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Original article

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 compound with higher affinity and selectivity for the 5-HT_3 over the 5-HT_4 receptor was the 3-amino-2-(4-benzyl-1-piperazinyl)-5,6-dimethyl-thieno[2,3-*d*]pyrimidin-4(3*H*)-one **28** (5-HT_3 $K_i = 3.92$ nM, 5-HT_4 not active), 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** (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. © 2000 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 recently been reported [4–6].

At present, our interest is focused on 5-HT_3 and 5-HT_4 receptors too.

Of the serotonin receptors, 5-HT_3 is the only one belonging to the ligand-gated ion channel receptor family; 5-HT_3 antagonists are used as antiemetic 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 with adenylate cyclase, first identified in 1988 and recently cloned [8], is localized in gastrointestinal, atrial and urinary bladder tissue, as well as in the central nervous system (CNS). Therapeutic applications, such as the treatment of irritable bowel syndrome, atrium arrhythmia

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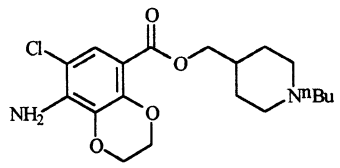
and urinary incontinence are proposed for the antagonists to the 5-HT_4 receptor, while the use of agonists (as prokinetic drugs) is suggested in the treatment of gastrointestinal motility disorders such as gastro-oesophageal reflux, functional dyspepsia and constipation.

Despite being so different, the same structural elements for the 5-HT_3 receptor characterize 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.

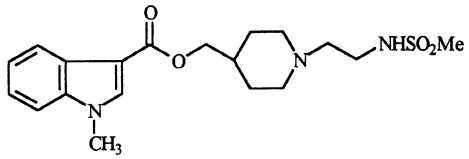
This work reports the synthesis, binding tests and molecular modelling of new derivatives A and B (*figure 1*), bearing the three key pharmacophoric elements, in order to identify potent and selective ligands for each receptor.

The thienopyrimidothioalkylpiperazine system was chosen because the compound **A**, with $R_1 = R_2 = CH_3$; R_3 = 2-pyrimidinyl, $X = NNH_2$, $Y = S(CH_2)_3$, shown in our earlier work on high-affinity and selective 5-HT_{1A} receptor ligands [1], has a good affinity for the 5-HT_3 receptor ($IC_{50} = 20$ nM).

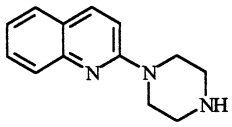
The structural modifications referred to this compound chosen as the lead were, the introduction in the R_1 and R_2 positions of a tetramethylene chain instead of two methyls and in the R_3 position of the



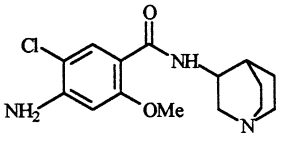
SB 204070



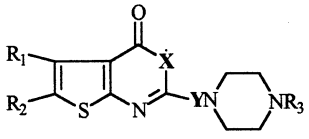
GR 113808



Quipazine

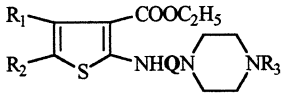


Zacopride



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**.

orthomethoxyphenyl and the benzyl groups instead of the 2-pyrimidinyl, the replacement of the NNH_2 group with the NH or S groups, together with the shortening and the elimination of the thioalkyl chain.

The compounds with the piperazine nucleus directly bound to the heterocyclic system, are related to quipazine (*figure 1*) and its derivatives, potent ligands for the 5-HT_3 receptor [9].

The compounds of type **B** (*figure 1*) were prepared and tested because the flexibility of structure might influence the affinity and selectivity for the 5-HT_4 receptor over the 5-HT_3 receptor [8]. On the most active compounds for the 5-HT_4 receptor (see Section 4), we carried out studies of conformational analysis.

