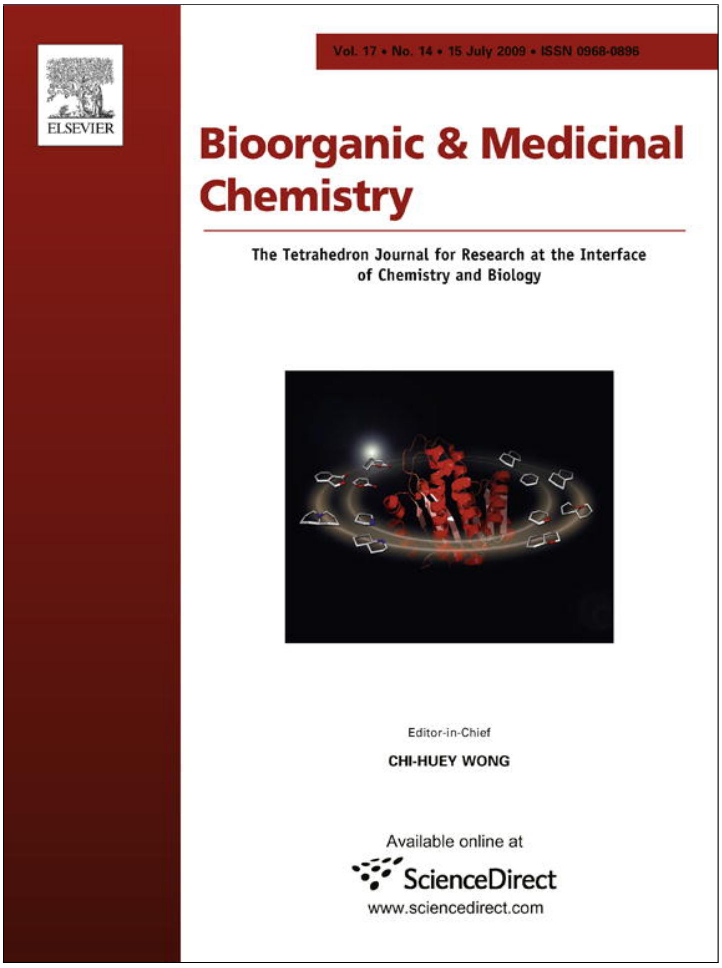
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Tetrahydroisoquinolines as dopaminergic ligands: 1-Butyl-7-chloro-6-hydroxy-tetrahydroisoquinoline, a new compound with antidepressant-like activity in mice

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ABSTRACT

Three series of 1-substituted-7-chloro-6-hydroxy-tetrahydroisoquinolines (1-butyl-, 1-phenyl- and 1-benzyl derivatives) were prepared to explore the influence of each of these groups at the 1-position on the affinity for dopamine receptors. All the compounds displayed affinity for D_1-like and/or D_2-like dopamine receptors in striatal membranes, and were unable to inhibit [^{3}H]-dopamine uptake in striatal synaptosomes. Different structure requirements have been observed for adequate D_1 or D_2 affinities. This paper details the synthesis, structural elucidation, dopaminergic binding assays, structure–activity relationships (SAR) of these three series of isoquinolines. Moreover, 1-butyl-7-chloro-6-hydroxy-tetrahydroisoquinoline (**1e**) with the highest affinity towards D_2-like receptors (K_i value of 66 nM) and the highest selectivity (49-fold D_2 vs D_1) by in vitro binding experiments was then evaluated in behavioral assays (spontaneous activity and forced swimming test) in mice. Compound **1e** increased locomotor activity in a large dose range (0.04–25 mg/kg). Furthermore, this lead compound produced reduction in immobility time in the forced swimming test at a dose (0.01 mg/kg) that did not modify locomotor activity. The haloperidol (0.03 mg/kg), a D_2 receptor preferred antagonist, blocked the antidepressant-like effect of compound **1e**.

1. Introduction

Dopamine-mediated neurotransmission plays an important role in several psychiatric and neurological disorders affecting several million people worldwide. Researchers have focused on various approaches towards modulating dopaminergic activity via the dopamine receptors (DR) as a potential means of treating schizophrenia and Parkinson's disease. For these reasons, much research has focused on the discovery of novel dopaminergic ligands as potential drug candidates.[1] DR can be classified into two pharmacological families (D_1 and D_2-like) that are encoded by at least five genes. The D_2-like DR antagonists are used in the treatment of schizophrenia (antipsychotics) and the agonists are utilized to treat Parkinson's disease.[2] Dopaminergic agonist actually used as antiparkinsonian drugs[3] could be classified into several categories in regard of their affinity and activity towards dopaminergic receptors[4,5] but all of them exhibited D_2-like agonist properties. Recent studies have also evidenced the potential role of D_2 agonists in the treatment of depression. Besides, even thought the pathophysiology of depression has been assigned to the noradrenalin and serotonin system, several results also support a role of the dopaminergic system in this mood disorder.[6] In particular, various selective D_2-type dopamine receptor agonists exert antidepressant-like actions in diverse rodent models suggesting a specific role of this subtype receptor in antidepressant-like activity.[6–9]

Substituted isoquinolines (IQ) represent a class of natural and synthetic compounds that has been evaluated for their ability to inhibit the dopamine transporter and to display affinity at D_1 and D_2-like DR binding sites in rat brain tissue.[10] Tetrahydroisoquinolines (THIQs), the most numerous naturally occurring alkaloids, include 1-benzyl-THIQs and aporphines, both of which have structural similarities to dopamine and can interact with DR.[11]

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Previous results in our group suggested that some natural and synthetic 1-benzyl-THIQs alkaloids were able to bind to DR.[12–14] In this way, we described the enantioselective syntheses of pairs of dopaminergic (1*S*)- and (1*R*)-benzyl-THIQs using (*R*)- and (*S*)-phenylglycinol as the chiral source. We observed that in these series of 1-benzyl-THIQs, their (1*S*)-enantiomers were 5–15 times more effective at the D_1-like and D_2-like DR than the (1*R*)-enantiomers.[15] Moreover the different synthesised 1-cyclohexylmethyl THIQs were able to displace the D_2-like DR radioligand from its specific binding sites in rat striatal membranes, while the N-methylated derivatives also showed affinity for the D_1-like DR.[16] We also determined the role of certain structural requirements to improve the affinity/selectivity for D_1 and D_2-like DR[15–20] and we have postulated that the presence of a hydroxyl (OH) and a halogen group (Cl) in the THIQ A-ring could contribute to obtain molecules which can bind selectively to one of the two groups of the aforementioned receptors.[18,19]

The aim of the present work was to profound in the determination of the structural features that define the affinity and selectivity of these compounds for D_1/D_2 receptors, analyzing the influence of the substitution at the 1-position over a 7-chloro-6-hydroxy-THIQ core, in order to obtain more specific dopaminergic ligands. We have prepared three series of 1-substituted: 1-butyl-, 1-phenyl- and 1-benzyl-THIQs, which support constant structural factors 6-chloro and 7-oxygenated substitutions, as well as a basic secondary (*N*H) or tertiary (*N*CH_3) amine. The structures of the resulting twelve 1-substituted-THIQs were determined on the basis of their NMR spectral data and mass spectrometry analysis. All the synthesised compounds were tested for their ability to displace the selective radioligands of D_1 and D_2-like DR from their specific binding sites in striatal membranes in order to establish their structure–activity relationships (SAR) as dopaminergic agents. Molecular modeling of the possible stereo-electronic requirements of dopamine D_2 receptoŕs ligand is also discussed with regard to the different affinity.

Furthermore, and based on the implication of D_2-like receptor ligands on spontaneous activity modulation,[21,22] the effects of the lead compound **1e** (with the highest D_2 receptor affinity and selectivity) were investigated with a photoactimetry test in a large dose range (0.01–25 mg/kg) in mice. Finally, in accordance with the in vivo D_2 receptor agonist activity observed and the therapeutic potential of D_2 receptor agonists in the treatment of depression[6–9] the antidepressant-like activity of this compound was evaluated in the forced swimming test in mice.

2. Results and Discussion

In the present work we have studied the influence of the substitution at the 1-position over a 7-chloro-6-hydroxy-THIQ core. By preserving the chlorine and hydroxyl (or methoxyl) groups at the C-6 and C-7 positions, respectively, of the THIQ A-ring with a secondary (*N*H) or a tertiary (*N*Me) amine, we decided to explore the

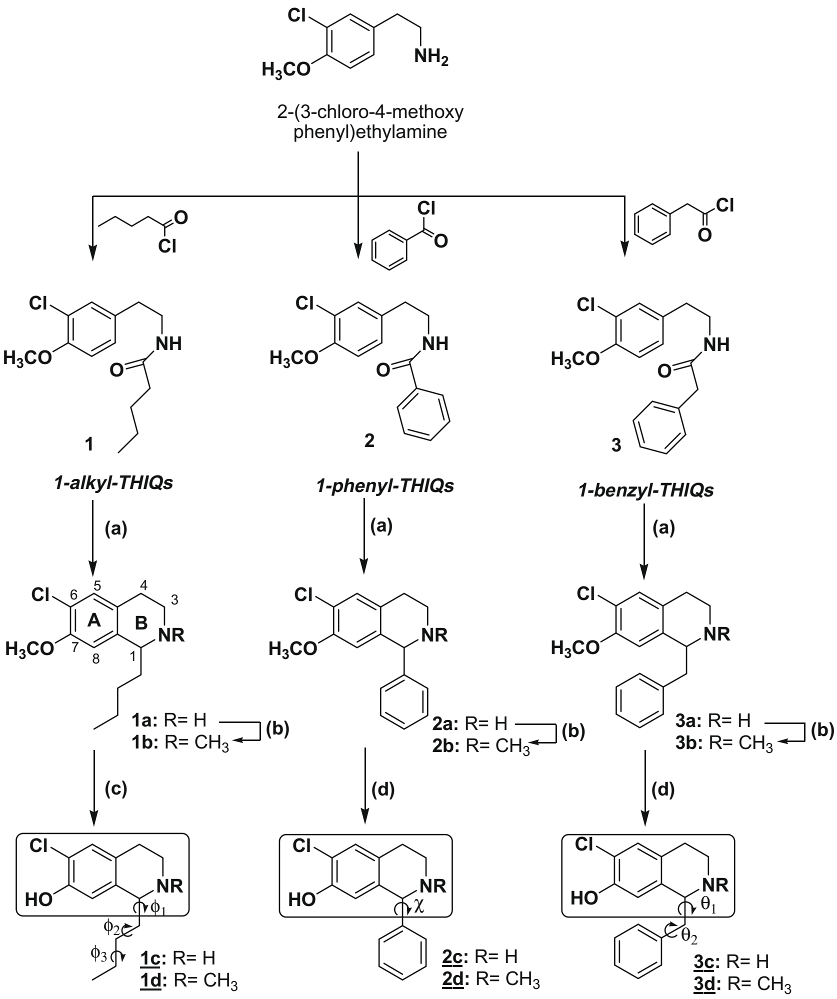


Figure 1. Synthesis of 1-substituted 6-chloro-THIQ analogues. (a) $POCl_3–P_2O_5$/toluene; $NaBH_4$/MeOH; (b) HCHO/$NaBH_4$; (c) BBr_3, 18 h; (d) BBr_3, 3 h.

impact of the inclusion at the 1-position of aliphatic and aromatic groups, such as butyl-, phenyl- or benzyl- moieties, to determine their influence on dopaminergic affinity. Thus, we prepared three series of 1-substituted-THIQs: 1-butyl-THIQs (**1a**–**d**), 1-phenyl-THIQs (**2a**–**d**) and 1-benzyl-THIQs (**3a**–**d**), which have enabled us to draw conclusions about the influence of each of these groups noted at the 1-position, a frequent occurrence in the structure of natural and/or synthetic drugs.

