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Study of the bioactive conformation of novel 5-HT$_4$ receptor ligands: influence of an intramolecular hydrogen bond

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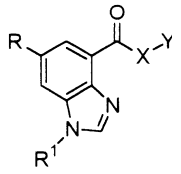
Abstract—By using NMR and IR techniques and theoretical methods, we have studied the prototropic equilibrium present in the benzimidazole ring of a series of derivatives acting at serotonin 5-HT$_4$ receptors. The structural study has allowed us to get insight into the bioactive conformation of the novel 5-HT$_4$ receptor ligands which has been supported by biological data. This will help the docking of the ligands into a 3-D model of the receptor binding site in order to guide the design and synthesis of new compounds with predetermined pharmacological activities.

To guide the design of new drugs with predetermined pharmacological properties, the study of the bioactive conformation is a crucial step necessary for the approach to the ligand–receptor recognition. In the course of a program aimed at the discovery of new serotonin 5-HT$_3$ and 5-HT$_4$ receptors agents, we have designed a series of new benzimidazole-4-carboxylic acid derivatives of general structure **1** (Scheme 1). These amides and esters have been characterized as potent and selective 5-HT$_3$ or 5-HT$_4$ receptor antagonists, depending upon the basic moiety Y linked to the acyl group.[1]

By using NMR and IR techniques and theoretical methods we have studied the prototropic equilibrium present in the benzimidazole ring of this family of benzimidazole derivatives **1**, in order to estimate the predominating tautomer of these molecules in solution. This study has allowed us to get insight into the bioactive conformation of the novel 5-HT$_4$ receptor ligands.

The annular tautomerism of azoles is such a fast process that in solution only averaged signals are observed in NMR spectra (^{1}H, ^{15}N, ^{13}C) at room temperature, and observation of separated signals for individual tautomers usually requires low temperature.[2] The influence of an intramolecular hydrogen bond on the prototropic equilibrium constant between two tautomers is a well-known phenomenon, and a number of examples have been reported.[2,3] Nevertheless, few of them concern the annular tautomerism of benzimidazoles.[4] Thus, the study of the prototropic equilibrium in benzimidazole derivatives **1**, which contain a hydrogen bond acceptor and/or donor group (CONH, COO) at 4-position of the ring, is of special interest.

^{13}C NMR is a very useful technique for the study of tautomerism in azole compounds.[2,3] The carbon chemical shifts of a supposed unsubstituted benzimidazole with no tautomerism have been previously calculated by considering the 1-methyl substituent effects in indole and indazole, and the experimental chemical shifts of 1-methylbenzimidazole.[5] Using these values and the substituent effects calculated by Ewing for monosubstituted benzenes,[6] we have calculated the expected chemical shifts for carbons in the two possible tautomers (I and II, see Table 1) of benzimidazole derivatives of general structure **1**. The



1

R = H, Cl
R^1 = H
X = NH, O
Y = basic moiety

Scheme 1.

Keywords: tautomerism; hydrogen bonding; benzimidazoles; 5-HT$_4$ receptor.

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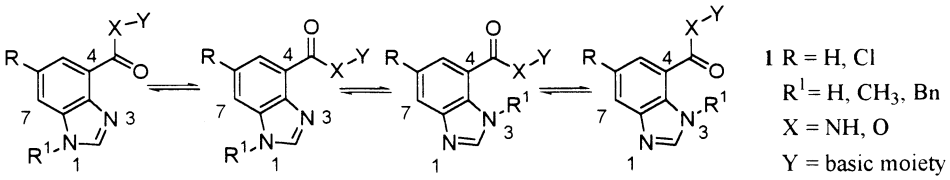
Table 1. ^{13}C NMR shifts (δ/ppm) for the homocycle carbons of amides and esters of general structure **1**