A conformational analysis using the Monte Carlo method as implemented in Macromodel (version 6.5) [10, 11] was used to obtain the main conformational and pharmacophoric requirements for receptor binding taking compound **41** as the template, 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 in the same

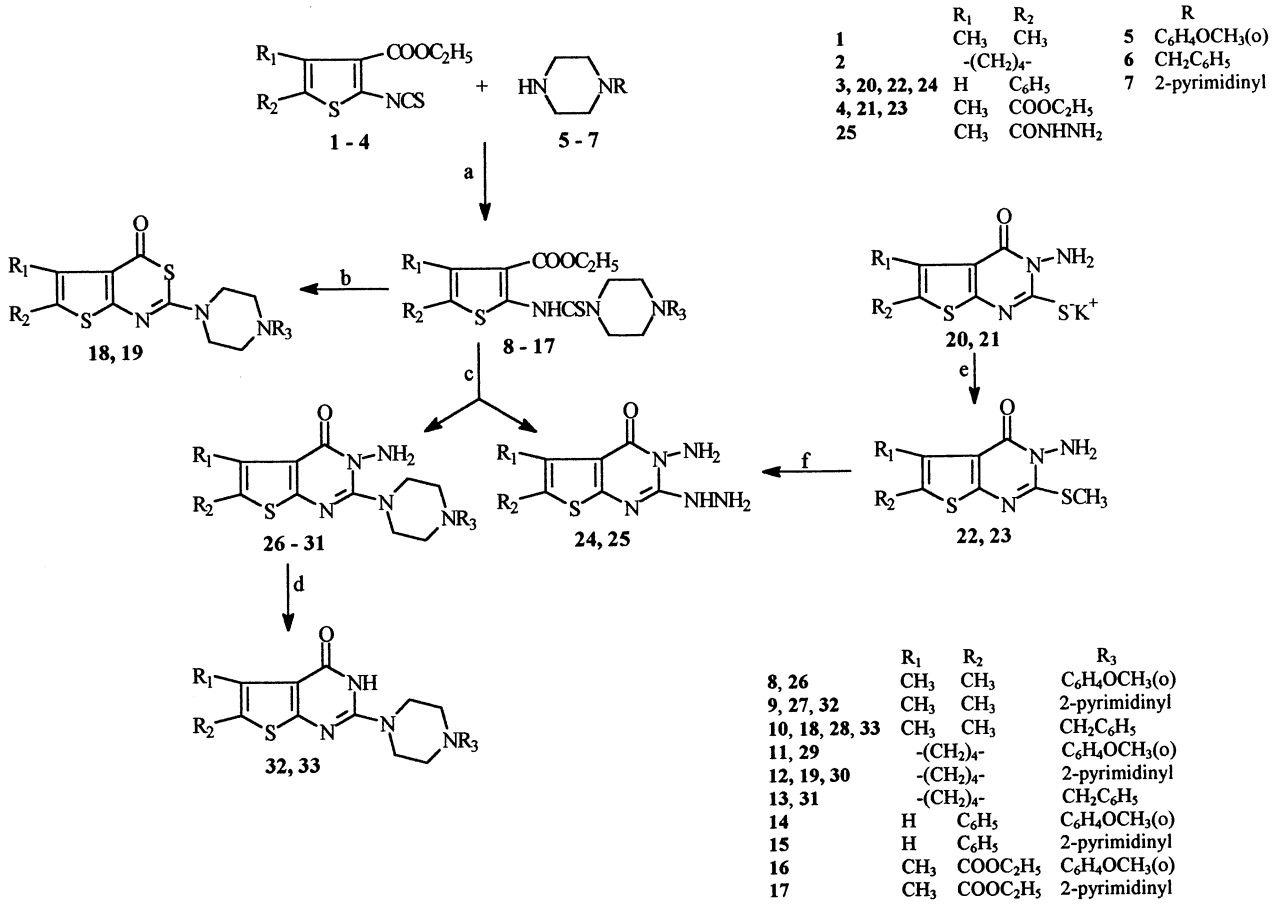


Figure 2. Synthetic procedure to compounds **18**, **19**, **24**–**33**. Reagents and conditions; (a) $CHCl_3$, reflux; (b) H_2SO_4 conc., r.t.; (c) $N_2H_4.H_2O$, EtOH, reflux; (d) HCl, $NaNO_2$, r.t.; (e) CH_3I, H_2O, r.t.; (f) $N_2H_4.H_2O$, 2-propanol, reflux.

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 were reported in section *figure 2*).

In the compounds **32** and **33**, the N3 amino group of the derivatives **27** and **28** was removed with an aqueous sodium nitrite solution and hydrochloric acid.

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

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-*d*]pyrimidinone **47** was prepared from the monopotassium salt **46** [1] and chloroethylpyrimidinylpiperazine **45**, which was obtained from 1-(2-pyrimidinyl)piperazine dihydrochloride **44** and 1-bromo-2-chloroethane (*figure 4*).