2.1. Chemistry

The general synthetic plan for these compounds focused on the preparation of the appropriate amides (**1**, **2**, and **3**) by standard methods. Thus, the previously synthesised 2-(3-chloro-4-methoxy)ethylamine was treated with three different acid chlorides, these being alkanoyl (valeryl chloride), benzoyl and phenylacetyl chloride, under Schotten–Baumann conditions (Fig. 1), affording the three amide derivatives with good yields: *N*-(3-chloro-4-methoxyphenethyl)pentanamide (**1**), *N*-(3-chloro-4-methoxy-phenethyl)benzamide (**2**) and the *N*-(3-chloro-4-methoxyphenethyl)-2-phenylacetamide (**3**). After a Bischler–Napieralski cyclodehydration reaction, each *N*-phenylethylamide was converted into the convenient 1-substituted-THIQ. At this stage, several points should be emphasized: (i) A classical Bischler–Napieralski cyclization failed in these compounds (refluxing with $POCl_3$ in CH_2Cl_2; which means having to use the most common Lewis acid for this reaction) because of the halogen substitution over the THIQ A-ring. A mixture of $POCl_3$ and P_2O_5 was needed to obtain the corresponding dihydroisoquinolines;[23–25] (ii) An unusual Bischler–Napieralski cyclodehydration was performed given a high concentration level of the P_2O_5 reagent.[26] Under these conditions, a mixture of THIQ isomers 6-OMe–7-Cl and 6-Cl–7-OMe was obtained, after the Bischler–Napieralski reaction followed by $NaBH_4$ reduction (Figs. 1 and 2); (iii) The indispensable O-demethylation of all synthesised isoquinolines was performed with an addition of 4 equiv of BBr_3 reagent, and a longer reaction time (18 h) was needed for the 1-butyl-THIQ series.

Concerning the synthesis, we observed the same fact during the Bischler–Napieralski cyclization, as reported by Doi et al. in 1997,[26] when we prepared the 1-butyl-THIQs starting from amide **1** (Fig. 2). It was necessary to add P_2O_5 (and $POCl_3$, in a molar ratio of 1:1) to the cyclodehydration reaction given the difficulty to cyclize the amide when there was a chlorine in the structure (originally at the C-6 position of the A-ring), and causes an aberrant cyclization by means of the formation of a nitrilium intermediate which gives two clearly identified positional isomers after the reduction step: 6-chloro-7-hydroxy-1-butyl-THIQ (**1c**: expected product), and 6-hydroxy,7-chloro-1-butyl-THIQ (**1e**: unexpected cyclization product), in a 1:2 ratio. This finding was less significant in series **2** and **3** where the mixture of isomers was 3:1 and 4:1, respectively. Each isomer was unambiguously determined by NOE DIFF experiments (Fig. 3). For compound **1c**, irradiation of H-8 (δ 6.78, s) caused the enhancement of H-1 (δ 3.88, dd) and the H-1′ (δ 1.80–1.67, m) signals, while irradiation of the more deshielded H-5 (δ 7.03, s) only affected H-4 (δ 2.73–2.65, m). However, NOE DIFF experiments for isomer **1e** exhibited signal enhancements of H-1 (δ 3.85, dd) and H-1′ (δ 1.80–1.63, m) upon irradiation of the more deshielded H-8 (δ 7.07, s), and irradiation of H-5 (δ 6.68, s) which influenced H-4 (δ 2.73–2.65, m).

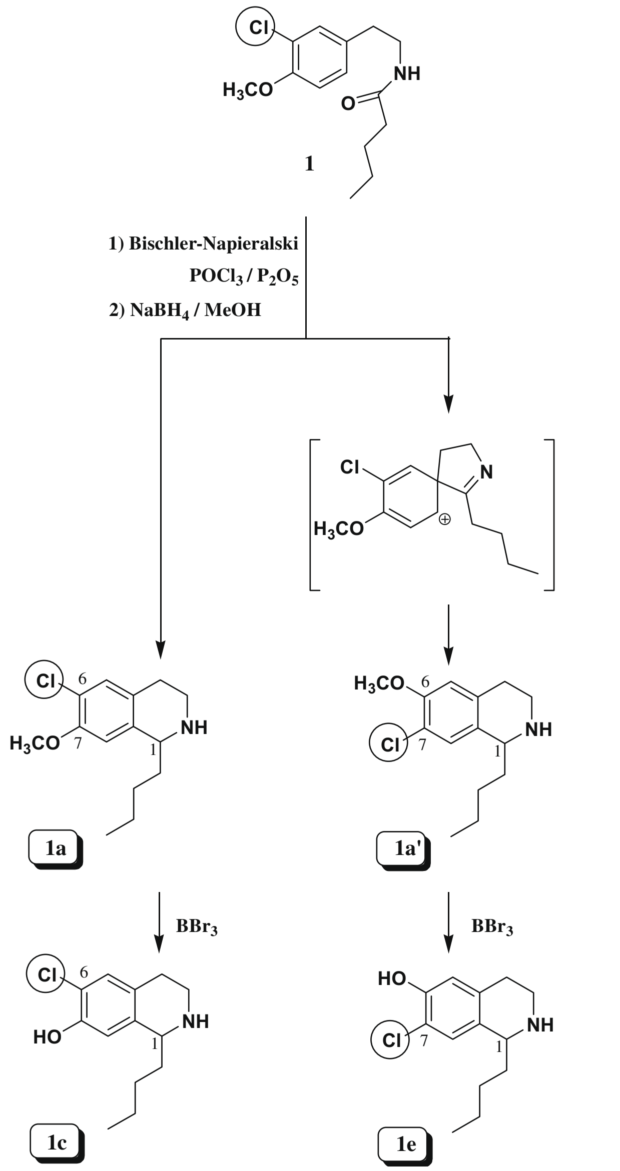


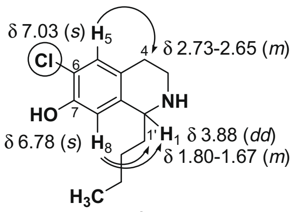
Figure 2. Abnormal BN reaction: synthesis of 1-butyl-7-chloro-THIQ's (**1e**).[26]

2.2. Binding affinities for dopamine receptors: SAR Studies

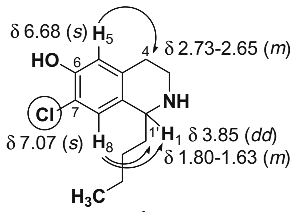
All the synthesised isoquinolines were assayed in vitro for their ability to displace the selective radioligands of D_1 and D_2 DR from their respective specific binding sites in rat striatal membranes. They were also tested for their ability to inhibit an in vitro [^{3}H]-dopamine uptake in rat striatal synaptosomes. Many of these compounds were able to displace both [^{3}H]-SCH 23390 and [^{3}H]-raclopride at nano- or micromolar (nM or μM) concentrations from their specific binding sites in the rat striatum, but all the compounds had a low or null effect on the [^{3}H]-dopamine uptake. The binding affinities for D_1 and D_2 DR are summarized in Table 1 and these results have illustrated some general trends of the SAR: the effect of the hydroxyl or methoxyl group at the C-7 position, the effect of the amine type (*N*H or *N*Me) and the effect of the butyl, phenyl and benzyl group at the C-1 position.

2.2.1. 7-Hydroxyl group and amine type effects

In the 1-substituted THIQs synthesised, the presence of a hydroxyl (OH) group at C-7 position positively influences their ability to displace the selective ligands of D_1 and D_2 DR from their specific binding sites in the striatal membranes (Table 1). Generally, all the tested 6-Cl and 7-OH-THIQs (**1c,d**; **2c,d** and **3c,d**) showed an affinity for DR 5–15 times higher than their corresponding 6-Cl and 7-OMe homologs. This result agrees with the established finding that a hydrophilic area, usually provided by the phenolic hydroxyl group in the THIQ A-ring, is required for a better binding



1c



1e

Figure 3. NOE DIFF effects of compounds **1c** and **1e**.

of the ligands to this type of receptors,[16,20] which also occurs in the molecule dopamine, a physiological mediator of the dopaminergic via. A similar behavior was noted in compound **1e** (**1c** isomer) with inverted ring-A substitutions: the 7-Cl and 6-OH groups.

To better understand the above experimental results we performed molecular modeling simulations combining both semiempirical and ab initio quantum mechanical calculations for different 1-butyl-, 1-phenyl- and 1-benzyl-THIQs/D_2 DR complexes. For such calculations we use a reduced model from the complete D_2 receptor model previously reported by Teeter et al.[27] Consistent with previous experimental[28] and theoretical[29] results, our simulations indicate the importance of the negatively charged aspartate 86 for the binding of these ligands. A highly conserved aspartic acid (Asp 86) in *trans*-membrane helix 3 (TM3) is important for the binding of both agonists and antagonists to the D_2 receptor,[28,29] and its terminal carboxyl group may function as an anchoring point for ligands with a protonated amino group.[27]

Our results indicate that in these complexes the strongest contributor to the network of serines was Ser 141 which is consistent with the experimental observation that a Ser 141 Ala mutated receptor completely lost dopamine induced activation.[28] It should be noted that in compounds **1c**, **1e**, **2c** and **3c** the hydroxyl group on the ring-A is acting as the proton–donor part; whereas the oxygen atom of the OH group of Ser 141 the proton–acceptor counterpart. In contrast, in the case of compounds **1a**, **2a** and **3a** the OH group of Ser 141 is the proton–donor and the methoxyl group on the ring-A is the acceptor.

Figure 4a shows the ligand **3c** interaction with the D_2 DR optimized using quantum mechanical calculations. The salt bridge between the protonated amino group and the carboxyl group of Asp 86 as well as the hydrogen bond between the 7-hydroxyl group with Ser 141 might be appreciated in this figure. From Figure 4a it is clear that a strong salt bridge takes place for this compound between the protonated amino groups and the carboxyl group of Asp 86 (calculated distance of 3.47 Å). The hydrogen bond between **3c** and Ser 141 is a bifurcated interaction in which the oxygen atom of hydroxyl group and the oxygen of carbonyl group of Ser 141 are the proton–acceptors, giving interatomic distances of 2.28 Å and 2.40 Å, respectively. Figure 4b gives the ligand **3a** interaction with the D_2 DR. In this case the 7-methoxyl group acts as proton–acceptor while the hydroxyl group of Ser 141 is the proton–donor displaying an interatomic distance of 2.32 Å.

Table 2 gives the energy binding calculated for the different complexes using RHF/6–31G(d). All the compounds possessing 7-methoxyl groups displayed higher binding energies with respect to the 7- hydroxyl homologs (compare **1a** with **1c**, **2a** with **2c** and **3a** with **3c**). Previously we reported that a 7-hydroxyl group acting as proton–donor gives stronger hydrogen bond than those derivatives possessing a 7-metoxyl group.[19] The present results are in agreement with those calculations previously reported for isolated and solvated molecules, as well as, with the experimental binding affinities reported here (Table 1).