Comp.	X	Y	R	R^1	C-4	C-5	C-6	C-7	C-3a	C-7a
I[a]	NH	H	H	H	125.2	119.9	121.9	114.4	141.1	133.8
I[b]	NH	H	H	H	124.3	119.7	122.3	115.1	140.9	134.2
II[a]	NH	H	H	H	117.6	121.2	120.6	122.0	133.1	141.8
II[b]	NH	H	H	H	116.7	121.0	121.0	122.7	132.9	142.2
1a[c]	NH	Quinuclidin-3-yl	H	H	122.1	122.2	122.2	116.0	139.6	134.3
1b[c]	NH	(1-Butyl-4-piperidyl)methyl	H	H	122.3[d]	122.3[d]	122.2[d]	116.0	139.6	134.8
1b[e]	NH	(1-Butyl-4-piperidyl)methyl	H	H	122.4	122.6[d]	123.1[d]	115.5	140.4	133.9
I[a]	NH	H	Cl	H	127.2	120.0	126.8	114.5	139.9	135.8
I[b]	NH	H	Cl	H	125.7	120.1	128.6	115.5	139.0	135.6
II[a]	NH	H	Cl	H	119.6	121.3	125.5	122.1	131.9	143.8
II[b]	NH	H	Cl	H	118.1	121.4	127.3	123.1	131.0	143.6
1c[c]	NH	Quinuclidin-3-yl	Cl	H	122.7[d]	121.7	126.5[d]	116.0	136.2[f]	137.8[f]
1d[e]	NH	(1-Isobutyl-4-piperidyl)methyl	Cl	H	123.5	123.5	128.7	115.1	139.1	133.5
1e[e]	NH	(1-Isobutyl-4-piperidyl)methyl	Cl	*N*1-CH_3	124.7	124.4	129.3	112.5	139.6	135.3
1f[c]	NH	(1-Isobutyl-4-piperidyl)methyl	Cl	*N*1-Bn	124.1[d]	122.7	127.1[d]	114.2	139.5	134.9
I[a]	O	Ethyl	H	H	121.1	121.7	122.7	116.7	142.9	134.6
I[b]	O	Ethyl	H	H	121.4	121.9	121.7	115.6	143.1	133.6
II[a]	O	Ethyl	H	H	113.5	123.0	121.4	124.3	134.9	142.6
II[b]	O	Ethyl	H	H	113.8	123.2	120.4	123.2	135.1	141.6
1g[c]	O	Quinuclidin-3-yl	H	H	114.2	124.4[d]	121.2	124.8[d]	132.7	144.4
1h[e]	O	[1-[2-[(Methylsulfonyl)amino] ethyl]-4-piperidyl]methyl	H	H	113.7	124.9[d]	121.7	125.5[d]	133.3	143.6
I[a]	O	Ethyl	Cl	H	123.1	121.8	127.6	116.8	141.7	136.6
I[b]	O	Ethyl	Cl	H	122.8	122.3	128.0	116.0	141.2	135.0
II[a]	O	Ethyl	Cl	H	115.5	123.1	126.3	124.4	133.7	144.6
II[b]	O	Ethyl	Cl	H	115.2	123.6	126.7	123.6	133.2	143.0
1i[c]	O	Quinuclidin-3-yl	Cl	H	115.2	123.4	125.3	123.4	132.0	144.2
1j[e]	O	(1-Butyl-4-piperidyl)methyl	Cl	H	114.5	125.0	127.4	125.0	131.9	144.5
1k[e]	O	(1-Butyl-4-piperidyl)methyl	Cl	*N*3-CH_3	117.1	126.0[d]	126.8	124.5[d]	131.5	146.8

Arbitrary numbering of benzimidazole ring was adopted to facilitate comparison of data.
[a] Values calculated from Ref. 5 and Ref. 6 for DMSO-d_6 solution.
[b] Values calculated from Ref. 5 and Ref. 6 for $CDCl_3$ solution.
[c] Values measured from the spectra in DMSO-d_6 solution.
[d] Unambiguous assignment between these two signals is not possible.
[e] Values measured from the spectra in $CDCl_3$ solution.
[f] Unambiguous assignment between these two signals is not possible.

experimental chemical shifts were then measured in $CDCl_3$ and/or DMSO-d_6 solutions, and the signals were assigned considering ^{13}C, DEPT and 2-D correlation spectra and the calculated values. Calculated and experimental carbon chemical shifts of representative compounds of general structure **1** are summarised in Table 1. By comparing the data for C-4 and C-7 and for C-3a and C-7a, we can observe that the experimental values of carboxylates (**1g–j**) are in accordance with those calculated for tautomer II, whereas in the case of carboxamides (**1a–d**) the experimental values are in accordance with those calculated for tautomer I. Furthermore, the experimental chemical shifts of the homocycle carbons do not exhibit significant difference in $CDCl_3$ and DMSO-d_6 spectra, indicating that in both solvents the tautomerism preference is similar.