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

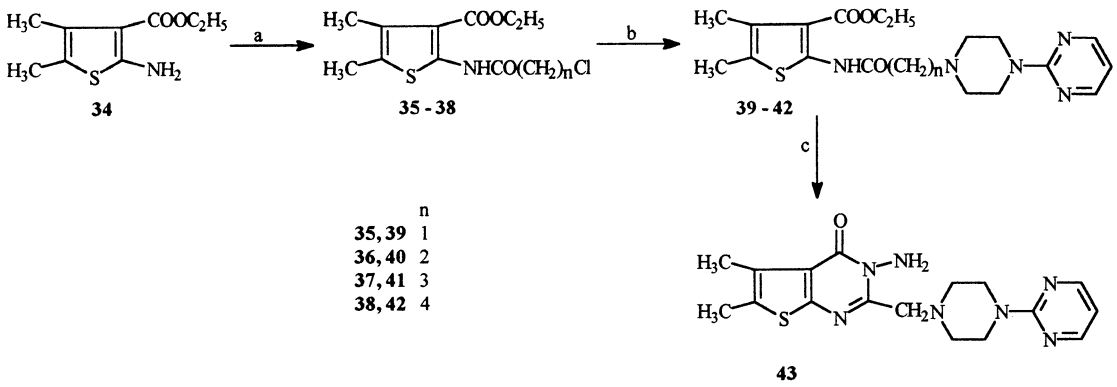


Figure 3. Synthetic procedure to compounds **35**–**43**. Reagents and conditions: (a) $ClCO(CH_2)_nCl$, $CHCl_3$, reflux; (b) 1-(2-pyrimidinyl)piperazine dihydrochloride, K_2CO_3, dimethylformamide, reflux; (c) $N_2H_4.H_2O$, 1-propanol, reflux.

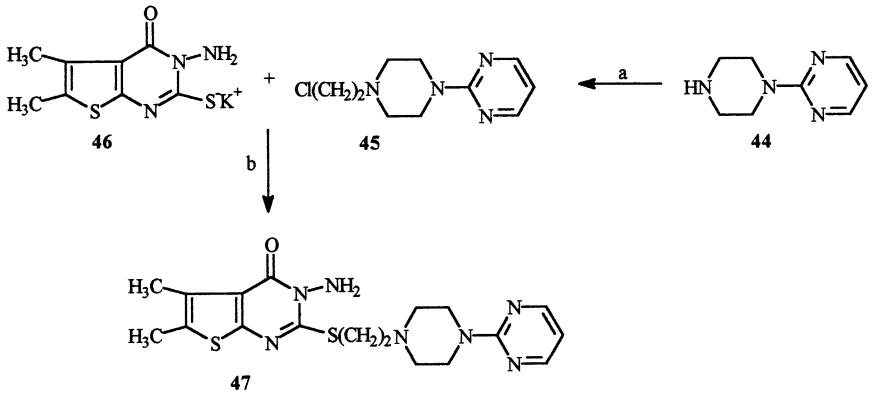
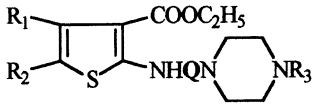


Figure 4. Synthetic procedure to compound **47**. Reagents and conditions; (a) $Cl(CH_2)_2Br$, K_2CO_3, dimethylformamide, r.t.; (b) EtOH, reflux.

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



Compound	R_1	R_2	Q	R_3	Melting point (°C)	Recrystallized 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

The thioxomethylamino derivatives **8**–**17**, the thienothiazinone derivatives **18** and **19**, the piperazinylacylamino derivatives **39**–**42**, the derivatives with the thienopyrimidinone system either directly bound to the piperazine (compounds **26**–**33**) or through a methylene (compound **43**) or a thiomethylene chain (compound **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

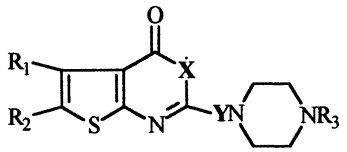
4.1. Structure–affinity relationships

The thienothiazinone derivative **18** and some compounds of the **26**–**33** series 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. Such a trend is compliant with what we already know, that is, those compounds where the basic nitrogen connected to the aromatic acyl group 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].

The compound with highest affinity for the 5-HT_3 receptor is the **28** ($K_i = 3.92$ nM), followed with a much lower affinity by the derivatives **33** ($K_i = 265$ nM) and **18** ($K_i = 377$ nM).

