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Short communication

Benzimidazole-2-carboxylic acid amides and esters: a new structural class of 5-HT_3 ligands

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Abstract – A series of novel benzimidazole-2-carboxylic acid amides and esters with a quinuclidine or a tropane moiety were synthesized and evaluated for in vitro affinity for the 5-HT_3, 5-HT_4 and D_2 receptors. Compounds **15a**, **13j** and **13h** exhibited affinity for the 5-HT_3 receptor (K_i = 20.2, 18.4 and 12.7 nM, respectively) and no significant affinity for both 5-HT_4 and D_2 receptors. The amide-ester replacement did not induce significant changes in the affinity profile. The enantioselectivity for the 5-HT_3 receptor was reversed with regard to the zacopride pattern and the (*R*)-enantiomer **13c** showed higher affinity (K_i = 56.4 nM) than the (*S*)-enantiomer **13d** (K_i = 242.3 nM). An increment of the steric hindrance around the nitrogen atom at the 1-position of the benzimidazole ring led to an improvement in the affinity. The 5-HT_3 receptor antagonist activity of compounds with higher affinity was performed by evaluating the inhibition of the 5-HT induced von Bezold-Jarisch reflex. They displayed moderate 5-HT_3 antagonist activity (ED_{50} = 10.6–29.1 μg/kg i.v.). © Elsevier, Paris

benzimidazole-2-carboxamide / benzimidazole-2-carboxylate / tropane / quinuclidine / 5-HT_3 ligand

1. Introduction

In recent years the 5-HT_3 receptor subtype has attracted much attention because of the effectiveness of 5-HT_3 receptor antagonists in the treatment of chemotherapy-induced emesis [1, 2]. In addition, they are expected to be useful for the treatment of anxiety, schizophrenia, migraine and irritable bowel syndrome [3–5]. Some of these compounds, which proved to behave as 5-HT_3 receptor antagonists, also show affinity at the 5-HT_4 serotonin receptor subtype [6, 7]. Several 5-HT_3 antagonist benzamides have also dopamine receptor antagonist properties, which induce unfavorable effects in man such as central nervous system depression and extrapyramidal syndrome [8].

Several studies have suggested that there are three structural requirements for 5-HT_3 receptor antagonism: an aromatic ring, a linking acyl function and a basic nitrogen group [9–11]. Many of the reported 5-HT_3 receptor antagonists include an azabicycloalkane moiety, indicating a particularly good fit between the receptor and these heterocycles, i.e. tropisetron **1**, granisetron **2** and zacopride **3** *(figure 1)*.

In order to find new 5-HT_3 receptor antagonists with selectivity over 5-HT_4 and D_2 receptors, we prepared a series of novel benzimidazole-2-carboxylic acid derivatives including a quinuclidine or a tropane moiety.

2. Chemistry

Benzimidazole-2-carboxylic acid **6** was synthesized as described in *figure 2*. 2-Hydroxymethylbenzimidazole **5** was prepared from 1,2-phenylenediamine **4** and glycolic acid, according to the Phillips procedure [12]. Oxidation of **5** with potassium permanganate afforded acid **6** [13]. 3-Aminotropane derivatives **9** and **10** were prepared using previously reported methods *(figure 3)* [14, 15]. The reaction of tropinone with hydroxylamine gave the oxime **8** which was reduced with lithium aluminum hydride to give the *endo* isomer **9**; sodium reduction of **8** afforded the *exo* isomer **10**. Other amines and alcohols used were commercially available. Amides **11** were

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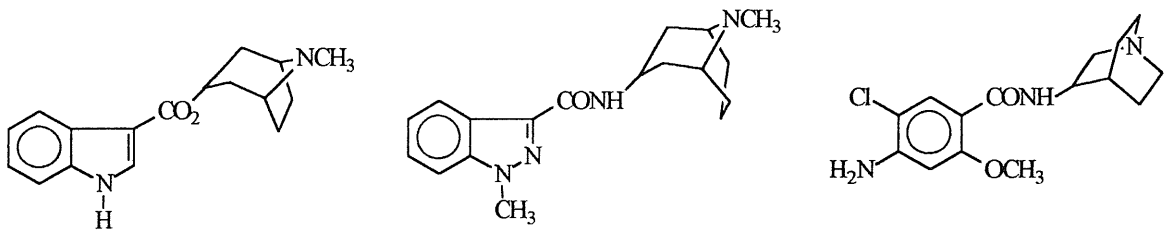