In order to get additional support to the tautomerism preference of general structure **1**, amide **1d** was alkylated to give *N*1-methyl and *N*1-benzyl derivatives **1e** and **1f**, respectively, and ester **1j** was alkylated to obtain *N*3-methyl product **1k**. Full structural assignment for **1e**, **1f** and **1k** was performed using usual procedures including DEPT and HMBC. The experimental values of ^{13}C NMR signals of alkylated derivatives listed in Table 1 give support to the method used to calculate the carbon chemical shifts of tautomers I and II of unalkylated analogues. We can conclude from the ^{13}C NMR analysis that tautomer I is more stable in the case of amides, while for esters tautomer II is predominant, both for unsubstituted and 5/6-chloro substituted derivatives **1**.

The stability of tautomer II over tautomer I in carboxylates could be attributed to an intramolecular hydrogen bond between the NH of the benzimidazole ring and the carboxylate group (see scheme in Table 1). The existence of this intramolecular hydrogen bond was demonstrated in ester **1h** [R=R^1=H, Y=[1-[2-[(methylsulfonyl)amino]-ethyl]-4-piperidyl]methyl] by concentration independent

Table 2. Energy difference (ΔV) and free energy difference in vacuum (ΔG) and solution ($\Delta G+\Delta\Delta G_{solv}$) between structures A and B, and energies of solvation of structures A (ΔG^{A}_{solv}) and B (ΔG^{B}_{solv}). Energies are in Kcal mol^{-1}

Comp	ΔV[a]	ΔG[b]	ΔG_{solv}[Ac]	ΔG_{solv}[Bc]	$\Delta G+\Delta\Delta G_{solv}$[d]
2	0.0	−0.3	−8.6	−6.7	−2.2
3	0.1	−0.4	−7.5	−6.8	−1.2
4	1.8	1.3	−4.4	−4.2	1.1

[a] Difference in energy ($V^{A}-V^{B}$) calculated at the B3LYP/6-311G**// B3LYP/6-31G** level.
[b] Thermal correction at 298.15 K to Gibbs free energy, calculated from normal mode vibrational frequencies, was added to ΔV.
[c] Calculated with polarised continuum model at the B3LYP/6-311G** level.[8]
[d] $\Delta\Delta G_{solv}$ is calculated as $\Delta\Delta G_{solv}=\Delta G^{A}_{solv}-\Delta G^{B}_{solv}$.

^{1}H NMR experiments at increments of temperature, from 20 to 100°C in DMSO-d_6. A linear correlation (r^2=0.998) was found between the shielding of benzimidazole NH signal (12.5 ppm at 20°C) and the temperature, while a similar behavior was observed for the amide NH chemical shifts [r^2=0.992; 9.9 ppm at 20°C for **1b**: R=R^1=H, Y=(1-butyl-4-piperidyl)methyl], indicating that structure IA is more stable in solution.

The presence of an intramolecular hydrogen bond in general structure **1** was additionally proved by the results of the registration of IR spectra at different concentrations (1, 10^{-1}, 10^{-2} M) in chloroform. The spectra of carboxamides show that the intensity of the associated band of the benzimidazole NH (3200 cm^{-1} for **1b**) is influenced by dilution, while the associated band of the amide NH (3300 cm^{-1} for **1b**) does not change with dilution. These results confirm the presence of an intramolecular hydrogen bond between the amide NH and the nitrogen of the benzimidazole ring. In contrast, the benzimidazole NH band in carboxylates (3200 cm^{-1} for **1g**) is not influenced by dilution, indicating that this NH is involved in an intramolecular hydrogen bond, as already shown by NMR experiments.