The result of the compound **28** shows that besides the three pharmacophoric elements, what is important in this series for the selectivity for the 5-HT_3 over the 5-HT_4 receptors is the presence of (i) the two methyls in position **5** and **6** of the thiophene nucleus; in fact, the substitution of the two methyls with a cyclic tetramethylene chain in compound **31** ($K_i = 2035$ nM)

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)	Recrystallized solvent	I.R. (KBr) ν(NH)	I.R. (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, NH2), 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.

leads to a remarkable decrease in affinity; (ii) the amino group bound to the N3 of the pyrimidinone, as may be seen from the results of the thienothiazinone and the thienopyrimidinone derivatives **18** and **33**; (iii) the benzyl 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 [9]. The N4 orthomethoxyphenyl and the N4 pyrimidine derivatives **26** and **27** do not show affinity for either receptor.

Finally, compound **28** acting as a full agonist at the 5-HT_3 receptor in the Bezold–Jarisch reflex (unpublished data) 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 3-D-QSAR).

The structural features which differentiate the compounds **39**–**42** among them, the most active and selective towards the 5-HT_4 receptor over the 5-HT_3 receptor, is the length of the amidomethylenic chain which links the thiophene to the piperazinylpyrimidine system. The one with higher affinity for the 5-HT_4 receptors is the 2-[4-[4-(2-pyrimidinyl)-1-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). Compound **41** has also been shown to have some selectivity since it has been found not to have affinity either for 5-HT_{1A} or α_1A receptors.

Due to the important result we obtained, further research is currently in progress for improving the affinity of these structures for the 5-HT_4 receptors.

4.2. Conformational analysis

The relative spatial disposition of the above listed functionalities as adopted by compound **41** ($n = 3$), showing the highest level of receptor affinity was first investigated and then considered to characterize the 'active conformation(s)', in which the molecule might interact at the receptor site both in its neutral (*figure 5*) and charged states (*figure 6*). The latter, which most likely is the bioactive form, is due to the protonation of the piperazine N1. Charged forms as bioactive are well documented for biomolecules with ionizable basic groups. Evidence coming out from the computational studies reported below 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 was identified which allows for the formation of a seven terms pseudo-cycle 'head-tail' in the most active compounds **41** and **42**, which includes the protonated piperazine nitrogen and the opposite amide oxygen atom in the side chain. This pseudo-cycle may act as a determinant of activity 'selecting' favourable/unfavourable conformations for binding of the molecule 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 ± 0.5 as the dihedral angle (τ), which defined by the S1-C2-Nam-Cam atom sequence. The less active deriva-