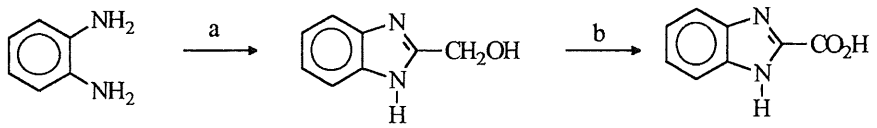
1 2 3

Figure 1. 5-HT_3 receptor antagonists.

obtained from the acid **6** following a standard procedure, by coupling with the appropriate amine using carbonyldiimidazole (CDI) *(figure 4)*. Amides **13** were synthesized by reaction of the corresponding amines with trimethylaluminium and subsequent treatment of the resulting dimethylaluminium amides with a carboxylic ester **12** [16]. Acid **6** was condensed with an alcohol using CDI and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) to give ester derivatives **14**; treatment of **14** with sodium hydride followed by reaction with an alkyl halide led to esters **15**.

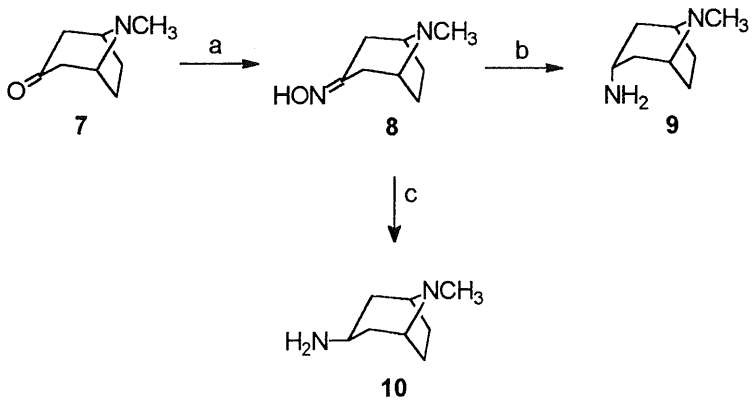
3. Pharmacology

Compounds were evaluated for their in vitro 5-HT_3, 5-HT_4 and D_2 receptor affinities by radioligand binding assays [17–19]. For each compound, the ability to displace the specific ligand ([^{3}H]-LY278584, [^{3}H]-GR113808, and [^{3}H]-Raclopride, respectively) from receptors was determined. The antagonist activity at the 5-HT_3 receptor was performed by evaluating the inhibition of the Bezold-Jarisch reflex evoked by 5-HT in urethane-anaesthetized rats *(table I)*.



4 5 6

Figure 2. Synthesis of benzimidazole-2-carboxylic acid **6**. Reagents: a) HO_2CCH_2OH, 4 N HCl, 40 min, reflux; b) $KMnO_4$, 10% NaOH, 3 h, room temperature.



7 8 9 10

Figure 3. Preparation of 3-aminotropane derivatives **9** and **10**. Reagents: a) NH_2OH, pyridine, EtOH, 2 h, reflux; b) $LiAlH_4$, 10% H_2SO_4, 2 h, reflux; c) Na, EtOH, 2 h, reflux.