From the NMR and IR studies, structure IA seems to be the bioactive conformation for carboxamides, while we cannot unambiguously discard conformation IIA or IIB for carboxylates since the intramolecular H-bond could be present in any of them. Nevertheless, we have undertaken quantum mechanical calculations to approach this problem. Full geometry optimization was performed for *N*-methylbenzimidazole-4-carboxamide and *N*-methyl-6-chlorobenzimidazole-4-carboxamide (**2** and **3**), and methyl benzimidazole-4-carboxylate (**4**), as models using the GAUSSIAN-98 system of programs.[7] Table 2 shows the energy difference (ΔV) and the free energy difference in both vacuum (ΔG) and aqueous solvent ($\Delta G+\Delta\Delta G_{solv}$) between structures A and B (see scheme of Table 1). Structure IA in carboxamides (**2** and **3**) is more stable in solution than structure IIB by −2.2 and −1.2 kcal mol^{-1}, respectively (see $\Delta G+\Delta\Delta G_{solv}$ in Table 2). This structural preference coincides with the above NMR and IR results and validates the employed theoretical methods.[8] These results suggest that the free carbonyl group of structure IA might act as a hydrogen bond acceptor in the interaction of amide ligands with the 5-HT$_4$ receptor. On the contrary, structure IIB of carboxylates (**4**) is more stable than structure IIA ($\Delta G+\Delta\Delta G_{solv}$=1.1 kcal mol^{-1}). This causes the carbonyl group of esters to hydrogen bond the benzimidazole NH. Thus, the common carbonyl moiety of carboxamides and carboxylates adopt different conformations in solution. Nevertheless, the energy required for the conformational change of carboxylates to adopt a similar bioactive conformation to carboxamides is estimated to be 1.3 kcal mol^{-1} in vacuum or 1.1 kcal mol^{-1} in solution (see Table 2), and these energies could be compensated by the ligand–receptor interaction.

Table 3. Binding affinities of benzimidazole derivatives at 5-HT$_4$ receptors

Comp.	K_i±SEM (nM)[a]
1d	0.29±0.04
1e	2.2±0.2
1j	2.9±0.4
1k	241±3
1l	>1000

[a] K_i values are means±SEM of two to four assays, performed in triplicate. Inhibition curves were analyzed by a computer-assisted-curve-fitting program (Prism Graph Pad), and K_i values were determined from the Cheng–Prusoff equation.

Biological data suggest the importance of the tautomeric bias of carboxamides (I) and carboxylates (II) in their interaction with the 5-HT$_4$ receptor. In vitro affinities at 5-HT$_4$ receptors have been determined by radioligand binding assays using [^{3}H]GR 113808 in rat striatum membranes (Table 3). Thus, a reduction of affinity for the 5-HT$_4$ receptor was observed after *N*3-methylation of **1j** [K_i (**1j**)= 2.9 nM vs. K_i (**1k**)=241 nM] suggesting that the intramolecular hydrogen bond present in **1j** not only fixes the tautomerism but also contributes to the coplanarity of the carbonyl group and the benzimidazole system, an important element for high affinity.[9] Consequently, *N*1-methylated analogue **1e** still exhibits high 5-HT$_4$ receptor affinity [K_i (**1e**)=2.2 nM vs K_i (**1d**)=0.29 nM]. Additional support to the proposed bioactive conformation was found upon examination of the 5-HT$_4$ receptor affinity of benzimidazole-5-carboxamide **1l**. Biological assays reveal that this regioisomer is completely inactive (K_i>1000 nM). The position of the amide moiety (C-5) in this derivative unables the formation of an intramolecular hydrogen bond which permits the tautomeric equilibrium at the benzimidazole ring[10] and allows the carbonyl group to be out of coplanarity with the aromatic benzimidazole system.

In summary, a structural study has been carried out for benzimidazole derivatives in order to estimate the bioactive conformation of these 5-HT$_4$ receptor ligands, which is being used in the docking of the ligands into a 3-D model of the 5-HT$_4$ receptor transmembrane domain, based on the crystal structure of rhodopsin.[11] The main molecular details of the ligand–receptor interaction are: an ionic interaction between the piperidine NH$^+$ group and Asp 3.32, a hydrogen bond between the carbonyl group and Ser 5.43, and a hydrogen bond between Asn 6.55 and the aromatic ring of carboxamides and additionally the O–Y group of carboxylates.[12] The derived computational model will be used to guide the design and synthesis of new compounds with predetermined affinities and selectivity.