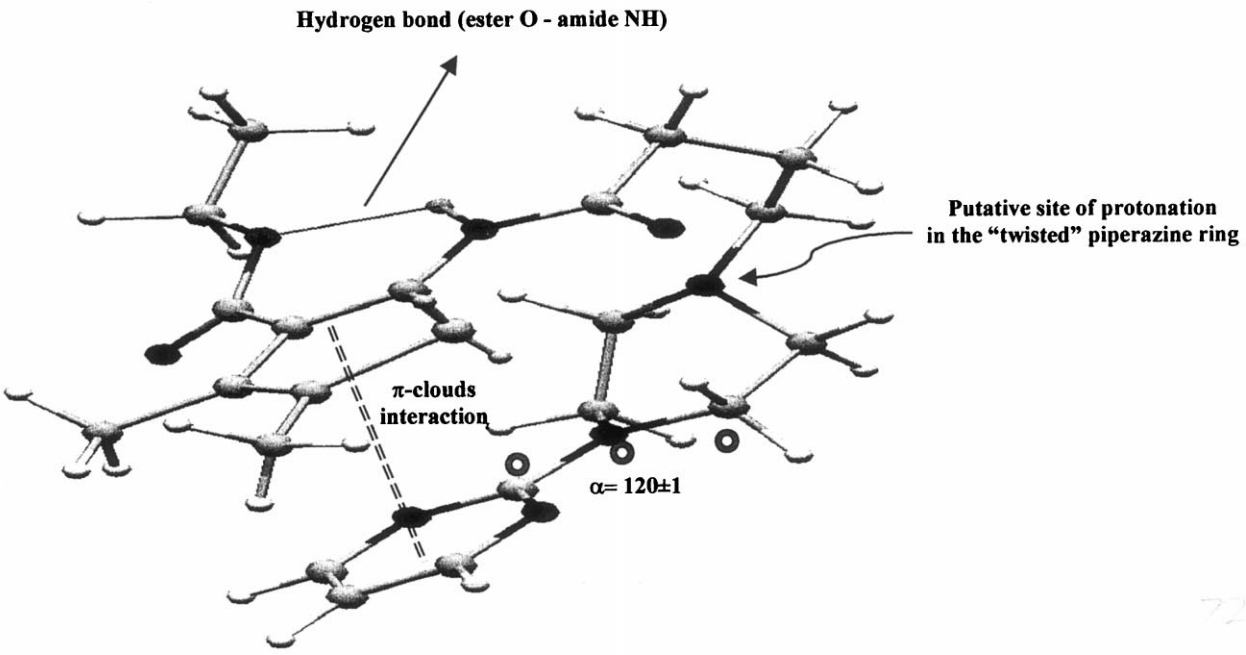


Figure 5. U-shape conformation of compound **41** in vacuo (see text for explanation). The piperazine ring is in a twisted boat conformation.

tives **39** and **40** show a less planar geometry as measured by a dihedral angle (τ) of 8.00 ± 0.6.

The above planar conformation allows the anti C4 carbonyl–amide NH groups in the side chain of compounds **39**–**42** to form a strong hydrogen bond (2.000 ± 0.1 Å), thus 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 compounds **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, so emphasizing the possible role of heteroaromatic interactions in the ligand-binding process.

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

X-ray studies are currently in progress to complement the computational in vacuo and aqueous results on a possible lowest 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 pseudo-cycle, formed by six terms instead of seven as for compounds **41**, negatively affects the activity of compound **40** ($n = 2$) making its structure too rigid, therefore, (*figure 7*) unfavourably locating the pyrimidine ring and precluding the ligand from key interactions in the ligand–receptor binding.

The better activity of compound **39** ($n = 1$) when compared with 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),

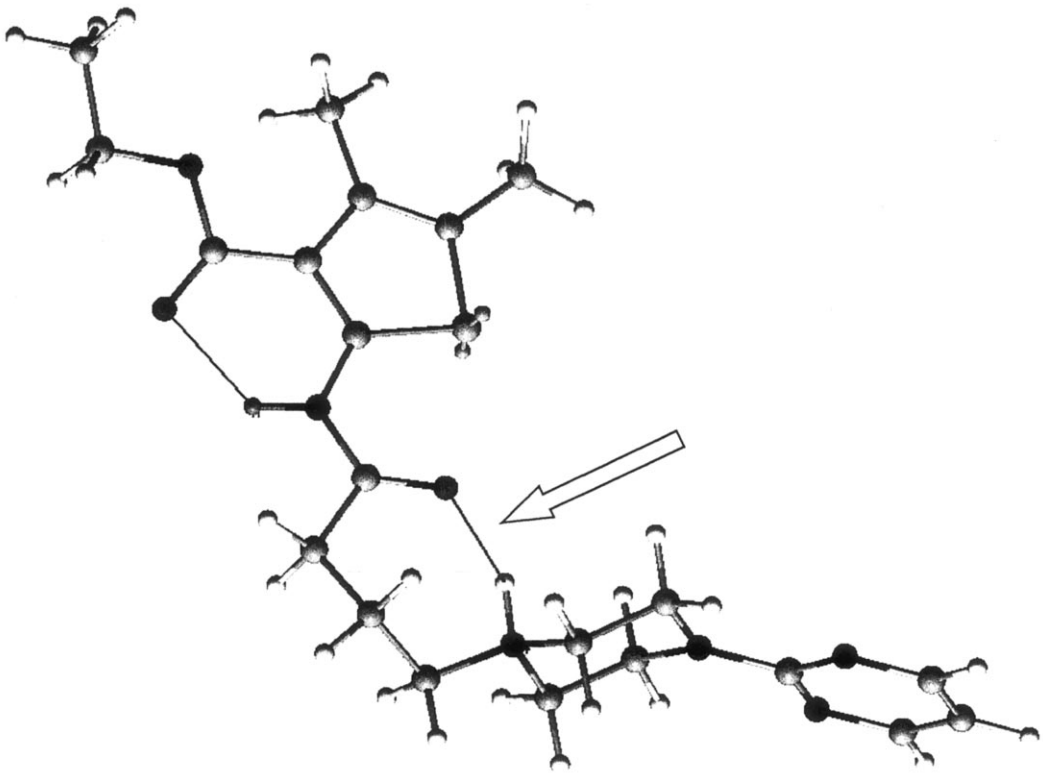


Figure 6. Ball-and-stick representation of compound **41** ($n = 3$) lowest energy conformation in the charged (protonated) form. Hydrogen bonds are displayed by dotted lines. Arrow points to the additional hydrogen bond (see text for explanation).