In general, the comparative K_i values between pairs of *N*H and *N*Me derivatives indicate that the attachment of a methyl group at the nitrogen atom (tertiary amine) appears to be important for improvement in the binding affinity to D_1 DR. Thus in the 1-phenyl-THIQ series (series **2**), compounds with *N*Me amine showed greater affinity for D_1 receptors than their corresponding *N*H derivatives. However in the 1-butyl- and 1-benzyl-THIQ series (series **1** and **3**, respectively), secondary amine (*N*H) compounds showed a greater affinity for D_2-like DR than their corresponding homologs with tertiary amine (*N*Me). Therefore, secondary amines **1c** and **3c** (K_i: 91 nM and 71 nM, respectively) displayed greater affinity and selectivity for D_2 receptors, whereas **2d** tertiary amine (K_i: 47 nM) presented greater affinity and selectivity for D_1 receptor (Fig. 5). This finding might be explained if we take into account the earliest conformational studies in which the *N*-methyl substi-

Table 1
Dissociation constants (pK_i) and selectivity of different compounds at the D_1-like and D_2-like dopaminergic receptors

Compounds	Series	Specific-D_1 ligand [^{3}H]-SCH 23390	Specific-D_2 ligand [^{3}H]-raclopride	Selectivity D_1/D_2
1a	1-Butyl-THIQ	4.763 ± 0.258	6.108 ± 0.165^{c}	20
1b	1-Butyl-THIQ	5.138 ± 0.256	5.424 ± 0.026^{d}	2.7
1c	1-Butyl-THIQ	5.918 ± 0.165^{i}	7.117 ± 0.151^{c,h}	17
1d	1-Butyl-THIQ	5.944 ± 0.09^{g}	6.403 ± 0.204^{e,h}	2.3
1e (**1c** isomer)	1-Butyl-THIQ	5.491 ± 0.036	7.220 ± 0.139^{c}	49
2a	1-Phenyl-THIQ	5.222 ± 0.222	5.212 ± 0.124	1.2
2b	1-Phenyl-THIQ	6.089 ± 0.292^{d}	5.670 ± 0.406	0.3
2c	1-Phenyl-THIQ	6.607 ± 0.205^{h}	5.950 ± 0.198	0.2
2d	1-Phenyl-THIQ	7.395 ± 0.176^{d,h}	6.298 ± 0.187^{b}	0.07
3a	1-Benzyl-THIQ	5.628 ± 0.397	6.014 ± 0.049	5
3b	1-Benzyl-THIQ	5.785 ± 0.359	5.816 ± 0.181	0.8
3c	1-Benzyl-THIQ	6.487 ± 0.075	7.178 ± 0.091^{a,h}	4.8
3d	1-Benzyl-THIQ	6.953 ± 0.149^{h}	6.683 ± 0.139^{g}	0.6

THIQ: tetrahydroisoquinoline.
The results are expressed as mean ± SEM from 3 to 6 experiments.
ANOVA, post Newmann Keuls multiple comparison test:
$^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$ versus D_1-like dopaminergic receptor.
$^{d}p < 0.05$, $^{e}p < 0.01$, $^{f}p < 0.001$ versus corresponding –NH derivatives (compounds **b** vs **a**, and **d** vs **c**).
$^{g}p < 0.05$, $^{h}p < 0.01$, $^{i}p < 0.001$ versus corresponding $–OCH_3$ derivatives (compounds **c** vs **a**, and **d** vs **b**).

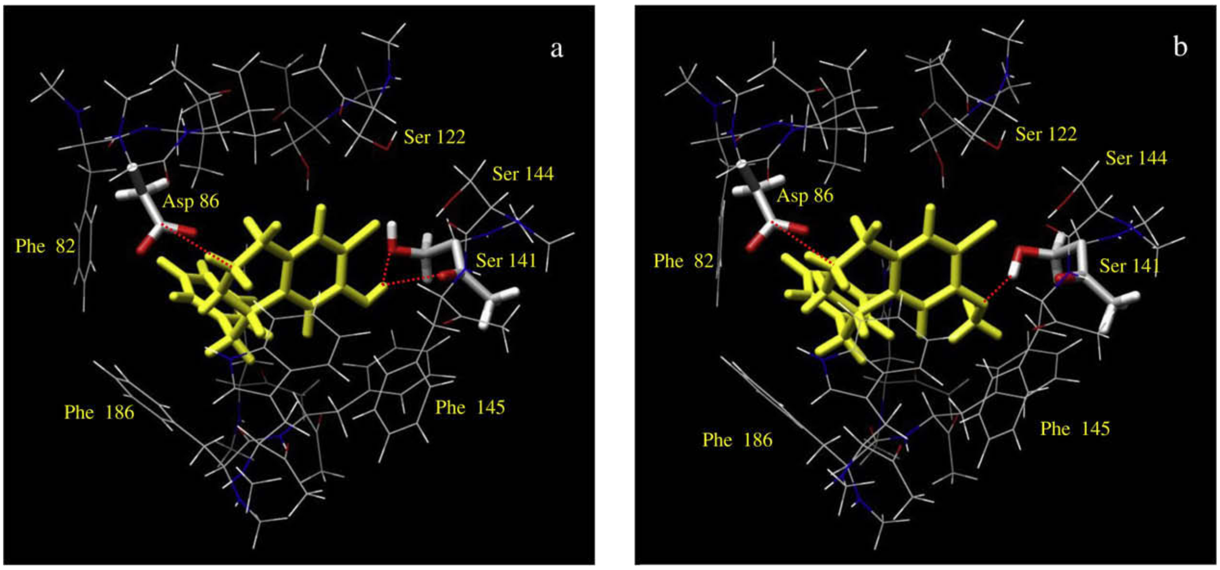


Figure 4. Interactions of compound **3c** (a) and **3a** (b) with the binding pocket D_2 DR. Spatial view of two interactions: salt bridge (Asp 86 with protonated amino group) to the right and hydrogen bond between hydroxyl groups with Ser 141 to the left.

Table 2
Relative binding energies obtained for the different complexes from RHF/6-31G(d) calculations

Compounds	Relative binding energy (BE) (kcal/mol)	
	$BE_{QM\ (RHF/6\text{-}31G(d))}$	$\Delta BE_{QM\ (RHF/6\text{-}31G(d))}$
1a	−98.91	17.45
1c	−111.92	4.43
1e	−114.03	2.32
2a	−75.46	40.89
2c	−90.48	25.87
3a	−99.94	16.41
3c	−116.35	0.00

tuent takes a more stable *equatorial* orientation in the *N*-methyl-THIQ*s*; therefore, the lone pair on the nitrogen is found at the axial position, thus favouring the interaction with D_1 receptor binding sites, whereas the nitrogen lone pair in the secondary amine precursors (*N*H) may be in equilibrium with axial and equatorial orientations.[19,25,30]

2.2.2. 1-Butyl, 1-phenyl and 1-benzyl group effects

Our results could indicate that the substituent at the C-1 position is an important factor to modulate the selectivity at dopamine receptors. A greater affinity towards D_2 receptors is evident in the 1-butyl-THIQ and 1-benzyl-THIQ series (series **1** and **3**, respectively). However, 1-phenyl-THIQ derivatives (series **2**) show selectivity for D_1 over the D_2 receptors (Fig. 5 and Table 1). Moreover, it is important to note that the compounds of series **1** (1-butyl-THIQs) show a lower D_1 affinity, and as a result, a more important D_2 selectivity than the compounds of the series **3** (1-benzyl-THIQs). The greater planar structure in series **2**, as well as the electronic influence on the tertiary amine nitrogen, could justify its higher selectivity for D_1 receptors.

Aromatic side chains are bulky, have low barriers for rotation, and are ideal to adjust to the changing conformation of the hydrophobic moiety of the ligand. In the dopamine D_2 receptor, the binding site proved to be lined with aromatic side chains and such residues can adjust to the different shapes and flexibility of the agonists in the binding site. Thus, Phe 82, Val 83 and Val 87(TM3); Phe 145 (TM5); and Trp 182, Phe 185, Phe 186 and His 189 (TM6) form a mostly hydrophobic pocket for ligands.

It is interesting to note that the only structural difference between compounds **1c**, **2c** and **3c** are the substituents at C-1. Whereas compound **2c** has a relatively rigidly held phenyl ring, the corresponding butyl and benzyl substituents on compounds **1c** and **3c**, respectively, are free to rotate allowing to better accommodate these hydrophobic moieties to interact with the cluster of aromatic and non polar residues located in the cleft. These results might be better appreciated from the different conformational behavior obtained for the torsional angles of their respective hydrophobic moieties from the calculations. Both 1-butyl and 1-benzyl groups displayed a high molecular flexibility from the calculations. In contrast, the conformational behavior obtained for the phenyl ring of compound **2c** displayed a very restricted molecular flexibility, keeping a spatial ordering almost perpendicular with respect to the rest of the molecule from our calculations (Fig. 6c). The different affinities obtained for compounds **2c** and **3c** suggest that the orientation of the substituent at C-1 may be the more important factor in the different effects on receptor affinity for the two ligands. This argument also applies to **1c** and **1e**, where the orientation of the butyl substituents is more favorable for hydrophobic interactions. Thus, the different affinities and selectivities obtained for these compounds might be explained, at least in part, by the different spatial orientations adopted by the varied hydrophobic portions located at C-1 which give different molecular interactions with the D_2 receptor. Figure 6a gives again the ligand **3c** interactions with the binding pocket. However, in this case a different spatial view is shown in order to better appreciate the hydrophobic interactions of this compound. From this figure we can observe that the benzyl group of **3c** adopts an adequate conformation to interact with Phe 186, Phe 82 and His 189. The butyl group of **1c** displays a spatial ordering very similar to that of the benzyl group of **3c**, giving also closely related hydrophobic interactions with the same hydrophobic residues (Fig. 6b). In contrast, the phenyl group of **2c** displayed a different spatial ordering giving an adequate distance to interact only with Phe 186 (Fig. 6c). Interestingly, the bonding energies obtained for these complexes are: **3c**/D_2 DR stronger than **1c**/D_2 DR and this complex stronger than **2c**/D_2 DR (Table 2), which is in agreement with the experimental results. The butyl portion of compound **1e** interacts with three aromatic residues: Trp 182, Phe 82 and Phe 186 (Fig. 6d).

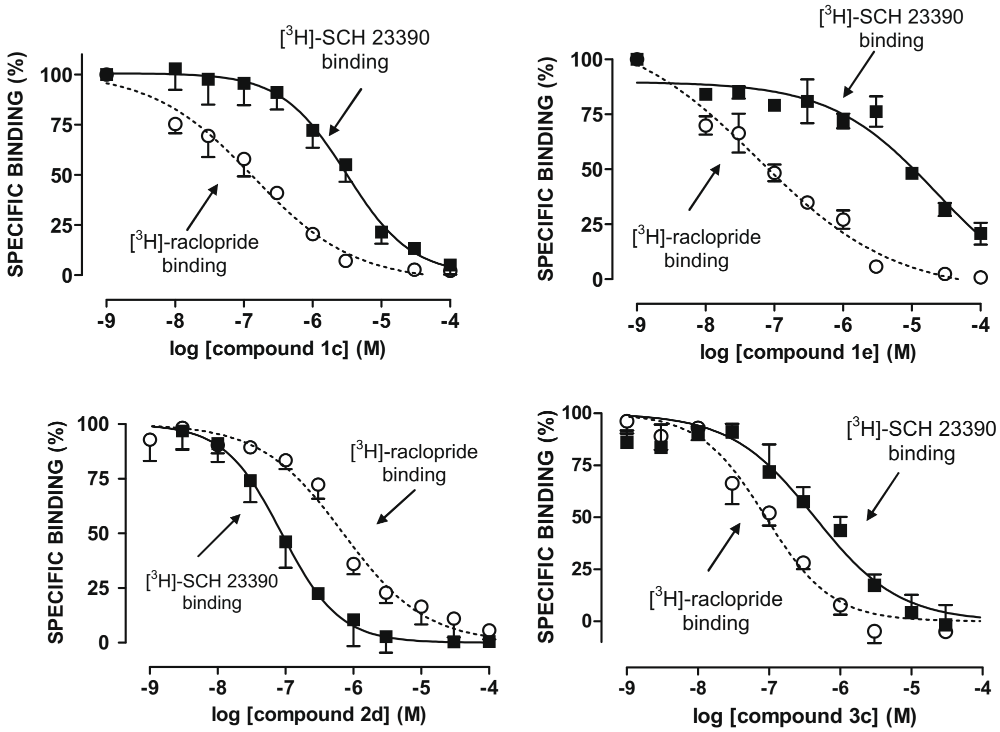


Figure 5. Displacement of specific binding of [^{3}H]-SCH 23390 (D_1-like DR specific ligand) and [^{3}H]-raclopride (D_2-like DR specific ligand) by compounds **1c**, **1e**, **2d** and **3c**. The results are expressed as mean ± SEM from 3 to 6 experiments.