1. Chemistry

1.1. General procedures

All reagents were commercially available products purchased from Aldrich or Fluka. All solvents were distilled prior to use. Anhydrous THF was obtained by distillation over sodium and benzophenone. Concentration dependent infrared spectra (IR) were registered at different concentrations (1, 10^{-1}, 10^{-2} M) in $CHCl_3$ on a double-beam spectrometer with subtraction of the solvent (FTIR-8300 Shimadzu spectrometer). Nuclear Magnetic Resonance spectra were recorded on a Varian VXR-300S, Bruker AM-300 and Bruker AC-200. Plots of δ (NH) vs. temperature for **1h** and **1b** were obtained recording consecutive ^{1}H NMR experiments at increments of temperature (20°C) from 20 to 100°C in DMSO-d_6 solutions. Elemental analyses were carried out on a Perkin–Elmer 2400 apparatus at the Facultad de Farmacia, UCM.

1.2. Synthesis of the *N*-alkylated derivatives 1e,f,k

1.2.1. *N*-[(1-Isobutyl-4-piperidyl)methyl]-6-chloro-1-methylbenzimidazole-4-carboxamide 1e. To a solution of 1 equiv. of **1d**[1c] in anhydrous THF was added 1.5 equiv. of *n*-BuLi (2.5 M in hexane) at −78°C under argon. The mixture was allowed to warm up to −10°C and 1 equiv. of methyl iodide was added dropwise, then the reaction mixture was stirred at room temperature for 3 h. The mixture was hydrolysed with brine and the organic layer was extracted with ethyl acetate, dried (Na_2SO_4) and concentrated under vacuum to give a crude oil which was purified by column chromatography on silica gel using a mixture of chloroform/methanol/ammonia (40:1:0.1) as eluent. Yield: 73%. Data for **1e**: mp 128–130°C (chloroform/hexane). IR ($CHCl_3$, cm^{-1}) 3298, 3095 (NH), 1655 (CONH), 1608, 1566, 1460 (Ar). ^{1}H NMR ($CDCl_3$, δ) 0.86 (d, J=6.5 Hz, 6H), 1.51 (q, J=11.8 Hz, 2H), 1.65–1.82 (m, 4H), 1.99 (br t, J=11.4 Hz, 2H), 2.13 (d, J=7.2 Hz, 2H), 2.93 (br d, J=11.8 Hz, 2H), 3.40 (t, J=6.0 Hz, 2H), 3.81 (s, 3H), 7.44 (d, J=2.0 Hz, 1H), 7.86 (s, 1H), 8.05 (d, J=2.0 Hz, 1H), 9.86 (br s, 1H). ^{13}C NMR ($CDCl_3$, δ) 21.0 (CH_3), 25.5 (NCH_2CH), 30.1 (C_3, C_5-piperidine), 31.4 (NCH_3), 36.3 (C_4-piperidine), 45.3 ($CONHCH_2$), 53.9 (C_2, C_6-piperidine), 67.2 (NCH_2), 112.5 (C_7), 124.4 (C_5), 124.7 (C_4), 129.3 (C_6), 135.3 (C_{7a}), 139.6 (C_{3a}), 143.9 (C_2), 164.2 (CONH). Elemental analysis calcd for $C_{19}H_{27}ClN_4O$: C, 62.88; H, 7.50; N, 15.44. Found: C, 62.74; H, 7.25; N, 15.26.