the overall view does not change. Likewise, their protonated counterparts — all the examined compounds **39**–**42**, are characterized by a six atoms pseudo-cycle formed by the hydrogen bonding between the sp^3 oxygen of the ester oxygen 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 stabilized by thiophene-phenyl π clouds 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 the molecules to establish 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, stabilizes 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 clouds interactions between the aromatic rings (thiophene-phenyl) are responsible for the stabilization 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° 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.

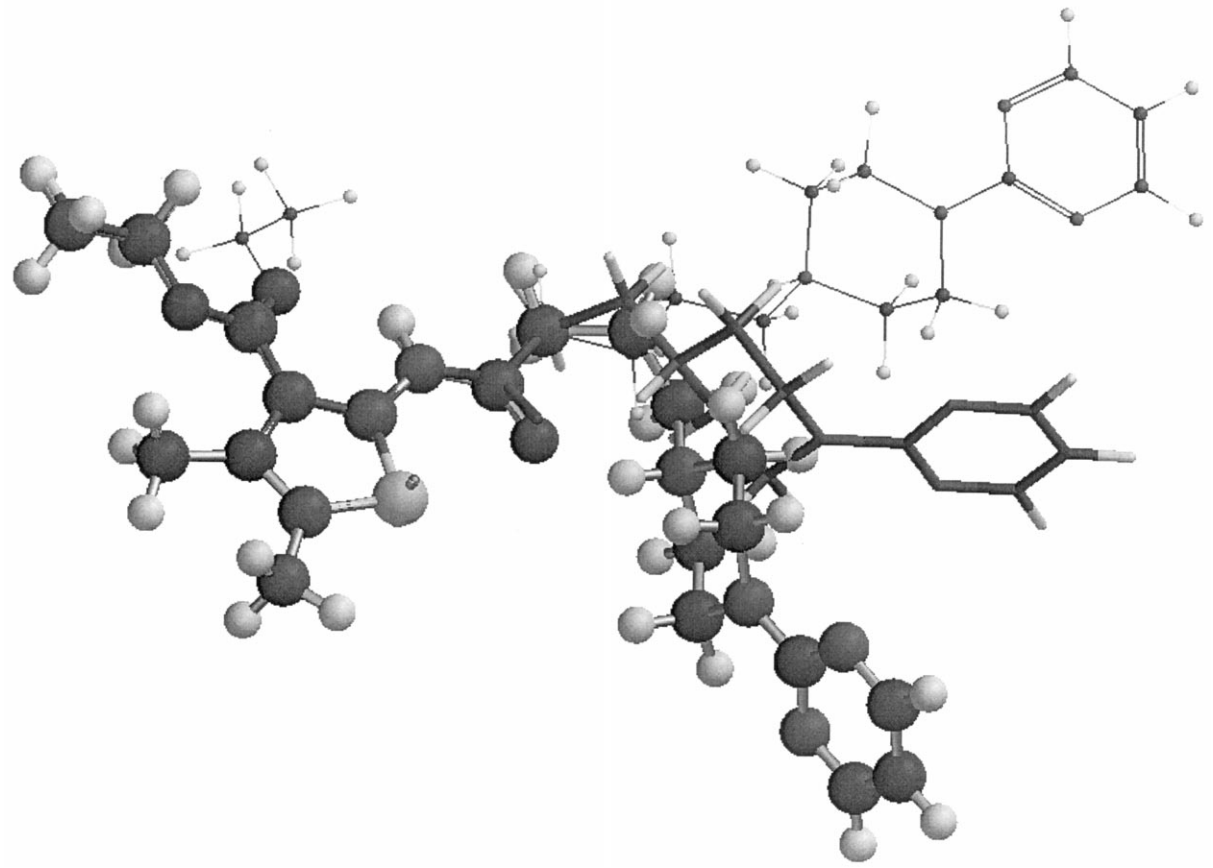


Figure 7. Superposition of compounds **40** ($n = 2$) (tube), **41** ($n = 3$) (ball-and-spoke) and **42** ($n = 4$) (ball-and-wire) lowest energy conformers in the charged (protonated) form, showing the different spatial arrangement of the piperazine ring.