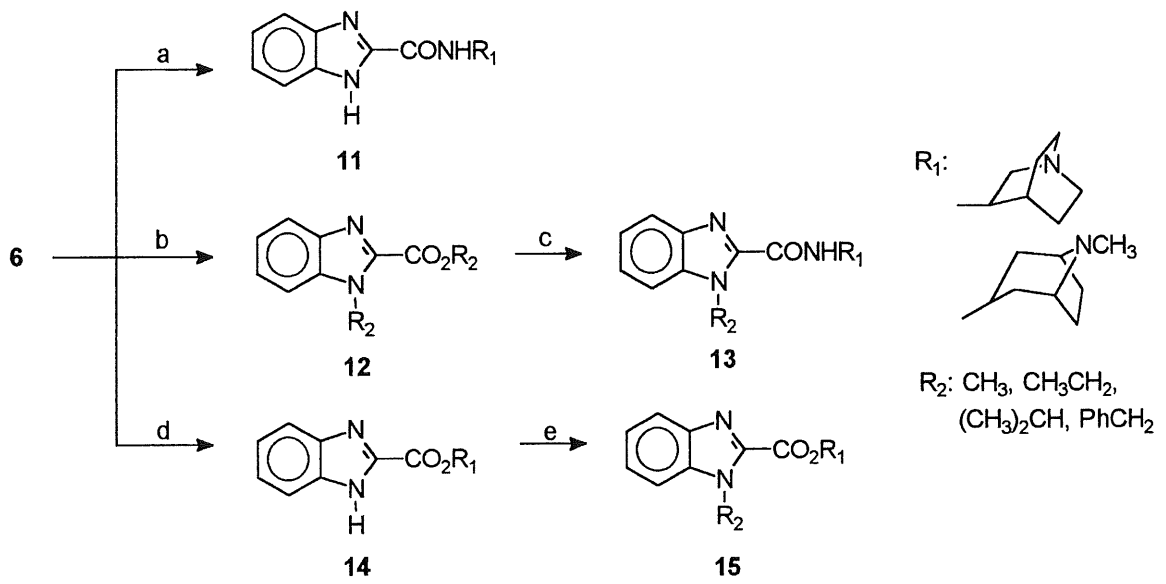


Figure 4. Preparation of compounds **11**, **12**, **13**, **14** and **15**.
Reagents: a) CDI, R_1NH_2, DMF, 20 h, room temperature; b) HNa, R_2Hal, DMF, 3 h, room temperature; c) Me_3Al, R_1NH_2, $PhCH_3$, 3 h, reflux; d) CDI, DMF, DBU, R_1OH, 24 h, 60 °C; e) HNa, R_2Hal, DMF, 3 h, room temperature.

4. Results and discussion

Most of the synthesized compounds exhibited moderate-to-high affinity for the 5-HT_3 receptor and no significant affinity for either the 5-HT_4 or the D_2 receptors.

The amide-ester replacement did not induce significant changes on the affinity. Compounds **11a** (K_i = 172.3 nM) and **13j** (K_i = 18.4 nM) showed a similar affinity value to **14a** (K_i = 171.7 nM) and **15a** (K_i = 20.2 nM), respectively. Only amide **11b** (K_i = 143.9 nM) displayed ca. 2-fold higher affinity than its related ester **14b** (K_i = 260.1 nM). A similar equipotency of amides and esters has been reported for several benzoic acid derivatives [20, 21].

With regard to the tropane moiety, the *endo*-derivative **11b** showed up to 1 000-fold more potent affinity for the 5-HT_3 receptor than the analogue *exo*-derivative **11c** (K_i > 1 000 nM). The inactivity of the axially substituted tropane derivatives is well known and has been reported [22]. On the other hand, the influence of the chirality of the quinuclidine moiety was examined. Whereas the (*R*)-enantiomer **13c** had higher affinity (K_i = 56.4 nM) than the racemate **13a** (K_i = 81.6 nM), the (*S*)-enantiomer **13d** showed weaker affinity (K_i = 242.3 nM). Consequently, the enantioselectivity for the 5-HT_3 receptor was reversed with regard to that of zacopride and related 3-quinuclidinyl amides in which the (*S*)-enantiomers have higher affinity for the 5-HT_3 receptor than the (*R*)-enantiomers [23–25]. A similar inversion of enantioselectivity has been reported for naphtalimides, benzoylureas, and indolyl-2-acetamides [26–28]. The increase in the potency of the (*R*)-enantiomer could indicate the existence of a different and relevant bond between the compound and the receptor site.

With respect to the introduction of an alkyl substituent at the 1-position of the benzimidazole ring, an increment of the steric hindrance around the nitrogen atom led to an improvement in the affinity for the 5-HT_3 receptor; e. g. compare **11b** (K_i = 143.9 nM) and **13j** (K_i = 18.4 nM); **11a** (K_i = 172.3 nM) and **13i** (K_i = 24.4 nM); **14b** (K_i = 260.1 nM) and **15a** (K_i = 20.2 nM). However, replacement of the hydrogen in **11b** by a methyl group led to **13b** (K_i = 158.5 nM), with no change in the affinity. Besides, the affinity of the N-benzyl derivative **13j** (K_i = 18.4 nM) was somewhat lower to that of the isopropyl analogue **13h** (K_i = 12.7 nM). Moreover, compounds **13a** (K_i = 81.6 nM), **13e** (K_i = 65.2 nM) and **13g** (K_i = 70.4 nM) did not show any remarkable difference in the affinity.