1.2.2. *N*-[(1-Isobutyl-4-piperidyl)methyl]-1-benzyl-6-chlorobenzimidazole-4-carboxamide 1f. To a suspension of 1 equiv. of **1d**[1c] and 1 equiv. of sodium hydride (60% in mineral oil) in DMF was added dropwise a solution of 1 equiv. of benzyl chloride in DMF (10 mL×mmol) and the reaction mixture was stirred at 30°C for 18 h. The mixture was concentrated under vacuum and the solid residue was redissolved in chloroform and washed with an aqueous saturated solution of Na_2CO_3. The organic layer was dried (Na_2SO_4) and concentrated under vacuum to give a crude oil which was purified by column chromatography on silica gel using a mixture of chloroform/methanol (95:5) as eluent. Yield: 90%. Data for **1f**: mp 150–151°C (ethyl acetate). IR (KBr, cm^{-1}) 3273 (NH), 1655 (CONH), 1607, 1560, 1487, 1472 (Ar). ^{1}H NMR (DMSO-d_6, δ) 0.86 (d, J=6.6 Hz, 6H), 1.28 (qd, J=11.7, 3.0 Hz, 2H), 1.52–1.86 (m, 6H), 2.01 (d, J=7.2 Hz, 2H), 2.83 (br d, J=11.1 Hz, 2H), 3.37 (t, J=6.3 Hz, 2H), 5.63 (s, 2H), 7.33–7.41 (m, 5H), 7.83 (d, J=2.1 Hz, 1H), 8.00 (d, J=2.1 Hz, 1H), 8.80 (s, 1H), 9.73 (t, J=5.7 Hz, 1H). ^{13}C NMR (Me_2SO-d_6, δ) 20.9 (CH_3), 25.1 (NCH_2CH), 29.9 (C_3, C_5-piperidine), 36.2 (C_4-piperidine), 44.7 ($CONHCH_2$), 48.1 (CH_2Ph), 53.6 (C_2, C_6-piperidine), 66.7 (NCH_2), 114.2 (C_7), 122.7 (C_5), 124.1, 127.1 (C_4, C_6), 127.6, 128.1, 128.9 (Ph), 134.9 (C_{7a}), 136.3 (Ph), 139.5 (C_{3a}), 146.3 (C_2), 163.1 (CONH). Elemental analysis calcd for $C_{25}H_{31}ClN_4O$: C, 68.40; H, 7.12; N, 12.76. Found: C, 68.13; H, 7.22; N, 12.56.

1.2.3. (1-Butyl-4-piperidyl)methyl-5-chloro-1-methylbenzimidazole-7-carboxylate 1k. To a suspension of 1 equiv. of **1j**[1c] and 1.2 equiv. of sodium hydride (60% in mineral oil) in DMF was added dropwise at 0°C a solution of 1.2 equiv. of methyl iodide in DMF and the reaction mixture was stirred at room temperature for 20 h. The mixture was concentrated under vacuum and the solid residue was redissolved in chloroform and washed with an aqueous saturated solution of Na_2CO_3. The organic layer was dried (Na_2SO_4) and concentrated under vacuum to give a crude oil which was purified by column chromatography on silica gel using a mixture of chloroform/methanol (50:1) as eluent. Yield: 25%. Data for **1k**: mp 194–195°C (ethyl acetate). IR ($CHCl_3$, cm^{-1}) 3390 (NH), 1718 (COO), 1662, 1504, 1472 (Ar). ^{1}H NMR ($CDCl_3$, δ) 0.92 (t, J=7.1 Hz, 3H), 1.22–1.59 (m, 6H), 1.84 (br d, J=8.3 Hz, 3H), 2.01 (br t, J=11.7 Hz, 2H), 2.38 (dd, J=8.1, 7.3 Hz, 2H), 3.04 (br d, J=11.5 Hz, 2H), 4.03 (s, 3H), 4.22 (d, J=6.1 Hz, 2H), 7.80 (d, J=2.0 Hz, 1H), 7.88 (s, 1H), 7.94 (d, J=2.2 Hz, 1H). ^{13}C NMR ($CDCl_3$, δ) 13.9 (CH_3), 20.7 (CH_2CH_3), 28.2 (C_3, C_5-piperidine), 29.6 (NCH_2CH_2), 35.0, 35.9 (NCH_3, C_4-piperidine), 52.9 (C_2, C_6-piperidine), 58.3 (NCH_2), 69.5 ($COOCH_2$), 117.1 (C_7), 124.5, 126.0 (C_4, C_6), 126.8 (C_5), 131.5 (C_{7a}), 146.8 (C_{3a}), 147.7 (C_2), 164.7 (COO). Elemental analysis calcd for $C_{19}H_{26}ClN_3O_2$: C, 62.71; H, 7.20; N, 11.55. Found: C, 62.50; H, 7.57; N, 11.50.