2.2.3. Behavioral assays

The major compound **1e** presents a similar dopaminergic profile to its isomer **1c**, and shows a similar affinity and selectivity towards D_2-like receptors (Table 1 and Fig. 4). For this reason, we have undertaken a large-scale synthesis of **1e** to perform in vivo tests (Fig. 7).

Acute administration of compound **1e** induced a dose-dependant hyperlocomotor activity (two-way ANOVA, $p < 0.05$, Table 3), that was observed as from 0.2 mg/Kg and which reached its maximum at 5 mg/Kg. Furthermore at the dose of 0.2 mg/Kg, this effect appeared as from the first 6 min of the test (post-hoc PLSD of Fischer, $p < 0.05$). In order to investigate the involvement of D_2 receptors in this hyperlocomotor activity, we examined the effect of a concomitant administration of compound **1e** with haloperidol at a non-sedative dose (Fig. 7). Haloperidol (0.03 mg/kg) totally reversed the hyperactivity induced by compound **1e** at 0.2 mg/Kg. Given that 1) compound **1e** displayed in vitro selectivity D_2 versus D_1 (49-fold), 2) haloperidol showed in vitro selectivity D_2 versus D_1 (at least, 20-fold), and 3) low doses of the two compounds were used, these facts suggest an agonist D_2-receptor activity in vivo for compound **1e**.

Forced swimming test: compound **1e** at 0.0 1 mg/Kg (the highest dose that did not increase spontaneous activity in the first 6 min) and imipramine at 16 mg/Kg (used as pharmacological reference), induced a significant reduction of the immobility time in the forced swimming test (Fig. 8). This antidepressant-like effect of **1e** was reversed by haloperidol (0.03 mg/Kg) (ANOVA followed by post-hoc PLSD of Fischer's test, $p < 0.05$, Fig. 9).

3. Conclusions

The replacement of THIQs at the C-1 position is an important factor to modulate the selectivity at DR. Series **1** and **3** show a greater affinity towards D_2 receptors when a butyl or benzyl moiety, respectively, is located in that position; however, 1-phenyl-THIQ derivatives (series **2**) show a selectivity for D_1 over D_2 receptors. The different activities and selectivity obtained for these compounds might be explained, at least in part, by the different spatial orientations adopted by the varied hydrophobic portions located at C-1 which lead to different molecular interactions with the D_2 receptors (quantum mechanics calculations). Nonetheless in the 1-butyl- and 1-benzyl-THIQ (series **1** and **3**), the greater flexibility of these substituents, along with their slight electronic effect towards the A-ring aromatic, confer more affinity for D_2.

1-Butyl-7-chloro-6-hydroxy-tetrahydroisoquinoline (**1e**), with the highest affinity towards the D_2 receptor (K_i value of 66 nM) and the highest selectivity (49-fold), was then evaluated by behavioral assays (spontaneous activity and forced swimming test) in mice. Compound **1e** increased the spontaneous activity as from 0.2 mg/kg and demonstrated an antidepressant-like activity at a low dose (0.01 mg/kg), which is likely because of an activation of the D_2-type receptor. Further experiments will be necessary to evaluate the real therapeutic potential of this compound as a new antidepressant drug.

4. Experimental

4.1. General instrumentation

Melting points were taken on a Cambridge microscope instruments coupled with a Reichert-Jung. EI and FAB mass spectra were recorded on a VG Auto Spec Fisons spectrometer instruments (Fisons, Manchester, United Kingdom). ^{1}H NMR and ^{13}C NMR spectra were recorded with $CDCl_3$ as solvent on a Bruker AC-300, AC-400 or AC-500. Multiplicities of ^{13}C NMR resonances were assigned by DEPT experiments. NOE DIFF irradiations, COSY, HMQC, HSQC and HMBC correlations were recorded at 400 MHz and 500 MHz (Bruker AC-400 or AC-500). All reactions were monitored by analytical TLC with Silica gel 60 F_{254} (Merck 5554). The residues were purified through Silica gel 60 (40–63 μm, Merck 9385)

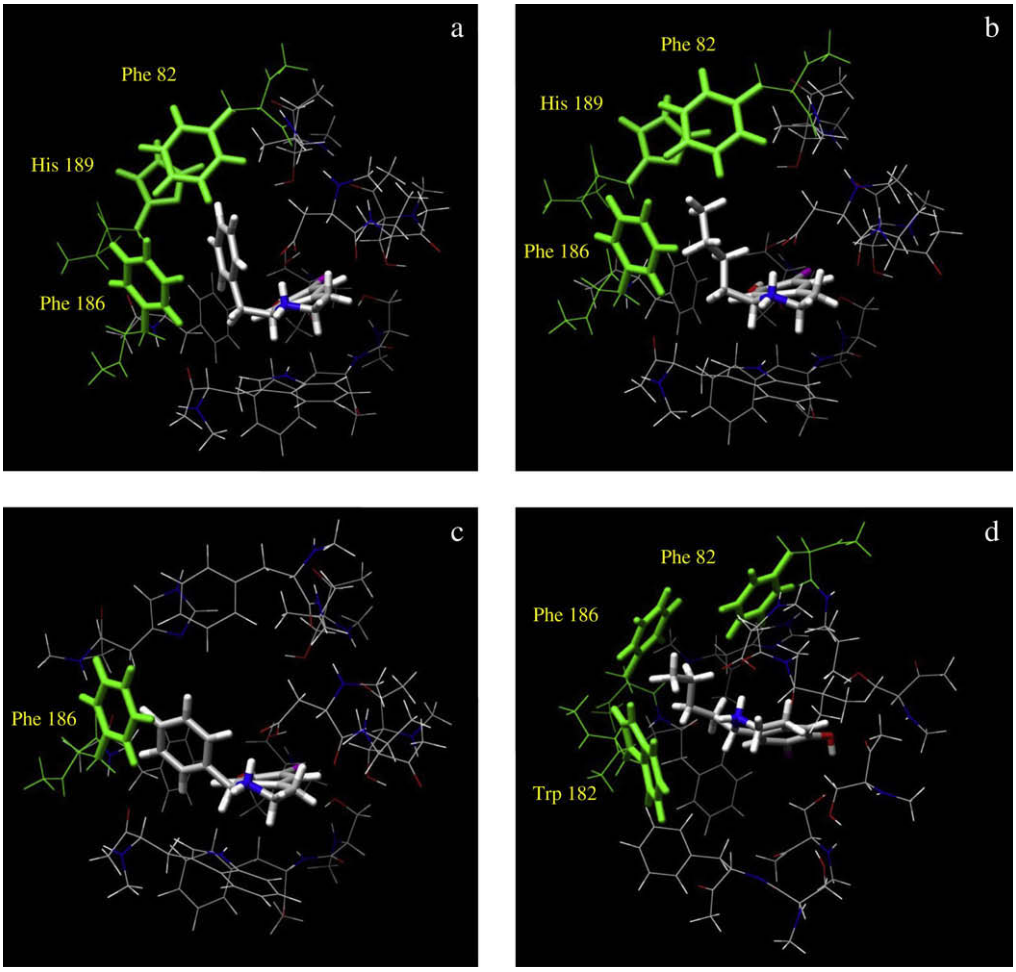


Figure 6. Interactions of compound **3c** (a), **1c** (b), **2c** (c) and **1e** (d) with the binding pocket D_2 DR. Different spatial views to show the hydrophobic interactions; the aromatic rings of Phe 82, His 189, Phe 186 and Trp 182 are denote in thick green lines in this figure.

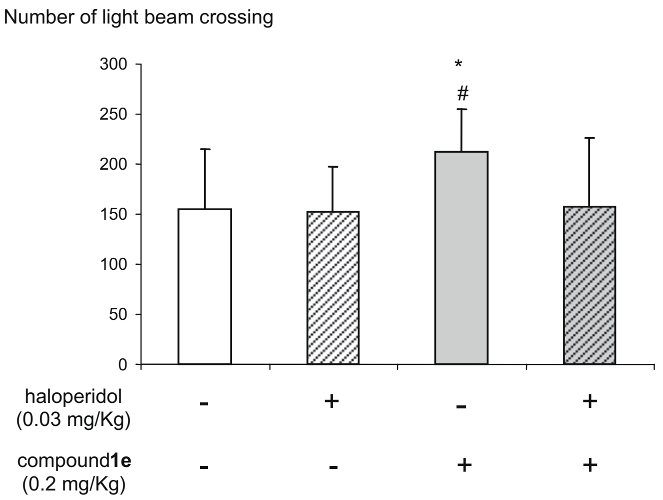


Figure 7. Effect of haloperidol (0.03 mg/kg; sc) on hyperlocomotor activity induced by compound **1e** (0.2 mg/kg; ip).*$p < 0.05$ versus control group, #$p < 0.05$ versus 'haloperidol + **1e**' group (PLSD of Fischer). The results are expressed as mean ± SD ($n = 10$ animals for each group).

column chromatography. Solvents and reagents were used as purchased from commercial sources. Quoted yields are of purified material. The HCl salts of the synthesised compounds were prepared from the corresponding base with 5% HCl in MeOH.

Table 3
Dose effect of compound **1e** on spontaneous locomotor activity assessed by the number of light beam crossing in a photoelectronic actimeter during a 30 min test session

	0–6 min	0–12 min	0–18 min	0–24 min	0–30 min
Vehicle	66 ± 29	102 ± 39	131 ± 55	153 ± 63	161 ± 70
0.01 mg/Kg	72 ± 38	105 ± 58	133 ± 81	147 ± 85	153 ± 88
0.04 mg/Kg	102 ± 24*	142 ± 39	170 ± 47	185 ± 42	200 ± 46
0.2 mg/Kg	115 ± 36*	186 ± 58*	219 ± 69*	255 ± 88*	277 ± 115*
1 mg/Kg	122 ± 29*	200 ± 43*	257 ± 48*	307 ± 74*	340 ± 87*
5 mg/Kg	153 ± 57*	276 ± 82*	345 ± 80*	411 ± 114*	464 ± 145*
25 mg/Kg	170 ± 39*	260 ± 73*	319 ± 106*	367 ± 139*	426 ± 164*

* ANOVA, PLSD of Fisher: $p < 0.05$ versus vehicle group, for each time section.