We evaluated the 5-HT_3 receptor-antagonist activity of the compounds with higher affinity (**13h**, **13i**, **13j** and **15a**) in the inhibition of the 5-HT induced von Bezold-Jarisch reflex assays in anaesthetized rats. They exhibited

5.1.1.1. N-(1-azabicyclo[2.2.2]octan-3-yl)-1H-benzimidazole-2-carboxamide **11a**

Yield: 67%; m.p.: 145–147 °C. ^{1}H NMR ($CDCl_3$) 1.54 (m, 1H, CH_2), 1.75 (m, 2H, CH_2), 1.89 (m, 1H, CH_2), 2.10 (m, 1H, CH_2), 2.72–3.02 (m, 5H, CH_2), 3.48 (m, 1H, CH), 4.21 (m, 1H, CH), 7.32–7.36 (m, 2H, ar), 7.51 (m, 1H, ar), 7.77 (m, 1H, ar), 7.89 (br d, 1H, CONH), 12.25 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) 19.97, 25.66, 25.88, 46.45, 47.14, 47.26, 55.05, 112.37, 120.19, 123.34, 124.75, 134.48, 142.65, 145.20, 158.47.

5.1.1.2. endo-N-(8-methyl-8-azabicyclo[3.2.1]octan-3-yl)-1H-benzimidazole-2-carboxamide **11b**

Yield: 73%; m.p.: 183–185 °C. ^{1}H NMR ($CDCl_3$) 1.76–2.43 (m, 8H, CH_2), 2.38 (s, 3H, CH_3), 3.25 (m, 2H, CH), 4.39 (m, 1H, CH), 7.28–7.34 (m, 2H, ar), 7.54 (m, 1H, ar), 7.81 (m, 1H, ar), 8.15 (br d, 1H, CONH), 12.30 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) 25.70, 36.17, 40.16, 41.83, 59.83, 112.28, 120.31, 123.07, 124.56, 134. 35, 142.76, 145.22, 158.82.

5.1.1.3. exo-N-(8-methyl-8-azabicyclo[3.2.1]octan-3-yl)-1H-benzimidazole-2-carboxamide **11c**

Yield: 71%; m.p.: 250–252 °C. ^{1}H NMR (DMSO-d_6) 1.42–2.14 (m, 8H, CH_2), 2.20 (s, 3H, CH_3), 3.06 (m, 2H, CH), 4.13 (m, 1H, CH), 7.24 (m, 2H, ar), 7.58 (m, 2H, ar), 8.70 (br d, 1H, CONH), 12.25 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6) 26.36, 35.46, 38.38, 39.91, 59.69, 112.94, 120.49, 123.37, 124.92, 134.84, 142.82, 145.06, 158.61.

5.1.2. General procedure for compounds **13**

To an ice-cooled solution of benzimidazole-2-carboxylic acid **6** (30 mmol) in 60 mL of DMF, HNa (60% suspension, 66 mmol) was added, and the mixture was stirred for 30 min. The appropriate alkyl halide (66 mmol) was added dropwise and the resulting suspension was stirred for 3 h at room temperature. Aqueous sodium hydroxide (10%, 20 mL) was slowly added and the mixture extracted with dichloromethane (3 × 20 mL). The combined organic layers were dried (Na_2SO_4) and concentrated, and compound **12** was isolated by silica gel chromatography (Cl_2CH_2:MeOH, 9:1).

To a solution of an appropriate amine (11 mmol) in 30 mL of toluene, a solution of trimethylaluminium in toluene (2 M, 11 mmol) was added, and the resulting mixture was stirred for 1 h at room temperature. Compound **12** (9 mmol) was added, and the mixture was heated to reflux for 3 h and then cooled. Water (10 mL) was added dropwise, and the resulting precipitate was filtered off. Evaporation of the filtrate under reduced pressure and further purification by silica gel chromatography (MeOH:NH_4OH, 98:2) afforded **13**.

5.1.2.1. N-(1-azabicyclo[2.2.2]octan-3-yl)-1-methyl-1H-benzimidazole-2-carboxamide **13a**

Yield: 64%; m.p.: 143–145 °C. ^{1}H NMR ($CDCl_3$) 1.58 (m, 1H, CH_2), 1.72 (m, 2H, CH_2), 1.83 (m, 1H, CH_2), 2.05 (m, 1H, CH_2), 2.53–2.99 (m, 5H, CH_2), 3.43 (m, 1H, CH), 4.15 (m, 1H, CH), 4.22 (s, 3H, CH_3), 7.31–7.42 (m, 3H, ar), 7.45–7.79 (m, 1H, ar), 7.89 (br d, 1H, CONH). ^{13}C NMR ($CDCl_3$) 20.18, 25.82, 25.84, 31.96, 46.64, 46.84, 47.49, 55.79, 110.37, 120.46, 123.43, 124.51, 136.95, 140.87, 143.25, 159.69.