1.2.4. *N*-[(1-Butyl-4-piperidyl)methyl]benzimidazole-5-carboxamide 1l. To a solution of benzimidazole-5-carboxylic acid (5 mmol, Aldrich) in dry DMF (5 mL) under an argon atmosphere was added 1,1′-carbonyldiimidazole (811 mg, 5 mmol). The mixture was stirred at 40°C for 1 h, then a solution of (1-butyl-4-piperidyl)methylamine[1c] and 1,8-diazabicyclo[5.4.0]undec-7-ene (761 mg, 5 mmol) in DMF (10 mL) was added dropwise, and the reaction mixture was stirred at 50°C for 20–24 h. The solvent was removed under reduced pressure, and the crude was taken up in $CHCl_3$ (50 mL) and washed with water (20 mL) and 20% aqueous K_2CO_3 (20 mL). The organic layer was dried over Na_2SO_4, and evaporated to afford the crude product, which was purified by column chromatography on silica gel using a mixture of chloroform/methanol (4:1) as eluent followed by recrystallization from ethyl acetate. Yield: 66%. Data for **1l**: mp 151–152°C (ethyl acetate). IR ($CHCl_3$, cm^{-1}) 3300, 3100 (NH), 1640 (CONH), 1550, 1470, 1450 (Ar). ^{1}H NMR ($CDCl_3$, δ)

0.86 (t, J=7.2 Hz, 3H), 1.22–1.48 (m, 6H), 1.64–1.66 (m, 1H), 1.72 (d, J=12.9 Hz, 2H), 1.88 (t, J=10.8 Hz, 2H), 2.29 (dd, J=8.1, 7.5 Hz, 2H), 2.93 (d, J=11.4 Hz, 2H), 3.33 (t, J=6.0 Hz, 2H), 7.19 (t, J=4.8 Hz, 1H), 7.46 (d, J=8.1 Hz, 1H), 7.58 (d, J=8.7 Hz, 1H), 8.02 (s, 1H), 8.05 (s, 1H). ^{13}C NMR ($CDCl_3$, δ) 14.0 (CH_3), 20.8 (CH_2CH_3), 28.8 (NCH_2CH_2), 29.8 (C_3, C_5-piperidine), 36.1 (C_4-piperidine), 45.6 ($CONHCH_2$), 53.3 (C_2, C_6-piperidine), 58.6 (NCH_2), 115.0 (C_4, C_7), 121.4 (C_6), 129.1 (C_5), 137.6, 140.0 (C_{3a}, C_{7a}), 143.1 (C_2), 168.9 (CONH). Elemental analysis calcd for $C_{18}H_{26}N_4O$: C, 68.75; H, 8.33; N, 17.82. Found: C, 68.51; H, 8.23; N, 17.41.

1.3. Radioligand binding assays at 5-HT$_4$ receptor

Male Sprague–Dawley rats (*Rattus norvegicus albinus*), weighing 180–200 g, were killed by decapitation and the brains rapidly removed and dissected. Tissues were stored at −80°C for subsequent use and homogenized on a Polytron PT-10 homogenizer. Membrane suspensions were centrifuged on a Beckman J2-HS instrument.

Binding assays were performed according to the procedure previously described by Grossman et al.[13] The striatum was homogenized in 15 volumes of ice-cold 50 mM HEPES buffer (pH 7.4 at 4°C) and centrifuged at 48000 g for 10 min. The pellet was resuspended in 4.5 mL of assay buffer (50 mM HEPES, pH 7.4 at 4°C). Fractions of 100 μL of the final membrane suspension were incubated at 37°C for 30 min with 0.1 nM [^{3}H]GR 113808 (85 Ci mmol^{-1}), in the presence or absence of six concentrations of the competing drug, in a final volume of 1 mL of assay buffer. Nonspecific binding was determined with 30 μM 5-HT and represented less than 40% of the total binding.

Competing drug, nonspecific, total and radioligand bindings were defined in triplicate. Incubation was terminated by rapid vacuum filtration through Whatman GF/B filters, presoaked in 0.05% poly(ethylenimine), using a Brandel cell harvester. The filters were then washed once with 4 mL of ice-cold 50 mM HEPES, pH 7.4 at 4°C and dried. The filters were placed in poly(ethylene) vials to which were added 4 mL of a scintillation cocktail (Aquasol), and the radioactivity bound to the filters was measured by liquid scintillation spectrometry. The data were analysed by an iterative curve-fitting procedure (program Prism, Graph Pad), which provided IC_{50}, K_i, and r^2 values for test compounds, K_i values being calculated from the Cheng–Prusoff equation.[14] The protein concentrations of the rat cerebral cortex and the rat striatum were determined by the method of Lowry,[15] using bovine serum albumin as the standard.

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