4.1.1. 2-(3-Chloro-4-methoxy-phenyl)ethylamine

A mixture of 3-chloro-4-methoxy-benzaldehyde (1.0 g, 5.87 mmol), nitromethane (1 mL, 18.41 mmol) and NH_4OAc (1.2 g, 15.57 mmol) in AcOH (15 mL) was refluxed for 4 h. After cooling, the mixture was diluted with H_2O (10 mL) and extracted with CH_2Cl_2 (3 × 10 mL). The organic solution was washed with brine (2 × 10 mL) and H_2O (2 × 10 mL), dried over anhydrous Na_2SO_4, and evaporated to dryness to obtain *3-chloro-4-methoxy-β-nitrostyrene* from EtOH as yellow needles (1.1 g, 88%) which was used in the following step; mp: 143–145 °C; ^1H NMR* (400 MHz, $CDCl_3$): δ 7.91 (d, $J = 13.74$ Hz, 1H, H-), 7.59 (d, $J = 2.16$ Hz, 1H, H-2), 7.52 (d, $J = 13.74$ Hz, 1H, H-), 7.44 (dd,

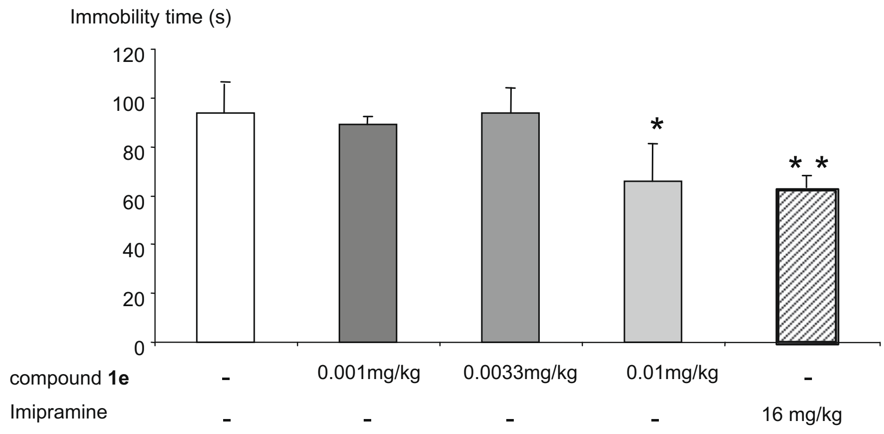


Figure 8. Effect of compound **1e** (0.001–0.0033–0.01 mg/kg; ip) and imipramine (16 mg/kg) on immobility time in the forced swimming test. * and ** illustrated statistical difference as compared to the control group (respectively, $p < 0.05$ and $p < 0.001$; PLSD of Fischer). The results are expressed as mean ± SD ($n = 10$ animals for each group).

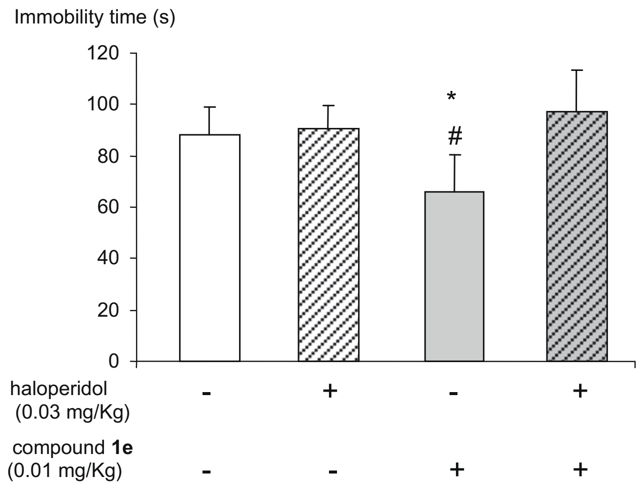


Figure 9. Effect of haloperidol (0.03 mg/kg; sc) on decrease of immobility time induced by compound **1e** (0.01 mg/kg ip) in the forced swimming test. *$p < 0.05$ as compared to the control group. #$p < 0.05$ versus 'haloperidol + **1e**' group (PLSD of Fischer). The results are expressed as mean ± SD ($n = 10$ animals for each group).

$J = 8.58$, 2.16 Hz, 1H, H-6), 6.97 (d, $J = 8.58$ Hz, 1H, H-5), 3.90 ppm (s, 3H, OCH_3-4); ^{13}C NMR* (100 MHz, $CDCl_3$) δ 158.4 (C-4), 138.4 (CH-β), 136.4 (CH-α), 130.9 (CH-2), 130.1 (CH-6), 124.2 (C-1), 123.7 (C-3), 112.7 (CH-5), 56.8 ppm (OCH_3); *The assignments were made by COSY and DEPT; MS (EI) *m/z* (%): 213 (55) $[M]^+$, 185 (100). A solution of 3-chloro-4-methoxy-β-nitrostyrene (1.0 g, 4.7 mmol) in anhydrous THF (14 mL) was added dropwise to a well-stirred suspension of $LiAlH_4$ (0.7 g, 18.5 mmol) in anhydrous Et_2O (20 mL) under nitrogen atmosphere, and after refluxed for 2 h. After cooling, the excess of reagent was destroyed by dropwise addition of H_2O and 15% aqueous NaOH. After partial evaporation of the filtered, the aqueous solution was extracted with CH_2Cl_2 (3×10 mL) and the organic layers were treated with 5% aqueous HCl. The resulting aqueous acid layer was made basic (5% aqueous NH_4OH, pH ≈ 9) and extracted with CH_2Cl_2. The organic solution was washed with brine (2×10 mL) and H_2O (2×10 mL), dried over Na_2SO_4, and the solvent was removed in vacuo to give *β-(3-chloro-4-methoxyphenyl)ethylamine* as a yellow powder (630 mg, 72%); The compound was used in further reaction without purification; ^1H NMR* (400 MHz, $CDCl_3$): δ 7.3 (d, $J = 2.24$ Hz, 1H, H-2), 7.2 (dd, $J = 8.46$, 2.24 Hz, 1H, H-6), 6.8 (d, $J = 8.46$ Hz, 1H, H-5), 3.9 (s, 3H, OCH_3-4), 3.1 (m, 2H, H-α), 2.8 ppm (m, 2H, H-); ^{13}C NMR* (100 MHz, $CDCl_3$): δ 153.8 (C-4), 133.3 (C-1), 130.8 (CH-2), 128.4 (CH-6), 122.6 (C-3), 112.5 (CH-5), 56.5 (OCH_3), 43.8 (CH_2-α), 39.1 ppm (CH_2-β); *The assignments were made by COSY and DEPT; MS (EI) *m/z* (%): 185 (45) $[M]^+$.

4.2. General procedure for synthesis of amides 1–3

Amides **1–3** were prepared under Schotten–Baumann conditions by condensation of the 2-(3-chloro-4-methoxyphenyl) ethylamine with the appropriate acid chloride: valeryl chloride (series 1), phenylacetyl chloride (series 2) or benzoyl chloride (series 3).

4.2.1. *N*-(3-Chloro-4-methoxyphenylethyl)butylacetamide (1)

An amount of valeryl chloride (0.44 mL, 3.75 mmol) was added dropwise at 0 °C to a solution of the β-(3-chloro-4-methoxyphenyl)ethylamine (580 mg, 3.13 mmol) in CH_2Cl_2 (20 mL) and 5% aqueous NaOH (4.5 mL), stirring at room temperature for 3 h. After, 2.5% aqueous HCl was added, and the organic solution was washed with brine (2×10 mL) and H_2O (2×10 mL), dried over Na_2SO_4 and evaporated to dryness. The residue was purified through silica gel column chromatography (hexane/EtOAc, 60:40) to afford the amide **1** as a yellow powder (730 mg, 2.71 mmol, 86.7%); ^1H NMR* (400 MHz, $CDCl_3$): δ 7.19 (d, $J = 2.07$ Hz, 1H, H-2), 7.04 (dd, $J = 8.28$, 2.07 Hz, 1H, H-6), 6.88 (d, $J = 8.28$ Hz, 1H, H-5), 3.88 (s, 3H, OCH_3-4), 3.47 (m, 2H, H-), 2.73 (t, $J = 6.97$ Hz, 2H, H-), 2.13 (t, $J = 7.62$ Hz, 2H, CH_2-1′), 1.59 (m, 2H, CH_2-2′), 1.30 (m, 2H, CH_2-3′), 0.91 ppm (t, $J = 7.26$ Hz, 2H, CH_3); ^{13}C NMR* (100 MHz, $CDCl_3$): δ 173.5 (CO), 154.0 (C-4), 132.1 (C-1), 130.4 (CH-2), 128.3 (CH-6), 122.7 (C-3), 112.5 (CH-5), 56.5 (OCH_3), 40.9 (CH_2-α), 36.8 (CH_2-1′), 34.9 (CH_2-β), 28.1 (CH_2-2′), 22.7 (CH_2-3′), 14.3 ppm (CH_3-4′); *The assignments were made by COSY and DEPT; MS (FAB) *m/z* (%): 270 $[M+H]^+$, 268 (35), 256 (21).

4.2.2. *N*-(3-Chloro-4-methoxyphenylethyl)benzylacetamide (2)

The title compound was prepared according to the procedure for **1**, using benzoyl chloride (0.43 mL, 3.75 mmol). The residue was purified through silica gel column chromatography (hexane/EtOAc, 50:50) to give the amide **2** as white powder (488 mg, 1.68 mmol, 54%); ^1H NMR* (300 MHz, $CDCl_3$): δ 8.13 (dd, $J = 8.46$, 1.14, 2H, H-2′, H-6′), 7.43 (m, 3H, H-3′, H-4′, H-5′), 7.25(d, $J = 2.20$ Hz, 1H, H-2), 7.08 (dd, $J = 8.42$, 2.20 Hz, 1H, H-6), 6.86 (d, $J = 8.42$ Hz, 1H, H-5), 3.94 (s, 3H, OCH_3-4), 3.65 (m, 2H, H-), 2.89 ppm (t, $J = 6.96$ Hz, 2H, H-); ^{13}C NMR* (75 MHz, $CDCl_3$): δ 168.0 (CO), 154.1 (C-4), 134.9 (C-1′), 132.6 (C-1), 132.0, 129.0, 127.8 (5CH-Ar), 128.8 (CH-2), 127.2 (CH-6), 122.3 (C-3), 112.6 (CH-5), 56.6 (OCH_3), 41.5 (CH_2-α), 34.9 ppm (CH_2-β); *The assignments were made by COSY and DEPT; MS (EI) *m/z* (%): 289 $[M]^+$, 275 (15), 168 (60).

4.2.3. *N*-(3-Chloro-4-methoxyphenylethyl)phenylacetamide (3)

The title compound was prepared according to the procedure for **1** and **2**, using the phenylacetyl chloride (0.5 mL, 3.75 mmol). The residue was purified through silica gel column chromatography (hexane/EtOAc, 60:40) to afford **3** as white powder (655 mg, 2.16 mmol, 69%); ^{1}H NMR* (400 MHz, $CDCl_3$): δ 7.35–7.14 (m, 5H, H–Ar), 7.05 (d, *J* = 2.7 Hz, 1H, H-2), 6.89 (dd, *J* = 8.5, 2.7 Hz, 1H, H-6), 6.78 (d, *J* = 8.5 Hz, 1H, H-5), 3.54 (s, 2H, CH_2CO), 3.90 (s, 3H, OCH_3-4), 3.4 (m, 2H, H-), 2.64 ppm (t, *J* = 6.79 Hz, 2H, H-); ^{13}C NMR* (100 MHz, $CDCl_3$): δ 171.3 (CO), 154.0 (C-4), 135.0 (C-1′), 132.2 (C-1), 127.7, 129.4, 129.5, 129.8, 130.8 (5CH–Ar), 128.2 (CH-2), 127.3 (CH-6), 122.7 (C-3), 112.5 (CH-5), 56.5 (OCH_3), 44.2 (CH_2-α), 41.0 (CH_2), 34.7 ppm (CH_2-β); *The assignments were made by COSY and DEPT; MS (EI) *m/z* (%): 303 (100) $[M]^+$, 289 (10).