5.1.2.2. endo-N-(8-methyl-8-azabicyclo[3.2.1]octan-3-yl)-1-methyl-1H-benzimidazole-2-carboxamide **13b**

Yield: 74%; m.p.: 143–145 °C. ^{1}H NMR (DMSO-d_6) 1.68–2.09 (m, 8H, CH_2), 2.15 (s, 3H, CH_3), 3.03 (m, 2H, CH), 4.05 (m, 1H, CH), 4.12 (m, 3H, CH_3), 7.28–7.38 (m, 2H, ar), 7.64–7.74 (m, 2H, ar), 8.32 (br d, 1H, CONH). ^{13}C NMR (DMSO-d_6) 25.51, 31.69, 35.30, 39.07, 40.74, 59.22, 111.22, 120.04, 123.03, 124.06, 136.68, 140.48, 143.88, 158.46.

5.1.2.3 N-(1-azabicyclo[2.2.2]octan-3-yl)-1-ethyl-1H-benzimidazole-2-carboxamide **13e**

Yield: 68%; m.p.: 120–122°C. ^{1}H NMR ($CDCl_3$) 1.48 (t, 3H, CH_3, J = 7.2 Hz), 1.50 (m, 1H, CH_2), 1.72 (m, 2H, CH_2), 1.85 (m, 1H, CH_2), 2.05 (m, 1H, CH_2), 2.55–2.99 (m, 5H, CH_2), 3.42 (m, 1H, CH), 4.14 (m, 1H, CH), 4.78 (q, 2H, CH_2, J = 7.2 Hz), 7.27–7.48 (m, 3H, ar), 7.77 (m, 1H, ar), 7.81 (br d, 1H, CONH). ^{13}C NMR ($CDCl_3$) 15.51, 20.20, 25.84, 25.85, 40.40, 46.67, 46.88, 47.41, 55.72, 110.45, 120.56, 123.36, 124.46, 135.96, 141.05, 142.78, 159.47.

5.1.2.4. endo-N-(8-methyl-8-azabicyclo[3.2.1]octan-3-yl)-1-ethyl-1H-benzimidazole-2-carboxamide **13f**

Yield: 57%; m.p.: 197–199 °C. ^{1}H NMR (DMSO-d_6) 1.43 (t, 3H, CH_3, J = 7.2 Hz), 2.02–2.45 (m, 8H, CH_2), 2.15 (s, 3H, CH_3), 3.63 (m, 2H, CH), 4.05 (m, 1H, CH), 4.81 (q, 2H, CH_2, J = 7.2 Hz), 7.28–7.48 (m, 2H, ar), 7.75–7.84 (m, 2H, ar), 8.82 (br d, 1H, CONH). ^{13}C NMR DMSO-d_6) 15.53, 23.68, 32.33, 37.56, 39.07, 40.74, 60.22, 111.19, 120.23, 123.04, 124.21, 135.51, 140.74, 143.78, 159.18.

5.1.2.5. N-(1-azabicyclo[2.2.2]octan-3-yl)-1-isopropyl-1H-benzimidazole-2-carboxamide (fumarate) **13g**

Yield: 51%; m.p.: 122 °C (dec). ^{1}H NMR (DMSO-d_6) 1.54 (d, 6H, CH_3, J = 6.9 Hz), 1.53 (m, 1H, CH_2), 1.73 (m, 2H, CH_2), 1.87 (m, 1H, CH_2), 2.06 (m, 1H, CH_2), 2.79–3.22 (m, 5H, CH_2), 3.42 (m, 1H, CH), 4.21 (m, 1H, CH), 5.64 (m, 1H, CH), 7.21–7.38 (m, 2H, ar), 7.68–7.87 (m, 2H, ar), 9.21 (br d, 1H, CONH). ^{13}C NMR (DMSO-d_6) 18.36, 21.01, 23.27, 25.26, 40.76, 45.16, 45.52, 48.15,

51.03, 110.45, 120.56, 123.36, 124.46, 135.96, 141.05, 142.78, 159.47.