5. Conclusions

The synthesis and binding tests on the 5-HT_3 and 5-HT_4 receptors of new thienopyrimidopiperazine and piperazinylacylaminodimethylthiophene derivatives have led to selective ligands for each receptor. The compound with higher affinity and selectivity for the 5-HT_3 over the 5-HT_4 receptor was the 3-amino-2-(4-benzyl-1-piperazinyl)-5,6-dimethyl-thieno[2,3-*d*]pyrimidin-4(3*H*)-one **28**, on the other hand, 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.

Based on the computational result, it might be speculated on a dynamic process of interaction by a change in the intramolecular stabilizing interaction from the electronic as in the uncharged form to that electrostatic (H bond) as in the protonated form, 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 because of the lack of key interactions.

6. Experimental protocols

6.1. Chemistry

Melting points were determined in open capillary tubes on a Gallenkamp Melting point apparatus and are uncorrected. Elemental analyses for C, H, N and S were obtained on an EA1108 Elemental Analyzer Fisons-Carlo Erba instrument and were within 0.4% of the theoretical values. The IR spectra were recorded with FT-IR 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 recrystallized by a suitable solvent. Compounds **12** and **15**, after cooling, 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 for 2 and 6 days, respectively. The solution was then poured into cold water and neutralized with a 10% NaOH solution. The solid was collected, washed with water, dried and recrystallized 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 room temperature for 0.5–1 h. The solid was collected, washed with water, dried and recrystallized by a suitable solvent.

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

Recrystallized 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**

Recrystallized 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. ($C_{11}H_{13}N_3O_3S_2$) 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 recrystallized. 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** were like those obtained from methylthio derivatives **22** and **23** (3.46 mmol), respectively, and hydrazine monohydrate (8 mL) boiled under reflux for 6 h in 2-propanol (15 mL), but in this preparation the yields were higher.

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

Recrystallized 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**

Recrystallized from dioxane, yield: 0.2 g, 43%; m.p. 284–286 °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 of compounds **24** and **25** from derivative **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 12 h. Compounds **10** and **13** were boiled under reflux for 2 h. After cooling, the solid was collected, washed with ethanol, dried and recrystallized 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 room temperature for 15 min, the solid was collected, resuspended in water, to the suspension were added some drops of a 10% NaOH solution, the solid was then collected, washed with water, dried and recrystallized 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, the organic layer was separated, dried over anhydrous sodium sulfate and 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. ($C_{12}H_{16}ClNO_3S$) 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. ($C_{13}H_{18}ClNO_3S$) C, H, N, S.

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

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 separated and the solvent was removed under reduced pressure. The solid obtained (compounds **39** and **40**) was collected, washed with water, dried and recrystallized 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 recrystallized (*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 room temperature in dimethylformamide (8 ml) for 22 h. The solid was separated and from the solution the solvent was removed 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. ($C_{10}H_{15}ClN_4.1/2H_2O$) 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 recrystallized 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 homogenized 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 homogenizer (2×20 s), and homogenates were centrifuged at $50\,000 \times g$ 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 $50\,000 \times g$ 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, 50 mM, pH 7.4, containing 10 μM pargyline for 5-HT4 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^{-1} 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 per sample), 0.25 mL of the [^{3}H] ligand (0.4 nM) and 0.01 mL of displacing agent or solvent, nonspecific binding was measured in presence of 1 μM quipazine. [^{3}H] GR 113 808 (*figure 1*) [18] (SA 84.0 Ci mmol^{-1} 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 per 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 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 unlabelled 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 earlier described [1].

6.3. Molecular modelling

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. Since quaternization can be tolerated, it can be assumed that the basic piperazine center is binding in its protonated form to 5-HT_4 receptor, although within reason, the basicity of the basic centre is not critical.

All minimizations 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 minimization 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) minimizer, allowing for a maximum of 500 iterations per structure, until a gradient of 0.05 kcal Å^{-1} was reached.

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

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

In consideration of the high flexibility of the ligands under investigation, an energy cut-off of 5.0 kcal mol^{-1}, high enough to map the conformational space including the bioactive conformation, was applied to the search results.

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