4.3. Synthesis of 1-butyl, 1-phenyl and 1-benzyl-THIQs series (compounds 1a–3d)

THIQs series were prepared in two steps by standard methods starting the corresponding amides **1–3.**

4.3.1. General procedure for Bischler–Napieralski cyclization. 1-Butyl-6-chloro-7-methoxy-1,2,3,4-tetrahydroisoquinoline (1a)

To a 250 mL three-neck round-bottomed flask under N_2, the corresponding amide **1** (500 mg, 1.86 mmol) was added in dry toluene (20 mL) and treated with P_2O_5 (5.2 g, 18.6 mmol) which was added in portions followed by the dropwise addition of $POCl_3$ (1.7 mL, 18.6 mmol). The mixture was stirred and refluxed under N_2 for 6–8 h and then, cooled to room temperature. The toluene was concentrated under reduced pressure and the reaction mixture was slowly poured into a mixture of crushed ice. The solid residue was triturated with 10% aqueous NaOH to afford a suspension (pH ≈ 8–9) and extracted with CH_2Cl_2 (3 × 15 mL). The combined CH_2Cl_2 extracts were dried over $NaSO_4$ and the solvent evaporated in vacuo to afford a reddish oil. The residue was dissolved in MeOH (20 mL), and then, cooled to −78 °C and treated with $NaBH_4$ (76 mg, 2 mmol). The reaction mixture was stirred for 2 h. Water (15 mL) was added and the volatiles were evaporated under reduced pressure. The aqueous phase was extracted with CH_2Cl_2 (3 × 15 mL), and the combined organic layers were dried over Na_2SO_4 and evaporated to dryness. The crude was purified by silica gel column chromatography (CH_2Cl_2/MeOH, 90:10) to furnish **1a** and **1a**′ (223 mg, 0.88 mmol, 47.3%); **1a**: ^{1}H NMR* (400 MHz, $CDCl_3$): δ 7.06 (s, 1H, H-5), 6.65 (s, 1H, H-8), 3.90 (m, 1H, H-1), 3.84 (s, 3H, OCH_3), 3.25–3.17 (m, 1H, H-3a), 2.97–2.88 (m, 1H, H-3b), 2.72–2.59 (m, 2H, H-4), 1.80–1.68 (m, 1H, H-1′), 1.47–1.28 (m, 4H, H-2′, H-3′), 0.92 ppm (t, *J* = 6.90 Hz, 3H, H-4′); ^{13}C NMR* (100 MHz, $CDCl_3$): δ 152.8 (C-7), 139.3 (C-8a), 130.3 (CH-5), 128.3 (C-4a), 119.9 (C-6), 109.9 (CH-8), 56.1 (OCH_3), 55.6 (CH-1), 40.7 (CH_2-3), 36.1 (CH_2-1′), 28.8 (CH_2-4), 28.2 (CH_2-2′), 22.8 (CH_2-3′), 14.0 ppm (CH_3-4′); *The assignments were made by COSY 45, DEPT, HSQC and HMBC; MS (FAB) *m/z* (%): 254 (100) $[M+H]^+$, 196 (73); HRMS-FAB *m/z* $[M+H]^+$ calcd for $C_{14}H_{21}NOCl$: 254.1311, found 254.1317; **1a**′: ^{1}H NMR* (400 MHz, $CDCl_3$): δ 7.07 (s, 1H, H-8), 6.57 (s, 1H, H-5), 3.87 (m, 1H, H-1), 3.80 (s, 3H, OCH_3), 3.18–3.15 (m, 1H, H-3a), 2.93–2.88 (m, 1H, H-3b), 2.78–2.66 (m, 2H, H-4), 1.76–1.60 (m, 1H, H-1′), 1.37–1.31 (m, 4H, H-2′, H-3′), 0.89 ppm (t, *J* = 6.90 Hz, 3H, H-4′); ^{13}C NMR* (100 MHz, $CDCl_3$): δ 152.7 (C-6), 134.7 (C-8a), 132.8 (C-4a), 127.5 (CH-8), 119.5 (C-7), 112.3 (CH-5), 55.9 (CH-1), 54.9 (OCH_3), 40.8 (CH_2-3), 35.9 (CH_2-1′), 29.8 (CH_2-4), 28.1 (CH_2-2′), 22.7 (CH_2-3′), 14.0 ppm (CH_3-4′); *The assignments were made by COSY 45, DEPT, HSQC and HMBC; MS (FAB) *m/z* (%): 254 (100) $[M+H]^+$, 196 (73); HRMS-FAB *m/z* $[M+H]^+$ calcd for $C_{14}H_{21}NOCl$: 254.1311, found 254.1313.

4.3.2. 1-Phenyl-6-chloro-7-methoxy-1,2,3,4-tetrahydroisoquinoline (2a)

The title compound was prepared according to the same procedure for **1a** using the corresponding amide **2** (500 mg, 1.73 mmol), P_2O_5 (4.9 g, 17.3 mmol) and $POCl_3$ (1.6 mL, 17.3 mmol). The residue was purified through silica gel column chromatography (CH_2Cl_2/MeOH, 90:10) to obtain **2a** as a yellow oil (110 mg, 0.403 mmol, 23.3%); ^{1}H NMR* (400 MHz, $CDCl_3$): δ 7.34–7.23 (m, 5H, H–Ar), 7.15 (s, 1H, H-5), 6.30 (s, 1H, H-8), 5.05 (s, 1H, H-1), 3.63 (s, 3H, OCH_3), 3.23–3.18 (m, 1H, H-4a), 3.05–2.87 (m, 2H, H-3), 2.76–2.71 ppm (m, 1H, H-4b); ^{13}C NMR* (100 MHz, $CDCl_3$): δ 152.7 (C-7), 144.1 (C-1′), 137.5 (C-8a), 135.1 (C-4a), 130.2 (CH-5), 128.9, 128.4, 127.5 (5CH–Ar), 120.4 (C-6), 111.6 (CH-8), 61.6 (CH-1), 56.0 (OCH_3), 41.6 (CH_2-3), 28.6 ppm (CH_2-4); *The assignments were made by COSY 45, DEPT, HSQC and HMBC; MS (FAB) *m/z* (%): 274 (100) $[M+H]^+$, 196 (13); HRMS-FAB *m/z* $[M]^+$ calcd for $C_{16}H_{16}NOCl$: 273.098, found 273.104.

4.3.3. 1-Benzyl-6-chloro-7-methoxy-1,2,3,4-tetrahydroisoquinoline (3a)

The title compound was prepared according to the same procedure for **1a** using the corresponding amide **3** (500 mg, 1.65 mmol), P_2O_5 (4.7 g, 16.5 mmol) and $POCl_3$ (1.5 mL, 16.5 mmol). The residue was purified through silica gel column chromatography (CH_2Cl_2/MeOH, 90:10) to obtain **3a** as a yellow oil (138 mg, 0.48 mmol, 29%); ^{1}H NMR* (400 MHz, $CDCl_3$): δ 7.35–7.24 (m, 5H, H–Ar), 7.11 (s, 1H, H-5), 6.63 (s, 1H, H-8), 4.18 (dd, *J* = 4.81, 9.22 Hz, 1H, H-1), 3.80 (s, 3H, OCH_3), 3.24–3.16 (m, 2H, H-αa, H-3a), 2.98–2.81 (m, 2H, H-αb, H-3b), 2.76–2.69 ppm (m, 2H, H-4); ^{13}C NMR* (100 MHz, $CDCl_3$): δ 152.6 (C-7), 138.7 (C-1′), 138.0 (C-8a), 129.3 (CH-5), 129.0, 128.6, 126, 5 (5CH–Ar), 128.3 (C-4a), 120.2 (C-6), 110.1 (CH-8), 57.0 (CH-1), 56.1 (OCH_3), 42.7 (CH_2-3), 40.3 (CH_2-α), 28.8 ppm (CH_2-4); *The assignments were made by COSY 45, DEPT, HSQC and HMBC; MS (FAB) *m/z* (%): 288 (84) $[M+H]^+$, 196 (100); HRMS-FAB *m/z* $[M+H]^+$ calcd for $C_{17}H_{19}NOCl$: 288.1155, found 288.1148.

4.3.4. General procedure for N-methylation. 1-Butyl-6-chloro-7-methoxy-*N*-methyl-1,2,3,4-tetrahydroisoquinoline (1b)

To a stirred solution of **1a** (500 mg, 1.98 mmol) in MeOH (20 mL), 37% formaldehyde (15 mL) and one drop of formic acid were added. The mixture was refluxed for 1 h, cooled to room temperature, treated with $NaBH_4$ (750 mg, 19.8 mmol), and refluxed an additional 1 h. The reaction mixture was warmed up to ambient temperature and the solvent was removed under reduced pressure. Water (3 mL) was added to the residue and the aqueous mixture was extracted with CH_2Cl_2 (3 × 15 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated under reduced pressure to give the crude product which was further purified by silica gel column chromatography (CH_2Cl_2/MeOH/NH_4OH, 98:2:0.2) to afford **1b** (480 mg, 1.8 mmol, 91%); ^{1}H NMR* (300 MHz, $CDCl_3$): δ 7.08 (s, 1H, H-5), 6.61 (s, 1H, H-8), 3.86 (s, 3H, OCH_3), 3.35 (t, 1H, *J* = 5.46 Hz, H-1), 3.12–3.06 (m, 1H, H-3a), 2.77–2.73 (m, 2H, H-4), 2.72–2.64 (m, 1H, H-3b), 2.42 (s, 3H, NCH_3), 1.75–1.68 (m, 2H, H-1′), 1.34–1.19 (m, 4H, H-2′, H-3′), 0.88 ppm (t, *J* = 6.97 Hz, 3H, H-4′); ^{13}C NMR* (75 MHz, $CDCl_3$): δ 152.6 (C-7), 134.3 (C-8a), 131.6 (C-4a), 128.6 (CH-5), 119.6 (C-6), 111.9 (CH-8), 62.8 (CH-1), 56.2 (OCH_3), 48.0 (CH_2-3), 42.6 (NCH_3), 34.4 (CH_2-1′), 27.56 (CH_2-2′), 26.06 (CH_2-4), 22.94 (CH_2-3′), 14.05 ppm (CH_3-4′); *The assignments were made by COSY 45, DEPT, HSQC and HMBC; MS (EI) *m/z* (%): 268 (100) $[M+H]^+$, 210 (68); HRMS-FAB *m/z* $[M+H]^+$ calcd for $C_{15}H_{23}NOCl$: 268.1468, found 268.1475.

4.3.5. 1-Phenyl-6-chloro-7-methoxy-*N*-methyl-1,2,3,4-tetrahydroisoquinoline (2b)

The title compound was prepared according to the same procedure for **1b** using the compound **2a** (500 mg, 1.83 mmol), 37% formaldehyde (15 mL), one drop of formic acid and $NaBH_4$ (700 mg, 18.3 mmol). The residue was purified by silica gel column chromatography (cyclohexane/OEtAc/Et_2N, 92:6:2) to obtain **2b** (460 mg, 1.60 mmol, 87.4%); ^1H NMR* (400 MHz, $CDCl_3$): δ 7.3–7.23 (m, 5H, H–Ar), 7.13 (s, 1H, H-5), 6.16 (s, 1H, H-8), 4.19 (s, 1H, H-1), 3.57 (s, 3H, OCH_3), 3.24–3.13 (m, 2H, H-4), 2.80–2.74 (m, 2H, H-3), 2.23 ppm (s, 3H, NCH_3); ^{13}C NMR* (100 MHz, $CDCl_3$): δ 152.9 (C-7), 143.2 (C-1′), 134.0 (C-8a), 131.8 (C-4a), 129.9 (CH-5), 129.5, 128.4, 127.5 (5CH–Ar), 119.8 (C-6), 111.4 (CH-8), 71.1 (CH-1), 56.1 (OCH_3), 51.9 (CH_2-3), 44.2 (NCH_3), 28.3 ppm (CH_2-4); *The assignments were made by COSY 45, DEPT, HSQC and HMBC; MS (FAB) *m/z* (%): 288 (100) $[M+H]^+$, 210 (48); HRMS-FAB *m/z* $[M+H]^+$ calcd for $C_{18}H_{20}NOCl$: 288.0013, found 288.1064.