5.1.2.6. endo-N-(8-methyl-8-azabicyclo[3.2.1]octan-3-yl)-1-isopropyl-1H-benzimidazole-2-carboxamide (fumarate) **13h**

Yield: 53%; m.p.: 183–185 °C. ^{1}H NMR (DMSO-d_6) 1.57 (d, 6H, CH_3, J = 6.92 Hz), 2.04–2.40 (m, 8H, CH_2), 2.15 (s, 3H, CH_3), 3.61 (m, 2H, CH), 4.01 (m, 1H, CH), 5.70 (m, 1H, CH), 7.24–7.35 (m, 2H, ar), 7.74–7.86 (m, 2H, ar), 8.77 (br d, 1H, CONH). ^{13}C NMR (DMSO-d_6) 21.01, 23.84, 32.44, 37.68, 40.75,48.16, 60.19, 113.39, 120.53, 122.62, 123.75, 134.03, 141.45, 144.44, 159.90.

5.1.2.7. N-(1-azabicyclo[2.2.2]octan-3-yl)-1-phenylmethyl-1H-benzimidazole-2-carboxamide **13i**

Yield: 64%; m.p.: 179–181°C. ^{1}H NMR (DMSO-d_6) 1.24 (m, 1H, CH_2), 1.59 (m, 2H, CH_2), 1.75 (m, 1H, CH_2), 1.86 (m, 1H, CH_2), 2.60–3.12 (m, 5H, CH_2), 3.40 (m, 1H, CH), 4.07 (m, 1H, CH), 5.96 (s, 2H, CH_2), 7.15–7.38 (m, 7H, ar), 7.63 (m, 1H, ar), 7.76 (m, 1H, ar), 8.87 (br d, 1H, CONH). ^{13}C NMR (DMSO-d_6) 19.88, 25.62, 25.86, 46.37, 46.62, 46.95, 47.46, 53.06, 111.68, 120.17, 123.13, 124.30, 126.98, 127.43, 128.53, 135.76, 137.31, 140.80, 143.93, 159.60.

5.1.2.8. endo-N-(8-methyl-8-azabicyclo[3.2.1]octan-3-yl)-1-phenylmethyl-1H-benzimidazole-2-carboxamide **13j**

Yield: 70%; m.p.: 125–127°C. ^{1}H NMR (DMSO-d_6) 1.68–2.09 (m, 8H, CH_2), 2.14 (s, 3H, CH_3), 3.02 (m, 2H, CH), 4.03 (m, 1H, CH), 5.95 (s, 2H, CH_2), 7.17–7.36 (m, 7H, ar), 7.60 (m, 1H, ar), 7.77 (m, 1H, ar), 8.41 (br d, 1H, CONH). ^{13}C NMR (DMSO-d_6) 25.48, 35.21, 39.91, 41.16, 47.53, 59.20, 111.72, 120.27, 123.24, 124.36, 126.95, 127.42, 128.52, 135.94, 137.23, 140.67, 143.63, 158.48.

5.1.3. General procedure for compounds **14**

To a solution of benzimidazole-2-carboxylic acid **6** (10 mmol) in DMF (40 mL), 1,1′-carbonyldiimidazole (11 mmol) was added. After stirring the reaction mixture for 3 h at room temperature, the corresponding alcohol (11 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (11 mmol) were added, and the mixture was heated at 60 °C for 18 h. Solvent was removed under reduced pressure and the residue was extracted with Cl_2CH_2 (3 × 20 mL). The combined organic layers were dried (Na_2SO_4) and evaporated to give a residue which was purified by silica gel chromatography (Cl_2CH_2: triethylamine, 95:5) to yield **14**.

5.1.3.1. 1-azabicyclo[2.2.2]octan-3-yl benzimidazole-2-carboxylate **14a**

Yield: 42%; m.p.: 128 °C (dec). ^{1}H NMR (DMSO-d_6) 1.40 (m, 1H, CH_2), 1.63 (m, 2H, CH_2), 2.88 (m, 1H, CH_2), 2.17 (m, 1H, CH_2), 2.61–2.87 (m, 5H, CH_2), 3.27 (m, 1H, CH), 5.12 (m, 1H, CH), 7.23–7.40 (m, 2H, ar), 7.60–7.79 (m, 2H, ar). ^{13}C NMR (DMSO-d_6) 19.12, 24.31, 25.21, 46.18, 46.93, 54.57, 73.68, 112.72, 114.65, 121.60, 124.51, 139.81, 142.06, 149.96, 159.92.

5.1.3.2. endo-8-methyl-8-azabicyclo[3.2.1]octan-3-yl benzimidazole-2-carboxylate **14b**

Yield: 49%; 106 °C (dec). ^{1}H NMR (DMSO-d_6) 1.92–2.32 (m, 8H, CH_2), 2.30 (s, 3H, CH_3), 3.21 (m, 2H, CH), 5.29 (m, 1H, CH), 7.27–7.40 (m, 2H, ar), 7.72 (m, 2H, ar), 12.21 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6) 25.92, 36.63, 40.52, 59.82, 70.58, 115.70, 116.67, 121.85, 123.91, 138.59, 142.16, 151.01, 159.80.

