 (s, 2H, NH_2), 7.24–7.68 (m, 6H, ArH and thiophene H), 8.47 (s, 1H, NH). Anal. ($C_{12}H_{11}N_5OS$) C, H, N, S.

*6.1.4.2. 5-Amino-6-hydrazino-3-methyl-5H,7H-thieno-[2,3-d]pyrimidin-4,6-dione-2-carboxyhydrazide (**25**)*

Recrystallised from dioxane, yield: 0.2 g, 43%; m.p. 284–86°C dec; IR (KBr) 3295 and 3205 (NH), 1685 (C=O) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ 2.63 (s, 3H, CH_3), 4.45 (s, 4H, NH_2), 5.32 (s, 2H, NH_2), 8.46 (s, 1H, NH), 9.22 (s, 1H, NH). Anal. ($C_8H_{11}N_7O_2S$) C, H, N, S.

*6.1.5. General procedure for compounds **26**–**31***

The procedure used was the same as for compounds **24** and **25** from derivatives **14**–**17**. A mixture of the appropriate derivative (**8**–**13**) (1.73 mmol) and hydrazine monohydrate (3.4 mL, 68 mmol) in ethanol (5 mL)

was boiled under reflux for 2 h (compounds **10** and **13**) or 12 h (compounds **8**, **9**, **11**, **12**). After cooling, the solid was collected, washed with ethanol, dried and recrystallised by a suitable solvent (*table II*).

*6.1.6. General procedure for compounds **32** and **33***

To a suspension of derivatives **27** and **28** (2.1 mmol), respectively, in 6 N hydrochloric acid (15 mL) an aqueous sodium nitrite solution (0.73 g, 10.58 mmol in 22 mL of water) was added dropwise with stirring at 0°C. The mixture was stirred at r.t. for 15 min and the collected solid was poured into water. The suspension was added with some drops of a 10% NaOH (p/v) solution and the solid was then collected, washed with water, dried and recrystallised by a suitable solvent (*table II*).

*6.1.7. General procedure for compounds **36** and **37***

A mixture of the amino ester **34** (20 mmol) and 3-chloropropionyl chloride or 4-chlorobutyryl chloride (20 mmol) was heated under reflux in chloroform (40 mL) for 2 h. After cooling, the solution was washed with water, and the combined extracts dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure to give a solid, that was used for the next step without further purification.

*6.1.7.1. 2-[(Chloropropionyl)amino]-4,5-dimethyl-3-thiophenecarboxylic acid ethyl ester (**36**)*

Yield: (3.3 g, 57%). m.p. 80–82°C; IR (KBr) 3245 (NH), 1705 and 1655 (C=O) cm^{-1} ^{1}H-NMR (DMSO-d_6) δ 1.32 (t, $J = 7$ Hz, 3H, CH_3CH_2), 2.19 (s, 3H, CH_3), 2.23 (s, 3H, CH_3), 3.03 (t, $J = 6.2$ Hz, 2H, $COCH_2$), 3.88 (t, $J = 6.2$ Hz, 2H, CH_2Cl), 4.30 (q, $J = 7$ Hz, 2H, CH_3CH_2), 10.99 (s, 1H, NHCO). Anal. ($C_{12}H_{16}C1NO_3S$) C, H, N, S.

*6.1.7.2. 2-[(Chlorobutyryl)amino]-4,5-dimethyl-3-thiophenecarboxylic acid ethyl ester (**37**)*

Yield: (1.8 g, 29%). m.p. 45–47°C; IR (KBr) 3300 and 3220 (NH), 1655 broad (C=O) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ 1.32 (t, $J = 7.2$ Hz, 3H, CH_3CH_2), 2.07 (m, 2H, CH_2), 2.17 (s, 3H, CH_3), 2.20 (s, 3H, CH_3), 2.64 (t, $J = 6.6$ Hz, 2H, $COCH_2$), 3.69 (t, $J = 6.6$ Hz, 2H, CH_2Cl), 4.28 (q, $J = 7$ Hz, 2H, CH_3CH_2), 10.96 (s, 1H, NHCO). Anal. ($C_{13}H_{18}C1NO_3S$) C, H, N, S.

*6.1.8. General procedure for compounds **39** and **42***

A mixture of the appropriate chloroderivative (**35**–**38**) (6.6 mmol), 1-(2-pyrimidinyl)piperazine dihydrochloride (1.6 g, 6.9 mmol) and potassium carbonate (1.9 g, 13.8 mmol) was boiled under reflux for 2 h in dimethylformamide (20 mL). After cooling, the solid was collected and the solvent was evaporated under reduced pressure. The solid obtained (compounds **39** and **40**) was collected, washed with water, dried and recrystallised by a suitable solvent. The sticky product obtained (compounds **41** and **42**) was purified by column chromatography (1:1, cyclohexane–ethyl acetate) (*table II*).

*6.1.9. 3-Amino-2-[1-[4-(2-pyrimidinyl)-1-piperazinyl]-methyl]-5,6-dimethyl-thieno[2,3-d]pyrimidin-4(3H)-one (**43**)*

A mixture of compound **39** (0.75 g, 1.86 mmol) and hydrazine monohydrate (1.8 mL, 36 mmol) was boiled under reflux for 24 h in 1-propanol (4.5 mL). After cooling, the solid was collected, washed with ethanol, dried and recrystallised by a suitable solvent (*table II*).