4.3.6. 1-Benzyl-6-chloro-7-methoxy-*N*-methyl-1,2,3,4-tetrahydroisoquinoline (3b)

The title compound was prepared according to the same procedure for **1b** using the compound **3a** (500 mg, 1.74 mmol), 37% formaldehyde (15 mL), one drop of formic acid and $NaBH_4$ (660 mg, 17.4 mmol). The residue was purified through silica gel column chromatography (cyclohexane/OEtAc/Et_2N, 98:1:1) to obtain **3b** (451 mg, 1.5 mmol, 86.2%); ^1H NMR* (400 MHz, $CDCl_3$): δ 7.29–7.09 (m, 5H, H–Ar), 7.07 (s, 1H, H-5), 5.92 (s, 1H, H-8), 3.77–3.74 (m, 1H, H-1), 3.47 (s, 3H, OCH_3), 3.25–3.15 (m, 2H, H-αa, H-3a), 2.88–2.71 (m, 3H, H-αb, H-3b, H-4a), 2.63–2.57 (m, 1H, H-4b), 2.54 ppm (s, 3H, NCH_3); ^{13}C NMR* (100 MHz, $CDCl_3$): δ 151.9 (C-7), 139.6 (C-1′), 136.7 (C-8a), 129.9, 129.9, 128.2 (5CH–Ar), 126.8 (C-4a), 126.1 (CH-5), 120.0 (C-6), 111.7 (CH-8), 65.1 (CH-1), 55.6 (OCH_3), 46.3 (CH_2-3), 42.5 (NCH_3), 40.7 (CH_2-α), 24.9 ppm (CH_2-4); *The assignments were made by COSY 45, DEPT, HSQC and HMBC; MS (EI) *m/z* (%): 301 (5) $[M]^+$, 210 (100); MS (FAB) *m/z* (%): 302 (100) $[M+H]^+$, 210 (43); HRMS-FAB *m/z* $[M+H]^+$ calcd for $C_{18}H_{20}NOCl$: 301.8105, found: 301.7903.

4.3.7. General procedure for O-demethylation. 1-Butyl-6-chloro-7-hydroxy-1,2,3,4-tetrahydroisoquinoline (1c) and 1-butyl-6-hydroxy-7-chloro-1,2,3,4-tetrahydro-isoquinoline (1e)

A solution of the appropriate isoquinoline **1a** and **1a**′ (260 mg, 1.02 mmol) in dry CH_2Cl_2 (10 mL) was cooled to −78 °C. To this stirring solution, BBr_3 (0.4 mL, 4.08 mmol) was added dropwise. After 15 min, the reaction mixture was warmed up to ambient temperature and stirred for 18 h. The reaction was terminated by the addition of MeOH (5 mL) dropwise and the mixture was stirred for another 30 min. The solvent was concentrated to dryness. The residue was dissolved in EtOAc (2 mL) and made alkaline with 37% aqueous NH_4OH to pH ≈ 11, and subsequently neutralized with 1 M HCl to pH ≈ 7–8. The aqueous layer was then extracted with the EtOAc (3 × 10 mL). The combined EtOAc extracts were dried over Na_2SO_4, and evaporated under reduced pressure. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH/NH_4OH, 100:8:0.5) to afford a mixture of the two isomers **1c** and **1e** (220 mg, 0.92 mmol, 90%) in a 1:2 ratio; **1c** (73 mg, 0.3 mmol, 30%) and **1e** (147 mg, 0.6 mmol, 60%); ^1H NMR* (300 MHz, $CDCl_3$) for **1c**: δ 7.03 (s, 1H, H-5), 6.78 (s, 1H, H-8), 3.88 (dd, *J* = 8.85, 3.75 Hz, 1H, H-1), 3.23–3.17 (m, 1H, H-3a), 2.98–2.90 (m, 1H, H-3b), 2.73–2.65 (m, 2H, H-4), 1.80–1.67 (m, 2H, H-1′), 1.40–1.33 (m, 4H, H-2′, H-3′), 0.93 ppm (t, *J* = 6.97 Hz, 3H, H-4′); ^{13}C NMR* (100 MHz, $CDCl_3$): δ 149.2 (C-7), 140.1 (C-8a), 129.0 (CH-5), 128.3 (C-4a), 117.5 (C-6), 113.6 (CH-8), 55.5 (C-1), 41.5 (CH_2-3), 35.9 (CH_2-1′), 28.9 (CH_2-4), 28.1 (CH_2-2′), 22.8 (CH_2-3′), 14.0 ppm (CH_3-4′); *The assignments were made by COSY 45, DEPT and NOE; MS (EI) *m/z* (%): 238 (5) $[M-H]^+$, 182 (100); HRMS-EI *m/z* $[M-H]^+$ calcd for $C_{13}H_{17}NOCl$: 238.0999, found: 238.0972.

^1H NMR* (400 MHz, CD_3OD) for **1e**: δ 7.07 (s, 1H, H-8), 6.68 (s, 1H, H-5), 3.85 (dd, *J* = 8.70, 3.60 Hz, 1H, H-1), 3.23–3.17 (m, 1H, H-3a), 2.98–2.90 (m, 1H, H-3b), 2.73–2.65 (m, 2H, H-4), 1.80–1.63 (m, 2H, H-1′), 1.40–1.33 (m, 4H, H-2′ and H-3′), 0.93 ppm (t, *J* = 6.97 Hz, 3H, H-4′); ^{13}C NMR* (100 MHz, CD_3OD): δ 152.60 (C-6), 135.62 (C-4a), 131.71 (C-8a), 128.32 (CH-8), 119.60 (C-7), 117.56 (CH-5), 56.12 (CH-1), 41.65 (CH_2-3), 36.72 (CH_2-1′), 29.47 (CH_2-4), 29.04 (CH_2-2′), 23.83 (CH_2-3′), 14.38 ppm (CH_3-4′); *The assignments were made by COSY 45, DEPT, HSQC, HMBC and NOE; MS (EI) *m/z* (%): 238 (3) $[M-H]^+$, 182 (100); HRMS-EI *m/z* $[M-H]^+$ calcd for $C_{13}H_{17}NOCl$: 238.0999, found: 238.0986.

4.3.8. 1-Butyl-6-chloro-7-hydroxy-*N*-methyl-1,2,3,4-tetrahydroisoquinoline (1d)

This compound was prepared as the same procedure for the synthesis of **1c** using the compound **1b** (260 mg, 0.97 mmol) and BBr_3 (0.39 mL, 3.9 mmol). The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH/NH_4OH, 100/6/0.5) to give **1d** (194 mg, 0.77 mmol, 79%); ^1H NMR* (300 MHz, $CDCl_3$): δ 7.03 (s, 1H, H-5), 6.72 (s, 1H, H-8), 3.35 (t, 1H, *J* = 5.46 Hz, H-1), 3.12–3.06 (m, 1H, H-3a), 2.77–2.63 (m, 3H, H-4, H-3b), 2.42 (s, 3H, NCH_3), 1.74–1.67 (m, 2H, H-1′), 1.33–1.26 (m, 4H, H-2′, H-3′), 0.88 ppm (t, *J* = 7.15 Hz, 3H, H-4′); ^{13}C NMR* (75 MHz, $CDCl_3$): δ 149.0 (C-7), 135.1 (C-8a), 131.8 (C-4a), 127.3 (CH-5), 117.2 (C-6), 115.7 (CH-8), 62.9 (CH-1), 47.8 (CH_2-3), 42.5 (NCH_3), 34.5 (CH_2-1′), 27.7 (CH_2-2′), 25.6 (CH_2-4), 22.9 (CH_2-3′), 14.1 ppm (CH_3-4′); MS (FAB) *m/z* (%): 254 (19) $[M+H]^+$; MS (EI) *m/z* (%): 196 (100); HRMS-FAB *m/z* $[M+H]^+$ calcd for $C_{14}H_{21}NOCl$: 254.1311, found: 254.1312.

4.3.9. 1-Phenyl-6-chloro-7-hydroxy-1,2,3,4-tetrahydroisoquinoline (2c)

This compound was prepared as the above procedure for the synthesis of **1c** using the compound **2a** (260 mg, 0.95 mmol) and BBr_3 (0.38 mL, 3.8 mmol). The residue was purified through silica gel column chromatography (CH_2Cl_2/MeOH/NH_4OH, 100:7:0.5) to give **2c** (170 mg, 0.66 mmol, 69%); ^1H NMR* (300 MHz, CD_3OD): δ 7.28–7.15 (m, 5H, H–Ar), 7.01 (s, 1H, H-5), 6.17 (s, 1H, H-8), 4.88 (s, 1H, H-1), 3.15–3.06 (m, 1H, H-3a), 2.94–2.79 (m, 2H, H-3b, H-4a), 2.70–2.60 ppm (m, 1H, H-4b); ^{13}C NMR* (75 MHz, CD_3OD): δ 150.0 (C-7), 142.6 (C-1′), 136.5 (C-8a), 128.6 (CH-5), 127.4, 127.3, 126.5 (5CH–Ar), 126.2 (C-4a), 114.6 (CH-8), 60.4 (CH-1), 40.6 (CH_2-3), 26.5 ppm (CH_2-4); HRMS-EI *m/z* $[M-H]^+$ calcd for $C_{15}H_{14}NOCl$: 259.0764, found: 259.0728.

4.3.10. 1-Phenyl-6-chloro-7-hydroxy-*N*-methyl-1,2,3,4-tetrahydroisoquinoline (2d)

This compound was prepared in a similar manner as described for the synthesis of **1c** using the compound **2b** (260 mg, 0.9 mmol) and BBr_3 (0.36 mL, 3.6 mmol). The residue was purified through silica gel flash column (CH_2Cl_2/MeOH/NH_4OH, 100:2:0.5) to give **2d** (130 mg, 0.47 mmol, 52%); ^1H NMR* (500 MHz, $CDCl_3$): δ 7.30–7.20 (m, 5H, H–Ar), 7.06 (s, 1H, H-5), 6.23 (s, 1H, H-8), 4.12 (s, 1H, H-1), 3.16–3.09 (m, 1H, H-4a), 3.07–3.04 (m, 1H, H-3a), 2.72–2.70 (m, 1H, H-4b), 2.55 (td, 1H, *J* = 11.13, 3.62 Hz; H-3b) 2.16 ppm (s, 3H, NCH_3); ^{13}C NMR* (75 MHz, $CDCl_3$): δ 149.07 (C-7), 143.06 (C-1′), 138.94 (C-8a), 129.48, 128.40, 127.52 (5CH–Ar), 128.25 (CH-5), 127.60 (C-4a), 118.02 (C-6), 115.94 (CH-8), 71.06 (CH-1), 52.16 (CH_2-3), 44.20 (NCH_3), 28.42 ppm (CH_2-4); *The assignments were made by COSY 45 and HSQC; HRMS-EI *m/z* $[M-H]^+$ calcd for $C_{16}H_{16}NOCl$: 273.0920, found: 272.0923.