5.1.4. endo-8-methyl-8-azabicyclo[3.2.1]octan-3-yl-1-phenylmethyl-1H-benzimidazole-2-carboxylate (fumarate) **15a**

To an ice-cooled solution of **14b** (4.56 mmol, 1.3 g) in DMF (40 mL), HNa (60% suspension, 6 mmol, 0.25 g) was slowly added, and the mixture was stirred for 30 min. Benzyl bromide (6 mmol, 0.7 mL) was added dropwise, and the solution was stirred at room temperature for 4 h. The reaction mixture was concentrated in vacuo and the residue was treated with H_2O (30 mL) and extracted with $CHCl_3$/^{i}PrOH (4/1). The organic layer was dried (Na_2SO_4) and evaporated under reduced pressure to give a residue which was purified by silica gel chromatography (Cl_2CH_2:triethylamine, 1:1) to provide **15a** (65%). ^{1}H NMR (DMSO-d_6) 1.91–2.42 (m, 8H, CH_2), 2.43 (s, 3H, CH_3), 3.71 (m, 2H, CH), 5.20 (m, 1H, CH), 5.89 (m, 2H, CH_2), 7.16–7.42 (m, 7H, ar), 7.67 (m, 1H, ar), 7.85 (m, 1H, ar). ^{13}C NMR (DMSO-d_6) 24.35, 33.52, 37.70, 48.20, 59.96, 67.23, 111.86, 121.21, 123.62, 125.46, 126.68, 127.51, 134.86, 136.01, 137.06, 140.86, 141.30, 158.85.

5.2. Pharmacology

5.2.1. 5-HT$_3$ receptor binding assay [17]

Adult male Wistar rats weighing 220–280 g, were used. Animals were killed by decapitation and the whole brain, with the exception of the brainstem and cerebellum, was quickly removed and the various areas dissected, weighed and immediately frozen at –70 °C. Enthorinal cortex used for the binding experiments was homogenized with an Ultra-Turrax (setting 5 for 20 s) in 10 volumes of ice-cold 0.32 M sucrose buffer, centrifuged at 1 000 *g* for 10 min (4 °C) and the supernatant was

recentrifuged at 17 000 *g* for 20 min (4 °C). The resulting pellet was resuspended in 50 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4), incubated at 37 °C for 10 min and then centrifuged three times more at 48 000 *g* for 10 min (4 °C). The final pellet was resuspended in 10 volumes of ice-cold 50 mM Tris-HCl buffer containing 5 mM $CaCl_2$ and 0.1% ascorbate, and was stored at –70 °C until use. At the time of the experiment, the membranes were diluted in the same ice-cold buffer with 10 μM pargyline (final dilution 1:40, wt/vol). Competition assays were performed in triplicate, in a final volume of 1 mL. To each assay tube were added the following: 0.1 mL of the displacer drug concentration (0.1 mL of vehicle if no competing drug was added) and 0.1 mL of [^{3}H]-LY278584 (Amersham, 60–85 Ci/mmol) in buffer (final concentration 2 nM). Non-specific binding was determined using 10 μM cold 5-HT. Binding experiments were initiated by addition of 0.8 mL of membrane suspension (500–600 μg of protein). After incubation for 30 min at 25 °C the reaction was stopped for vacuum filtration through Whatman GF/B glass pretreated filters (1% polyethylenimine in 50 mM Tris-HCl buffer), using a Brandel Cell Harvester, followed by two washes with 5 mL of ice-cold 50 mM Tris-HCl (pH 7.4) buffer. Filters were placed in polyethylene scintillation vials (with 5 mL of scintillation cocktail), equilibrated, and filter-retained radioactivity measured in a liquid scintillation counter (Kontron Beta V). IC_{50} and K_i values were calculated using the computer program EBDA (McPherson).