*6.1.10. 1-(2-Chloroethyl)-4-(2-pyrimidinyl)piperazine (**45**)*

A mixture of 1-(2-pyrimidinyl)piperazine dihydrochloride (**44**) (1.5 g, 6.3 mmol), 1-bromo-2-chloroethane (0.58 mL, 6.97 mmol) and K_2CO_3 (2.6 g, 18.9 mmol) was stirred at r.t. in dimethylformamide (8 mL) for 22 h. The solid was collected and the solvent was evaporated under reduced pressure. The sticky product was purified by column chromatography (7.5:2.5, ethyl acetate–MeOH) to give compound (**45**) (0.3 g, 20%). m.p. 61–63°C; ^{1}H-NMR (DMSO-d_6) δ 2.47 (m, 4H, piperazine H), 2.68 (t, $J = 6.6$ Hz, 3H, CH_2N), 3.74 (m, 6H, CH_2Cl and piperazine H), 6.62 (t, $J = 4.8$ Hz, 1H, ArH), 8.35 (d, $J = 4.8$ Hz, 2H, ArH). Anal. ($C_{10}H_{15}ClN_4 \cdot 1/2H_2O$) C, H, N, S.

*6.1.11. 3-Amino-2-[[2-[4-(2-pyrimidinyl)-1-piperazinyl]-ethyl]thio]-5,6-dimethyl-thieno[2,3-d]pyrimidin-4(3H)-one (**47**)*

A mixture of potassium salt **46** (1.16 g, 3.6 mmol) and 1-(2-chloroethyl)-4-(2-pyrimidinyl)piperazine (**45**) (1.16 g, 4.32 mmol) was boiled under reflux for 6 h in ethanol (15 mL). The suspension was cooled, the solid was collected, washed with ethanol, dried and recrystallised by a suitable solvent (*table II*).

6.2. Pharmacology

6.2.1. In vitro binding assays

Male CRL:CD(SD)BR-COBS rats (about 150 g, Charles River, Italy) and male CRL:(HA)BR albino

guinea pigs (about 300 g, Charles River, Italy) were killed by decapitation; their brains were rapidly dissected into the various 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.

Tissues were homogenised in 50 vol. of ice-cold Tris HCl, 25 mM, pH 7.4 for 5-HT_3 or Hepes HCl, 50 mM, pH 7.4, for 5-HT_4, using an Ultra Turrax TP-1810 homogeniser (2×20 s), and homogenates were centrifuged at 50 000xg 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 000xg 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, 50 mM, pH 7.4, containing 10 μM pargyline for 5-HT_4 and Tris HCl, 25 mM, pH 7.4, containing 10 μM pargyline for 5-HT_3) just before the binding assay.

[^{3}H]Zacopride (*figure 1*) [17] (SA 85.0 Ci/mmol Amersham, for 5-HT_3) binding was assayed in a final incubation volume of 0.5 mL, consisting of 0.25 mL of tissue (10 mg/sample), 0.25 mL of the [^{3}H]ligand (0.4 nM) and 0.01 mL of displacing agent or solvent, non-specific binding was measured in presence of 1 μM quipazine. [^{3}H]GR 113808 (*figure 1*) [18] (SA 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, non-specific binding was measured in the presence of 10 μM serotonin. Incubations (30 min at 25°C) were 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, 25 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 LKB1214 RACKBETA liquid scintillation spectrometer with a counting efficiency of about 60%. Drugs were tested in triplicate at different concentrations (from 10^{-5} to 10^{-10} M) and dose-inhibition curves were analysed by the ALLFIT [19] 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 [20]. 5-HT_{1A} and α_1A receptors binding assays were performed as previously described [1].

6.3. Molecular modeling

6.3.1. Conformational analysis

Starting structures were built using the fragment library within MACROMODEL 6.5 [10, 11]. All calculations were performed on a Silicon Graphics O2 R/5000 workstation running IRIX 6.5.6.

Conformational analyses were performed with MACROMODEL version 6.5 using the Monte Carlo Multiple Minimum Search protocol. All the active ligands were considered both in their neutral and protonated, positively charged forms. Because quaternisation can be tolerated, it may be assumed that the basic piperazine center is binding in its protonated form to 5-HT_4 receptor, although the basicity of the basic centre is not critical.

All minimisations were performed using the AMBER* force-field as implemented in MACROMODEL 6.5 using the GB/SA continuum water model to check for the hydrogen bond stability.

Prior to submitting the ligands to the Monte Carlo protocol, a minimisation was carried out using the AMBER* force field and the GB/SA continuum water model. Default options were used with the Full Matrix Newton–Raphson (FMNR) minimiser, allowing for a maximum of 500 iterations per structure, until a gradient of 0.05 kJ/Å-mol was reached.

To search the conformational space, 5000 MC steps were performed on each starting conformation.

Least squares superposition of all non-hydrogen atoms was used to eliminate duplicate conformations.

Considering the high flexibility of the ligands under investigation, an energy cut-off of 25.0 kJ mol^{-1}, high enough to map the conformational space including the bioactive conformation, was applied to the search results.

7. Note added in proof

Ab initio studies (RHF/STO3G) carried out during the publication process of this manuscript by SPARTAN 5.1.3 [21] have confirmed the reliability of using the MM (Molecular Mechanics) module as implemented in MACROMODEL.

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