4.3.11. 1-Benzyl-6-chloro-7-hydroxy-1,2,3,4-tetrahydroisoquinoline (3c)

This compound was prepared in a similar manner as described for the synthesis of **1c** using the compound **3a** (260 mg, 0.9 mmol) and BBr_3 (0.36 mL, 3.6 mmol). The residue obtained was purified through silica gel column (CH_2Cl_2/MeOH/NH_4OH, 100:6:0.5) to give **3c** (183 mg, 0.77 mmol, 74%); ^1H NMR* (300 MHz, $CDCl_3$): δ 7.34–7.22 (m, 5H, H–Ar), 7.06 (s, 1H, H-5), 6.85 (s, 1H, H-8), 4.11 (dd, J = 10.17, 3.75 Hz, 1H, H-1), 3.22–3.14 (m, 2H, H-αa, H-3a), 2.92–2.81 (m, 2H, H-αb, H-3b), 2.75–2.65 ppm (m, 2H, H-4); ^{13}C NMR* (75 MHz, $CDCl_3$): δ 149.7 (C-7), 138.2 (C-1′), 138.2 (C-8a), 129.4 (CH-5), 129.3, 128.8, 126.7 (5CH–Ar), 127.8 (C-4a), 118.5 (C-6), 114.1 (CH-8), 56.7 (CH-1), 42.0 (CH_2-3), 40.2 (CH_2-α), 28.5 ppm (CH_2-4); *The assignments were corroborated by NOE DIFF; HRMS-EI m/z $[M-H]^+$ calcd for $C_{16}H_{15}NOCl$: 272.0842, found: 272.0804.

4.3.12. 1-Benzyl-6-chloro-7-hydroxy-*N*-methyl-1,2,3,4-tetrahydroisoquinoline (3d)

This compound was prepared in a similar manner as described for the synthesis of **1c** using the compound **3b** (260 mg, 0.86 mmol) and BBr_3 (0.34 mL, 3.4 mmol). The residue was purified through silica gel column chromatography (CH_2Cl_2/MeOH/NH_4OH, 100:3:0.5) to give **3d** (150 mg, 0.52 mmol, 61%); ^1H NMR* (300 MHz, $CDCl_3$): δ 7.28–7.05 (m, 5H, H–Ar), 7.01 (s, 1H, H-5), 6.28 (s, 1H, H-8), 3.75 (m, 1H, H-1), 3.27–3.09 (m, 2H, H-αa, H-3a), 2.87–2.62 (m, 2H, H-αb, H-4a, H-3b), 2.58–2.52 (m, 1H, H-4b), 2.48 ppm (s, 3H, NCH_3); ^{13}C NMR* (75 MHz, $CDCl_3$): δ 148.79 (C-7), 139.44 (C-1′), 137.95 (C-8a), 129.56, 128.14, 126.10 (5CH–Ar), 128.69 (CH-5), 127.31 (C-4a), 117.86 (C-6), 115.29 (CH-8), 64.67 (CH-1), 46.52 (CH_2-3), 42.51 (NCH_3), 41.01 (CH_2-α), 24.67 ppm (CH_2-4); HRMS-FAB m/z $[M+H]^+$ calcd for $C_{17}H_{19}NOCl$: 288.1155, found: 288.1151.

4.4. Pharmacological in vitro assays

4.4.1. Animals

Female Wistar rats (200–220 g) bred in a standard experimental animal room of the Faculty of Pharmacy were used for $[^3H]$-dopamine uptake assays and radioligand binding experiments. The rats were housed under a 12-h light/dark cycle at 22 °C and 60% humidity. All protocols complied with European Community guidelines for the use of experimental animals and were approved by the Ethics Committee of the University of Valencia.

4.4.2. $[^3H]$-Dopamine uptake assay

$[^3H]$-Dopamine uptake was studied using a preparation of rat striatal synaptosomes. All experimental procedures for the synaptosomes preparation were carried out at 0–4 °C. The rat striatum was dissected, homogenized in 10 volumes (w/v) of 0.32 M sucrose with an ultraturrax T25 (Janke & Kinkel) (4 s, maximal scale) and centrifuged at 1000g for 10 min. The supernatant was stored and the pellet was resuspended in 10 volumes of 0.32 M sucrose and recentrifuged at 1000g for 10 min. The two supernatants were combined and the mixture centrifuged at 16,000g for 30 min. The resultant pellet was suspended in 10 volumes of ice-cold Krebs medium (pH 7.6) contained (mM): 118 mM NaCl; 4.75 mM KCl; 1.2 mM KH_2PO_4; 1.8 mM $CaCl_2$; 1.2 mM $MgCl_2$; 25 mM $NaHCO_3$; and 11 mM glucose. Aliquots were preincubated during 10 min at 37 °C in Krebs buffer containing 10 μM pargyline (to block metabolism of dopamine by monoamine oxidase), $[^3H]$-dopamine (47 Ci/mmol, Amersham) was added to a final 0.5 nM concentration and the incubation was continued for another 10 min. Compounds were screened at 100 μM. Incubation was terminated by dilution into ice-cold Krebs medium and the samples were filtered rapidly through fiberglass filters (Schleicher & Schuell Grade 30) using a Brandel cell harvester (model M-24, Biochemical Research and Development Laboratories, Inc.). Filters were washed twice with 3 mL cold Krebs medium and dried. Non-specific $[^3H]$-dopamine uptake was determined in the presence of 10 μM nomifensine (dopamine uptake inhibitor). Filters were placed into scintillation mixture (Optiphase 'Hisafe' 2, Perkin Elmer) and radioactivity was determined by scintillation spectrometry[12,15] Protein concentrations were determined using the Bradford protein assay (Biorad).

4.4.3. Radioligand binding assays

$[^3H]$-SCH23390 and $[^3H]$-raclopride binding experiments were performed on rat striatal membranes. The rat striatum was homogenized in 10 volumes (w/v) of TRIS–HCl buffer (50 mM, pH 7.4 at 22 °C) with an ultraturrax T25 (Janke & Kinkel) (4 s, maximal scale). The homogenate was centrifuged twice at 49,000g for 15 min at 4 °C with resuspension in the same volume of TRIS–HCl buffer between each centrifugation. The final pellet was resuspended in TRIS-ions buffer containing 120 mM NaCl; 2 mM $CaCl_2$, 5 mM KCl; 1 mM $MgCl_2$; and 0.1% ascorbic acid (pH 7.4). For D_1-like receptor binding assays, membranes (100 μg/mL) were incubated with $[^3H]$-SCH 23390 (0.25 nM; 66 Ci/mmol, Amersham, GE Healthcare, UK) and various concentrations of competition compound (10^{-10}M–10^{-4}M) for 1 h at 23 °C. Non-specific binding was determined in the presence of 30 μM SK&F38393. For D_2-like receptor binding assays, membranes (200 μg/mL) were incubated with $[^3H]$-raclopride (0.5 nM; 62.2 Ci/mmol, Perkin Elmer) and various concentrations of competition compound (10^{-10}M–10^{-4}M) for 1 h at 23 °C. Non-specific binding was determined in the presence of 50 μM apomorphine (Sigma). In both cases, incubations were stopped by the addition of 3 mL ice-cold TRIS-ions buffer followed by rapid filtration through fiberglass filters (Schleicher & Schuell Grade 30) using a Brandel cell harvester (model M-24, Biochemical Research and Development Laboratories, Inc.). Filters were washed twice with 3 mL cold TRIS-ions buffer. After the filters had been dried, radioactivity was counted in 4 mL scintillation liquid (Optiphase 'Hisafe' 2, Perkin Elmer). All the compounds were used as hydrochloride salts. Data were analyzed by Prim (Graph Pad Software; San Diego, California, USA) and K_i values were determined using the K_D value for $[^3H]$-SCH23390 of 0.36 nM and for $[^3H]$-raclopride of 1,25 nM. Values are expressed at the mean ± SEM of three to six independent determinations performed in duplicate.

4.5. Behavioral studies

4.5.1. Animals

Naïve male NMRI mice (Centre d'Elevage René Janvier) weighing 25–30 g were used for all experiments. Mice were housed by groups of 10 animals in standard polycarbonate cages and maintained in a regulated environment (22 ± 1 °C) under 12–12 h light/dark cycle (light on between 20:00 and 8:00) with food and water freely available in the home cage. Behavioral tests were conducted during the dark phase of the cycle, between 10 h and 16 h. Each animal was used only once. All experiments complied with the European Community guidelines and the French law on animal experimentation (personal authorization n° 14–17 and 14–26 for MB and TF, respectively).

4.5.2. Drug administration

Animals were randomly divided into groups (n = 10/group). All injections (10 mL/Kg) were realized 30 min before behavioral tests. In dose–response study, mice were injected intra-peritaneously, by either the **1e** compound (0.001–25 mg/kg), or vehicle (saline water, 0.9% NaCl). In order to investigate the implication of D_2-receptors in hyperlocomotor and anti-depressant like activities, a D_2-receptor antagonist (haloperidol) was used. In both cases, haloperidol was injected sub-cutaneously, just before ip administration (**1e** compound or saline water), at the dose of 0.03 mg/Kg on basis preliminary study (data not shown).

4.5.3. Spontaneous locomotor activity

In order to assess the spontaneous horizontal activity, mice were tested using a photoelectronic actimeter (APELAB®), initially designed by Boissier and Simon (1965).[31] The apparatus consisted of a perspex enclosure (25.5 × 20.5 × 9 cm^3). Two infrared perpendicular light beams emerging from two consecutive walls, crossed in the center of the box and were connected to two photoelectric cells. Locomotor activity was recorded as the number of times the mouse crossed each beam, and consecutively interrupting the light beam. Each mouse was tested for a total period of 30 min and the numbers of light beams interruptions were noted all 6 min. In this condition, chlorpromazine (4 mg/Kg, ip) and amphetamine (10 mg/Kg, ip), used as pharmacological references (depressive and stimulative, respectively), induce, respectively a decrease (490%, PLSD of Fisher, $p < 0.001$) and an increase (260%, PLSD of Fisher, $p < 0.001$) of spontaneous activity measured during 30 min.

4.5.4. Forced swimming test

The FST[32,33] was carried out in mice which were individually forced to swim during 6 min in an open cylindrical container (diameter 12 cm, height 20 cm) with a water depth of 13 cm at 23–24 °C. The duration of immobility, after a delay of 2 min, was measured during the last 4 min. Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. Animals were not pre-tested. In this condition, imipramine (16 mg/kg), used as pharmacological reference, significantly decreased the immobility time (32% versus control group, $p < 0.001$—PLSD of Fisher).

4.6. Statistical analyses

All quantitative data were expressed as mean ± standard deviation and were analyzed (statview®) using analysis of variance (ANOVA) followed, in case of significant effects, by a post-hoc multiple comparison tests (PLSD of Fisher). *P*-values less than 0.05 were considered to be significant.

4.7. Theoretical calculations

Binding pocket of the D_2 L–R (ligand–receptor) was defined according to Teeter et al.[27] and Neve et al.[34] In our reduced model system, only 13 aminoacids were included for the molecular calculations. The size of the molecular system simulated and the complexity of the structures under investigation restricted the choice of the quantum mechanical method to be used. Consequently the semiempirical AM1 method was selected combined with ab initio calculations (RHF/6-31G(d)). The torsional angles of the ligands and the flexible side-chains of the amino acids as well as the bond angles and bond lengths of the moieties involved in the potential intermolecular interactions were optimized at semiempirical level. Next the torsional angles of the ligands and the flexible side-chains of the amino acids as well as the potential intermolecular interactions were optimized at RHF/6-31G(d). In contrast, the torsional angles of backbones as well as the bond angles and bond lengths of non-interacting residues were kept frozen during the calculations.

The binding energy of the complexes was calculated with the approximation neglecting the superimposition of error due to the difference between the total energies of the complex with the sum of the total energies of the components:

$$BE_{QM} = E_{L/D2DR} - (E_{D2DR} + E_L)$$

where BE_{QM} is the binding energy, $E_{L/D2DR}$ the complex energy, E_{D2DR} the energy of the reduced receptor model (binding pocket) and E_L is the energy of the ligand.

All the simulations and optimizations reported here have been carried out on the R isomer of each compound which displayed the better stereo-electronic complementarities with the D_2 DR.

All the calculations reported here were carried out using the GAUSSIAN 03 program.[35]

Spatial views shown in Figures 4 and 6 were constructed using the UCSF CHIMERA program[36] as graphic interface.

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