5.2.2. 5-HT$_4$ receptor binding assay [18]

Adult male Dunkin-Hartley guinea-pigs weighing 350–400 g were used. Animals were killed by decapitation and the whole brain, with the exception of the brainstem and cerebellum, was quickly removed and the various areas dissected, weighed and immediately frozen at –70 °C. Striatum used for the binding experiments was homogenized with an Ultra-Turrax (setting 5 for 20 s) in 15 volumes of ice-cold 50 mM Hepes buffer (pH 7.4) and centrifuged at 48 000 *g* for 20 min (4 °C). The resulting pellet was resuspended in 10 volumes of ice-cold 50 mM Hepes buffer (pH 7.4) and was stored at –70 °C until use. At the time of the experiment, the membranes were diluted in the same ice-cold buffer (final dilution 1:80, wt/vol). Competition assays were performed in triplicate, in a final volume of 1 mL. To each assay tube were added the following: 0.1 mL of the displacer drug concentration (0.1 mL of vehicle if no competing drug was added) and 0.1 mL of [^{3}H]GR-113808 (Amersham, 60–85 Ci/mmol) in buffer (final concentration 0.1 nM). Non-specific binding was determined using 10 μM cold 5-HT. Binding experiments were initiated by addition of 0.8 mL of membrane suspension (800–900 μg of protein). After incubation for 30 min at 25 °C the reaction was stopped for vacuum filtration through Whatman GF/B glass pretreated filters (1% polyethylenimine in 50 mM Hepes buffer), using a Brandel Cell Harvester. Filters were placed in polyethylene scintillation vials (with 5 mL of scintillation cocktail), equilibrated, and filter-retained radioactivity measured in a liquid scintillation counter (Kontron Beta V). IC_{50} and K_i values were calculated using the computer program EBDA (McPherson).

5.2.3. D$_2$ receptor binding assay [19]

Adult male Wistar rats weighing 220–280 g were used. Animals were killed by decapitation and the whole brain, with the exception of the brainstem and cerebellum, was quickly removed and the various areas dissected, weighed and immediately frozen at –70 °C. Striatum used for the binding experiments was homogenized with an Ultra-Turrax (setting 5 for 20 s) in 50 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.7), centrifuged at 48 000 *g* for 10 min (4 °C). The resulting pellet was resuspended in 50 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.7), incubated at 37 °C for 10 min and then centrifuged once more at 48 000 *g* for 10 min (4 °C). The resulting pellet was resuspended in 10 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and was stored at –70 °C until use. At the time of experiment, the membranes were diluted in the same ice-cold buffer with 10 μM pargyline (final dilution 1:150, wt/vol). Competition assays were performed in triplicate, in a final volume of 1 mL. To each assay tube were added the following: 0.1 mL of the displacer drug concentration (0.1 mL of vehicle if no competing drug was added) and 0.1 mL of [^{3}H]Raclopride (NEN, 60-87 Ci/mmol) in buffer (final concentration 1 nM). Non-specific binding was determined using 1 μM cold (+)-butaclamol. Binding experiments were initiated by addition of 0.8 mL of membrane suspension (300–400 μg of protein). After incubation for 60 min at 25 °C the reaction was stopped for vacuum filtration through Whatman GF/B glass filters, using a Brandel Cell Harvester, followed by two washes with 5 mL of ice-cold 50 mM Tris-HCl (pH 7.7) buffer. Filters were placed in polyethylene scintillation vials (with 5 mL of scintillation cocktail), equilibrated, and filter-retained radioactivity measured in a liquid scintillation counter (Kontron Beta V). IC_{50} and K_i values were calculated using the computer program EBDA (McPherson).

5.2.4. Inhibition of the von Bezold-Jarisch reflex

Adult male Wistar rats weighing 220–300 g and fasted for 18 h were used. Rats were anaesthetized with urethane (1.25 g/kg i.p.) and placed on a heating table (Hugo

Sachs Elektronik, Freiburg, Germany) to maintain body temperature at 37 °C. The trachea and right jugular vein were cannulated to facilitate respiration and drug administration, respectively. The carotid artery was also cannulated and connected to a Goul Statham P23Db pressure transducer to record blood pressure. The heart rate was measured using the blood pressure signal and a cardiotachometer coupler and recorded on a Graphtec Linearcorder WR3101 (Hugo Sachs Elektronik, Freiburg, Germany). The B-J reflex was evoked by rapid bolus i.v. injection of 5-HT (30 μg/kg). When 5-HT-induced bradycardia (65–75% fall of heart rate from control heart rate) returned to pretreatment levels (within 5 min), either antagonist or saline were administered (1 mL/kg) and 5-HT-induced bradycardia was elicited again 5 min later. Drugs were prepared daily and were dissolved in distilled water or 0.1 M tartaric acid, with subsequent dilutions in physiological saline. ED_{50} values were calculated from 3–5 doses (4–5 rats per dose) by a linear-regression analysis.

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